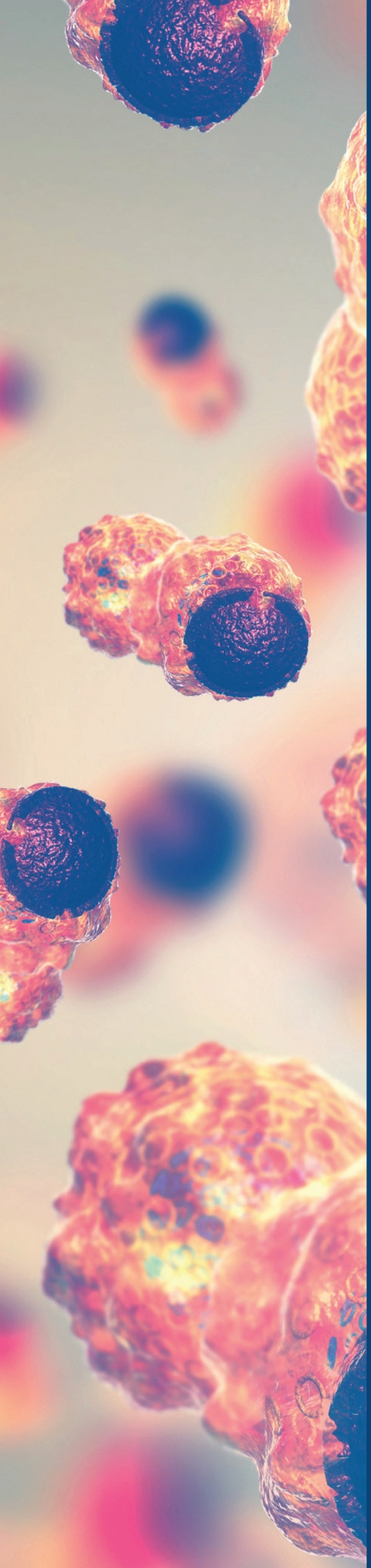

Volume 1
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THIRD EDITION

Encyclopedia *of* CANCER

Edited by Paolo Boffetta and Pierre Hainaut

ENCYCLOPEDIA OF CANCER

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ENCYCLOPEDIA OF CANCER

THIRD EDITION

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Paolo Boffetta, MD, MPH, graduated in Medicine from the University of Turin and obtained a Master in Public Health from Columbia University. He worked at the American Cancer Society, the International Agency for Research on Cancer, a specialized agency of the World Health Organization located in Lyon, France, and at the German Cancer Research Center in Heidelberg, Germany. In 2010 he moved to Icahn School of Medicine at Mount Sinai, New York, where he is Professor of Medicine, Global Health, Oncological Sciences, and Preventive Medicine, and Associate Director for Global Oncology of the Tisch Cancer Institute, a NCI-designated Cancer Center.

Dr. Boffetta also holds a full professorship at the University of Bologna and adjunct appointments at Harvard School of Public Health, Vanderbilt University, Catholic University of Rome, and University of South Carolina. Since 2017 he is Senior Advisor for Research at Vinmec Health System. His main fields of research are cancer epidemiology and cancer prevention, with emphasis on modifiable risk factors (environmental exposure and personal behaviors), gene–environment interactions, molecular epidemiology, and evidence integration. He is the initiator and coordinator of several large-scale international consortia of molecular cancer epidemiology studies, including ILCCO (lung cancer), INHANCE (head and neck cancer), PANC4 (pancreatic cancer), StoP (stomach cancer), and ILCEC (liver cancer).

Dr. Boffetta is the editor or associate editor of 5 scientific journals and member of the editorial board of 10 additional journals; he is a member of review panels of NIH and several medical research agencies in Europe. He has edited 13 books and is Editor in Chief of the new edition of Elsevier's *Encyclopedia of Cancer*. He has published over 1250 peer-reviewed publications; his publications have been quoted more than 90000 times; his h-index is 148.

Pierre Hainaut is Professor of Exceptional Class in Cancer Biology at University Grenoble-Alpes, France and Director of the Institute of Advanced Biosciences (IAB), a joint research center of Institut National de la Santé et de la Recherche Médicale (Inserm), Centre National de la Recherche Scientifique (CNRS), and University Grenoble-Alpes. He also heads the IAB research team on Molecular Biology and Biomarkers.

Pierre Hainaut holds a PhD in Biology (Zoology) from University of Liège, Belgium (1987). After postdocs in Nice (France, 1988–90), Cambridge, and York (United Kingdom, 1990–94), he joined the International Agency for Research on Cancer (IARC, World Health Organization) in 1995, where he held the post of Head of Molecular Carcinogenesis from 1999 to 2011. In 2012, he joined the International Prevention Research Institute (Lyon, France) and became Professor at the Strathclyde Institute of Pharmacy and Biomedical Science (Glasgow, United Kingdom). In 2014, he was awarded a Chair of Excellence in Translational Research from University Grenoble-Alpes (Grenoble, France).

His research focuses on *TP53* mutations and p53 protein regulation in cancer and chronic diseases. From 1994 to 2011, he has led the development of the international IARC TP53 database, a source of information on the causes and consequences of mutations affecting the *TP53* suppressor gene in cancer. His work addresses the mechanisms of *TP53* mutagenesis as well as the prognostic and predictive significance of *TP53* mutations in lung, liver, and oesophageal cancers. His studies on p53 regulation have focused on the role of environmental mutagens in *TP53* mutagenesis, on the biochemical mechanisms of p53 control by oxidation-reduction and by metabolism, and on the identification of p53 isoforms as factors acting as dominant inhibitors of p53 functions in cancers without *TP53* mutations. His current activities focus on germline *TP53* mutation and on the diversity of genetic and nongenetic factors that modulate the penetrance of the Li–Fraumeni Syndrome, as well as on the mechanisms that maintain optimal p53 protein balance in cells and tissues over lifetime. He is the author of over 450 publications and 50 book chapters. He is Editor of the Cancer Biology section of *Current Opinion in Oncology*. He has co-edited books on p53 (*25 Years of p53 Research*, 2005, 2007, *p53 in the Clinics*, Springer), a textbook on molecular epidemiology (*Molecular Epidemiology: Principle and Practice*, IARC Press, 2011) and two-volume textbook on human biobanking (*Human Biobanking, Principle and Practice*, 2017, 2018).

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Fred T. Bosman MD PhD, studied Medicine at the University of Leiden (MD 1971), where he also earned his PhD degree (in cytogenetics, 1976), and trained as a pathologist. He was staff pathologist at the University of Leiden, Professor and Chair of Pathology at the Faculty of Medicine of the University of Maastricht, Faculty of Medicine of the Erasmus University in Rotterdam, Director of the University Institute of Pathology, and Professor of Pathology at the University Medical Center (CHUV) of Lausanne in Switzerland, now emeritus. He is Honorary Fellow of the Royal College of Pathologists (United Kingdom) and foreign correspondent of the Royal Netherlands Academy of Sciences.

Fred Bosman's research activities (combining diagnostic and experimental pathology) focused on the biology of digestive tract cancer, notably Barrett's esophagus and colorectal cancer, with a strong emphasis on the development of molecular diagnostics. He has written over 350 original publications and over 50 book chapters. Fred Bosman was Series co-editor of the 4th edition of the WHO Series *Classification of Human Tumours*, the international standard for tumor classification, and co-editor of the Volume on *Tumours of the Digestive Tract*.

Graham A. Colditz, MD, DrPH, FAFPHM, is an internationally recognized leader in cancer prevention. As an epidemiologist and public health expert, he has a longstanding interest in the preventable causes of chronic disease, particularly among women. He focuses his research on early life and adolescent lifestyle, growth, and breast cancer risk. He is also interested in approaches to speed translation of research findings into prevention strategies that work. Dr. Colditz developed the award-winning Your Disease Risk website (www.yourdiseaserisk.wustl.edu) which communicates tailored prevention messages to the public. He has published over 1100 peer-reviewed publications, six books and six reports for the Institute of Medicine, National Academy of Sciences. His h-index is over 220.

In October 2006, on the basis of professional achievement and commitment to public health, Dr. Colditz was elected to membership of the National Academy of Medicine, an independent body that advises the US government on issues affecting public health. In 2011, he was awarded the American Cancer Society Medal of Honor for cancer control research. In 2012 he received the AACR-American Cancer Society Award for Research Excellence in Cancer Epidemiology and Prevention. He also received awards in 2014 for cancer prevention research from ASCO and from AACR. During 2016 he served on the Implementation Science Work Group of the Blue-Ribbon Panel to advise the National Cancer Moonshot. He received the 2018 Daniel P. Schuster Award for Distinguished Work in Clinical and Translational Science, Washington University School of Medicine. He was also elected as a Fellow, American Association for the Advancement of Science

Carlo La Vecchia received his MD from the University of Milan and a Master of Science degree in Medicine (epidemiology) from Oxford University. Presently, he is Professor of Medical Statistics and Epidemiology at the Faculty of Medicine at the University of Milan. Dr. La Vecchia serves as an editor for numerous clinical and epidemiologic journals. He is among the most renowned and productive epidemiologists in the field with over 2040 peer-reviewed papers in the literature and is among the most highly cited medical researchers in the world, according to ISI HighlyCited.com, the developer and publisher of the Science Citation Index (2003, 2017, H index 153, H10 index 1543, second Italian in Clinical Medicine). Dr. La Vecchia is Adjunct Professor of Medicine at Vanderbilt Medical Center and the Vanderbilt-Ingram Cancer Center (2002-18).

Gerd. P. Pfeifer received a PhD degree in biochemistry from Goethe University in Frankfurt, Germany. After postdoctoral training, he became a faculty member at the Beckman Research Institute of the City of Hope in Duarte, California, where he spent much of his career working on cancer research. In 2014, Dr. Pfeifer joined the new Center for Epigenetics at the Van Andel Research Institute in Grand Rapids, MI, United States, as a Professor of Epigenetics. Dr. Pfeifer has authored more than 300 publications, has held an NIH MERIT award, and was elected Fellow of the American Association for the Advancement of Science in 2015. Research in Dr. Pfeifer's laboratory has been concerned with genetic and epigenetic mechanisms of human carcinogenesis, with emphasis on DNA methylation and genetic toxicology.

Marco Alessandro Pierotti graduated in 1973 in Biological Sciences at the University of Milan, Italy, and started working at the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) in Milan. From 1978 to 1980 he was Visiting Investigator at the Laboratory of Chemical Cancerogenesis of the NCI-NIH Bethesda (MD, United States) and Postdoctoral Research Fellow at the Laboratory of Viral Oncology of the Memorial Sloan-Kettering Institute in New York. In 2006, Dr. Pierotti was appointed Scientific Director of the Fondazione IRCCS Istituto Nazionale dei Tumori in Milan, where, since 1970, he had already held various positions, including Director of the Department of Experimental Oncology.

Since 1988, he has been Professor of Molecular Genetics of Cancer at the Postgraduate School of Oncology, University of Milan Medical School and co-director of the Laboratory of Molecular Diagnosis at the INT. In September 2014 he resigned from his position at INT to take the position of President and CEO of Nerviano Medical Sciences (NMS) srl, one of the biggest oncological pharma companies in Europe. In April 2015 he left the company and took the position of Scientific Coordinator of the Institute of Pediatric Researches (IRP) in Padua, Italy, devoted to study molecular aspects of the main pediatric diseases with particular focus on pediatric onco-hematology. The Institute was created by a private Charity, The City of Hope Foundation of Monte Malo (Vi).

Since 2000 he is Senior Group leader of the Molecular Genetics of Cancer group at the Institute FIRC of Molecular Oncology (IFOM, Milan). Past President (2006–08) of the Italian Cancer Society, Dr. Pierotti is a member of the American Association for Cancer Research and of its Advisory Board and the Laboratory Research Awards Selection Committee. He has also been President (2006–08) of the European Association for Cancer Research (EACR) and in this role was among the founders of the European CanCer Organisation (ECCO) where he was appointed as member of the Policy Committee.

In recent years, he was the Italian Representative at the Scientific Committee of the International Agency for Research on Cancer (IARC), Lyon. From 2006 to 2014 he was Scientific Secretary of Alleanza Contro il Cancro (ACC), promoted by the Italian Ministry of Health. In 2008 he was Member of the Evaluation of the Research Program Functional and Structural Genomics for DKFZ. He was an expert for the Oncology Research Projects of the European Community and was a consultant in oncology research for the Ministries of Research of different Countries. From 2006 to 2014 he was Chair of the regional Project, The Region Lombardy Oncological Network (ROL), selected in 2014 by the EC as one of the best examples of oncological network. His appointments in the Organisation of European Cancer Institutes (OECI) started in 2007 when he took the position of Vice-President. From 2008 he was the President-elect and then the President of the Organization. Finally, in 2014 he was appointed OECI Executive Secretary.

Over the years, Dr. Pierotti has been Principal Investigator or Head of several national and international research grants, funded by both private and public bodies. His authorship includes over 470 publications that deal with various aspect of experimental oncology including studies on immunology, biochemistry, and molecular biology using both experimental and human tumors.
In addition, since its fifth edition he is the first author of the chapter on "Oncogenes" in the most reputed textbook *Cancer Medicine* (Holland-Frei). The metrics of his scientific activity is summarized by an H index of 96 and total citations of 37.256 (Google scholar June 2018).

Professor **Thomas Tursz**, born in Kraków, Poland, in 1946, died in Paris, France, on April 27 2018. He was Professor of Oncology at the Faculty of Medicine Paris-Sud since 1986 and General Director of the Institut Gustave Roussy (1994–2010). He was the leader of the French Doctoral School of Oncology which he founded in 1999, and President of the French Federation of Comprehensive Anticancer Centres (FNCLCC) from 2004 to 2010. He was highly involved in the European Organization for the Research and Treatment of Cancer (EORTC) as both Chairman of the Scientific Advisory Committee (2003–06) and Vice President of the Board (2006–09). His experience as President of the FNCLCC was crucial for the Organization of European Cancer Institutes (OECI) when he acted as President from 2002 to 2005.

His scientific interests included the biology of virus-induced tumors, as well as immunological responses including the role of thioredoxin in lymphocytes infected by Epstein–Barr virus. In the clinical research area, he conducted a number of important clinical trials in breast cancer, lung cancer, and soft-tissue sarcoma. He had a particular interest in cytokines and gene therapy, and his clinical research activities were further disseminated to the European level when he was the Chairman of the Sarcoma Group of the EORTC (1993–96).

Prof. Tursz received several prestigious awards, such as the Prix de Cancérologie from the French National League Against Cancer (1979), the Bernard Halpern Immunology Award (1983), the Rosen Oncology Award (1989), the Grand Prix in Oncology from the Academy of Medicine (1992), the Hamilton Fairley Award for clinical research (1998), and the Prix de Rayonnement Français (2001). He was the author of 350 international scientific publications. He was also an esteemed member of the Editorial Board of *Molecular Oncology* ever since its creation in 2007.

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HOW TO USE THE ENCYCLOPEDIA

Structure of the Encyclopedia

All articles in the encyclopedia are arranged alphabetically as a series of entries.

There are four features to help you easily find the topic you are interested in: an alphabetical contents list, cross references, a full subject index, and contributors.

1. Alphabetical contents list: The alphabetical contents list, which appears at the front of each volume, lists the entries in the order that they appear in the encyclopedia. So that they can be easily located, entry titles generally begin with the key word or phrase indicating the topic, with any generic terms following. For example, "Multiple Myeloma: Pathology and Genetics" is the entry title rather than "Pathology and Genetics of Multiple Myeloma".
2. Cross references: Virtually all the entries in the encyclopedia have been extensively cross-referenced. The cross references which appear at the end of an entry, serve three different functions:
   i. To draw the reader's attention to related material on other entries
   ii. To indicate material that broadens and extends the scope of the article
   iii. To indicate material that covers a topic in more depth

Example
The following list of cross-references appears at the end of the entry "Carcinogen—DNA Adducts".

> ***See also:*** Cancer Risk Reduction Through Lifestyle Changes. Cell Responses to DNA Damage. Genetic Instability. Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2. Molecular Epidemiology and Cancer Risk. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics.

3. Index: The index appears at the end of volume 3 and includes page numbers for quick reference to the information you are looking for. The index entries differentiate between references to a whole entry, a part of an entry, and a table or figure.
4. Contributors: At the start of each volume there is a list of the authors who contributed to all volumes.

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SUBJECT CLASSIFICATION

Causes of Cancer

Aflatoxins
Aging and Cancer
Cancers as Ecosystems: From Cells to Population
Diabetes and Cancer
Dietary Factors and Cancer
Helicobacter Pylori-Mediated Carcinogenesis
HIV (Human Immunodeficiency Virus)
Obesity and Cancer: Epidemiological Evidence
Opisthorchis Viverrini, *Clonorchis Sinensis*, and Cholangiocarcinoma
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Physical Inactivity and Cancer
Radiation Therapy-Induced Metastasis and Secondary Malignancy
Sleep Disturbances and Misalignment in Cancer

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Acute Myelogeneous Leukemia: Diagnosis and Treatment
Bladder Cancer: Pathology, Genetics, Diagnosis, and Treatment
Bone and Soft Tissue Sarcoma: From Molecular Features to Clinical Applications
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Chromatin Dynamics and Cancer: Epigenetic Parameters and Cellular Fate
Chronic Myelogenous Leukemia: Pathology, Genetics, Diagnosis, and Treatment
Colorectal Cancer: Diagnosis and Treatment
End of Life Support
Esophageal Cancer: Diagnosis and Treatment
Glioblastoma: Biology, Diagnosis, and Treatment
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Kidney Cancer: Diagnosis and Treatment
Laryngeal Cancer: Diagnosis and Treatment
Malignant Tumors of the Eye, Conjunctiva, and Orbit: Diagnosis and Therapy
Myelodysplastic Syndromes: Mechanisms, Diagnosis, and Treatment
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New Rationales and Designs for Clinical Trials in the Era of Precision Medicine
Non-Hodgkin Lymphoma: Diagnosis and Treatment
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Bladder Cancer: Pathology, Genetics, Diagnosis, and Treatment
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Colorectal Cancer: Pathology and Genetics
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Uterine Cervix Cancer: Pathology and Genetics
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Prevention and Control

Aspirin and Cancer
Cancer Disparities
Cancer in Populations in Transition
Cancer in Sub-Saharan Africa
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Prevention and Control: Nutrition, Obesity, and Metabolism

PREFACE

Cancer holds a special status in biology, medicine, and society. It offers formidable challenges to basic, clinical, and population science; it ranks at the top of medical research priorities and healthcare costs in most countries. The general public is continuously exposed to news of discoveries promising to defeat the disease in the near future. Indeed, ground-breaking advances are being made, from preventive vaccines to genome-guided personalized medicine, sophisticated imaging and surgical technologies, and systemic therapies aimed at awakening natural immune responses against cancer. These novel therapeutic approaches make cure a real possibility for a growing number of patients. They also launch cancer care into a new era of maintaining the disease under control for an indefinite period of time, turning it into a form of chronic disease. At the same time, evidence-based prevention and early detection strategies have opened a new window on the natural history of the disease, enabling effective intervention well ahead of diagnosis. As a result, the first two decades of this millennium have witnessed a marked decrease in the mortality and, in some instances, the incidence of cancers that have dominated the death toll in more developed countries during the second half of the 20th century.

A turning point in our understanding of cancer is the deciphering of the human genome and its spin-off endeavors aimed at exploring the genomic landscape and architecture of human cancers. These discoveries are causing a major overhaul of our vision of cancer as a dynamic, rapidly evolving, and heterogeneous disease at the individual level. Harnessing this complexity requires mastering increasingly complex sources of data at molecular, cellular, systemic, personal, environmental, and societal level, heralding the emergence of big-data science in cancer diagnosis and treatment. However, this exceptional acceleration in knowledge and solutions cannot hide the fact that cancer remains a global scourge that exerts a massive burden on humankind and societies worldwide, in particular in societies in transition and in low-resource contexts.

Today, cancer crystallizes many of the major societal challenges pertaining to lifestyles, sustainable development and environmental policies, demography and population aging, access to education and healthcare, sharing of resources and knowledge, and protection of persons and personal information. The information on cancer available at a fingertip is overwhelming in volume, complexity, veracity, and velocity. We worked on the Third Edition of Elsevier's *Encyclopedia of Cancer* with this rapidly changing background. Rather than aiming at developing a comprehensive framework encompassing all aspects, we attempted to address the literal meaning of the greek terms ἐγκύκλιος παιδεία, which means "general education". While we retained some articles from the previous edition, which, at the time of the publication, represented an exceptional achievement of Dr. J. Bertino, we largely modified the structure and the list of chapters, and the possibility of continuous update of the articles has been a great incentive for us and for the authors of the chapters. This new edition of the Encyclopedia consists of six major parts: (i) mechanisms of cancer, (ii) hallmarks of cancer, (iii) causes of cancer, (iv) cancer prevention and control, (v) diagnosis and treatment of specific cancers, and (vi) pathology and genetics of specific cancers. This repartition is necessarily artificial and is complemented by the extensive cross-references between articles. The repartition, however, reflects our effort to identify discrete topics that would best address the needs of a wide community of readers.

The primary target readership of the Encyclopedia comprises medical and other health science students, as well as non-specialized physicians and other health practitioners. Cancer researchers, oncologists, and other cancer professionals may find the articles pertaining to their specific field to be too short, over-simplistic, and perhaps obsolete; they too, however, may benefit from articles on topics other than their own. The Encyclopedia also offers an easy way to navigate across concepts and topics that should be appealing to readers from other communities, including social sciences or stakeholders in public decision-making.

We were fortunate to work with a formidable team of section editors, including Fred Bosman, Graham Colditz, Carlo La Vecchia, Gerd Pfeifer, and Marco Pierotti. An additional section editor was Professor Thomas Tursz, who passed away prematurely during the preparation of the Encyclopedia. Thomas was a great colleague and mentor, and a major figure in oncology in France and internationally. We had the privilege to involve him in the last project of his long career, and we want to dedicate this work to him. We wish to thank the many article authors, who agreed to contribute to the success of this international endeavor, and in particular Dr. Katarzyna Szymańska, who drafted several cancer-specific articles. Finally, we want to thank the staff at Elsevier, whose patience and perseverance helped us bringing the project to the final stage. All these individuals are responsible for the many strengths of the new edition of the *Encyclopedia of Cancer*, while weaknesses are mainly ours.

Paolo Boffetta
Pierre Hainaut

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Acute Lymphocytic Leukemia: Diagnosis and Treatment

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Abbreviations

6-MP 6-Mercaptopurine
ALL Acute lymphocytic (lymphoblastic) leukemia
AYA Adolescents and young adults
B-ALL B-lymphoblastic leukemia
B-LBL B-lymphoblastic lymphoma
CAR-T Chimeric antigen receptor T-cells
CBC Complete blood count
CD Cluster of differentiation
CGH Comparative genomic hybridization
CNS Central nervous system
COG The Children's Oncology Group
CR Complete remission
CT Computed tomography
DIC Disseminated intravascular coagulation
FAB French–American–British (histopathologic classification of acute leukemia)
FDA US Food and Drug Administration
FISH Fluorescence in situ hybridization
HSCT Hematopoietic stem cell transplantation
ICD-O International Classification of Diseases for Oncology
IGH Immunoglobulin heavy locus
LDH Lactate dehydrogenase
MRD Minimal residual disease
NCCN US National Comprehensive Cancer Network
NK Natural killer
PET Positron emission tomography
Ph Philadelphia chromosome
RT-PCR Reverse-transcriptase polymerase chain reaction
SNP Single nucleotide polymorphism
T-ALL T-lymphoblastic leukemia
TCR T-cell receptor
TdT Terminal deoxynucleotidyl transferase
TK Tyrosine kinase
TKI Tyrosine kinase inhibitor
TMPT Thiopurine methyltransferase
WBC White blood cells
WHO World Health Organization

Definition and Classification

Acute lymphocytic leukemia (also called acute lymphoblastic leukemia; ALL) is a neoplasm of immature B- or T-cells (lymphoblasts). In the most recent World Health Organization (WHO) classification of hematopoietic and lymphoid tumors, it is classified under "precursor B-cell lymphoblastic leukemia/lymphoma" and "precursor T-cell lymphoblastic leukemia/lymphoma." This

classification has replaced the French–American–British (FAB) histopathologic classification of acute leukemia, originally proposed in 1976, which classified ALL into three entities: adult, childhood, and Burkitt type ALL. The new WHO classification system takes into account not only morphological and cytochemical characteristics of the disease but also its cytogenetic and clinical diversity. All in all, the widely used term "ALL" encompasses several entities as classified by WHO, defined below.

B-lymphoblastic leukemia (B-ALL) not otherwise specified (NOS; ICD-O code: 9811/3) is a neoplasm of precursor lymphoid cells committed to the B-cell lineage, typically composed of small to medium-size blast cells with scant cytoplasm, moderately condensed to dispersed chromatin and inconspicuous nucleoli, involving bone marrow and blood. By convention, the term B-lymphoblastic lymphoma (B-LBL), which is included under the same ICD-O code, is used when a process is confined to a mass lesion with no or minimal evidence of blood and bone marrow involvement (generally defined as less than 20% of lymphoblasts in the marrow). The term B-ALL does not encompass Burkitt leukemia/lymphoma.

B-ALLs with specific recurrent genetic abnormalities associated with particular clinical and phenotypic features, and/or of prognostic significance are classified as separate entities. These are as follows:

B lymphoblastic leukemia with *t*(*9;22*)(*q34;q11.2*); *BCR-ABL1* (ICD-O code: 9812/3)
B lymphoblastic leukemia with *t*(*v;11q23*); *MLL* (also called *KMT2A* or *MLL1*) rearranged (9813/3)
B lymphoblastic leukemia with *t*(*12;21*)(*p13;q22*); *TEL-AML1* (*ETV6-RUNX1*) (9814/3)
B lymphoblastic leukemia with hyperdiploidy (9815/3)
B lymphoblastic leukemia with hypodiploidy (Hypodiploid ALL; 9816/3)
B lymphoblastic leukemia with *t*(*5;14*)(*q31;q32*); *IL3-IGH* (9817/3)
B lymphoblastic leukemia with *t*(*1;19*)(*q23;p13.3*); *E2A-PBX1* (*TCF3-PBX1*) (9818/3)
B lymphoblastic leukemia, *BCR-ABL1*-like (9819/3)

Additionally, B lymphoblastic leukemia with *iAMP21* has been depicted as a provisional entity with no separate ICD-O code (it is coded under B-ALL, NOS; ICD-O code: 9811/3).

T-lymphoblastic leukemia (T-ALL) is a neoplasm of precursor lymphoid cells with the same cellular characteristics as B-ALL but concerning precursor cells committed to the T-cell lineage.

Presentation and Diagnosis

The symptoms of ALL are usually nonspecific and may appear only weeks or even days before diagnosis. Fatigue and weakness are among the most common nonspecific symptoms. Fever, with or without night sweats, and weight loss are frequent. The cause of fever is often not found, although granulocytopenia may lead to rapidly progressing and potentially life-threatening bacterial infections. Many patients present with thrombocytopenia and/or neutropenia, easy bruising and/or bleeding (bleeding gums, epistaxis, purplish patches in the skin or petechiae), and anemia as a result of bone marrow failure and disrupted hematopoiesis. Dyspnea, chest pain, and dizziness may also occur. Bone and joint pain due to bone marrow and periosteal infiltration is frequent in children in whom it may be the only presenting symptom. Headaches, vomiting, irritability, cranial nerve palsies, seizures, papilledema, and blurred vision are manifestations of the central nervous system (CNS) involvement and/or leukemic meningitis but initial CNS involvement is uncommon.

A sensation of abdominal fullness and/or discomfort may appear due to hepatomegaly and/or splenomegaly. Hepatomegaly, splenomegaly, and lymphadenopathy at physical examination are found in about 20% of all patients and in approximately 50% of adult ALL presentations. Extramedullary infiltration may also lead to leukemia cutis (a raised, nonpruritic rash). Oliguria may occur as a result of dehydration, uric acid nephropathy, or disseminated intravascular coagulation (DIC).

The initial workup includes complete blood count (CBC) with peripheral blood smear, blood chemistry profile, liver and kidney function tests, coagulation analysis (including measurement of prothrombin time, partial thromboplastin time, and fibrinogen), and a tumor lysis syndrome panel (including measurement of serum lactate dehydrogenase (LDH), potassium, uricemia, calcium, and phosphorus). Computed tomography (CT) scans of the neck, chest, abdomen, and pelvis are recommended for detecting possible organ involvement, lymphadenopathy, and organomegaly. If any extramedullary involvement is suspected, a positron emission tomography (PET)/CT is used for diagnosis.

The leukocyte count in ALL patients may be elevated (majority of cases), normal, or decreased. T-ALL patients typically present with a high leukocyte count and often with a large mediastinal mass or other tissue mass. Circulating blast cells are present in virtually all cases. However, the percentage may be very low in some patients.

ALL diagnosis is based on a hematopathological evaluation of bone marrow aspirate and biopsy material, with a demonstration of at least 20% of bone marrow lymphoblasts confirming a definitive ALL diagnosis. According to the guidelines of the US National Comprehensive Cancer Network (NCCN), the diagnostic workup should include a morphologic assessment of Wright/Giemsa-stained bone marrow aspirate smears and of hematoxylin-and-eosin-stained bone marrow core biopsy and clot sections, a comprehensive immunophenotyping using flow cytometry, and a baseline characterization of the leukemic clone(s) (see "**Pathology and Genetics**" section for more) to facilitate subsequent analysis of the minimal residual disease (MRD). For the latter, cytogenetic testing by fluorescence in situ hybridization (FISH) with a panel of the acute leukemia probes and reverse-transcriptase polymerase chain reaction (RT-PCR) for *BCR-ABL1* fusions (and other fusions in case of *BCR-ABL1* negativity), including determining the transcript size, are commonly used. In cases of aneuploidy, array comparative genomic hybridization (CGH) may also be useful.

Epidemiology and Risk Factors

Burden

ALL is mainly a childhood disease, with 75% of cases diagnosed in children under 6 years of age. It is also the most common form of childhood leukemia, accounting for 75%–80% of all childhood leukemia cases, while it accounts for only 20% of cases in adults. The estimated annual incidence worldwide is 1–4.8 cases per 100,000 population. In the United States, the age-adjusted incidence rate of all ALL is 1.58 per 100,000 population per year, with approximately 5970 new cases and 1440 deaths estimated in 2017. The prevalence of specific B-ALL types and of T-ALL is summarized in **Table 1**.

Etiology and Risk Factors

The etiology of both B- and T-ALL is not well known. However, some environmental exposures have been associated with an increased risk. Radiation is a well-documented leukemogenic factor in humans, with a direct relationship between cumulative radiation dose and the incidence of some leukemia types. ALL may develop following exposure to ionizing radiation as well as radiotherapy. Also chemotherapy has been associated with an increased risk of developing the disease. Moreover, some chemicals, like benzene and toluene, contribute to the risk of acute leukemias, including ALL, likely by mutating or ablating the bone marrow stem cells. Acute leukemia usually develops 1–5 years after exposure and is often preceded by bone marrow hypoplasia, dysplasia, and pancytopenia. Age over 70 years is also a known risk factor for ALL.

Moreover, several hereditary conditions are associated with an increased risk of ALL, in particular Down syndrome but also less frequent disorders: neurofibromatosis type 1, Bloom syndrome, Klinefelter syndrome, Schwachman–Diamond syndrome, Fanconi anemia, and ataxia telangiectasia.

Genome-wide association studies have identified several single nucleotide polymorphisms (SNPs) associated with an increased risk of B-ALL, including in *GATA2*, *ARID5B*, *IKZF1*, *CEBPE*, and *CDKN2A*. In true familial ALL cases, even though rare, mutations in *PAX5*, *ETV6*, and *TP53* have been described.

Pathology and Genetics

In smear preparations, B-ALL lymphoblasts present variably, from small blasts with scant cytoplasm, condensed nuclear chromatin and indistinct nucleoli to larger cells with moderate amounts of light-blue to bluish-grey cytoplasm, sometimes vacuolated, dispersed chromatin and multiple variably prominent nucleoli. Nuclei are round or convoluted. Coarse azurophilic granules are present in some lymphoblasts in about 10% of cases. In bone marrow specimens, B-ALL lymphoblasts have a relatively uniform appearance, with round or oval, indented or convoluted nuclei. Nuclei range from inconspicuous to prominent, the chromatin is finely dispersed and the number of mitotic figures varies. Lymphoblasts in B-ALLs with specific genetic abnormalities usually do not have any specific morphological or cytochemical features that would allow a more specific diagnosis. Also T-ALL lymphoblasts are morphologically indistinguishable from B-ALL lymphoblasts.

B-ALL lymphoblasts are nearly always positive for the B-cell markers CD19, cCD79a, and cCD22. None of these molecules on its own is a specific diagnostic marker but their combination or high intensity strongly supports the B-ALL diagnosis. In most cases, the expression of CD10, surface CD22, CD24, PAX5, and terminal deoxynucleotidyl transferase (TdT) is also found. The myeloid-associated markers CD13 and CD33 may be expressed and this does not exclude the B-ALL diagnosis. The PAX5 expression is

Table 1 The prevalence of different acute lymphoblastic leukemia (ALL) entities

Entity	*Epidemiology*
B-ALL with *t*(*9*;*22*)(*q34*;*q11.2*); *BCR-ABL1*	More common in adults (25% of all adult ALLs) than in children (2%–4%)
B-ALL with *t*(*v*;*11q23*); *MLL* rearranged	The most common leukemia in infants under 1 year of age; less common in older children; incidence increases with age into adulthood
B-ALL with *t*(*12*;*21*)(*p13*;*q22*); *TEL-AML1* (*ETV6-RUNX1*)	Common in children (25% of all B-ALLs in children) but not seen in infants; incidence decreases with age (this entity is uncommon in adults)
B-ALL with hyperdiploidy	Common in children (25% of all B-ALLs in children) but not seen in infants; incidence decreases with age; accounts for 7%–8% of adult B-ALL cases
B-ALL with hypodiploidy	1%–5% of cases, both adults and children (nearly-haploid ALL likely in children only)
B-ALL with *t*(*5*;*14*)(*q31*;*q32*); *IL3-IGH*	Less than 1% of all ALL cases, both adults and children
B-ALL with *t*(*1*;*19*)(*q23*;*p13.3*); *E2A-PBX1* (*TCF3-PBX1*)	About 6% of childhood B-ALL cases, uncommon in adults
B-ALL, *BCR-ABL1*-like	10%–25% of all ALL cases; incidence increases with age
B-ALL with *iAMP21*	About 2% of ALL cases in children; no data on the incidence in adults
T-ALL	About 15% of childhood ALL cases (more common in adolescents than in younger children) and 25% of adult ALL cases; more common in males than in females

B-ALL, B-lymphoblastic leukemia; *T-ALL*, T-lymphoblastic leukemia.

considered to be the most specific and sensitive marker for the B-cell lineage in tissue sections but it is also expressed in some variants of acute myeloid leukemia. The expression profile of cell surface markers within the B-cell lineage varies with the B-cell differentiation (maturation stage). The immunophenotypes characterizing B-ALLs with specific recurrent genetic aberrations are summarized in **Table 2**.

T-ALL lymphoblasts are usually positive for TdT and variably express CD1a, CD2–CD5, CD7, and CD8, with CD3 (cytoplasmic) and CD7 being positive most frequently. Only CD3, however, is specific for the T-lineage. CD4/CD8 coexpression is common but it is not specific to T-ALL. TdT expression combined with that of CD34, CD1a, and CD99 may help to establish the precursor nature of T-lymphoblasts. CD52 may be expressed in 30%–50% of adult T-ALL cases. One or both of the myeloid markers may also be expressed but, like for B-ALL, this does not exclude the T-ALL diagnosis. Moreover, many markers characteristic for premature T-cells (e.g., CD2 or CD7) may also be expressed by immature natural killer (NK) cells. Therefore, it may be very difficult to distinguish the true NK-cell ALL (which is rare) and T-ALL associated with the expression of immature T-cell markers.

Clonal rearrangements of the *immunoglobulin heavy* locus (*IGH*) gene and rearrangements of the *T-cell receptor* (*TCR*) gene are found in both B-ALL and T-ALL, albeit at different frequencies. In B-ALL, *IGH* clonal rearrangements are found in nearly all cases and *TCR* rearrangements in up to 70% of cases, whereas in T-ALL, clonal *TCR* rearrangements are seen in most cases and simultaneous *IGH* rearrangements in about 20%.

Moreover, cytogenetic abnormalities are seen in a majority of B-ALL cases, many of them defining specific entities with unique phenotypic and prognostic features (**Table 2**). In addition to those "classifying" aberrations, deletions of chromosome 6q, 9p, and 12p are frequent in B-ALL but they do not seem to impact prognosis. Some other abnormalities observed in B-ALL may emerge as having some clinical utility. For example, the rare *(17;19)(q22;p13.3)* translocation is associated with a very poor prognosis but there are too few cases to validate this alteration as a prognostic marker and classify these B-ALL cases as a separate entity.

In T-ALL, abnormal karyotype is found in 50%–70% of cases. The most common recurrent cytogenetic abnormality involves the *alpha* and *delta TCR* loci (14q11.2) as well as the *beta* (7q34) and *gamma* locus (7p14.1), with a multitude of partner genes, most common of them being the *TLX1* (also called *HOX11*; 10q24.3) and *TLX3* (*HOX11L2*; 5q35.1) transcription factors. The most prevalent deletions are those of chromosome 9p, encompassing the locus of the *CDKN2A* gene.

Alterations at a gene level are also found in ALL. In particular, a large number of recurrent genetic alterations (copy number alterations and specific intragenic mutations) are found in B-ALL. Many of them, such as those in the *PAX5* gene, are found in most of the subtypes, suggesting that they play a fundamental role in leukemogenesis. Others, like those in *RAS* and *IKFZ1*, tend to be associated with specific subtypes (**Table 2**). In T-ALL, the most frequently mutated gene is *NOTCH1* which encodes a protein critical for early T-cell development (mutated in 50% of cases). In 30% of cases, mutations in *FBXW7*, a negative regulator of *NOTCH1*, are found. According to data in the Cosmic database, the genes that are most frequently mutated in ALL are the following: *IKFZ1* (35% of cases), *ABL1* (21%), *NRAS* (10%), *TP53* (10%), *CDKN2A*, *PAX5*, and *KMT2D* (9% each). However, the data do not distinguish between B-ALL and T-ALL, which—given remarkable differences between the subtypes—is a serious limitation.

Management and Therapy

The introduction of new therapies has resulted in a dramatic improvement of cure rates, in particular in B-ALL patients. ALL has a relatively good prognosis in children (5-year survival rates up to 89%). However, the prognosis for adults remains less favorable, especially for those of older age.

Risk assessment in ALL is an essential part of treatment planning and the criteria, widely debated, have been evolving. Patient age, initial white blood cells (WBC) count, disease subtype, presence of CNS disease, and rapidity of response to induction therapy are recognized as important factors in assessing prognosis. For years, clinicians used to stratify children, adolescents, and young adults (AYA) with ALL into two groups: standard risk and high risk, based mainly on age, WBC count, and disease subtype. The Children's Oncology Group (COG) has refined risk assessment criteria, creating four groups: very high, high, standard, and low risk. The very high risk category comprises all B-ALL patients with the *t(9;22)* translocation (Ph-positive ALL) and/or presence of the BCR-ABL1 fusion protein, B-ALL patients with hypodiploidy (defined as less than 44 chromosomes), *BCR-ABL1*-like, or *iAMP21* subtype, and those with *KMT2A* alterations or failure to achieve remission with induction therapy, regardless of age and WBC count. About 4% of nonadult B-ALL patients fall into this category. Risk stratification for T-ALL has been more difficult and this entity is frequently categorized as very high risk. However, newer treatments have resulted in improved outcomes also for T-ALL patients. According to the current NCCN guidelines, the initial risk stratification of all ALL patients should be based on the presence of the *t(9;22)* chromosomal translocation and/or BCR-ABL fusion protein (Ph-positive versus Ph-negative). Further stratification may be based on patient age, WBC count, comorbidities, and the presence of minimal residual disease (MRD).

The treatment of ALL is extremely complex, with treatment regimens, selection of drugs, doses, and schedules which differ according to the age group of patients (children, AYA, or older adults), risk stratification, and ALL subtype. However, all treatment protocols follow the same basic principles, with three main treatment phases: induction, consolidation, and maintenance. CNS prophylactic and/or therapeutic treatment is also a mandatory element of ALL management.

The goal of the induction therapy, the first phase of the ALL treatment, is to reduce tumor burden by clearing as many leukemic cells as possible from the bone marrow, that is to induce remission. Most inductions regimens are based on a combination of vincristine, anthracycline (e.g., daunorubicin or doxorubicin, in particular for higher risk patients), and a corticosteroid (e.g.,

Table 2 The genetic and immunophenotypic profiles of different B-lymphoblastic leukemia (B-ALL) entities as well as their clinical significance

Entity	*Genetic profile*	*Immunophenotype*	*Prognosis and predictive factors*
B-ALL with *t*(*9*;*22*)(*q34*;*q11.2*); *BCR-ABL1*	The *t(9;22)* translocation resulting from fusion of *BCR* at 22q11.2 and *ABL1* at 9q34.1, with production of a BCR-ABL1 fusion protein (p190 BCR-ABL1 in most childhood cases and in about half of adult cases); the resulting aberrant chromosome 22 harboring the fusion *BCR-ALB1* gene locus is called Philadelphia (Ph) chromosome	Typically positive for CD10, CD19, and TdT, and negative for CD117 (KIT); CD25 expression highly associated with this entity, especially in adults; myeloid-associated markers CD13 and CD33 frequently expressed; T-cell precursor phenotype in rare cases	The worst prognosis of all types, particularly in adults; patients potentially responsive to tyrosine kinase inhibitors (e.g. imatinib), so prognosis may change with the introduction of targeted therapies
B-ALL with *t*(*v*;*11q23*); *MLL* rearranged	Fusions of the *KMT2A* (*MLL*) gene with multiple genes (over 100 identified fusion partners), the most common partner being *AFF1* (*AFF4*; 4q21), followed by *MLLT1* (19p13.3), and *MLLT3* (9p21.3); none of these fusions is specific to this entity; frequent overexpression of *FTL3*; very few additional mutations in infants, usually in the RAS pathway	Typically positive for CD19 and CD15, and negative for CD10 and CD24; the NG2 homolog usually expressed and relatively specific	*KMT2A-FFA1* translocation associated with a very poor prognosis, particularly in infants under the age of 6 months
B-ALL with *t*(*12*;*21*)(*p13*;*q22*); *TEL-AML1* (*ETV6-RUNX1*)	The *ETV6-RUNX1* translocation with production of the fusion protein (a putative negative regulator of the transcription factor RUNX1), likely an early event in the leukemogenesis	Positive for CD10 and CD19, and usually also for CD34; characteristic nearly complete absence of CD9, CD20, and CD66c (relatively specific); myeloid-associated markers, especially CD13, frequently expressed	Very favorable prognosis (cure in $>$90% of childhood cases); relapses later than for other B-ALL types
B-ALL with hyperdiploidy	Increased chromosome number (usually without structural aberrations), most commonly extra copies of chromosome 21, X, 14, and 4; the least common: chromosome 2 and 3	Positive for CD10 and CD19, and other typical B-ALL markers; most cases positive for CD34 and negative for CD45	Very favorable prognosis in children (cure in $>$90% of cases); not enough data for adults; trisomies may have more significance as prognostic factors than the actual number of chromosomes; simultaneous trisomy of chromosome 4 and 10 being associated with the best prognosis
B-ALL with hypodiploidy	Loss of one or more chromosomes and nonspecific structural aberrations in the other chromosomes (rare in near-haploid cases)	Typically positive for CD10 and CD19 but no other distinct features	Poor prognosis (likely even without MRD); the worst prognosis for nearly-haploid cases suggested by some studies
B-ALL with *t*(*5*;*14*)(*q31*;*q32*); *IL3-IGH*	A functional rearrangement between *IL3* (chromosome 5) and *IDH* (chromosome 14) leading to constitutional overexpression of IL3 and eosinophilia	Typically positive for CD10 and CD19; even small numbers of blasts expressing these two markers in a patient with eosinophilia strongly suggests this diagnosis	No data

(Continued)

Table 2 The genetic and immunophenotypic profiles of different B-lymphoblastic leukemia (B-ALL) entities as well as their clinical significance—cont'd

Entity	*Genetic profile*	*Immunophenotype*	*Prognosis and predictive factors*
B-ALL with *t*(*1*;*19*)(*q23*;*p13.3*); *E2A-PBX1* (*TCF3-PBX1*)	*TCF3-PBX* translocation with production of the fusion protein (oncogenic); the fusion gene is located on chromosome 19, may be associated with loss of chromosome 1 (unbalanced translocation); an alternative *TCF3* translocation involving the *HLF* gene (chromosome 17) in rare cases	Typically positive for CD10, CD19, and cytoplasmic mu heavy chain (pre-B phenotype); typical strong expression of CD9 with lack or very limited expression of CD34 (with these features, this diagnosis may be suspected even if cytoplasmic mu is undetermined)	The *TCF3* translocation associated with very poor prognosis; possibly an increased risk of CNS relapses in patients with this entity
B-ALL, *BCR-ABL1*-like	Various chromosomal rearrangements of multiple genes with various partners; *CRLF2* rearrangements in approximately half of the cases; the tyrosine kinase-type translocations often involve *ABL1* with partners other than *BCR*, as well as other kinases; additionally frequent deletions and point mutations in other genes, particularly *IKZF1* (not specific to this entity) and *CDKNA2A*/*B*; mutations in *JAK1* or *JAK2* found in about a half of cases with *CRLF2* rearrangements	Typically positive for CD10 and CD19; very high levels of the translocated *CRLF2* gene product (detectable by flow cytometry) in a subset of cases with these translocations	Overall poor prognosis; this may be due to an increased risk of MRD (controversial); children resistant to induction therapy usually have a translocation involving *PDGFRB* (most often with *EBF1*) and are very responsive to ABL-class tyrosine kinase inhibitors (e.g. imatinib or dasatinib); patients with *JAK* mutations or translocations may be candidates for treatment with JAK inhibitors (to be tested)
B-ALL with *iAMP21*	Intrachromosomal amplification of chromosome 21 (*iAMP21*) detectable with FISH probes recognizing the *ETV6-RUNX1* translocation; multiple other chromosomal abnormalities (most commonly involving chromosome X and 7) in 80% of cases; deletions of *RB1* and *ETV6 as well as CRLF2* rearrangements more frequent than in other ALLs	Unknown	Unclear

CNS, central nervous system; *FISH*, fluorescence in situ hybridization; *MRD*, minimal residual disease; *t*, translocation; *TdT*, terminal deoxynucleotidyl transferase.

dexamethasone or prednisone) with or without L-asparaginase and/or cyclophosphamide, given over the course of 4–6 weeks. An alternative is the so-called Hyper-CVAD regimen which alternates cycles of hyperfractionated cyclophosphamide, vincristine, doxorubicin (adriamycin), and dexamethasone with those of high-dose methotrexate and cytarabine.

Most children achieve complete remission (CR) within 4 weeks of the induction therapy. If CR is not achieved within 6 weeks, alternative treatments are started. In adults, a regimen based on a combination vincristine/prednisone/anthracycline is associated with 75% CR rates. Dexamethasone has been shown to give better outcomes compared to prednisone. It has a better penetration into the CNS, which explains its efficacy in preventing CNS relapse. However, it is associated with severe toxicities and its advantage for the overall survival has yet to be demonstrated.

CNS prophylaxis after induction chemotherapy aims at preventing CNS disease or early CNS relapse. The CNS is the initial site of ALL relapse in more than half of children unless prophylaxis is given and it is also a frequent site of relapse in adults. The goal of CNS prophylaxis and/or treatment is to clear leukemic cells within sites that cannot be easily accessed with systemic chemotherapy because of the blood–brain barrier. Different regimens of CNS-directed therapy exist. Many authorities recommend intrathecal methotrexate, often combined with cranial irradiation. Triple intrathecal therapy (methotrexate, hydrocortisone, cytarabine), with or without high-dose systemic chemotherapy (e.g., methotrexate or cytarabine), may substitute for cranial irradiation which can lead to late intellectual disabilities when used in children. For adults, prophylactic intrathecal chemotherapy alone is considered sufficient. CNS prophylaxis is typically administered to all patients throughout the entire course of ALL therapy.

The consolidation treatment aims at eliminating any leukemic cells remaining after induction therapy, further eradicating residual disease. The postremission induction phase of treatment may also be referred to as intensification therapy. It typically involves an intensive multidrug regimen. A variety of regimens are used in different centers and trials, although frequently containing similar drug combinations to those that were used during the induction treatment. High-dose methotrexate, cytarabine, 6-mercaptopurine (6-MP), cyclophosphamide, vincristine, corticosteroids, and L-asparaginase are frequently incorporated into consolidation/intensification regimens. For treatment of younger children, adjusted doses and frequencies are used.

Maintenance therapy aims to prevent disease relapse after postremission induction and consolidation therapy. Most regimens are based on daily 6-MP and weekly methotrexate, typically with a periodic addition of vincristine and corticosteroids, for 2–3 years. Of note, the efficacy of the maintenance therapy is determined by the metabolism of 6-MP. In particular, the efficacy of the active metabolite production is modified by polymorphisms in thiopurine methyltransferase (TMPT). Genotyping TMPT allows to anticipate decreased bioavailability or increased toxicity of 6-MP and adjust the dose. The optimal duration of the maintenance therapy is not clearly defined and the therapy is frequently stopped due to associated toxic effects.

The emergence of targeted drugs offers hope for more effective treatments of ALL. In particular, adding tyrosine kinase inhibitors (TKIs) to the induction treatment regimens of Ph-positive ALL patients has significantly improved their prognosis. Imatinib (also called imatinib mesylate; brand name: Gleevec®, Novartis Pharmaceuticals), an inhibitor of the BCR-ABL tyrosine kinase, has been approved by the US Food and Drug Administration (FDA) for treatment of relapsed or refractory Ph-positive ALL adult patients and of all untreated Ph-positive pediatric patients. Dasatanib, a second-generation TKI which inhibits both the BCR-ABL tyrosine kinase and kinases of the Src family, seems to be even more effective than imatinib due to its capacity to inhibit several TK-related signaling pathways. It has also been shown to maintain activity against cells harboring several (but not all) *ABL* domain mutations which confer resistance to imatinib. Furthermore, it has a better penetration of the blood–brain barrier and so it may prove useful in the CNS prophylaxis. Dasatinib (under the brand name of SPRYCEL, Bristol-Myers Squibb Co) has been approved by FDA for treatment of pediatric patients with Ph-positive chronic myeloid leukemia and it is being extensively trialed (phase II and III) for treatment of ALL. Single-agent TKI therapy in Ph-positive ALL patients has been shown to yield improved response to induction therapy as compared to chemotherapy. However, the remission-free periods are relatively short for both imatinib and dasatinib alone. TKIs have been shown to provide most benefit, with significantly improved disease-free and overall survival rates, when combined with corticosteroids. Patients with CD20-positive B-ALL benefit from the addition of rituximab, an anti-CD20 monoclonal antibody, to standard ALL chemotherapy regimens. All these agents may be incorporated as part of the induction, consolidation, and/or maintenance therapy in the initial treatment of ALL, or in regimens for treating relapsed and/or refractory disease.

Allogeneic hematopoietic stem cell transplantation (HSCT) used to be considered the standard of care in AYA and adults with Ph-positive ALL, and for relapsing ALL patients. However, with the advent of BCR-ABL-targeted TKIs, its role has been questioned. Still, it may offer some benefit to specific groups of Ph-positive patients who relapse after initial remission.

Treatment of relapsed ALL remains a challenge. Combining standard chemotherapy regimens with emerging targeted drugs offers much hope to this effect. In particular, mitoxantrone (Novantrone®, Immunex Corporation; an anthracycline derivative), cytarabine, fludarabine/cytarabine/granulocyte colony-stimulating factor/idarubicin (FLAG-IDA), methotrexate/asparaginase (CAPIZZI), and blinatumomab (a monoclonal antibody against CD19 and CD3) have been shown to provide benefit to patients with CD19-positive ALL, while inotuzumab ozagamicin (FDA-approved since August 2017) has been shown to be useful in patients with CD22-postiive disease. Several first-, second-, and third-generation TKIs are being currently tested in clinical trials and their approval for treatment of relapsed/refractory ALL, and in particular for *BCR-ABL1*-like ALL, is likely just a matter of time. However, as of now, bone marrow transplant remains the only cure for relapsed/refractory ALL.

For patients who are not eligible for transplant, generation of chimeric antigen receptor T-cells (CAR-T) targeting specific molecules on the surface of cancer cells may be a solution. In August 2017, FDA approved the anti-CD10 CAR-T therapeutic agent tisagencleucel (KYMRIAH®, Novartis Pharmaceuticals) for treatment of refractory or relapsed B-ALL patients (at least second relapse) below 25 years of age. However, CAR-T therapy is associated with severe potentially fatal toxicities (like cytokine release syndrome, B-cell aplasia, and cerebral edema) and its use should be preceded by a specific risk evaluation. FDA also requires a special

certification for centers administering this therapy. For relapsed or refractory T-ALL, nelarabine, a purine nucleoside analog, is currently an approved treatment.

Assessing the response to treatment also remains a challenging issue. Complete remission (CR) is defined as the absence of circulating blasts and extramedullary disease. However, patients who achieved CR according to morphological assessment can potentially harbor leukemic cells in the bone marrow, giving rise to a low-level disease which is not detectable by conventional cytomorphological techniques (minimal residual disease, MRD). Many trials have confirmed that MRD is the strongest prognostic factor in both children and adults. Therefore, applying reliable sensitive methods to detect MRD is of utmost importance for further treatment planning and monitoring of treatment response at all stages following the induction therapy. MRD can be detected by multicolor flow cytometry or sensitive molecular techniques targeting characteristic genetic or chromosomal aberrations, like RT-PCR or next-generation sequencing.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.

International Agency for Research on Cancer, 2017. In: Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. (Eds.), WHO classification of tumours of hematopoietic and lymphoid tissues, Revised 4th edn. International Agency for Research on Cancer, Lyon.

Kotrova, M., Brüggemann, M., 2017. Minimal residual disease in adult ALL: Technical aspects and implications for correct clinical interpretation. Blood Advances 1 (25), 2456–2466.

National Comprehensive Cancer Network (NCCN), 2017. NCCN clinical practice guidelines in oncology: Acute lymphoblastic leukemia, Version 5.2017. October 27, 2017. https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Relevant Website

https://clinicaltrials.gov/—Clinical trial information at the US National Library of Medicine, National Health Institutes.

Acute Myelogeneous Leukemia: Diagnosis and Treatment

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Abbreviations

AML Acute myeloid (myelogeneous) leukemia
APL Acute promyelocytic leukemia
APLDS APL differentiation syndrome
ATO Arsenic trioxide
ATRA All-*trans*-retinoic acid
CD Cluster of differentiation
CNS Central nervous system
CR Complete remission
CT Computed tomography
DIC Disseminated intravascular coagulation
FAB French–American–British [histopathologic classification of acute leukemia]
FDA US Food and Drug Administration
FISH Fluorescence in situ hybridization
GO Gemtuzumab ozogamicin
HiDAC High-dose cytarabine
HSCT Hematopoietic stem cell transplantation
ICD-O International Classification of Diseases for Oncology
IDH Isocitrate dehydrogenase
ITD Internal tandem duplication
MDS Myelodysplastic syndrome
MDS/MPN Myelodysplastic/myeloproliferative neoplasm
MRD Minimal residual disease
MRI Magnetic resonance imaging
NCCN US National Comprehensive Cancer Network
PET Positron emission tomography
t-AML Therapy-related AML
WBC White blood cells
WHO World Health Organization

Definition and Classification

Acute myelogenous leukemia (AML), also called acute myeloid leukemia and acute nonlymphocytic leukemia, is a heterogeneous hematological malignancy characterized by clonal expansion of myeloid blasts in the peripheral blood, bone marrow, and/or other tissues. The term encompasses a number of entities whose basic characteristics are summarized in Table 1.

Of note, the current classification of acute leukemia is the 2016 classification by the World Health Organization (WHO). It has replaced the French–American–British (FAB) histopathologic classification of acute leukemia, originally proposed in 1976, and it takes into account not only morphological and cytochemical characteristics of the disease but also its cytogenetic and clinical diversity. However, the "historical" names of some leukemia types referring to FAB categories have persisted and they are frequently used in clinical practice and related publications. These names are listed among synonyms in Table 1. Moreover, the WHO classification has lowered the diagnostic threshold of blasts which defines AML to 20% (compared to 30% in the FAB classification) and it also allows the AML diagnosis regardless of the blast count in patients with abnormal hematopoiesis and specific cytogenetic abnormalities.

Table 1 Classification and defining characteristics of acute myeloid leukemia (AML) entities

Entity name [synonyms]	*ICD-O code*	*Defining characteristics other than cytogenetic/genetic abnormalities reflected in the name of the entity*
AML with balanced translocations/inversions		
AML with *t(8;21)(q22;q22); RUNX1-RUNX1T1* [FAB M2, *t(8;21)(q22;q22);* FAB M2, *AML1(CBF-alpha)/ETO*]	9869/3	Generally shows maturation in the neutrophil lineage; Bone marrow and peripheral blood show large myeloblasts with abundant basophilic cytoplasm, often containing azurophilic granules; Classified as AML regardless of the blast count
AML with abnormal marrow eosinophils [AML, *t(16;16)(p 13;q 11)*; AML, *CBF-beta/MYH11;* acute myelomonocytic leukemia with abnormal eosinophils; FAB M4Eo; AML, *inv(16)(p13;q22)*]	9871/3	Usually shows monocytic and granulocytic differentiation and characteristically abnormal eosinophil component in the bone marrow; Classified as AML regardless of the blast count
Acute promyelocytic leukemia (APL) with *PML-RAR*-alpha [acute promyelocytic leukemia, *t(15;17)(q22;q11-12)*; acute promyelocytic leukemia, NOS; FAB M3; AML, *PML/RAR*-alpha; AML with *t(15:17)(g22;q11-12)*]	9866/3	Predomination of abnormal promyelocytes; Two types exist: hypergranular and microgranular (hypogranular); Classified as AML regardless of the blast count
AML with *t(9;11) (p22;q23); MLLT3-MLL*	9897/3	Usually associated with monocytic features; It is controversial whether cases with blast count $<20\%$ should be classified as AML
AML with *t(6;9)(p23;q34); DEK-NUP214*	9865/3	At least 20% of peripheral blood or bone marrow blasts with or without monocytic features; often associated with basophilia and multilineage dysplasia
AML with *inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1* [AML with *inv(3)(q21q26.2) or t(3;3)(q21;q26.2); GATA2-MECOM*]	9869/3	At least 20% of peripheral blood or bone marrow blasts; often associated with normal or elevated platelet counts and showing increased dysplastic megakaryocytes with unilobed or bilobed nuclei and multilineage dysplasia in the bone marrow
AML (megakaryoblastic) with *t(1;22)(p13;q13); RBM15-MKL1*	9911/3	Usually shows maturation in the megakaryocyte lineage; It is controversial whether cases with blast count $<20\%$ should be classified as AML
AML with *BCR-ABL1*	9912/3 (provisional entity)	De novo AML in patients with no evidence of chronic myeloid leukemia that do not meet the criteria for other AML types
AML with gene mutations		
AML with mutated *NPM1*	9877/3	Frequently monocytic or monomyelocytic features
AML with biallelic mutations of *CEBPA*	9878/3	Usually meets the criteria for AML with maturation or for AML without maturation; myelomonocytic or monoblastic features in some cases
AML with mutated *RUNX1*	9879/3 (provisional entity)	None; cases that fulfil the criteria for any other AML type, should not be classified in this category
Other AMLs		
AML with myelodysplasia-related changes [AML with multilineage dysplasia; AML with prior myelodysplastic syndrome; AML without prior myelodysplastic syndrome]	9895/3	At least 20% peripheral blood or bone marrow blasts with morphological features of myelodysplasia, or a prior history of a myelodysplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasm, or MDS-related cytogenetic abnormalities, and absence of the specific genetic abnormalities of AML with recurrent genetic abnormalities; patients should not have a history of prior cytotoxic or radiation therapy for an unrelated disease; cases assigned to this category for one or more of the following reasons: AML arising from previous MDS or myelodysplastic/myeloproliferative neoplasm, AML with an MDS-related cytogenetic abnormality, or AML with multilineage dysplasia

Therapy-related AML (t-AML), NOS [therapy-related AML, alkylating agent related; therapy-related AML, epipodophyllotoxin-related]	9920/3	t-AML is part of a category grouping all therapy-related myeloid neoplasms (under one ICD-O code) and is distinguished from other subentities in this category by the number of blasts; cases which progressed to AML from myeloproliferative neoplasms or MDS are excluded from this category (categorized as "secondary AML")
AML, NOS [acute nonlymphocytic leukemia; acute granulocytic leukemia; acute myelogenous leukemia; acute myelocytic leukemia]	9861/3	AML which does not fulfil the criteria for any of the entities listed above; Includes several subtypes, classified/coded separately because they require different diagnostic criteria (below)
Acute myeloblastic leukemia [AML with minimal differentiation; FAB M0]	9872/3	AML with no morphological or cytochemical evidence of myeloid differentiation; the myeloid nature of the blasts is shown by immunophenotyping
AML without maturation [FAB M1]	9873/3	High percentage of bone marrow blasts without significant evidence of maturation to more mature neutrophils; nucleated bone marrow cells comprise $<10\%$ of maturing cells of the granulocytic lineage; the myeloid nature of the blasts is demonstrated by MPO or Sudan black B (SBB; 3% or more of blasts) positivity and/or the presence of Auer rods
AML with maturation [FAB M2, NOS]	9874/3	At least 20% blasts in the bone marrow or peripheral blood and evidence of maturation ($\geq 10\%$ maturing cells of the granulocytic lineage); cells of monocyte lineage comprise $<20\%$ of bone marrow cells
Acute myelomonocytic leukemia [FAB M4]	9867/3	Proliferation of both neutrophil and monocyte precursors; peripheral blood or bone marrow has $\geq 20\%$ blasts (including promonocytes); neutrophils and their precursors, and monocytes and their precursors, each comprise at least 20% of bone marrow cells; the threshold of 20% distinguishes this entity from cases of AML with or without maturation in which some monocytes may be present
Acute monocytic and monoblastic leukemia [FAB M5]	9891/3	At least 20% of blasts (including promonocytes) in peripheral blood or bone marrow, and 80% or more of the leukaemic cells being of monocytic lineage, including monoblasts, promonocytes and monocytes; a minor neutrophil component ($<20\%$) may be present; Acute monoblastic leukemia and acute monocytic leukemia are distinguished by the relative proportions of monoblasts and promonocytes (most of the monocytic cells are monoblasts (typically $>80\%$) in the first case, and promonocytes or monocytes in the latter)
Pure erythroid leukemia [acute erythroid leukemia; erythroleukemia; FAB M6; Di Guglielmo disease]	9840/3	Neoplastic proliferation of immature cells (undifferentiated or proerythroblastic in appearance) committed exclusively to the erythroid lineage ($\geq 80\%$ of bone marrow cells) with no evidence of a significant myeloblastic component
Acute megakaryoblastic leukemia [megakaryocytic leukemia; FAB M7]	9910/3	At least 20% of blasts of which at least 50% are of megakaryocyte lineage; this category excludes cases of AML with myelodysplasia-related changes, AML with *t(1;22)(p13;q13)*, *inv(3)(q21q26.2)*, *t(3;3)* (*q21;q26.2*), and Down syndrome-related cases
Acute basophilic leukemia	9870/3	AML with the primary differentiation to basophils
Acute panmyelosis with myelofibrosis (APMF) [malignant myelosclerosis; acute myelofibrosis; acute myelosclerosis, NOS; acute panmyelosis, NOS]	9931/3	Acute panmyeloid proliferation with increased blasts (at least 20%) and accompanying fibrosis of the bone marrow that does not meet the criteria for any other AML
AML associated with Down Syndrome	9898/3	Most frequently acute megakaryoblastic leukemia, often following a prolonged MDS-like phase

inv, inversion; *MDS*, myeslodysplastic syndrome; *t*, translocation.

Table 2 Prevalence of different acute myleoid leukemia (AML) entities

Entity	*Prevalence*
AML with balanced translocations/inversions	
AML with *t(8;21)(q22;q22); RUNX1-RUNX1T1*	1%–5% of all AML cases, mostly in younger patients
AML with abnormal marrow eosinophils	5%–8% of younger AML cases; less among older adults
Acute promyelocytic leukaemia (APL) with *PML-RAR*-alpha	5%–8% of younger AML cases; less frequent among elderly patients; most patients are middle-aged adults
AML with *t(9;11) (p22;q23); MLLT3-MLL*	More common in children (9%–12% of pediatric cases compared to 2% of adult cases)
AML with *t(6;9)(p23;q34); DEK-NUP214*	0.7%–1.8% of AML cases, both childhood (median age: 13 years) and adult
AML with *inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1*	1%–2% of AML cases, occurs mostly in adults
AML (megakaryoblastic) with *t(1;22)(p13;q13); RBM15-MKL1*	Less than 1% of AML cases; most common in infants, predominantly female
AML with *BCR-ABL1*	Less than 1% of all AML cases and less than 1% of all *BCR-ABL1*-positive acute and chronic leukemias; mostly in adults, possibly with a male predominance
AML with gene mutations	
AML with mutated *NPM1*	2%–8% of childhood AML and 27%–35% of adult AML cases (45%–64% of cases with a normal karyotype), with a female predominance
AML with biallelic mutations of *CEBPA*	4%–9% of childhood AML cases, probably less in adults
AML with mutated *RUNX1*	4%–16% of all AML cases; more frequent in Fanconi anemia and congenital neutropenia patients
Other AMLs	
AML with myelodysplasia-related changes	24%–36% of all AML cases; occurs mainly in elderly patients, rare in children
Therapy-related AML (t-AML), NOS	10%–20% of all AML, MDS, and MDS/MPN cases taken together; 80%–95% of cases develop t-AML following previous treatment of a malignancy, either solid (most commonly breast cancer), or hematological (most commonly non-Hodgkin lymphoma); the risk associated with alkylating agents or radiotherapy increases with age
Acute myeloblastic leukemia	Less than 5% of all AML cases; occurs mostly in infants and older adults
AML without maturation	5%–10% of AML cases; most patients are adults (mean age: 46 years)
AML with maturation	10% of AML cases; occurs in all age groups, with 25% of patients under the age of 25 and 40% over 60
Acute myelomonocytic leukemia	5%–10% of AML cases; more common in older individuals (median age: 50 years)
Acute monocytic and monoblastic leukemia	Less than 5% of AML cases; acute monocytic leukemia is more common in adults, with the male-to-female ratio of 1.8:1, whereas acute monoblastic leukemia is more common in young individuals
Pure erythroid leukemia	Extremely rare; occurs in all age groups
Acute megakaryoblastic leukemia	Less than 5% of AML cases, both childhood and adult
Acute basophilic leukaemia	Very rare
Acute panmyelosis with myelofibrosis (APMF)	Rare; occurs mostly in adults
AML associated with Down Syndrome	20%–30% of pediatric AML cases; about 1%–2% of children with Down syndrome develop AML, mostly under 5 years of age

MDS, myelodysplastic syndrome; *MDS/MPN*, myelodysplastic/myeloproliferative neoplasm.

Epidemiology and Risk Factors

Burden

AML is the most common adult leukemia (90% of all AML cases) and the first cause of leukemia-associated death. An estimated 19,520 new cases will be diagnosed and 10,670 people will die of the disease in the United States in 2018. As over a half of the cases are diagnosed in older adults (mean age at diagnosis in the United States: 67 years; 54% of cases diagnosed in individuals over 65 years of age), the incidence of AML seems to be rising with aging of the population. However, some AML subtypes are more common in children. The prevalence of particular AML subtypes is summarized in Table 2.

Etiology and Risk Factors

The environmental factors that are well known to increase the risk of developing AML include prolonged exposure to petrochemicals, solvents (like benzene or toluene), pesticides, and ionizing radiation. The chemicals probably contribute to the risk of AML by mutating or ablating the bone marrow stem cells. The disease usually develops 1–5 years after exposure and is often preceded by bone marrow hypoplasia, dysplasia, and pancytopenia.

Another risk factor for developing AML is radiation which is a well-documented leukemogenic factor in humans, with a direct relationship between cumulative radiation dose and the incidence of some leukemia types. AML may develop following exposure to ionizing radiation as well as radiotherapy.

Also chemotherapy used for treatment of certain primary tumors (including breast and gynecological cancers, and lymphomas) has been associated with an increased risk of developing AML. In particular, alkylating agents (e.g. cyclophosphamide) and topoisomerase II inhibitors (e.g. doxorubicin) have been clearly shown to be leukemogenic agents. Treatment with antimetabolites, such as the purine analog fludarabine, has also been associated with therapy-related AML in patients with lymphoproliferative disorders, especially when administered in combination with alkylating agents.

Finally, several hereditary conditions are also associated with an increased risk of MDS and AML. These include Fanconi anemia, congenital dyskeratosis, Diamond–Blackfan anemia, Shwachman–Diamond syndrome, and the Down syndrome (individuals with the Down syndrome usually develop acute megakaryoblastic leukemia). Moreover, recent genomic analyses of families with multiple cases of MDS or AML have identified several genes which confer inherited risk for MDS and/or AML as the primary malignancy, including *RUNX1*, *ANKRD26*, *DDX41*, *ETV6*, *GATA2*, and *SRP72*.

Presentation and Diagnosis

AML patients present with signs and symptoms resulting from bone marrow failure or organ infiltration with leukemic cells, or both, many of them nonspecific. The time course is variable. In some cases, in particular in younger individuals, acute symptoms develop within a few days to up to 2 weeks, whereas others may experience symptoms that last for months. A longer course may suggest an antecedent hematological disorder, such as MDS.

Fatigue and weakness are among the most common nonspecific symptoms and are related to anemia, neutropenia, and thrombocytopenia resulting from bone marrow failure. Other symptoms of anemia include dyspnea upon exercise, dizziness, and—in patients with coronary artery disease—anginal chest pain. Myocardial infarction may actually be the first presenting symptom in older AML patients. Physical signs of anemia, including pallor and a cardiac flow murmur, are frequent in all AML patients. Many patients have decreased neutrophil levels (neutropenia) despite an increased total white blood cell (WBC) count. Patients generally present with fever which may occur without an apparent infection. However, a history of upper respiratory infection symptoms that have not improved despite empiric treatment with oral antibiotics is common. Thrombocytopenia and coagulopathy resulting from disseminated intravascular coagulation (DIC) are also frequent. This manifests as easy bruising and/or bleeding, including bleeding gums, petechiae (particularly on the lower extremities) and multiple ecchymoses, and may also lead to potentially life-threatening bleeding in the lungs, gastrointestinal tract, and central nervous system.

AML manifestations may as well result from organ infiltration by leukemic cells, the most common infiltration sites being the spleen, liver, gums, and skin. A sensation of abdominal fullness and/or discomfort, and early satiety may appear due to hepatomegaly and/or splenomegaly. Lymphadenopathy on physical examination is less common. Gingivitis and swollen gums due to gum infiltration and also neutropenia often lead AML patients to present at their dentist's first. Organ infiltration occurs most commonly in patients with the monocytic subtypes of AML. Skin rashes due to infiltration of the skin with leukemic cells (leukemia cutis) may occasionally appear.

Patients with markedly elevated WBC counts can present with symptoms of leukostasis, such as respiratory distress and altered mental status. Leukostasis is a medical emergency that calls for immediate intervention. Patients with a high leukemic cell burden may present with bone pain caused by increased pressure in the bone marrow.

Headaches, vomiting, irritability, cranial nerve palsies, seizures, papilledema, and blurred vision are manifestations of central nervous system (CNS) involvement and/or leukemic meningitis. However, initial CNS involvement, like other extramedullary presentations, is uncommon in most AML subtypes.

Rapidly distinguishing AML from other, less urgent, hematological conditions is a major challenge. The initial workup includes complete blood count (CBC) with peripheral blood smear and a differential, blood chemistry profile, liver and kidney function tests, coagulation analysis (including measurement of prothrombin time, partial thromboplastin time, and fibrinogen), and a tumor lysis syndrome panel (including measurement of serum lactate dehydrogenase (LDH), potassium, serum uric acid, calcium, and phosphorus). The leukocyte count in AML patients may be elevated (majority of cases), normal, or decreased. Circulating blasts are present in nearly all cases, however their proportion may be very low in some patients.

The AML diagnosis is made based on the presence of at least 20% of leukemic blasts in the bone marrow or peripheral blood (with the exception of a few subentities; see **Table 1** for definitions). Myeloblasts, monoblasts, and megakaryoblasts are included in the blast count. In AML with monocytic or myelomonocytic differentiation, monoblasts and promonocytes, but not abnormal monocytes, are counted as blast equivalents. The accurate AML diagnosis requires multidisciplinary studies using immunochemistry and/or cytochemistry as well as molecular genetics. Flow cytometry immunophenotyping of bone marrow or peripheral blood samples can be used to help distinguish AML from acute lymphocytic leukemia (ALL) and further classify the AML subtype. The most commonly used diagnostic AML immunomarkers include precursor markers of the myeloid lineage (CD34, CD117 (c-KIT), CD33, CD13, and HLA-DR), granulocytic markers (CD65 and cytoplasmic MPO), monocytic markers (CD14, CD36, and CD64), megakaryocytic markers (CD41 and CD61), and erythroid markers (glycophorin A and CD36).

Cytogenetic analysis of the bone marrow (karyotype), with or without fluorescence in situ hybridization (FISH), is necessary for risk stratification and to guide therapy. A comprehensive analysis of several molecular markers for risk assessment and prognostication, and for confirming diagnosis of an AML with genetic abnormalities in some cases, is also an important element of the initial workup (see also the section "Genetics and Prognostic Biomarkers"). Circulating blasts form peripheral blood can be used to detect molecular abnormalities in patients with blast counts over 1000/mcL.

If an extramedullary involvement is suspected, positron emission tomography with computed tomography (PET/CT) is recommended. Patients presenting with signs or symptoms suggesting the central nervous system (CNS) involvement should be evaluated using proper imaging techniques, like CT or magnetic resonance imaging (MRI), followed by a lumbar puncture if no CNS mass or lesion is detected by imaging. However, CNS involvement is uncommon in AML patients.

Genetics and Prognostic Biomarkers

Like most sporadic human malignancies, AML is a complex disease, characterized by multiple somatically acquired driver mutations, coexisting competing clones, and molecular evolution over time. However, AML genomes seem to have fewer mutations than those of many other adult cancers. A comprehensive genomic analysis of 200 AML samples by the Cancer Genome Atlas (TCGA) Research Network has concluded that adult AML genomes contain a median of only one somatic copy-number variant and an average of less than one gene fusion event (generally caused by translocations). The study identified 23 significantly mutated genes, including genes that had been well established as being relevant to AML pathogenesis (e.g., *DNMT3A*, *FLT3*, *NPM1*, *IDH1*, *IDH2*, and *CEBPA*), along with genes that have only recently been implicated in AML pathogenesis, such as *U2AF1*, *EZH2*, *SMC1A*, and *SMC3*. *FLT3* mutations were identified in 28% of samples and an additional 31% were found to have mutations in genes encoding other kinases, phosphatases, or *RAS* family proteins. However, most of these genes were mutated in only one to three samples (with the exception of *KIT*, *KRAS*, *NRAS*, and *PTPN11*). In total, 59% of samples had a mutation in a gene encoding a signaling protein. A recent analysis of mutational patterns in 1540 AML samples has segregated AML cases into nonoverlapping classes, each with a distinct clinical phenotype and outcome. Beyond known disease classes, three additional heterogeneous classes emerged: AML with mutations in chromatin and RNA-splicing regulators, AML with *TP53* mutations and/or chromosomal aneuploidies, and, provisionally, AML with *IDH2* mutations. Recent studies in large population-based cohorts have identified recurrent mutations in epigenetic regulators (*DNMT3A*, *ASXL1*, *TET2*) and—less frequently—in splicing factor genes (*SF3B1*, *SRSF2*), associated with clonal hematopoietic expansion in elderly seemingly healthy subjects. The most common chromosomal and genetic alterations found in AML are summarized in Fig. 1.

Genetic abnormalities have been shown to be powerful prognostic factors in AML. However, the prognostic value of most of them is context-dependent, which calls for comprehensive analyses of genetic profiles in AML patients. Current guidelines, in

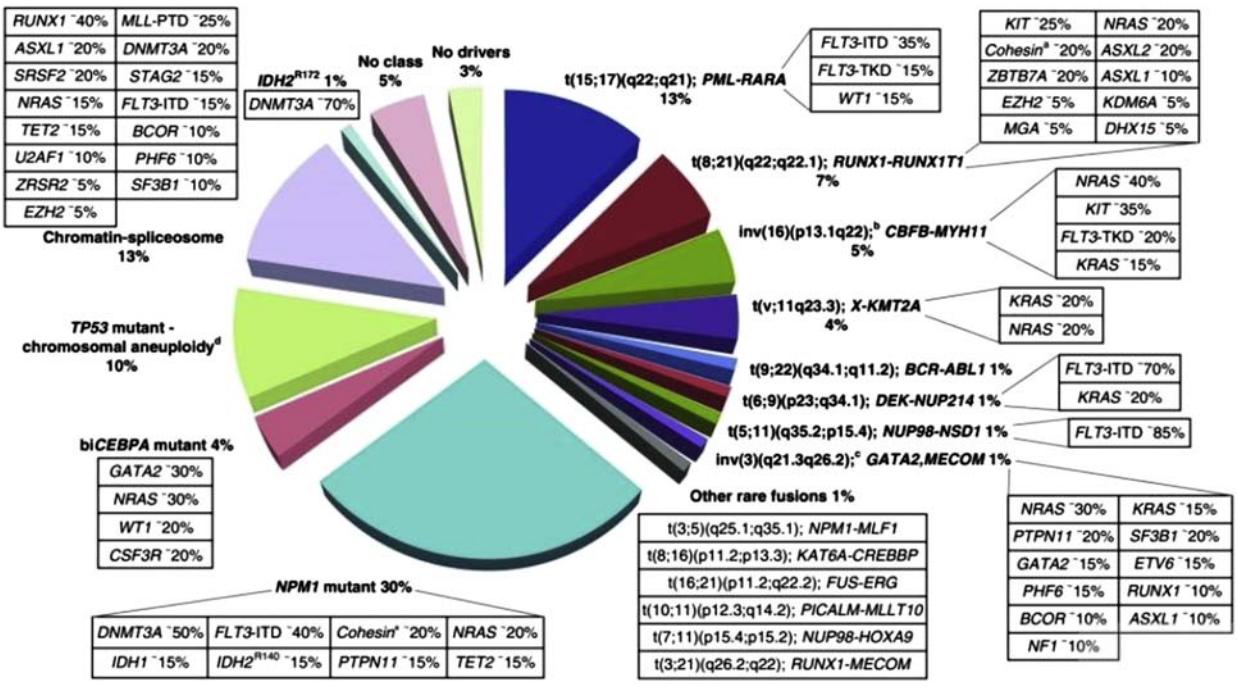

Fig. 1 Molecular classes of AML and concurrent gene mutations in adult patients up to the age of ~65 years. For each AML class denoted in the pie chart, frequent cooccurring mutations are shown in the respective boxes. Data on the frequency of genetic lesions are compiled from the databases of the British Medical Research Council (MRC), the German–Austrian AML Study Group (AML SG), and from a few selected studies; a: indicates cohesin genes including *RAD21* (~10%), *SMC1A* (~5%), and *SMC3* (~5%); b: *inv(16)(p13.1q22)* or *t(16;16)(p13.1; q22)*; *CBFB-MYH11*; c: *inv(3)(q21.3q26.2)* or *t(3;3)(q21.3;q26.2)*; *GATA2*, *MECOM*(*EV11*); and d: *TP53* mutations (found in ~45% of this class), and complex karyotypes (~70%). The structure of the pie chart is generated by Adam Ivey (King's college London, London, United Kingdom). Reprinted with permission from Döhner, H., et al. (2017). Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* **129**(4), 424–447.

Table 3 Prognostic factors in patients with acute myeloid leukemia (AML)

Prognostic biomarkers	
Favorable prognosis	*Adverse prognosis*
Cytogenetic and genetic factors	
t(8;21)(q22;q22.1); RUNX1-RUNX1T1	*t(6;9)(p23;q34.1); DEK- NUP214*
inv(16)(p13.1q22) or *t(16;16)(p13.1;q22) ; CBFB-MYH11*	*t(v;11q23.3); KMT2A* rearranged
Mutated *NPM1* without *FLT3-ITD* or with low allelic ratio (<0.5) *FLT3-ITD*	*t(9;22)(q34.1;q11.2); BCR- ABL1*
Biallelic mutated *CEBPA*	*inv(3)(q21.3q26.2)* or *t(3;3)(q21.3;q26.2); GATA2, MECOM(EVI1)*
	− 5 or del(5q); − 7; − 17/abn(17p)
	Complex karyotype anomalies or monosomal karyotype (very poor prognosis if combined with *TP53* mutations)
	Wild-type *NPM1* and high allelic ratio *FLT3-ITD*
	Mutated *RUNX1* (very poor prognosis if combined with mutated *ASXL1*)
	Mutated *ASXL1* (very poor prognosis if combined with mutated *RUNX1*)
	mutated *TP53* (very poor prognosis if combined with a complex karyotype)
Demographic and other patient-related factors	
Age below 50 years	Age above 60 years
	High WBC count

inv, inversion; *ITD*, internal tandem duplications; *t*, translocation; *WBC*, white blood cells.

addition to routine screening for cytogenetic abnormalities, recommend screening for mutations in *NPM1*, *CEBPA*, *RUNX1*, and *FLT3* (both for internal tandem duplications (ITDs) together with the mutant–to–wild-type allelic ratios, and for tyrosine kinase domain mutations at codons D835 and I836) as well as in *TP53* and *ASXL1* within the diagnostic workup, even though in many countries these results are not yet used to guide treatment decisions. With the ongoing research, other genes/genetic alterations should soon extend that list. **Table 3** lists the currently established prognostic AML biomarkers.

Management and Therapy

Treatment options for acute myeloid leukemia (AML) traditionally comprise a backbone of anthracyclines and aracytine, and stem cell transplantation. However, the cure rates of standard chemotherapy regimens could be improved and so including AML patients in clinical trials as the main therapeutic option is highly recommended.

AML treatment is divided into the induction phase aiming at achieving remission and the postremission phase consisting of the consolidation therapy aiming at "consolidating" remission, that is obtaining durable disease control. Except for the APL subtype, maintenance therapy is not included in current regimens as the available data do not support the notion that it gives any additional benefit.

A standard induction treatment consists of infusional cytarabine (arabinosylcytosine, ara-C) and an anthracycline (daunorubicin or idarubicin) or anthracenedione (mitoxantrone). A complete remission (CR) is usually achieved in about 1 month of treatment. In adult patients with *FLT3*-mutated AML, adding midostaurin (Rydapt), an orally administered multitargeted kinase inhibitor to the standard cytarabine and daunorubicin induction regimen has been shown to improve survival. Midostaurin and its major active metabolites inhibit the activity of wild-type FLT3, *FLT3*-mutant kinases (ITD and TKD), KIT (wild type and D816V mutant), PDGFR-alpha/beta, VEGFR2, and members of the serine/threonine kinase protein kinase C (PKC) family. In April 2017, it was approved for treatment of newly diagnosed adult AML patients by the US Food and Drug Administration (FDA). Moeover, it has been suggested that adding purine analogs, such as cladribine or fludarabine, to the backbone induction regimen may improve patient outcomes. Purine analogs increase intracellular uptake of cytarabine by promoting the accumulation of cytarabine triphosphate (ara-CTP) in leukemia blasts. However, the results are still preliminary and using these agents is not a number one recommendation in any of the current clinical guidelines. The use of high-dose cytarabine (HiDAC) combined with an anthracycline for induction therapy remains controversial. The US National Comprehensive Cancer Network (NCCN) recommends this option as the first choice only for patients under 45 years of age.

The induction therapy is associated with severe toxicities, including—among others—tumor lysis syndrome, cardiac abnormalities, and pancytopenia. As patients who do not receive postremission therapy usually relapse within 6–9 months, it is important that patients complete the induction therapy in a condition to tolerate subsequent more aggressive treatments of the consolidation therapy. Therefore, treating patients over 60 years of age is controversial as they often cannot tolerate the toxicities of the intensive induction therapy. Moreover, they frequently develop AML with adverse prognostic features. Those older adults who are candidates for intensive induction therapy may be treated with standard infusional cytarabine and anthracycline or—in case of cardiac or other contraindications—with alternative nonanthracycline and lower intensity regimens, such as those with hypomethylating agents (e.g. 5-azacytidine or decitabine), or with the purine analog clofarabine. However, clinical trials are the most recommended option.

A successful induction therapy should clear all visible signs of leukemia in the bone marrow and restore normal hematopoiesis in patients with a de novo AML. The response to the induction therapy is evaluated based on a bone marrow aspirate and a biopsy. In case of residual disease, additional standard-dose cytarabine with anthracycline (and midostaurin in *FLT3*-mutant cases) may be considered.

Postremission consolidation therapy aims to reduce the number of residual abnormal cells to a level which can be contained by immune surveillance. Most patients below 60 years of age are evaluated for allogeneic hematopoietic stem cell transplantation (HSCT), except for patients with favorable cytogenetic and molecular markers and those with significant comorbidities who are offered further chemotherapy. HiDAC therapy is standard for patients under 60 with favorable prognosis (in particular AML with *CEBPA* mutations). One cycle of HiDAC may also be a reasonable option for older patients.

Gemtuzumab ozogamicin (GO; Mylotarg), a monoclonal antibody against CD33 conjugated with a toxin (calicheamicin) offers hope for targeted treatment of CD33-positive AML. In September 2017, this drug was approved by FDA for treatment of adults with newly diagnosed CD33-positive AML and for treating relapsed or refractory CD33-positive AML in patients older than 2 years.

Patients with relapsed AML have an extremely bad prognosis. Most of them respond poorly to salvage therapies (e.g. patients with an initial CR duration of less than 1 year or with no initial CR have a 14% complete response rate to the initial salvage therapy) and should be referred to investigational therapies (clinical trials) whenever possible. If that is not possible, standard regimens with high-dose cytarabine or with hypomethylators are the remaining options. These include cladribine combined with high doses of ara-C, with granulocyte colony-stimulating factor and mitoxantrone (CLAG-M), and mitoxantrone/etoposide/intermediate-dose cytarabine (MEC). If remission is obtained, an allogeneic transplantation should follow in these patients whenever feasible.

A number of novel targeted therapeutics that emerge give hope for the treatment of relapsed and refractory AML. A monotherapy with venetoclax (VEN), a selective BCL2 inhibitor, has been shown to have an activity in relapsed and refractory AML, and also efficacy in lower intensity combinations for treatment-naive elderly AML patients. Different venetoclax regimens are currently tested in several phase II and III clinical trials. A very promising class of targeted therapeutics are inhibitors of IDH1 and IDH2. Mutations in genes encoding these two enzymes are key events in AML pathogenesis, leading to accumulation of the oncometabolite (*R*)-2-hydroxyglutarate (2HG) and a block in cell differentiation. In view of recent genomic data, AML with *IDH2* mutations is likely to be classified as a separate entity. In August 2017, an oral inhibitor of mutant IDH2, enasidenib (IDHIFA, Celgene Corp.) was approved by FDA for treatment of relapsed or refractory *IDH2*-mutant AML. Enasidenib is also tested for treatment of newly diagnosed AML patients in combination with the induction and consolidation therapy, or with azacitidine in patients who are not candidates for intensive chemotherapy. Other IDH inhibitors, like AG-120 or FT-2102 (both IDH1 inhibitors), are in clinical development. Another class of targeted molecular therapeutics are agents that restore the function of mutant p53 protein. Among those, the small molecule PRIMA-1 and its methylated derivative PRIMA-1(MET; APR-246) can restore the wild-type conformation of the mutant p53 protein and thus restore its normal function, which results in apoptosis of tumor cells. The safety and efficacy of APR-246 is currently tested in a phase Ib/II clinical trial and it is actually the first compound targeting mutant p53 which has entered into a clinical trial.

Acute promyelocytic leukemia (APL) is a particularly aggressive AML subtype with distinctive clinical features and its management substantially differs form that for the other AML subtypes. APL is commonly associated with coagulopathy due to DIC and fibrinolysis. Therefore, aggressive supportive care is an important component of the APL treatment. This diagnosis used to be associated with a particularly poor prognosis. However, incorporation of all-*trans*-retinoic acid (ATRA) and precise risk stratification based on WBC count into the management strategies have resulted in dramatically improved outcomes. ATRA has the capacity to induce differentiation in APL blasts and thus reverse the coagulopathy which is the major cause of death during APL induction therapy. Patients with suspected APL are put on the ATRA therapy immediately, without waiting for a cytogenetically confirmed diagnosis, as this disease is rapidly fatal, usually due to early hemorrhage. If the APL diagnosis is not confirmed, the ATRA therapy is discontinued and patients are switched to standard AML induction therapy.

Following the APL diagnosis, patients are stratified according to their WBC count. Low-risk patients (WBC below 10×10^9/L) receive "chemo-free therapy," for example ATRA plus arsenic trioxide (ATO) until remission. ATO has been shown to be a potent inducer of apoptosis in APL cells. For higher risk patients, alternative regimens include ATRA with daunorubicin and cytarabine, or ATRA with idarubicin. Combining ATRA with an anthracycline for induction therapy has been shown to give CR rates exceeding 90%. Consolidation therapy for APL is most often ATRA with ATO for 4 weeks out of 8 weeks for 4 cycles, followed by ATRA 2 weeks on, 2 weeks off for 7 cycles. Other regimens using various combinations of ATRA, ATO and chemotherapy are also used. High-risk patients (WBC above 10×10^9/L) receive induction therapy with ATRA plus chemotherapy (daunorubicin and cytarabine or idarubicin) with or without ATO. Consolidation therapy consists of combinations of ATRA, ATO, and chemotherapy.

Following consolidation therapy, patients are assessed for molecular remission based on the analysis of bone marrow samples. Patients who have achieved CR are usually put on a maintenance regimen of ATRA with 6-mercaptopurine and methotrexate. In patients who do not achieve CR following the complete induction plus consolidation treatment and in relapsed patients, subsequent treatment with ATO is recommended, either as a single agent or in combination with chemotherapy. However, the optimal consolidation strategy following ATO-based treatment of patients with relapsed APL remains to be defined.

A serious complication associated with ATRA and ATO therapy is the APL differentiation syndrome (APLDS; also called the retinoic acid syndrome). It results from rapid differentiation of leukemic promyelocytic cells into mature polynuclear cells and is characterized by fever, weight gain, pleural and pericardial effusions, respiratory distress, episodic hypotension, and acute renal failure. This syndrome occurs in about 25% of patients treated with ATRA alone. However, concurrent administration of chemotherapy

(idarubicin and cytarabine) seems to reduce the APLDS incidence. Moreover, corticosteroids (dexamethasone) can be effective both as prophylaxis and therapy.

Assessing the response to treatment remains a challenging issue in the management of both APL and other AML subtypes. Complete remission (CR) is defined as the absence of circulating blasts and of extramedullary disease. However, patients who achieved CR according to morphological assessment can potentially harbor leukemic cells in the bone marrow, giving rise to low-level disease which is not detectable by conventional cytomorphological techniques (minimal residual disease, MRD). Many trials have confirmed that MRD is the strongest prognostic factor. Therefore, applying reliable sensitive methods to detect MRD is of utmost importance for further treatment planning and monitoring of treatment response at all stages following the induction therapy. MRD can be detected by multicolor flow cytometry or sensitive molecular techniques targeting characteristic genetic or chromosomal aberrations, like RT-PCR or next-generation sequencing. The current clinical guidelines have not incorporated MRD detection into postremission monitoring strategies, except for APL. However, including it in the guidelines for all AML subtypes is only a matter of time.

Further Reading

Bannon, S.A., DiNardo, C.D., 2016. Hereditary predispositions to myelodysplastic syndrome. International Journal of Molecular Sciences 17 (6), E838.

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.

Döhner, H., et al., 2017. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood 129 (4), 424–447.

International Agency for Research on Cancer, 2017. In: Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. (Eds.), WHO classification of tumors of hematopoietic and lymphoid tissues, Revised 4th edn. World Health Organization, Geneva.

National Comprehensive Cancer Network (NCCN). *NCCN Clinical Practice Guidelines in Oncology*, *Acute Myeloid Leukemia*. Version 1.2018: February 7, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Papaemmanuil, E., et al., 2016. Genomic classification and prognosis in acute myeloid leukemia. The New England Journal of Medicine 374 (23), 2209–2221.

Saygin, C., Carraway, H.E., 2017. Emerging therapies for acute myeloid leukemia. Journal of Hematology & Oncology 10, 93.

The Cancer Genome Atlas Research Network, 2013. Genomic and Epigenomic landscapes of adult De novo acute myeloid leukemia. The New England Journal of Medicine 368, 2059–2074.

Adrenal Glands Tumors: Pathology and Genetics

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Pheochromocytoma and Paraganglioma

Definitions

Pheochromocytomas and paragangliomas (PPGLs) are neurally derived neoplasms arising from the adrenal medulla and extra-adrenal paraganglia respectively.

Paraganglia are anatomically dispersed neuroendocrine organs which are divided into two groups. The first group, known as "sympathetic" or "sympathoadrenal" paraganglia, is normally distributed along the prevertebral and paravertebral sympathetic chains, and along sympathetic nerve branches that innervate retroperitoneal and pelvic organs. The second group, known as "parasympathetic" or "head and neck" paraganglia, is associated with supradiaphragmatic branches of the vagus and glossopharyngeal nerves, predominantly in the middle ear and carotid bodies. The adrenal medulla is a sympathetic paraganglion and, by definition, pheochromocytoma is an intraadrenal sympathetic paraganglioma. Older literature frequently refers to sympathetic paragangliomas as extra-adrenal pheochromocytomas. However, current nomenclature reserves the name pheochromocytoma for tumors in the adrenal.

Sympathetic paragangliomas are almost always biochemically functional, that is, capable of catecholamine synthesis, and usually are also clinically functional, as defined by signs and symptoms of catecholamine excess. Pheochromocytomas are biochemically distinctive in that they often produce epinephrine, which is very rare in other paragangliomas. Parasympathetic paragangliomas are primarily located in the head and neck, and are usually clinically nonfunctional. A subset of these clinically silent tumors produces small quantities of norepinephrine and/or dopamine, but many do not express tyrosine hydroxylase which is required for catecholamine synthesis, and therefore they are also biochemically silent.

Burden

Paraganglionic tumors occur in patients of any age, but mainly in the fourth to fifth decades of life with approximately equal sex distribution. Hereditary disease commonly manifests before the age of 40 years. The annual incidence of pheochromocytoma is estimated at 0.4–9.5 cases per million population and that of head and neck paragangliomas at 1 case per 30,000–100,000 population. The prevalence of PPGL in hypertensive adults is approximately 0.1%–0.6% and higher in pediatric population (0.5%–2%). There is a higher prevalence of hereditary, extra-adrenal, multifocal, noradrenergic, and metastatic PPGLs in children than in adults.

Risk Factors

Hereditary susceptibility is the main causative factor for PPGLs, while chronic hypoxic states, that is, high altitude environment and cyanotic heart disease, are implicated as additional risk factors for head and neck PGLs. Prolonged hypoxic conditions have been suggested to contribute to development of sympathetic paragangliomas.

Pathology

The first histological description and histochemical contribution to diagnosis of pheochromocytoma was performed by pathologist Max Schottelius, who was acknowledged but not listed as an author of the classic 1886 paper by his clinical colleague Felix Fränkel. Paraganglionic tumors usually exhibit the classic histoarchitecture of an alveolar "Zellballen" pattern, comprising nests of polygonal neoplastic cells with peripheral sustentacular cells, separated by a delicate fibrovascular framework. Variations in architecture, unusual histological features and/or degenerative changes can be present, confounding the diagnosis. Unusual sites of occurrence, co-occurrence with other neuroendocrine tumors particularly in syndromic settings, and nonendocrine tumors in various anatomic sites might pose additional diagnostic challenges. Immunohistochemistry (IHC) is accordingly utilized as a valuable aid to diagnosis in challenging cases: PPGLs express generic neuroendocrine markers, that is, chromogranin A, synaptophysin and CD56, as well as specific paraganglionic markers, such as catecholamine-synthesizing enzymes, such as tyrosine hydroxylase and dopamine beta-hydroxylase, while usually negative for keratins, CEA and calcitonin. S100 and/or GFAP highlight the sustentacular cell population.

Molecular Pathology and Genetics

Genetic Profile

Pheochromocytomas and paragangliomas carry the highest degree of heritability among all human neoplasms. Approximately 40% of PPGL patients carry a causal germline mutation, while at least 30% of the remaining "sporadic" cases harbor a somatic mutation in a predisposing gene. Consequently, the American Society of Clinical Oncology recommends offering genetic testing to all PPGL-affected patients and the Endocrine Society has published clinical practice guidelines concerning genetic assessment by accredited laboratories. In the recent WHO Classification of Tumours of Endocrine Organs, 17 hereditary PPGL susceptibility genes are mentioned, that is, *VHL*, *RET*, *NF1*, *SDHD*, *SDHAF2*, *SDHC*, *SDHB*, *SDHA*, *TMEM127*, *MAX*, *EPAS1/HIF2A*, *PHD1/EGLN2*, *PHD2/EGLN1*, *FH*, *MDH2*, *KIF1B*, and *MEN1*.

The catalogue of genes implicated in PPGL pathogenesis is continuously expanding, including many genes of low mutational frequency. Tumor-suppressor genes and oncogenes reported at the germline and/or somatic level, in addition to the aforementioned ones, now include: *IDH1*, *IDH3B*, *GOT2*, *SLC25A11*, *HRAS*, *BRAF*, *CSDE1*, *IRP1*, *ARNT*, *SETD2*, *FGFR1*, *IGF1*, *EZH2*, *H3F3A*, *CDKN2A*, *CDKN2C*, *GNAS*, *TP53*, *BAP1*, *MSH2*, *ATRX*, *MET*, *MERTK*, *KMT2D*, *KMT2B*, *JMJD1C*, *STAG2*, *PALB2*, *STAT3*, *CREBBP*, *EP300*, *WHSC1L1*, *SMARCA4*, *SMARCA5*, *SMARCAL*, *SMARCE1*, and *ARID1B*. Mutations in *IDH1*, *HRAS*, *BRAF*, *CSDE1*, *IRP1*, *ARNT*, *SETD2*, *FGFR1*, *TP53*, *ATRX*, *CDKN2A*, *CDKN2C*, *GNAS*, *STAG2*, *PALB2*, *STAT3*, *CREBBP*, *EP300*, *WHSC1L1*, *SMARCA4*, *SMARCA5*, *SMARCAL*, *SMARCE1*, and *ARID1B* have been reported thus far at the somatic level only, *HRAS* and *BRAF* mutations displaying a mean frequency of about 9% in sporadic paraganglionic tumors. *NF1* inactivation is more common at the somatic level in contrast to the *SDHx* mutations, which are exceedingly rare in the absence of germline mutations. *NF1*, *HRAS*, *EPAS1/HIF2A*, *ATRX*, *VHL*, *RET*, and *CSDE1* are frequent somatically mutated genes.

In addition to low mutation frequency of significant genes, PPGLs have a low mutational load and some mutations are mutually exclusive. As an example, somatic *HRAS* mutations have not been detected in cases with known PPGL susceptibility gene mutations. In contrast, other somatic variants do co-occur with germline mutations. These include *ATRX* with *SDHx/VHL/RET/NF1/FH* mutations, *CDKN2C* with *RET* mutations, *NF1* with *EZH2* mutation, *EPAS1/HIF2A* with *IGF1* mutation, *KDM2B/TP53* with *SDHB* mutation, *KIF1Bb/MET* with *NF1* mutation and *VHL* with *SDHA* mutation. In this context, a subset of aggressive SDH-deficient extra-adrenal PGLs is enriched for mutations in non-coding functional genomic regions, such as the promoter of the telomerase reverse transcriptase (*TERT*) gene. At the somatic level, co-occurrence of mutations in PPGL susceptibility genes, that is, *RET/VHL/MAX/MET* co-occurring with *NF1* mutation, has been reported. In the era of massively parallel sequencing of PPGL patients, potential cases of double germline mutations or *M*ultilocus *I*nherited *N*eoplasia *A*lleles *S*yndrome (MINAS) were found, for example, germline *PTEN* and *SDHC* mutations in a patient suffering from multifocal papillary thyroid carcinoma and carotid PGLs. These findings, along with the fact that PPGLs of different genetic backgrounds share overlapping territories of genomic gain or loss, suggest that co-segregation of susceptibility mutations might play a pathogenetic role, in keeping with other paradigms of molecular tumourigenesis.

Integrative genomic analysis has provided strong concordance between the mutation status of the well-known PPGL susceptibility genes and multiomics data, adding further to the initial transcription profile-based PPGL classification into a pseudohypoxic cluster 1 (*VHL*, *SDH-x*, *FH*, *MDH2*, *EPAS1/HIF2A*, *PHD1/EGLN2*, *PHD2/EGLN1*) and a kinase receptor-signaling cluster 2 (*RET*, *NF1*, *TMEM127*, *MAX*, *KIF1B*). The close link between gene-expression subtypes of PPGL and tumor genotype has been subsequently confirmed with *HIF2A*-mutated PGLs displaying a characteristic expression signature within the pseudohypoxic cluster. A consensus clustering analysis of multi-omics data classified paraganglionic tumors into five molecular subgroups with distinct driver mutations, copy number changes, DNA methylation aberrations, miRNA dysregulations, expression signatures, and clinical characteristics. The following clusters emerged: (1) cluster C1A comprising *SDHx*- and *FH*-mutated tumors; (2) cluster C1B comprising *VHL*-mutated tumors; (3) cluster C2A comprising tumors containing *RET*, *NF1*, *TMEM127*, *MAX*, *HRAS* mutations; (4) cluster C2B comprising tumors containing *MET* mutations; and (5) cluster C2C. Clusters C2B and C2C were enriched in sporadic tumors.

A Cancer Genome Atlas project (TCGA) study classified PPGLs by comprehensive integrated analysis into four molecularly defined groups: (1) pseudohypoxia subtype, (2) kinase signaling subtype, (3) Wnt-altered subtype, driven by *MAML3* and *CSDE1*, and (4) a cortical admixture subtype possibly representing adrenal cortical contamination of PCCs belonging to other groups. An important discovery in the TCGA study was the presence of fusion genes in PPGLs, involving *MAML3*, *BRAF*, *NGFR*, and *NF1*, adding to the diversity of mechanisms that influence PPGL pathogenesis through a broad range of biological pathways. In addition to germline/somatic mutations, these include postzygotic mosaicism, epigenetic alterations (e.g., extensive genome methylation in *SDHx*-mutated tumors), fusions as well as large and recurrent structural alterations/copy number changes (1p/3q, 11p, 11q, 6q, 17p, 22 losses; 9q, 17q, 19p13.3, and 20q gains).

Genetic Susceptibility

Germline mutations in almost all PPGL susceptibility genes lead to an autosomal dominant pattern of inheritance. In those familial cases associated with *SDHD*, *SDHAF2*, and *MAX* mutations, there is a parent-of-origin expression phenotype, with paraganglioma development almost always dependent on inheritance via the paternal line. However, PCC-affected patients have inherited the *SDHD* mutation via the maternal line. The Hensen model, the Muller threshold model and the Baysal methylated boundary

element model have been proposed to explain the mechanism underlying the paternal transmission of *SDHD* and/or *SDHAF2* mutations.

Gain-of-function mutations in the *RET* proto-oncogene and *EPAS1* do not require a second hit, in contrast to tumor suppressor genes characterized by inactivation or loss of the wild-type allele in the tumors. Nonetheless, double *RET* mutations at the somatic/germline level, duplication of the mutated *RET* allele, double *EPAS1* mutations at the somatic and/or somatic/germline level as well as somatic *EPAS1* mutation co-occurring with *EPAS1* locus amplification have been documented in paraganglionic tumors. Most *EPAS1* mutations associated with PPGL are somatic/mosaic. This expands the spectrum of endocrine syndromes that can involve mosaicism, including neurofibromatosis type 1 (NF1) and McCune-Albright syndrome. Although mosaicism is caused by a postzygotic de novo mutational event, usually resulting in somatic mosaicism, the mutation can potentially also be present in germinal cells (germline mosaicism) and therefore be transmitted to the offspring. This possibility creates genetic counseling implications. Postzygotic mutation in the histone 3.3 encoding gene (*H3F3A*) was recently linked to a new paraganglioma syndrome, encompassing paraganglionic tumors and aggressive giant cell tumor of bone, and without a family history of pheochromocytoma or paraganglioma.

Another nonhereditary genetic disorder, possibly characterized by mosaicism, is the Carney Triad, a syndrome of tumors affecting at least five organs, that is, GIST in the stomach, pulmonary chondroma, PCC/PGL of adrenal medulla/extra-adrenal paraganglia, adrenal cortical adenoma and leiomyoma of the esophagus. Although approximately 10% of Carney Triad patients harbor germline variants in the *SDHA*, *SDHB* or *SDHC* genes, most cases display SDH down-regulation through site-specific aberrant DNA hypermethylation of the *SDHC* gene promoter. Germline and/or somatic mosaicism for *SDHC* promoter methylation has been documented in GIST-affected patients as well as in patients suffering from PGLs and adrenocortical adenoma and/or PCCs and GIST. Although it remains speculative whether these might represent Carney Triad cases with incomplete phenotypic expression, epigenetic changes should be taken into consideration in the evaluation of PPGL-affected patients. Of note, *SDHC* epimutations have been documented exclusively in females.

Awareness of syndromic associations (**Table 1**) and genotype-phenotype correlations, provides clues to occult hereditary disease, which has important implications both for the patients and their families in terms of genetic testing and counselling, active surveillance programs and management. Specific tumor combinations, that is, PPGLs and enteropancreatic neuroendocrine tumors, pituitary adenomas, adrenocortical adenomas, renal tumors, gastrointestinal stromal tumors (GISTs) or peripheral neuroblastic tumors (**Table 2**) should raise a suspicion of occult hereditary disease. Although these neoplasms and/or related conditions such as polycythemia do arise within different genetic syndromes, it remains elusive whether particular dysregulated pathways, for example, defects in oxygen sensing pathways for polycythemia, and/or inherently susceptible tissues explain distinctive combinations of findings associated with different genotypes.

MEN2- and TMEM127-related adrenal gland pathology is characterized by bilateral and/or multicentric micro- and macro-PCCs, different from this seen in VHL with a thick vascular capsule, small- to medium-sized tumor cells with amphophilic clear cytoplasm and numerous interspersed small vessels. The qualification of micro-PCCs to nodular adrenomedullary hyperplasia has been recently expanded in the MAX-deficient state. Worth mentioning is also SDH-deficient GIST with its particular gastric location (predilection for the distal stomach/antrum), common multifocality, a multinodular/plexiform growth pattern, epithelioid cytomorphology, either pure or combined with a spindle-cell component, SDHB and/or SDHA immunonegativity, metastatic potential, a relatively indolent clinical course, even in the presence of metastatic disease (often to lymph nodes), and insensitivity to imatinib. Finally, pathological features of SDH- and FH-deficient renal cell carcinoma (RCC) merit to be mentioned. SDH-deficient RCC exhibits uniform low-grade cytology, cytoplasmic vacuoles, eosinophilic or flocculent cytoplasm, focal cystic change, solid to lobulated growth with peripherally entrapped renal tubules and absence of immunoreactivity for SDHB and/or SDHA. FH-deficient RCC exhibit predominantly papillary morphology combined with additional architectural patterns, and at least focal presence of eosinophilic macronucleoli with perinucleolar halos, reminiscent of viral inclusions, and display loss of FH expression and aberrant S-(2-succinyl) cysteine (2SC) immunoreactivity.

In parallel with recent advances in molecular genetics, IHC has emerged as a screening tool for inferring the presence of mutations in patients presenting with tumors associated with hereditary cancer predisposition syndromes. Since IHC detects a phenotype rather than a genotype, it should ideally be regarded as a preliminary screen to triage patients for optimal genetic testing. In particular, IHC has been shown to complement morphological or clinical suspicion of hereditary PPGL predisposition syndromes.

Currently, three panels of IHC staining are in use: (i) IHC staining for SDHB/SDHA/SDHD to evaluate SDH deficiency (**Fig. 1**) (ii) staining for FH and 2SC to evaluate FH deficiency; and (iii) staining for MAX to evaluate MAX deficiency. Loss of SDHB protein expression is seen in PPGLs either harboring a germline mutation in any of the *SDHx* genes (**Fig. 2**) or with somatic hypermethylation of the promoter region of *SDHC* gene, whereas loss of both SDHB and SDHA immunoreactivity is exhibited only in the context of an *SDHA* mutation. Loss of FH expression and 2SC overexpression in cases containing germline *FH* mutations, while immunohistochemical expression of MAX is negative in cases harboring *MAX* truncating mutations.

SDHB/SDHA IHC has been integrated either in genetic testing algorithms along with other parameters, that is, tumor location and biochemical/secretory phenotype, or in genetic testing strategies encompassing massively parallel sequencing (NGS panels) and copy number variation (CNV) analysis. In the presence of syndromic presentation and/or known familial variant, targeted sequencing is accordingly recommended. Despite the availability of genetic testing of PPGLs at an ever-decreasing cost, IHC still remains an important integrated component of algorithms to guide either subsequent Sanger sequencing or biological validation of variants of uncertain significance (VUS) identified by massive parallel sequencing. Correct immunohistochemical evaluation is critical, given that false-positive or false-negative interpretation can result in failure to identify PCC/PGL affected individuals at

Table 1 Clinical presentations of pheochromocytoma and paraganglioma associated with germline mutations and somatic mosaicism

Gene	*Mutation rate (%)*[a]	*Predominant tumor site*	*Tumor number*	*Nonparaganglionic tumors and other related features*
VHL	7	PCC > PGL	Multiple	Retinal and CNS hemangioblastoma, clear cell RCC and renal cysts, pancreatic neuroendocrine tumor and pancreatic cyst, endolymphatic sac tumors, papillary cystadenoma of the epididymis/broad ligament and mesosalpinx
RET[b]	6	PCC > PGL	Multiple	Medullary thyroid carcinoma, multiglandular parathyroid disease, marfanoid habitus, mucocutaneous neuromas, gastrointestinal ganglioneuromatosis, cutaneous lichen amyloidosis
NF1	3	PCC > PGL	Single	Neurofibroma, malignant peripheral nerve sheath tumor, gastrointestinal stromal tumor, optic nerve and other brain stem glioma, neuroendocrine tumor (somatostatinoma) of the periampullary duodenum, cafe-au-lait spots and axillary or inguinal freckling, Lisch nodules, dysplasia of sphenoid bone or long bones, kyphoscoliosis, cerebral arteriopathy, pulmonary artery stenosis, learning disabilities
SDHA	1–3	PGL >> PCC > PGL/PCC	Single > multiple	Gastrointestinal stromal tumor, pituitary adenoma, SDH-deficient RCC, melanoma?
SDHB	6–8	PGL > PCC	Multiple	Gastrointestinal stromal tumor, SDH-deficient RCC, pituitary adenoma
SDHC	1–2	PGL > PCC	Multiple	Gastrointestinal stromal tumor, SDH-deficient RCC, pituitary adenoma
SDHD	5–6	PGL > PCC	Multiple	Gastrointestinal stromal tumor, SDH-deficient RCC, pituitary adenoma, pancreatic neuroendocrine tumor?
SDHAF2	0.1	PGL > PCC	Multiple	None reported
FH	1	PCC > PGL	Single	Hereditary leiomyomatosis and RCC (HLRCC) phenotypic manifestations including cutaneous and uterine leiomyomas and/or leiomyosarcomas? and FH-deficient RCC encompassing papillary RCC Type 2, tubulo-papillary RCC and/or tubulocystic RCC with poorly differentiated foci, and collecting duct RCC; and leydig cell tumor of the testis?
MDH2	<1	PGL?	?	?[c]
TMEM127	2	PCC > PGL > PCC/PGL	Multiple	Clear cell RCC
MAX	1	PCC > PGL	Multiple	Pituitary adenoma, Renal oncocytoma? RCC?
KIF1B (*s* > *g*)	<1	PCC?	?	Neuroblastoma? ganglioneuroma? Lung and colorectal adenocarcinomas? Endometrial carcinoma? Leiomyosarcoma?
EPAS1/HIF2a[b] (*s* and *s/m* >> *g*)[d]	1	PGL > PCC >> PGL/PCC	Multiple > single	Polycythemia with markedly elevated EPO levels, duodenal somatostatinoma, hamangioblastoma, ocular manifestations, that is, dilated capillaries, retinal neovascularization and fibrosis overlying the optic disc
H3F3A (m)	<0.1	PCC/PGL	?	Giant cell tumor of the bone; recurrent/metastatic forms
PHD1/EGLN2	<1	PCC/PGL	?	Polycythemia with borderline or mildly elevated EPO levels
PHD2/EGLN1	<1	PGL∼PCC	?	Polycythemia with borderline or mildly elevated EPO levels?
BAP1	<1?	PGL?	?	Uveal melanoma, malignant mesothelioma, cutaneous melanoma, atypical spitz tumors/naevi, basal cell carcinoma, RCC
MEN1	<1?	PCC > PGL	Single	Parathyroid adenoma/hyperplasia, entero-pancreatic neuroendocrine tumor (gastrinoma > nonfunctioning/ PPoma > Insulinoma > Glucagonoma/VIPoma), pituitary adenoma (prolactinoma > somatotrophinoma > corticotrophinoma/ nonfunctioning), adrenal cortical tumor, brochopulmonary neuroendocrine tumor, thymic neuroendocrine tumor, gastric neuroendocrine tumor, angiofibromas, collagenomas, lipomas, meningiomas
MET[b] (*s* > *g*)[d]	<1?	PCC > PGL	?	Germline *MET* mutations are associated with Hereditary Papillary RCC[e]
MERTK[b] (*g*∼s)?	<1?	PCC/PGL	?	Medullary thyroid carcinoma?

Abbreviations: CNS, central nervous system; EPO, erythropoietin; g, germline mutation; PGL, paraganglioma; PCC, pheochromocytoma; RCC, renal cell carcinoma; s, somatic mutation; s/m, somatic mosaicism.

[a]Combined data for PPGL prevalence (Favier et al., Nature Review Endocrinology, 2014; Bausch et al., JAMA Oncology, 2017; Dahia PL, Nature Review. Cancer, 2014).

[b]Gain-of-function mutations; loss-of-function mutations in RET result in intestinal aganglionosis and Hirschprung disease.

[c]One truncating MDH2 mutation is recorded in The Catalogue of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

[d]Indicating low level constitutional mosaicism.

[e]Paraganglionic tumors are not features of this disorder.

Table 2 Tumor combinations of pheochromocytoma and paraganglioma (PPGL) raising the suspicion of occult hereditary disease

Tumor combinations	*Syndromes and/or germline mutations*
PPGL and entero-pancreatic neuroendocrine tumor	*Duodenal somatostatinoma* Polycythemia-PPGL-somatostatinoma syndrome (or Pacak-Zhuang syndrome) Neurofibromatosis type 1 (NF1) *Pancreatic neuroendocrine tumor* Von Hippel-Lindau (VHL) syndrome Multiple endocrine neoplasia type 1 (MEN1) PGL1 (or SDHD-related PPGL syndrome)
PPGL and pituitary adenoma	Multiple endocrine neoplasia type 1 (MEN1) SDH- and MAX-related PPGL syndromes
PPGL and adrenal cortical adenoma	Multiple endocrine neoplasia type 1 (MEN1) Carney Triad
PPGL and gastrointestinal stromal tumor	SDH-related PPGL syndromes Carney-Stratakis syndrome Carney Triad Neurofibromatosis type 1 (NF1)
PPGL and renal tumor	Von Hippel-Lindau (VHL) syndrome SDH-related PPGL syndromes MAX- and TMEM127-related PPGL syndromes Hereditary leiomyomatosis and renal cell cancer (HLRCC)
PPGL and peripheral neuroblastic tumor	*KIF1b, SDHB, SDHC* Composite tumor *NF1*, *VHL*, *MEN2A*, *MEN2*, *SDHB*

Table 3 Adrenocortical tumor staging criteria as proposed in children

Stage I	Completely resected tumors <100 g and <200 cm^3 with normal postoperative hormone levels
Stage II	Completely resected tumors ≥ 100 g or ≥ 200 cm^3 with normal postoperative hormone levels
Stage III	Unresectable, gross or microscopic residual disease Tumor spillage Patients with stage I and II tumors who fail to normalize hormone levels after surgery Patients with retroperitoneal lymph node involvement
Stage IV	Presence of distant metastatic disease

Ribeiro, R. C., Pinto, E. M., Zambetti, G. P., Rodriguez-Galindo, C. (2012). The international pediatric adrenocortical tumor registry initiative: Contributions to clinical, biological, and treatment advances in pediatric adrenocortical tumors. *Molecular and Cellular Endocrinology* **351**(1), 37–43, Copyright 2012, with permission from Elsevier.

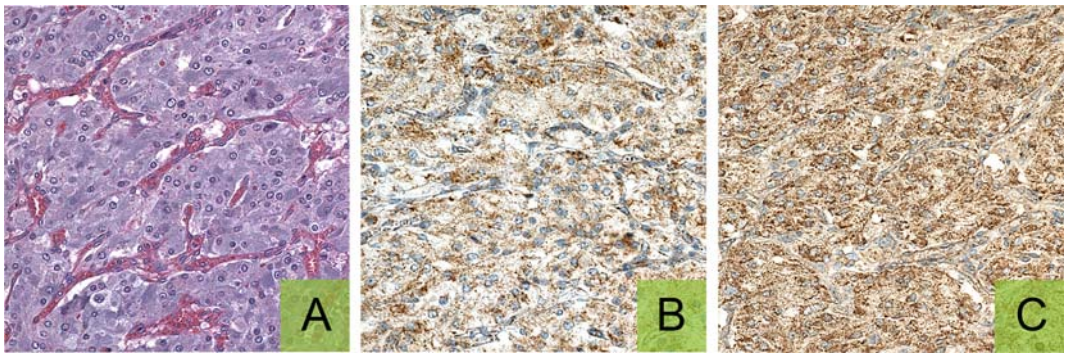

Fig. 1 Sporadic pheochromocytoma (A; H&E) with wild type SDH showing identical patterns of granular cytoplasmic staining for SDHB (B) and SDHA (C) corresponding to the mitochondrial localization of the enzyme. For proper interpretation, staining should be granular and of the same intensity as in endothelial cells, which serve as an essential endogenous control.

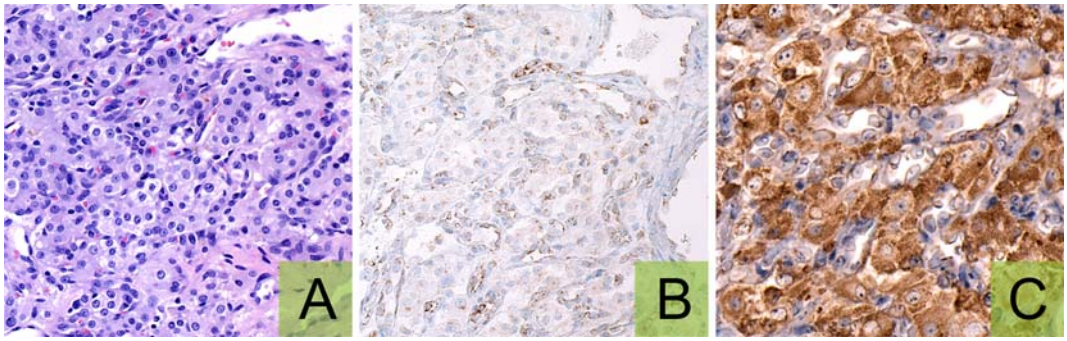

Fig. 2 Extra-adrenal paraganglioma (A; H&E) arising in the context of germline *SDHB* mutation. Granular cytoplasmic staining for SDHB (B) is maintained in endothelial cells and lost in tumor cells. Note that some tumor cells show a faint blush of non-granular background stain.

increased risk for other than endocrine-related neoplasia (e.g., GIST and/or RCC), incorrect interpretation of the pathogenicity of genetic VUS and inappropriate genetic testing.

In order for clinical practice to keep pace with the rate of discovery as well as to advance the diagnosis and optimize PPGL-affected patient care, a comprehensive reporting template has been proposed taking into consideration clinicopathologic correlations and SDHB/SDHA IHC. A simpler pathology reporting template based on current established evidence for clinical relevance of each of the pathology parameters is being developed under the auspices of the International Collaboration on Cancer Reporting (http://www.iccr-cancer.org/) and major pathology societies.

Microenvironment Including Immune Response

The antitumor immune response has been largely unexplored in PPGLs. In an attempt to evaluate prevalence and clinico-pathologic significance of programmed cell death (PD) ligands expression in PPGLs, evidence has emerged, supporting differential regulation of PD-L1 and PD-L2, the latter having a more predominant role in shaping the immune-tolerogenic environment given a preferential co-expression of immune-inflammatory genes in association only with PD-L2.

Experimental studies have shown that the microenvironment, as represented by primary cancer-activated fibroblasts, plays a pivotal role in enhancing collective migration/invasion in spheroids of mouse Pheo *SDHB*-silenced cells.

Staging and Grading

Staging

TNM staging of tumors of the adrenal medulla and extra-adrenal paraganglia (PPGLs), has been recently introduced in the American Joint Committee on Cancer (AJCC) staging manual, 8th edition. Stage 1 and stage 2 are defined as strictly localized pheochromocytomas with a size of <5 cm or ≥ 5 cm in greatest dimension, respectively. Stage 2 includes sympathetic paragangliomas of any size, but without invasion into surrounding tissue. Stage 3 tumors are characterized by infiltration into surrounding tissue (e.g., liver, pancreas, spleen, kidney) or positive regional lymph nodes. Stage 4 is restricted to patients with distant metastasis to bone (M1a), to only distant lymph nodes/liver or lung (M1b) or to bone plus multiple other sites (M1c). According to this staging system, parasympathetic paragangliomas are not staged as they are regarded as largely benign.

Grading

Currently, no histologic grading system is widely utilized or endorsed. The Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) scoring system has been proposed for stratifying risk of metastasis only for pheochromocytomas and based on histological features, while the GAPP (Grading of Adrenal Pheochromocytoma and Paraganglioma) system has been proposed for predicting metastatic disease for both pheochromocytomas and paragangliomas and is based on histologic and non-histologic parameters.

Prognostic and Predictive Biomarkers

In contrast to the WHO 2004 classification of endocrine neoplasia which defined malignancy by development of metastatic disease, current thinking as stated in the WHO 2017 classification assumes metastatic potential in all PPGLs and adopts an approach based on risk stratification. At present, the major risk factors are *tumor location*, *size*, *genotype*, and *biochemical phenotype*, all of which are interrelated. Local invasion, even if extensive, is a poor predictor of metastasis when considered alone, but is one of several putative adverse features in proposed grading systems. The overall risk of metastasis in PPGLs is approximately 10%–15%. In decreasing order of frequency, around 40% of extra-adrenal sympathetic PGLs, 5%–10% of pheochromocytomas and 4%–6% of head and

neck PGLs metastasize. In order to differentiate between metastases and multiple primary tumors, the diagnosis of metastasis requires the presence of tumor at anatomic sites devoid of normal paraganglionic tissue, that is, nodes, bone, liver, and lung. Rarely, mostly solitary primary paragangliomas occur in lung or near the hilum of the liver.

Although metastases can develop decades after resection of a primary tumor, 35%–40% of patients whose tumors are destined to metastasize have synchronous metastases at initial presentation and 65% of patients develop metachronous metastases at a median of 5.5 years. The clinical course of patients with metastatic PPGL is heterogeneous with metastases not necessarily signifying a poor short-term prognosis, in accordance with variable mortality rates. R a p i d disease progression has been associated with male sex, older patient age at initial diagnosis, synchronous metastases, tumor size >5 cm, elevated dopamine and unresectability of primary tumor. Noradrenergic and/or dopaminergic biochemical phenotype appears to be related to metastatic potential, while distant metastases seem to be associated with overall survival rate. In particular, patients with bone metastases only have better survival than liver and/or lung metastatic disease regardless of co-existence of skeletal metastases.

In support of a close link between prognosis and genetic profile, up to 55% of pediatric and/or adult PPGL patients with metastatic disease have been reported to be germline *SDHB* mutation carriers. Despite the fact that children show a higher prevalence of metastatic PPGLs, *SDHB*-mutated children survive longer than adult patients with germline *SDHB* mutations. Lower survival rates have been documented in the latter compared with apparently sporadic counterparts further adding to the concept that SDHB status is predictive of patient outcome.

The enrichment of Krebs cycle-related gene mutations, i.e. succinate dehydrogenase subunits (*SDHA/B/C/D*) and assembly factor (*SDHAF2*), fumarate hydratase (*FH*), malate dehydrogenase 2 (*MDH2*), isocitrate dehydrogenases (*IDH1/IDH3B*), as well as mitochondrial genetic defects i.e. mitochondrial 2-oxoglutarate/malate carrier (*SLC25A11*) or glutamic-oxaloacetic transaminase 2 (*GOT2*), highlights a link between metabolism and downstream signaling pathways involved in PPGL tumorigenesis. Based on metabolic profiling, genotype-specific differences were illustrated in PPGLs with a specific succinate-glutamate signature of *SDH*-mutated PGLs and/or ratios of succinate:fumarate and other metabolites proposed as a method to identify patients for testing of *SDHx* mutations and to validate VUS with prognostic implications. In this context, it has been shown that mass spectrometric profiling can identify PPGLs with metastatic potential based on differential expression of specific asparagine-linked glycan (N-glycan) structures.

Recent TCGA analysis revealed differences in mutations and mRNA expression between metastatic and non-metastatic PPGLs and MAML3 fusion gene was found to correlate with metastatic potential. A significantly higher mutation density was detected in malignant than in benign tumors. Structural *TERT* rearrangements underlying *TERT* activation were documented in metastatic pheochromocytomas, in the absence of *TERT* promoter mutations or promoter DNA methylation. As histological and/or multiparameter scoring systems are not currently endorsed, biomarkers emerging from transcriptomic profiling and/or DNA methylation analysis hold promise for stratifying PPGL patients according to risk of developing metastasis.

Adrenal Cortical Carcinoma

Definition

Adrenal cortical carcinoma (ACC) is a malignant epithelial neoplasm arising from the mesodermal-derived cortex of the adrenal gland.

Burden

ACC is a rare endocrine malignancy with an annual incidence of 0.5–2.0 cases per 1 million population. ACC is even more uncommon in the pediatric population, except in geographic regions with a high prevalence of founder mutations which account for a high incidence, e.g. *TP53* p.R337H mutation in Southern Brazil with a penetrance of adrenal cortical tumors estimated approximately at 10%. The median age at diagnosis is in the fifth and six decades with a female preponderance and a F:M ratio ranging from 1.5:1–2.5:1. A bimodal distribution has been suggested with a second incidence peak during childhood and a striking female predominance observed at a young age (3:1), but not in children ≥ 10 years old (1:1). Differences in age of onset (≤ 5 years and/or >5 years) and hormonal secretion pattern of pediatric adrenocortical tumors might be *tissue* (fetal or transient embryonic adrenal cortex)- and/or *genotype* (germline *TP53* mutation)-dependent, possibly reflecting perturbations of sex- and age-specific developmental processes in the adrenal cortex.

Risk Factors

Hereditary susceptibility is the main causative factor (see *Genetic Susceptibility*). Subsets of ACC exhibit mutation signature 1, featuring C > T substitution in a CG context and resembling the age- and DNA-mismatch repair-deficiency signatures. They also exhibit mutation signature 2, featuring A > T substitution in a CG context and resembling the smoking signature. The latter is in accordance with data derived from the 1986 National Mortality Followback Survey suggesting heavy cigarette smoking as a risk factor in men. The same study supported a role of using oral contraceptives before the age of 25 as risk factor in women. This is consistent with clinical data indicating that proliferation and/or secretion of adrenocortical neoplasms might be affected by the

hormonal context of pregnancy. It is also supported by experimental evidence: a growth-inhibitory effect of selective estrogen receptor modulators on human adrenocortical cell line H295R and on human tumors xenografted in athymic nude mice.

Pathology

For a diagnosis of malignancy (i.e., the ability to invade adjacent structures and metastasize) of an adrenal cortical neoplasm, multiparametric histopathological scoring systems are used, including parameters such as cell type, tumor architecture, tumor cell invasion, and cellular proliferation. These systems include the Weiss scoring system, Hough scoring system, van Slooten scoring system, Weiss revisited index and/or diagnostic algorithms, including the stepwise discriminate diagnostic system and the "reticulin" diagnostic algorithm. The latter defines malignancy in a two-step process, the presence of a disrupted reticulin framework constituting the first step of this diagnostic approach, and subsequent identification of at least 1 out of 3 parameters: a high mitotic rate ($>5/50$ HPFs), necrosis, and venous invasion. Both the "reticulin" algorithm and the Helsinki score, a novel model for prediction of metastases in ACCs based on mitotic index ($>5/50$ HPFs), necrosis and Ki67 labeling index, have been subsequently validated.

The Weiss system is the most popular among all multifactorial scoring systems, comprising nine histologic parameters, that is, *high nuclear grade*, *mitotic rate* (>5 mitotic figures/50 HPFs), *atypical mitoses*, *<25% clear cells*, *diffuse architecture*, *tumor necrosis*, *capsular invasion*, *venous invasion*, and *sinusoidal invasion*, with presence of ≥ 3 indicating malignant behaviour. Nevertheless, no particular system is currently endorsed in the 2017 WHO classification. Various limitations of the Weiss system include lack of reproducibility of several criteria, borderline tumors with a Weiss score of 2 or 3, oncocytic and myxoid variants of adrenocortical neoplasms, and pediatric adrenocortical tumors, which are frequently overdiagnosed as malignant given the presence of impressively atypical histologic features. With regard to the latter, a specialized classification system has been proposed by Wieneke et al., while the Lin-Weiss-Bisceglia system has been established for oncocytic adrenocortical neoplasms given the inherent presence of three definitional criteria and overdiagnosis of malignancy if Weiss system is applied.

On a large ACC series, assessment of the most frequent diagnostic pitfalls resulted in a disagreement level as high as 9% of referral cases. Misclassification of metastatic and/or primary intra-adrenal tumors (PCC, ACA, and soft tissue neoplasm) as ACCs and vice versa accounted for such discrepancies, which significantly modified clinical management. An immunohistochemically determined Ki67 proliferation index $>5\%$ and/or over-expression of IGF2 with a characteristic juxtanuclear (Golgi) dot-like pattern are useful for accurate classification of adrenocortical neoplasms. From a differential diagnostic perspective, ACCs usually exhibit reactivity for steroidogenic factor-1 (SF-1), Melan-A, inhibin-α, calretinin and synaptophysin, while they are negative for cytokeratins, EMA, CEA, PAX-8, HMB-45 and chromogranin-A. Hence, this immunohistochemical panel can exclude *intraadrenal tumors*, for example, PCC and primary soft-tissue neoplasms, *metastatic tumors*, for example, lung/breast/colorectal adenocarcinomas, urothelial carcinomas and melanomas, and *adrenal gland involvement by other malignancies*, for example, renal cell carcinoma, hepatocellular carcinoma and retroperitoneal sarcomas.

Despite these advances, some adrenocortical neoplasms defy classification into benign and malignant categories. In such cases, the diagnosis germ of *adrenocortical neoplasm of uncertain/undetermined malignant potential* can be used.

Molecular Pathology and Genetics

Genetic Profile

Driver-gene mutations and activation of key cellular signaling pathways they control appear to suffice for adrenal tumorigenesis. By adapting methods from molecular evolution to the study of cancer genomes, it has been shown that positive selection outweighs negative selection during cancer development, while the number of driver mutations increases, but not linearly, with increasing mutation burden. Multistep tumorigenesis within ACCs was further corroborated by data emerging from pan-genomic analysis of TCGA based on variant allele fractions, genotype and LOH patterns and from a comprehensive genomic analysis of pediatric adrenocortical tumors based on timing of copy-neutral-LOH patterns and the temporal order of somatic single nucleotide variations (SNV). The former displayed widespread chromosomal loss with whole genome doubling (WGD) as important genomic event in ACC progression, while the latter highlighted copy-neutral-LOH of chromosomes 11/17 as early driver events with subsequent SNV acquisition in these regions.

Whether ACCs arise de novo or as result of an adenoma-to-carcinoma sequence is still not settled, although clinicopathologic and molecular evidence in support of a progression model is accumulating. Molecular evidence in support of stepwise progression derives from isolated reports, single nucleotide polymorphism (SNP) array profiling, comparative genomic hybridization, X-chromosome inactivation pattern analysis and microsatellite allelotyping. Nevertheless, this sequence is currently regarded as exceptionally rare with an extremely low progression rate given molecular and/or epidemiological data.

In the pediatric context, a similar model was also proposed for *TP53*-associated adrenocortical tumors, arising from a simpler genomic background and progressing to acquire complex genomic aberrations. Given shared molecular attributes in pediatric adrenal cortical tumors, that is, germline *TP53* mutations, chromosome 11p15 abnormalities, and IGF2 overexpression, it has been recently proposed that adrenocortical adenomas, tumors of undetermined histology and ACCs represent a spectrum of the same disease.

The comprehensive TCGA study has recently provided detailed insight in molecular events in ACC oncogenesis. ACC displayed a heterogeneous somatic mutation density with a median of 0.9 per Mb, which is higher than all genitourinary malignancies, with

the exception of urothelial carcinoma of the bladder. Significantly mutated genes were *TP53*, *CTNNB1*, *MEN1*, *PRKAR1A*, and *RPL22*. The catalogue of known ACC driver genes was expanded to include *TERF2*, *CCNE1*, and *NF1*. A low frequency of gene fusions was found without recurrent events. Copy number alterations were significant with WGD as hallmark mechanism for disease progression. Expression level of telomerase reverse transcriptase (*TERT*) was high but telomere length decreased, highlighting the role of telomerase reactivation and telomere maintenance. Finally, *p53 apoptosis/Rb1 cell-cycle* pathway and *Wnt/β-catenin* pathway as well as *PKA subnetwork* were significantly modified. Despite the relative lack of targetable hotspot mutations, a subset of ACCs demonstrated approximately 50 potentially actionable alterations, encompassing mutations and copy-number changes.

The TCGA study was based on a near-global ACC sample and in continuity with previous efforts on adult and pediatric adrenocortical cancer from Europe, North America and Brazil. This confirmed (i) highly prevalent IGF2 over-expression; (ii) high frequency of *TP53* mutations, which are dominant in the pediatric setting; (iii) high frequency of diverse defects resulting in deregulation of the *Wnt/β-catenin* pathway, that is, *CTNNB1* point mutations, homozygous deletion of Wnt repressors *ZNRF3/KREMEN1* and/or deactivating *APC/MEN1* alterations; (iv) evidence for epigenetic deregulation, for example, alterations of chromatin remodeling (*ATRX/DAXX*) and histone modification (*MLL/MLL2/MLL4*) genes; and (v) significant copy number changes with recurrent focal involvement of the *ZNRF3* and/or *TERT* loci. Along these lines, *TERT* promoter mutations were also detected in a small subset of ACCs in accordance with previously published data. In addition, the TCGA study expanded the transcriptomics classification and refined the multi-omics classification of two distinct molecular entities C1A/C1B driven by different oncogenic alterations into three molecular Cluster of Cluster (CoC) subtypes I/II/III with distinct clinical outcomes based on integrated analysis of DNA copy number, mRNA expression, DNA methylation and miRNA expression.

The occurrence of genome near-haploidisation with or without subsequent *endo*-reduplication has been demonstrated in thyroid, parathyroid and adrenocortical tumors with oncocytic features and without any associations with mitochondrial (mt) DNA mutations. In another study, the 4977 bp mtDNA "common deletion" was detected in approximately 50% of oncocytic adrenocortical neoplasms and with a relatively high degree of intercellular and intracellular heteroplasmy. A subset of sarcomatoid ACC was shown to be monoclonal in origin with dysregulation of *Wnt/β-catenin signaling* pathway and mutational *TP53* inactivation as frequent genetic events. In this context, SF1 immunonegativity and/or loss of expression of steroidogenic enzymes provided further evidence of a dedifferentiation process in this ACC variant.

Molecular diagnostic markers that differentiate ACCs from adenomas have been stemming from gene expression, microRNA and DNA methylation profiling analyses. Further studies are warranted in order to validate and define the level of diagnostic accuracy for such molecular tests especially in borderline adrenocortical tumors.

Genetic Susceptibility

Hereditary transmission of ACCs is uncommon: most tumors are sporadic. Nonetheless, the spectrum of genes associated with familial cancer susceptibility syndromes encompasses *TP53* (Li-Fraumeni syndrome), *IGF2/CDKN1C/H19* locus alterations on 11p15 (Beckwith-Wiedemann syndrome), mismatch repair (MMR) genes, that is, *MSH2*, *MSH6*, *MLH1*, *PMS2* (Lynch syndrome), *APC* (Familial Adenomatous Polyposis syndrome), *NF1* (Neurofibromatosis type 1), *MEN1* (Multiple Endocrine Neoplasia type 1), and *PRKAR1A* (Carney Complex). Pathogenic *MUTYH* mutations were also present in ACC patients of two series analyzed by whole-exome sequencing and with the corresponding tumors displaying loss of the wild-type *MUTYH* allele. Germline *MUTYH* mutations are associated with MUTYH-associated polyposis and of interest is that mostly benign adrenal lesions are relatively frequent in patients with polyposis, that is, Familial Adenomatous Polyposis, Attenuated Familial Adenomatous Polyposis and/or MUTYH-Associated Polyposis, who undergo abdominal imaging.

Exceedingly rare examples of ACCs have been reported arising in patients with germline *SDHC/SDHA* mutations (PGL3/PGL5), *FH* (Hereditary Leiomyomatosis and Renal Cell Cancer), *BRCA2 (*BRCA2 hereditary breast and ovarian cancer syndrome*)* and in the context of Werner syndrome, a rare genetic disease caused by pathogenic *WRN* mutations and often accompanied by various types of malignancy. However, ACC is not considered component of the tumor spectrum of the aforementioned syndromes, while evidence for biallelic inactivation in the tumor was documented only in two cases, that is, *SDHC* and *BRCA2*.

Adrenal cortical carcinoma is a core malignancy of the classical tumor spectrum of Li-Fraumeni syndrome with germline *TP53* mutations accounting for 50%–80% of ACC cases in the pediatric population. Nonetheless, only 4%–6% of carriers develop adrenal cortical tumors, indicative of co-operating genetic alterations in the tumorigenic process. Hotspot *TP53* p.R337H mutation exerts founder effect and was found in one out of 375 individuals within a total population of ~100 million in Southern and Southeastern Brazil. Those carriers exhibit variable tumor susceptibility ranging from isolated pediatric ACC cases to Li-Fraumeni or Li-Fraumeni-like syndromes.

Genotype-phenotype correlations were reported in the setting of this pleiotropic hereditary cancer syndrome with a proposed clinical gradient of germline *TP53* mutations: (i) dominant-negative missense mutations as the most severe aberrations significantly associated with early tumor onset and representing the main germline alterations in carriers who develop childhood cancers apart from ACC; (ii) nondominant-negative missense mutations which define an intermediate class with regard to clinical severity; and (iii) loss of function mutations, that is, nonsense mutations, frameshift mutations, or genomic rearrangements, as the less severe aberrations associated with later tumor onset, and mainly detected in pedigrees characterized by cancers occurring in adults. Gene alterations other than dominant-negative missense mutations were detected in the majority of pediatric ACCs; with the low-penetrant *TP53* p.R158H mutation as the predominant mutation in childhood ACC and adult tumors.

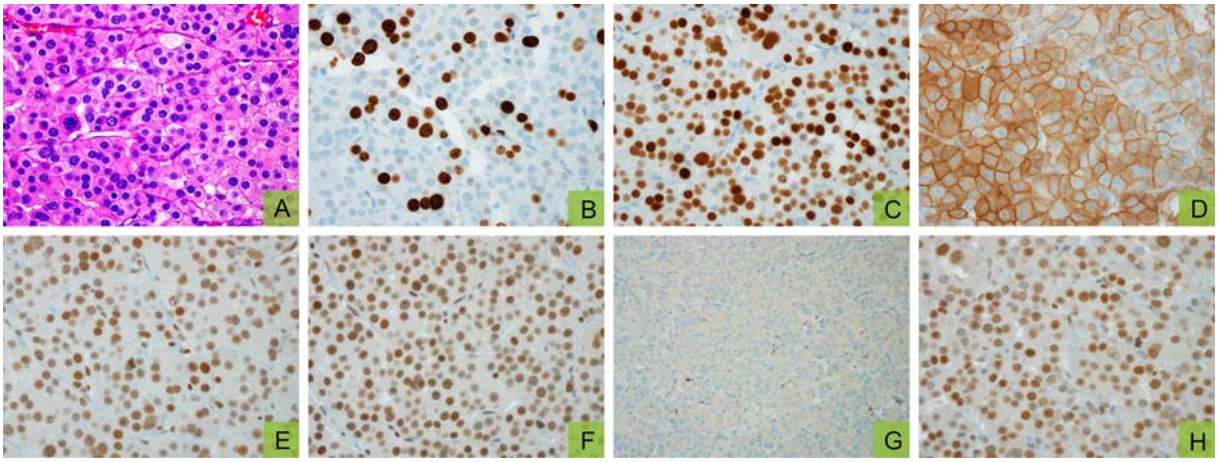

Fig. 3 Immunohistochemistry stains of a mismatch repair (MMR)-deficient adrenocortical carcinoma (A; H/E stain) including the proliferation marker Ki67 (B), p53 (C), beta-catenin (D), and the MMR panel of four markers, that is, MLH1 (E), MSH2 (F), MSH6 (G) and PMS2 (H). This tumor displays nuclear p53 and membranous beta-catenin immunoreactivity with Ki67 labeling index estimated at 22% by manual counting. As in this case a germline MSH6 mutation had been identified. MSH6 nuclear staining is absent, while nuclear immunoreactivity for MLH1, MSH2 and PMS2 is retained.

Analysis of the International Agency for Research on Cancer (IARC) TP53 database to re-evaluate age and variant-dependent tumor patterns showed that pathogenic *TP53* variants in the germline have different consequences according to cell, tissue, context and age. This indicates *variable penetrance* according to age, sex and mutation type and *temporal tumor patterns* displaying distinct phases. At 0–15 years, 22% of all malignancies includes ACC, rhabdomyosarcoma, choroid plexus carcinoma, and medulloblastoma. At 16–50 years this is 51%, encompassing breast cancer, osteosarcoma, soft tissue sarcomas, leukemia, astrocytoma and glioblastoma, colorectal and lung cancer. At 51–80 years this is 27%, comprising prostate and pancreatic cancer.

In the adult population, germline *TP53* mutations and *MMR* gene mutations account for 3%–7% and 3% of ACC cases respectively. Of note is that the prevalence of Lynch syndrome among patients with ACC is comparable to that in colorectal and endometrial cancer. Irrespective of family history and/or restriction of age at diagnosis, it is recommended that all patients with newly diagnosed ACC shall be offered genetic counseling and assessed for inherited predisposition, i.e. germline testing for *TP53* mutations and/or immunohistochemical analysis for MMR deficiency (**Fig. 3**) and subsequent molecular genetic testing for germline *MMR* gene mutations and/or *EPCAM* deletion. Given that MMR-deficient ACCs were found to be microsatellite stable, microsatellite instability analysis might not be needed as a complementary test to the IHC screening.

Microenvironment Including Immune Response

Adrenocortical carcinomas are regarded as relatively pure tumors with low fractions of tumor infiltrated stromal cells and lymphocytes in cortisol-secreting tumors; the latter mainly attributable to suppressive action exerted by glucocorticosteroids.

Staging and Grading

Staging

TNM staging of adrenal cortical carcinoma, as introduced in the 8th edition of AJCC staging manual, is consistent with the European Network for the Study of Adrenal Tumors (ENSAT) staging system. Accordingly, stage 1 and stage 2 are defined as strictly localized tumors with a size of ≤ 5 cm or > 5 cm, respectively. Stage 3 tumors are characterized by infiltration into surrounding tissue or invading adjacent organs, that is, kidney, diaphragm, pancreas, liver or great vessels (renal vein or vena cava), or positive regional lymph nodes. Stage 4 is restricted to patients with distant metastasis.

In the pediatric population, the staging system for ACCs (**Table 3**) is based on a modification of the work of Sandrini et al. by investigators from Children's Oncology Group (COG) and relies on the clinical data from the International Pediatric Adrenocortical Tumour Registry.

Grading

The mitotic rate has been incorporated in all multiparametric systems and/or diagnostic algorithms for evaluating the biological behaviour and also included in the grading of ACCs, further adding to the prognostic value of proliferative activity (see *Prognostic*

and Predictive Biomarkers). Proliferation grading of ACCs is based on mitotic rate, that is, low grade ≤ 20 mitoses per 50 high-power fields and high grade > 20 mitoses per 50 high-power fields.

The TCGA study measured adrenal cortical differentiation using a single metric, namely Adrenocortical Differentiation Score (ADS), based on 25 genes, which are of importance for adrenal function, i.e. cholesterol transporters, steroidogenic enzymes and their transcriptional regulator SF1, and with very high expression levels in the adult adrenal cortex. Of interest, higher ADS values were noted in functional tumors, but without any association with histopathological parameters, that is, Weiss score.

Prognostic and Predictive Biomarkers

The prognosis of ACC is poor, but heterogeneous, with a 5-year overall survival rate varying between 37% and 47%. The resection status and the proliferation marker Ki67 constitute the most relevant prognostic parameters in both localized and advanced ACCs. Modified ENSAT stages (stages IVa, IVb, or IVc) along with G R A S parameters [Grade (Weiss > 6 and/or Ki67 $\geq 20\%$), resection status, age ≥ 50 years and tumor- or hormone-related symptoms] correlated with overall survival in stage III–IV ACCs. Clinically relevant hypercortisolism has an adverse effect on survival in patients with completely resected ACC. Surgical margin status has an impact on long-term outcome, roughly two-thirds of ACC patients experience disease recurrence. Patients with combined locoregional and distant recurrence have worse survival than those with distant-only or locoregional-only recurrence.

Some studies confirmed Ki67 as a powerful tool for prognostic stratification, and it has been integrated into a model for prediction of metastatic disease. However, preanalytical variation, lack of standardized assessment methods and significant levels of interobserver variation pose important challenges. This is reflected in the variable threshold (10%–30%) used to incorporate the parameter into treatment flow charts to guide therapy decision making. In the pediatric setting, Ki67 proliferation marker has recently emerged as a strong prognostic indicator with Ki67 LI $\geq 15\%$ independently associated with poor prognosis.

In an effort to refine prognostic prediction in ACC, it has been recently shown that combined assessment of Ki67 LI and VAV2 expression improved patient stratification to low-risk and high-risk groups. VAV2 is a guanine nucleotide exchange factor for small GTPases that control the cytoskeleton, and that is driven by increased expression of the gene encoding SF-1 (also known as *NR5A1*) in ACC. SF1 is a marker of adrenal cortical derivation, but also of biological aggressiveness as reflected in immunohistochemical marker staining patterns. Other proliferation-based (immunohistochemical) scoring methods and/or routine histology, that is, assessment of mitotic activity, angioinvasion, and Weiss score, can provide valuable aid in determining prognosis.

In addition to the aforementioned methods, -omics derived parameters seem very promising to recognize aggressive behaviour in adrenocortical cancer. Two distinct molecular groups have been identified: the C1A subgroup with poor outcome, characterized by numerous mutations as well as alterations of DNA methylation, and the C1B subgroup with good prognosis, exhibiting specific deregulation of two microRNA clusters. Furthermore, three ACC subtypes have been identified with distinct clinical outcome, based solely on integrated multi-platform molecular profiling: Cluster of Cluster (CoC) I/II/III. CoC I ACCs showed better outcome, significantly unregulated genes in immune-mediated pathways and lower Ki67 expression levels, whereas CoC III tumors showed dismal outcome, significant disregulation of genes in mitotic pathways and higher Ki67 defined proliferative activity. CoC II ACCs had more heterogeneous outcome.

A recent TCGA study showed that high mutation density and a specific copy-number phenotype, characterized by a high number of chromosomal breaks and frequent loss of 1p (with 1q intact), are associated with aggressive disease. Whole genome doubling, associated with *TERT* expression along with telomere shortening, predict dismal outcome in ACC. This is consistent with recent immunohistochemical data suggesting a better prognosis of ACCs with loss of expression of the telomere regulator DAXX.

Methylation profiles support a correlation between CpG island hypermethylation and poor survival. Tumor DNA methylation has recently emerged as independently prognostic for disease-free survival (along with stage) and overall survival (along with stage and Ki67 proliferative index) in ACC. miRNA expression signatures (high levels of miR-483-5p and/or low levels of miR-195) have been reported to predict worse overall survival. Expression of TOP2A, EZH2 and BARD1, identified through gene expression studies, might provide additional prognostic information. Overexpression of histone methyltransferase EZH2, a result of deregulated *P53/RB/E2F* pathway activity, has been associated with increased proliferation and poor prognosis.

With regard to pediatric adrenocortical cancer, recent National Cancer Data Base (NCDB) and European Cooperative Study Group on Pediatric Rare Tumors (EXPeRT) studies showed that age ≥ 4 years, tumor size ≥ 10.0 cm in diameter, extension of primary disease into adjacent structures, metastatic disease, and margin status were significantly associated with poor long-term survival. Furthermore, distant metastatic disease and large tumor volume > 200 cm^3 were the main unfavorable prognostic factors. Of note, a cut-off ≥ 10.0 cm in tumor diameter, corresponding to a tumor volume of 524 cm^3, was found to be of prognostic relevance, consistent with data generated by the Surveillance, Epidemiology, and End Results (SEER) program study on pediatric ACC. This challenges the 200 cm^3 threshold currently used in the COG modification of the Sanrini et al. staging system.

In pediatric cases, concomitant germline *TP53* and somatic *ATRX* mutations as well as associated genomic aberrations, such as massive structural variations and/or frequent background mutations, are associated with a dismal outcome. Such cases showed high tumor weight and advanced disease (COG stage III/IV). Glucose transporter 1 (GLUT1) was found to be more strongly expressed in clinically malignant pediatric adrenocortical tumors and associated with shorter overall and disease-free survival. Of note, metabolic reprogramming towards a hyperglycolytic and acid-resistant phenotype is also found in adult cases, with GLUT1 emerging as a stage-independent predictor of outcome. Accordingly, ACC patients with a high metabolic tumor volume, total lesion glycolysis and maximum standardized uptake value by 18F-FDG PET/CT prior to treatment, had worse overall survival.

Human cytochrome P450 2W1 (CYP2W1), ribonucleotide reductase large subunit 1 (RRM1) and sterol-O-acyl-transferase 1 (SOAT1) might represent predictive markers for the response to mitotane. This agent confers adrenal-specific cytotoxicity and down-regulates steroidogenesis. However, mitotane is significantly toxic. Topoisomerase II alpha (TOP2A) and thymidylate synthase (TS) were not found predictive of mitotane efficacy in ACC patients. These markers await further validation in prospective studies.

Further Reading

Assié, G., Letouzé, E., Fassnacht, M., Jouinot, A., Luscap, W., Barreau, O., et al., 2014. Integrated genomic characterization of adrenocortical carcinoma. Nature Genetics 46 (6), 607–612.

Bausch, B., Schiavi, F., Ni, Y., Welander, J., Patocs, A., Ngeow, J., et al., 2017. Clinical characterization of the pheochromocytoma and paraganglioma susceptibility genes SDHA, TMEM127, MAX, and SDHAF2 for gene-informed prevention. JAMA Oncology 3 (9), 1204–1212.

Carney, J.A., 2013. Carney triad. Frontiers of Hormone Research 41, 92–110.

Castro-Vega, L.J., Lepoutre-Lussey, C., Gimenez-Roqueplo, A.P., Favier, J., 2016. Rethinking pheochromocytomas and paragangliomas from a genomic perspective. Oncogene 35 (9), 1080–1089.

Castro-Vega, L.J., Letouzé, E., Burnichon, N., Buffet, A., Disderot, P.H., Khalifa, E., et al., 2015. Multi-omics analysis defines core genomic alterations in pheochromocytomas and paragangliomas. Nature Communications 6, 6044.

Dahia, P.L., 2014. Pheochromocytoma and paraganglioma pathogenesis: Learning from genetic heterogeneity. Nature Reviews Cancer 14 (2), 108–119.

de Krijger, R.R., Papathomas, T.G., 2012. Adrenocortical neoplasia: Evolving concepts in tumorigenesis with an emphasis on adrenal cortical carcinoma variants. Virchows Archiv 460 (1), 9–18.

de Reyniès, A., Assié, G., Rickman, D.S., Tissier, F., Groussin, L., René-Corail, F., et al., 2009. Gene expression profiling reveals a new classification of adrenocortical tumors and identifies molecular predictors of malignancy and survival. Journal of Clinical Oncology 27 (7), 1108–1115.

Else, T., Kim, A.C., Sabolch, A., Raymond, V.M., Kandathil, A., Caoili, E.M., et al., 2014. Adrenocortical carcinoma. Endocrine Reviews 35 (2), 282–326.

Favier, J., Amar, L., Gimenez-Roqueplo, A.P., 2015. Paraganglioma and phaeochromocytoma: From genetics to personalized medicine. Nature Reviews Endocrinology 11 (2), 101–111.

Fishbein, L., Leshchiner, I., Walter, V., Danilova, L., Robertson, A.G., Johnson, A.R., et al., 2017. Comprehensive molecular characterization of pheochromocytoma and paraganglioma. Cancer Cell 31 (2), 181–193.

Giordano, T.J., Kuick, R., Else, T., Gauger, P.G., Vinco, M., Bauersfeld, J., et al., 2009. Molecular classification and prognostication of adrenocortical tumors by transcriptome profiling. Clinical Cancer Research 15 (2), 668–676.

Huang KL, Mashl RJ, Wu Y, Ritter DI, Wang J, Oh C, et al. (2018) Pathogenic germline variants in 10,389 adult cancers. *Cell* 173(2): 355–370.e14.

Jimenez, C., Libutti, S.K., Landry, C.S., et al., 2017. Adrenal—Neuroendocrine tumours. In: AJCC cancer staging manual, 8th edn. Springer International Publishing.

Lloyd, R.V., Osamura, R.Y., Kloppel, G., Rosai, J. (Eds.), 2017. WHO classification of tumours of endocrine organs, 4th edn. IARC, Lyon.

Papathomas, T.G., de Krijger, R.R., Tischler, A.S., 2013. Paragangliomas: Update on differential diagnostic considerations, composite tumors, and recent genetic developments. Seminars in Diagnostic Pathology 30 (3), 207–223.

Phan, A.T., Grogan, R.H., Rohren, E., Perrier, N.D., 2017. Adrenal cortical carcinoma. In: AJCC cancer staging manual, 8th edn. Springer International Publishing.

Pinto, E.M., Chen, X., Easton, J., Finkelstein, D., Liu, Z., Pounds, S., et al., 2015. Genomic landscape of pediatric adrenocortical tumors. Nature Communications 6, 6302.

Zheng, S., Cherniack, A.D., Dewal, N., Moffitt, R.A., Danilova, L., Murray, B.A., et al., 2016. Comprehensive pan-genomic characterization of adrenocortical carcinoma. Cancer Cell 29 (5), 723–736.

Aflatoxins

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Introduction

As one of the most potent naturally occurring known carcinogens, aflatoxins represent a special case in the study of cancer etiology (Squire, 1981). Research spanning more than five decades has created a detailed corpus of histological, biochemical, and molecular toxicology, which comprehensively and convincingly characterizes the carcinogenic mechanisms following aflatoxin exposure (Kensler et al., 2011; Wogan et al., 2012). These efforts have produced a suite of non- and minimally-invasive biomarkers, the use of which have allowed the mechanistic relation of aflatoxin exposures to internal dose, DNA adduct formation, and a signature carcinogenic mutation in the tumor suppressor p53. Harnessing these tools, epidemiological studies have consistently demonstrated a significantly increased risk of hepatocellular carcinoma (HCC) from dietary aflatoxin exposure (Liu et al., 2012) and, most dramatically, a synergistic increase in risk for those also chronically infected with hepatitis B virus (HBV) (Wild et al., 2009). Consequently, out of total global HCC incidence (nearly 800,000 new cases per year (Ferlay et al., 2015)), it is estimated that approximately one quarter is attributable to aflatoxin exposure, either alone or in combination with chronic viral hepatitis (Liu et al., 2010, 2012). The International Agency for Research on Cancer (IARC) declared aflatoxins, as a group, to be human carcinogens in 1987 (International Agency for Research on Cancer, 1987), a classification that has been confirmed in each of three subsequent reviews (International Agency for Research on Cancer, 1993, 2002, 2012). Concordantly, cancer prevention efforts have benefitted from the same half-century of molecular toxicology that enabled the determination of aflatoxins' carcinogenicity. Biomarkers of aflatoxin exposure, with their mechanistically defined relationships to HCC development, unshackled scientists from the necessity to directly link exposures to cancer outcomes, greatly reducing the time and resources required to test novel preventative measures. As a result, a wide array of promising primary and secondary prevention strategies have been identified (Groopman et al., 2008; International Agency for Research on Cancer, 2015), some of which have now been associated with reductions in HCC mortality (Chen et al., 2013). The goals of this chapter are not only to describe the carcinogenic effects of aflatoxin exposure and to recount major discoveries in aflatoxin research, but also to highlight throughout the strength and breadth of this body of literature.

Sources of Aflatoxin Exposure

Aflatoxins are secondary metabolites produced by members of the fungal genus *Aspergillus*, which contains approximately 250 known species (Klich, 2007). However, less than a dozen are known to produce aflatoxins (International Agency for Research on Cancer, 2002; Klich, 2007; Bennett et al., 2003) and only *A. flavus* and *A. parasiticus* are truly relevant to human health through contamination of the food supply. *A. flavus* is found in soils across the globe, but is more common in tropical and sub-tropical climates between latitudes 35 degrees north and south of the equator (Klich, 2007; Abbas et al., 2009; Council for Agricultural Science and Technology, 2003). Regardless, due to globalized trade, dietary aflatoxins are a worldwide concern. A major distinction between these two aflatoxigenic species are their profiles of toxin production—while *A. parasiticus* produces both the B and G series of aflatoxins, *A. flavus* produces only B toxins (Dorner et al., 1984; Klich et al., 1988).

Many factors can influence the crop colonization, growth, and toxin production of *Aspergillus* species, notably heat, humidity, pest or environmental host stressors, and post-harvest practices (Abbas et al., 2009; Pitt et al., 2013; Diener et al., 1987). Maize, groundnuts, cottonseed, and tree nuts represent the most common sources of food supply contamination worldwide, as these crops are preferred by *Aspergillus* for colonization pre-harvest, while also being susceptible to contamination due to improper drying and storage conditions post-harvest. However, although other crops like wheat, sorghum, and rice are much less susceptible to pre-harvest colonization, poor post-harvest handling can also encourage aflatoxin contamination in these stored commodities and levels of contamination vary widely (International Agency for Research on Cancer, 2002; Council for Agricultural Science and Technology, 2003).

Aflatoxin Discovery and Structural Elucidation

The aflatoxins were first discovered as a result of their role in the acute intoxication and death of many thousand British poultry in 1960 (Blount, 1961). The toxic outbreaks were linked to moldy groundnut meal used in animal feed (Blount, 1961) and crude extracts revealed the presence of a potent substance produced by *A. flavus*, which was able to recapitulate the lethal and histological effects of the original toxic feed (Sargeant et al., 1961). The carcinogenic potential of the as-yet-unidentified agent was quickly suspected, however, as liver tumors were found in rats fed the toxic groundnut meal (Lancaster et al., 1961). Crude extracts were revealed to contain at least two primary constituents (van der Zijden et al., 1962; De Iongh et al., 1962), which, given their mycological source and intense blue or green fluorescence, were designated as aflatoxin (*A. flavus* toxin) B and G, respectively (Nesbitt et al., 1962). Büchi, Wogan, and colleagues proposed the structures of AFB_1 and AFG_1 in 1963 (Asao et al., 1963), followed by

a longer report 2 years later (Asao et al., 1965), in which they identified the structures of AFB_2 and AFG_2. In 1967, Büchi's group produced the first total synthesis of AFB_1 (Büchi et al., 1967). The structures of the naturally occurring aflatoxins are shown in Fig. 1.

Aflatoxin Metabolism and Molecular Mechanisms of Carcinogenicity

Human exposure to aflatoxins occurs through the diet, however, these parent compounds are not carcinogenic per se. Rather, as procarcinogens, they must first be transformed in vivo to their carcinogenic derivative (Smith et al., 2016; Rendic et al., 2012). The ultimate carcinogenic metabolite of AFB_1, AFB_1-*exo*-8,9-epoxide, is produced in the liver through oxidation of AFB_1 by cytochrome P450 (CYP) enzymes. If not conjugated by GSTs, the AFB_1-*exo*-8,9-epoxide intercalates with DNA, facilitating electrophilic attack on guanine residues at the N^7 position, leading to mutagenesis.

Structure–Activity Relationships Driving Aflatoxin Carcinogenicity

Mixtures of the four naturally occurring aflatoxins (AFB_1, AFG_1, AFB_2, and AFG_2) represent the relevant form of aflatoxin exposure for humans and have been classified by IARC as a Group 1 carcinogen, indicating sufficient evidence for carcinogenicity in humans (Ostry et al., 2017). However, while carcinogenic as mixed species (Carnaghan, 1967; Norred et al., 1983), in their purified forms, these four aflatoxins are not equally potent. In bacterial mutagenicity assays, with AFB_1 as the reference, AFG_1 is 3% as potent, and both AFB_2 and AFG_2 retain $<1\%$ activity (Wong et al., 1976). In general, carcinogenesis studies agree both with this order and the relative decrements in potency, to the point where AFG_2 is essentially non-toxic (Wogan et al., 1971, 1974a; Bailey et al., 1988; Ayres et al., 1971; Butler et al., 1969). AFB_1, by far the most carcinogenic of the group, displays a dose–response in carcinogenicity in rats down to 1 ppb in the diet ($\sim$0.2 μg/kg bw) with long-term dietary exposure (Wogan et al., 1974a), while very large studies in trout suggest that it may retain carcinogenic potency well below the 1 ppb level (Williams et al., 2009). Single large doses of purified AFB_1 cause substantial rates of hepatocarcinogenesis in rats (Carnaghan, 1967), while even a single 15 minute exposure to 0.5 ppm AFB_1 is carcinogenic in trout (Bailey et al., 1988).

Appreciation of critical structural differences between these similar compounds reveals structure–activity relationships responsible for their differing carcinogenicities. As shown in Fig. 1, all four naturally occurring aflatoxins share a common structural feature—the methoxylated difuranocoumarin backbone. Very strong biochemical evidence supports the 8,9-*exo*-epoxide (Fig. 2) as the ultimately carcinogenic DNA-adducting metabolite of AFB_1 (Swenson et al., 1973, 1974, 1977) and its generation depends on the oxidation potential of the 8,9 carbon–carbon bond of the outermost furan ring (Guengerich et al., 1998) (or the 9,10 bond in G series aflatoxins). A blunt illustration of this requirement is provided by synthetic analogues entirely lacking the furofuran moiety (e.g., 5,7-dimethoxycyclopentenone coumarins)—these agents are completely inactive as liver carcinogens in the trout (Ayres et al., 1971) or at high doses in the rat (Wogan et al., 1971). Comparison of AFB_2 and AFB_1 provides a more relevant perspective. Saturation of the 8,9 double bond in AFB_1 significantly reduces the carcinogenicity of AFB_2: doses of AFB_2 115-fold greater than that of AFB_1 induced fewer tumors in rats (Wogan et al., 1971), while equivalent doses of AFB_1 and AFB_2 in trout induced liver tumors at drastically different rates (78% vs. 5%) (Ayres et al., 1971). Molecular evidence supports these studies, showing that administration of radiolabeled AFB_2 to rats results in hepatic DNA adduct formation at levels approximately 1% that of an equivalent dose of radiolabeled AFB_1 (Swenson et al., 1977).

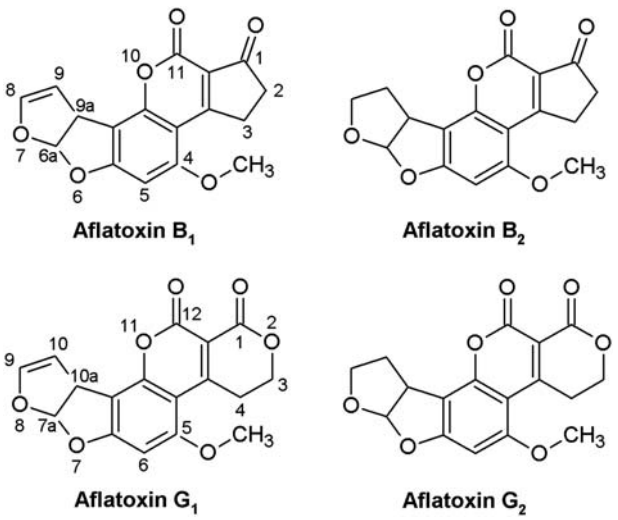

Fig. 1 Structures of the naturally occurring aflatoxins.

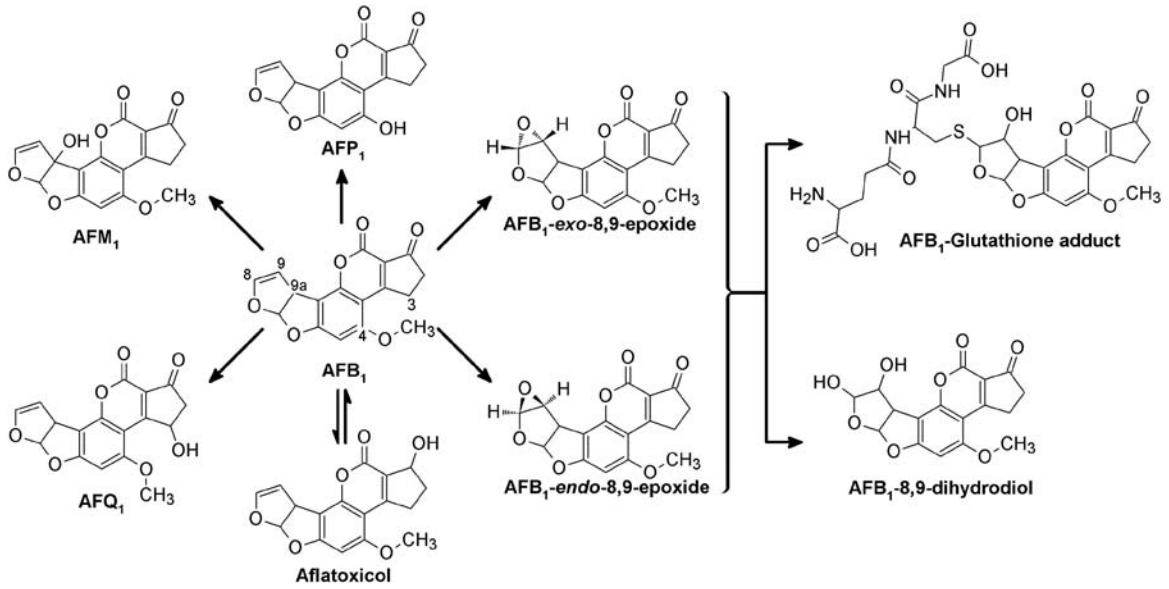

Fig. 2 AFB_1 metabolism.

Oxidation of AFB_1 by cytochrome P450s produces two stereoisomers: the AFB_1-8,9-*exo*- and AFB_1-8,9-*endo*-epoxides (Guengerich et al., 1998; Dohnal et al., 2014). However, the *exo* epoxide is $\sim 10^3$ times as mutagenic as the *endo* (Iyer et al., 1994; Ueng et al., 1995), which exhibits negligible reactivity towards double-stranded DNA (Iyer et al., 1994). Intercalation of the 8,9-epoxide into DNA dramatically enhances adduction—single-stranded DNA is 6.5-fold less reactive with the epoxide than is double-stranded DNA (Johnson et al., 1997a), while adduction to free deoxyguanosine is 2000-fold less efficient (Brown et al., 2009). While both the *exo*- and *endo*-epoxides intercalate into double-stranded DNA (Gopalakrishnan et al., 1989, 1990), structural differences result in differing rates of adduct formation. After intercalation, the oxirane moiety of the *exo*-epoxide faces away from the guanine N^7, providing optimal orientation for S_N2 attack (Iyer et al., 1994). In contrast, the position of the *endo*-epoxide precludes the reaction, and consequently, adduction. Secondly, calculations from molecular modeling suggest that steric hindrance from solvating water molecules during the reaction with guanine is systematically greater in the *endo* configuration than the *exo* (Okajima et al., 2000; Bren et al., 2007). These mechanisms likely explain why the *exo*-epoxide is a highly potent carcinogen, while the *endo* isomer is non-toxic.

Finally, additional structural differences between aflatoxin species affects their intercalation into DNA and, thus, carcinogenic potency. Compared to the cyclopentenone ring of AFB_1 and AFB_2, the lactone substituent of the G series aflatoxins is less planar (Cheung et al., 1964); as a result, AFG_1 and AFG_2 intercalate into DNA with an affinity approximately one order of magnitude lower than AFB_1 and AFB_2 (Raney et al., 1990). AFB_1-8,9-epoxide forms more guanine adducts than its equivalent AFG_1 epoxide at equivalent concentrations, and, accordingly, AFG_1 has been shown to be less carcinogenic than AFB_1 in both rats (Wogan et al., 1971) and trout (Ayres et al., 1971).

To summarize, saturation of the furofuran ring is the largest determinant of carcinogenic activity and separates the relatively nontoxic AFB_2 and AFG_2 from the carcinogenic AFB_1 and AFG_1. Further refinement in potency is provided by substitution of the cyclopentenone ring for a lactone ring, resulting in the reduced intercalation efficiency and DNA adduction of AFG_1 compared to AFB_1.

Aflatoxin Metabolism

Phase I activation

As with procarcinogen metabolism more generally (Rendic et al., 2012), the P450s are the major players in aflatoxin oxidation (Guengerich et al., 1998; Dohnal et al., 2014). Multiple CYPs have been shown to activate AFB_1 (Pelkonen et al., 1997; Aoyama et al., 1990), but CYP3A4 appears to be the enzyme of greatest relevance for AFB_1 epoxidation in humans (Guengerich et al., 1998; Dohnal et al., 2014; Ueng et al., 1995; Shimada et al., 1989); one report in human liver microsomes suggests that CYP3A4 accounts for 79%–95% of total AFB_1-8,9-epoxide formation (Kamdem et al., 2006). This has been somewhat controversial, though, as some epidemiological data suggests that polymorphisms in CYP1A2 modify HCC risk (Chen et al., 2006), and experimental reports suggested CYP1A2 as the dominant enzymatic source of AFB_1 epoxidation, rather than 3A4 (Gallagher et al., 1994, 1996). However, CYP1A2 hepatic protein content is 10–25 times lower than CYP3A4 and, even granting the former an advantage in reaction rate, 1A2 is likely far outstripped by 3A4 in total AFB_1 epoxidation capacity (Kamdem et al., 2006). Moreover, while CYP3A4 produces only the mutagenic AFB_1-8,9-*exo*-epoxide but not the non-toxic *endo*-epoxide, CYP1A2-derived AFB_1 epoxides are split roughly equally between the *exo* and *endo* stereoisomers (Ueng et al., 1995). Finally, higher contribution of other CYP enzymes to

AFB_1 epoxidation has been shown to depend on concurrently low CYP3A4 activity (Kamdem et al., 2006; Wojnowski et al., 2004).

Genetic differences may impact AFB_1 epoxidation and risk of carcinogenesis. Several reports have demonstrated very high inter-individual variation in hepatic P450 protein content—up to 57-fold in CYP3A4 (Kamdem et al., 2006; Doi et al., 2002; Kirby et al., 1993). Accordingly, microsomal AFB_1 epoxide formation exhibits high variability (Kamdem et al., 2006) and correlates with CYP3A4 expression (68). Male rats fed AFB_1 demonstrated two- to threefold higher hepatic microsomal P450 content and AFB_1-DNA adduct formation than AFB_1-fed female rats, but castration of males lowered these values to those in females, while testosterone administration in castrated males rescued values to those of intact males (Gurtoo et al., 1976). A similar result was shown with hypophysectomy (Swenson et al., 1977; Goodall et al., 1969), while testosterone administration to female rats resulted in a phenotype very similar to that of intact males (Gurtoo et al., 1976). In the analysis of human hepatic microsomes by Kamdem et al., males exhibited a median rate of aflatoxin epoxidation nearly 50% higher than females (Kamdem et al., 2006). These mechanisms may explain, at least in part, the epidemiological evidence showing that men are at two- to threefold higher risk of HCC incidence than women (Petrick et al., 2016).

Once formed, the 8,9-*exo*-epoxide is highly unstable in solution ($t_{½} \approx 1$ s), rapidly undergoing hydrolysis to the 8,9-dihydrodiol (Johnson et al., 1996) (**Fig. 2**). Hydrolysis of the carcinogenic *exo*-epoxide is catalyzed enzymatically by epoxide hydrolase, but in vitro evidence suggests that only supra-physiological expression of the enzyme would be able to provide added protection from genotoxicity (Johnson et al., 1997b). Despite this, epidemiological studies have revealed elevated AFB_1 adduct levels (McGlynn et al., 1995; Dash et al., 2007) or an increased risk of HCC in individuals carrying mutant alleles of the enzyme (McGlynn et al., 1995; Tiemersma et al., 2001). An alternate possibility is that epoxide hydrolase status may impact HCC risk independently from aflatoxin metabolism (Tiemersma et al., 2001; Rahat et al., 2012).

Major metabolites of AFB_1

AFB_1 is also detoxified through conversion to less-potent metabolites (**Fig. 2**). CYP3A4 indeed produces the AFB_1-8,9-*exo*-epoxide, but also catalyzes hydroxylation of the cyclopentenone ring to yield aflatoxin Q_1 (AFQ_1). The yield for each of these products is vastly different, with production of AFQ_1 being up to 8-fold greater than the epoxide (Ueng et al., 1995; Wang et al., 1998). Similarly, another major metabolite, AFM_1, is produced though 9a-hydroxylation by CYP1A2, at a ratio of up to 5:1 AFM_1:epoxide (Ueng et al., 1995). Additionally, AFB_1 can be demethylated to aflatoxin P_1 (AFP_1) (Dalezios et al., 1971, 1973) or subjected to ketoreduction at the cyclopentenone ring to form aflatoxicol. Notably, these major detoxification products (AFM_1, AFQ_1, AFP_1, and aflatoxicol) all retain the crucial 8,9 double bond and thus themselves can be epoxidized at this site, capable of DNA adduct formation (Bujons et al., 1995; Neal et al., 1998; Eaton et al., 1988; Essigmann et al., 1980). However, it appears that very little epoxidation occurs relative to AFB_1 (Neal et al., 1998; Eaton et al., 1988), limiting their mutagenicity. Indeed, while AFM_1 is carcinogenic in its own right (International Agency for Research on Cancer, 2002; Hsieh et al., 1984; Cullen et al., 1987; Wogan et al., 1974b), AFM_1, AFQ_1, and AFP_1 are all at least 10 times less potent than AFB_1 (Wong et al., 1976). An exception is aflatoxicol, which retains ~20%–50% the potency of its parent (Wong et al., 1976; Garner et al., 1972). Interestingly, although the epoxide of aflatoxicol can form DNA adducts in vitro, aflatoxicol-DNA adducts are undetectable after dietary aflatoxicol treatment in vivo, while AFB_1 adducts are readily observed (Bailey et al., 1994). This, as well as the high potency of aflatoxicol relative to the other major AFB_1 metabolites, can be explained by the in vivo conversion of aflatoxicol back to AFB_1 (Salhab et al., 1976). In total, nearly 90% of cytochrome P450 oxidation products may be diverted away from epoxidation during initial metabolism of AFB_1 (Kamdem et al., 2006).

Phase II conjugation

Phase II detoxification involves the conjugation of phase I-activated electrophiles to endogenous nucleophiles, to create polar metabolites that may be safely passed in the urine or excreted through biliary secretion into feces. In general, most of this work is performed through hepatic glucuronidation by UDP-glucuronosyl transferases (UGT) or adduction to glutathione by glutathione S-transferase (GST) enzymes (Rendic et al., 2012). As discussed above, the bulk of ingested AFB_1 is converted to its hydroxylated metabolites (AFM_1, AFQ_1, AFP_1), which are subsequently glucuronidated or sulfonated and excreted (Dalezios et al., 1973; Wei et al., 1985a, b; Wogan et al., 1967; Raj et al., 1984). However, as for the carcinogenic epoxide, GST-dependent conjugation to glutathione (**Fig. 2**) appears to be the most critical phase II detoxification pathway (Appleton et al., 1982; Monroe et al., 1988; Lotlikar et al., 1980; Raj et al., 1986), as well as a significant source of variation for between- and within-species aflatoxin sensitivity (Dohnal et al., 2014; Hayes et al., 1991). With the exceptions of GSTM1 and GST1, most human GST isoforms do not conjugate the AFB_1 epoxide well, or at all (Raney et al., 1992; Johnson et al., 1997c). However, primary hepatocytes from GSTM1-positive subjects—but not GSTM1-null—have been shown to be competent in glutathione conjugation of AFB_1-8,9-epoxide (Langouët et al., 1995; Gross-Steinmeyer et al., 2010), GSTM1-null individuals were more likely than GSTM1-positive to have elevated AFB_1 adduct levels (McGlynn et al., 1995), and GSTM1 expression and GST conjugating activity have been shown to be lower in liver tumors than neighboring normal tissue (Kirby et al., 1993). Accordingly, GST polymorphisms—especially in GSTM1—have been shown to increase HCC risk in numerous epidemiological studies, particularly in the context of high aflatoxin exposure (Sun et al., 2001; Kirk et al., 2005a; Long et al., 2006; Shen et al., 2014).

Due to metabolic differences throughout the aflatoxin detoxification pathway (Dohnal et al., 2014), animal species exhibit substantial variability in sensitivity to aflatoxin (Newberne et al., 1969). However, the capacity to detoxify the AFB_1-epoxide through conjugation to glutathione appears to be the largest determinant of a species' sensitivity to aflatoxins, and thus the

most crucial of the metabolic idiosyncrasies between clades. Beginning with the paper from Degen et al. (1981), many reports have characterized the divergent susceptibilities of adult rats (sensitive) and mice (resistant) to be the result of a striking difference in AFB_1-epoxide detoxification capacity (Monroe et al., 1987, 1988; O'Brien et al., 1983). Subsequent interspecies comparisons revealed that the significant kinetic advantage of murine hepatic cytosolic fractions over those from rats (7–50-fold) and humans (300–3500-fold) (Raney et al., 1992; Slone et al., 1995) was not simply due to greater enzymatic abundance, but that reaction rates for purified mouse GSTs were orders of magnitude greater than their rat and human homologues (Raney et al., 1992; Johnson et al., 1997c; Buetler et al., 1992). However, differences in GST expression may impact aflatoxin sensitivity *within* a species—relative to male rats, female rats are more resistant to aflatoxin-induced DNA adduction (Gurtoo et al., 1976), apparently due to 10-fold higher expression of GSTA5 (Hayes et al., 1994) and greater epoxide detoxification (Degen et al., 1981). Additionally, temporal shifts in aflatoxin metabolism may dramatically impact the sensitivity of an organism throughout its development. While adult mice are resistant to aflatoxin-induced carcinogenesis, infant mice have long been known to be much more sensitive (Vesselinovitch et al., 1972). Recent work has shown that prenatal/neonatal AFB_1 exposure induced 20 times more mutations than exposures during adulthood (Chen et al., 2010; Chawanthayatham et al., 2015). Moreover, Shupe et al. have shown that murine hepatic GST content rose fivefold from 4 days postnatal to the end of the first year of life, while DNA adducts from AFB_1 gavage decreased 13-fold over the same period (Shupe et al., 2004).

Targeting Nrf2 for chemoprotective modulation of aflatoxin metabolism

Nuclear factor erythroid 2 like 2 (Nrf2) is a crucial regulator of the endogenous antioxidant response, which protects cells from oxidative damage by reactive oxygen species and electrophiles (Suzuki et al., 2015). This is accomplished through binding of Nrf2 to the antioxidant response element (ARE) motif within the promoter regions of phase II detoxifying enzymes, including GSTs (Itoh et al., 1997; Nguyen et al., 2000; Kensler et al., 2007). Indeed, loss of *Nrf2* in rodents increases sensitivity to chemical carcinogenesis (Ramos-Gomez et al., 2001; Taguchi et al., 2016), while stabilization of Nrf2 through disruption of its negative regulator, Keap1, results in increased expression of cytoprotective Nrf2-target genes (Thimmulappa et al., 2002; McWalter et al., 2004; Yates et al., 2007). Mice lacking the Nrf2 target GSTA3 (Jowsey et al., 2003) are rendered hypersensitive to dietary AFB_1 treatments, with AFB_1-DNA adducts accumulating to levels >100-fold greater than in wild-type mice (Kensler et al., 2014; Ilic et al., 2010). However, neither simultaneous deletion of Keap1 nor pharmacological activation of Nrf2 was able to rescue adduct levels in $GSTA3^{-/-}$ mice back to control levels, despite the presence of an active Nrf2 signaling pathway (Kensler et al., 2014).

Similar approaches have been used to identify chemopreventative strategies for translation to at-risk human populations. Studies with oltipraz, a dithiolthione previously studied as an antischistosomal agent, have demonstrated it to be an inducer of GST expression and activity (Davidson et al., 1990; Kensler et al., 1985, 1987; Primiano et al., 1995), an inhibitor of AFB_1-DNA adduct formation (Kensler et al., 1985, 1987), and protective against AFB_1-induced hepatocarcinogenesis (Kensler et al., 1997). While oltipraz does appear to be a modulator of cytochrome P450-dependent AFB_1 oxidation (Langouët et al., 1995; Kensler et al., 1985, 1987), DNA adduction by AFB_1 was inversely and highly correlated with induction of GST activity (Kensler et al., 1985, 1987), suggesting this to be the predominant effect on AFB_1 metabolism. Another inducer of carcinogen detoxification, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), has been shown to exhibit exceptional chemoprotective effects in animals exposed to AFB_1. Demonstrating that CDDO-Im acts through Nrf2, Taguchi et al. reported that in wild-type rats—but not Nrf2-knockouts—CDDO-Im potently induced Nrf2, GSTA3, and GSTA5, while reducing hepatic AFB_1-DNA adducts (Taguchi et al., 2016). Others have shown that CDDO-Im dose-dependently induced GST expression and inhibited hepatic AFB_1-DNA adduct formation (Yates et al., 2006), while another report revealed complete protection against AFB_1-induced hepatocarcinogenesis with simultaneous administration of CDDO-Im (Johnson et al., 2014). Finally, natural agents, such as the isothiocyanate sulforaphane, are able to provide protection against aflatoxin exposure through the same Nrf2-dependent mechanism (Kensler et al., 2013; Wakabayashi et al., 2004). Although not as potently as CDDO-Im, sulforaphane induces phase II detoxification genes (Hu et al., 2004) in an Nrf2-dependent manner (Thimmulappa et al., 2002) and inhibits the formation of mutagenic AFB_1-DNA adducts (Fiala et al., 2011).

DNA Adduct Formation

Prior to DNA adduction, the AFB_1-8,9-*exo*-epoxide must remain stable long enough to enter the nucleus and interact with DNA after formation in the endoplasmic reticulum by P450s. Given the rapid solvolysis of the AFB_1-*exo*-epoxide in aqueous solution (Johnson et al., 1996), a pertinent consideration is a comparison of the rate of epoxide hydrolysis with its anticipated rate of encounter with DNA. Based on estimates of diffusion rate and distance within the cell, Johnson et al. have calculated the epoxide's rate of interaction with DNA to be roughly 10^{11}-times greater than its spontaneous degradation (Johnson et al., 1997a). As described above in the section "Structure–activity relationships driving aflatoxin carcinogenicity", intercalation of the AFB_1-8, 9-*exo*-epoxide into double-stranded DNA facilitates S_N2 attack on the N^7 position of guanine residues; specifically, Kobertz et al. have shown that this intercalation must occur to the 5′ side of guanine (Kobertz et al., 1997). The AFB_1-N^7-guanine adduct (N^7-guanine; **Fig. 3**) was first identified in vitro by Wogan and colleagues (Essigmann et al., 1977), followed by their demonstration of its dose-dependent formation in vivo (Croy et al., 1978). Due to its positively charged imidazole ring, the N^7-guanine DNA adduct is unstable and susceptible to either spontaneous depurination or base-catalyzed hydrolysis and imidazole ring-opening (**Fig. 3**). In the former case, this produces an apurinic (AP) site and the free AFB_1-N^7-guanine adduct, which is excreted and can be detected in the urine (Bennett

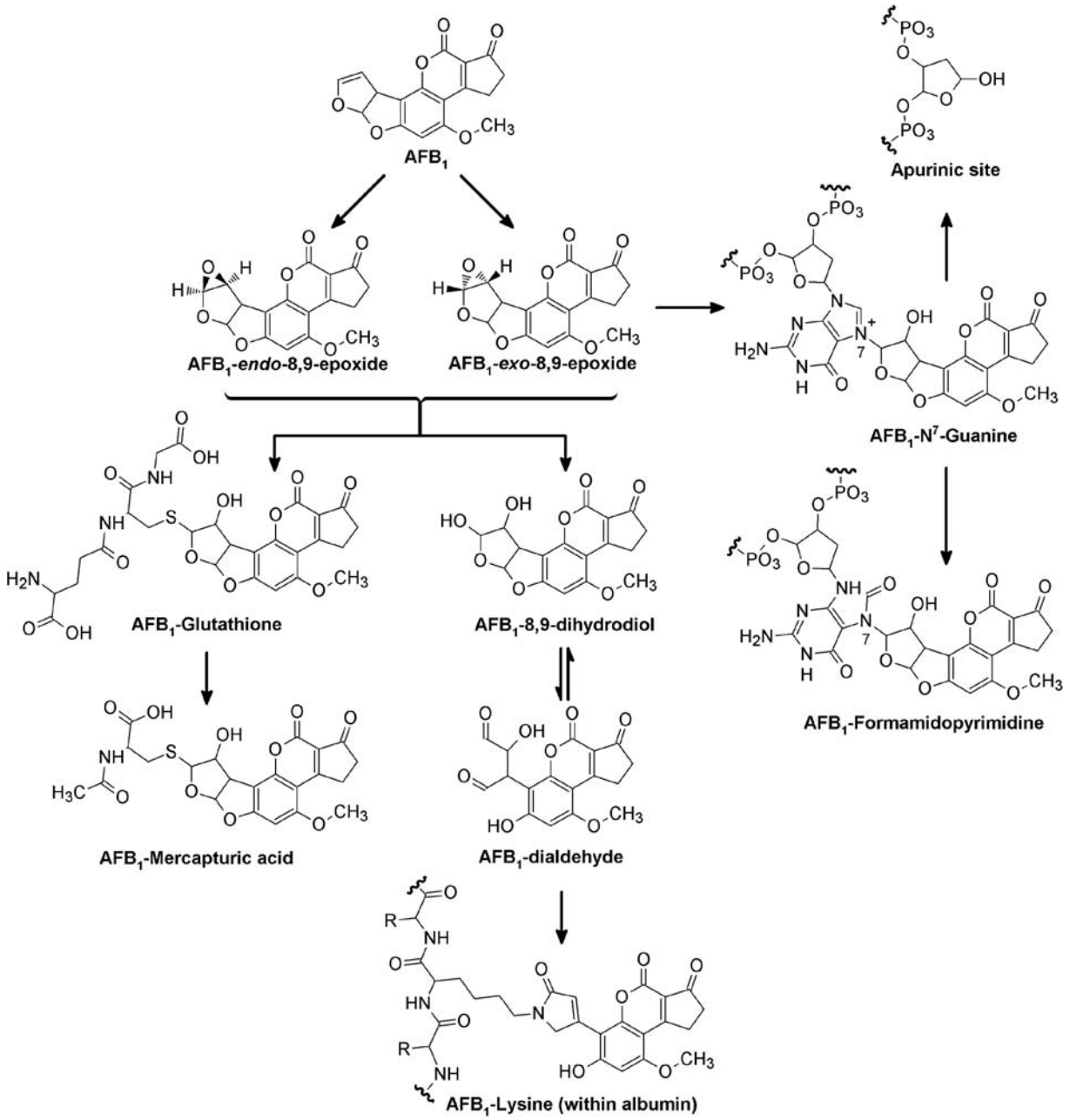

Fig. 3 Formation of major AFB_1 adducts.

et al., 1981). Spontaneous depurination is not uncommon in mammalian cells in general (Loeb et al., 1986) and dose–response experiments in rats suggest that approximately 30% of the initial AFB_1-N^7-guanine DNA adducts may be depurinated and excreted within 48 hours of initial dosing (Bennett et al., 1981). Regarding base-catalyzed ring-opening, hydrolysis of the AFB_1-N^7-guanine imidazole ring results in formation of an AFB_1-formamidopyrimidine (FAPyr) adduct that is much more stable than the initial N^7-guanine lesion. This accounts for the observed temporal shifts in adduct profiles in AFB_1-perfused livers or single AFB_1 doses in vivo. Two hours after dosing, N^7-guanine adducts represent $>80\%$ of total AFB_1 adducts (at a rate of ~ 10 AFB_1-N^7-guanine per 10^{11} nucleotides), while FAPyr adducts (~ 1 per 10^{11}) account for less than 10% (Essigmann et al., 1980; Croy et al., 1981a, b). However, the chemical instability of the N^7-guanine DNA adduct and the persistence of the FAPyr adduct results in the predominance of FAPyr at 24 hours and a reversal of the initial profile by 48–72 hours (Croy et al., 1981a; Hertzog et al., 1980). This shift is delayed with repeated AFB_1 exposure—for example, dosing to rats over 14 days (Croy et al., 1981a)—but eventually results in an accumulation of FAPyr at $\sim 80\%$ of total DNA adducts.

Adduct Repair in DNA

Enzymatic repair of AFB_1 lesions in DNA is carried out through both nucleotide excision repair (NER) and base excision repair (BER) mechanisms. Typically, NER is considered to act on a variety of bulky, helix-distorting lesions, with BER utilized for small lesions, such as AP sites (Bedard et al., 2006). However, both NER and BER have been shown to contribute to N^7-guanine and FAPyr adduct repair. In human fibroblasts treated with AFB_1, deficiency of the NER gene *XPA* resulted in lower efficiency of N^7-guanine adduct repair (Leadon et al., 1981) and higher rates of mutation frequency (Levy et al., 1992), while AFB_1-dosed $Xpa^{-/-}$ mice had greater hepatic carcinoma incidence and multiplicity than similarly treated wild-type controls (Takahashi et al., 2002). While the FAPyr adduct has been shown to be repaired by NER mechanisms in bacteria (Alekseyev et al., 2004), this adduct is persistent in mammals (Croy et al., 1981a; Hertzog et al., 1980), independently from NER status (Leadon et al., 1981). However, the FAPyr adduct appears to be less helix-distorting than the N^7-guanine adduct and prokaryotic NER mechanisms are less sensitive to helix distortion than their mammalian homologues (Bedard et al., 2006). Thus, poor recognition of FAPyr adducts by mammalian NER mechanisms may increase its persistence and explain differences between assays performed in prokaryotic and eukaryotic systems. Several BER enzymes are capable of DNA glycosylase activity at guanine formamidopyrimidine adducts, allowing for specific excision of these mutagenic lesions (Dizdaroglu et al., 2008). One of these, *Neil1*, has recently been shown to catalyze excision of the AFB_1-FAPyr adduct (Vartanian et al., 2017), while AFB_1-treated newborn mice deficient in *Neil1* exhibit more FAPyr adducts 48 hours post-dosing and are more susceptible to HCC than wild-type animals (Vartanian et al., 2017).

In humans, epidemiological evidence suggests that defects in adduct repair may increase AFB_1-related HCC risk. Polymorphisms of several DNA repair genes (XRCC1, XRCC3, XRCC4, XRCC7, ERCC2, XPC) have been shown to increase HCC risk (Kirk et al., 2005a; Long et al., 2006, 2008, 2013; Yao et al., 2014), especially in individuals with high AFB_1 exposure (Long et al., 2006, 2008, 2013; Yao et al., 2014). In particular, ERCC2 and XPC are involved in NER, while XRCC1 is involved in BER.

Mutagenesis

AFB_1 exposure induces G → T transversion mutations in humans and animal models (Hsu et al., 1991; Bressac et al., 1991a; Smela et al., 2001; Chawanthayatham et al., 2017). While the AP, N^7-guanine, and FAPyr adducts all result from AFB_1 exposure, they may not contribute equally to the resulting mutational spectra. AP sites are well-known to be mutagenic, as they induce incorporation of non-complementary bases opposite to the adducted base (Loeb et al., 1986). Given the origination of AFB_1-induced AP sites from adducted guanine residues and a predominance of AP→T mutations (Bailey et al., 1996), AP sites may logically contribute to AFB_1 mutations. However, although most AFB_1-induced mutations occur at the site of adduction, a minor fraction (7%–13%) occur at the residue 5′ to the adducted guanine (Bailey et al., 1996). Although these mutations can be caused by intercalation of the bulky N^7-guanine or FAPyr adducts, they cannot result from AP sites, which only form mutations at the lesioned base (Smela et al., 2001; Bailey et al., 1996). Furthermore, while N^7-guanine and FAPyr adducts exhibit the same mutagenic profiles (>80% G→T, 10% G→A, <5% G→C, <5% deletion), the FAPyr is at least twice as mutagenic as the N^7-guanine adduct (Lin et al., 2014a, b; Smela et al., 2002). Thus, combined with observed differences in stability and repair, the FAPyr adduct is likely responsible for the majority of AFB_1 mutagenesis.

In 1991, two reports in the same issue of Nature reported the presence of a G→T transversion hotspot at the third position of codon 249 (5′-CGG **AGG** CCC-3′) of the human *TP53* gene (Hsu et al., 1991; Bressac et al., 1991a), resulting in the non-synonymous substitution of arginine with serine (R249S) within the p53 protein (Li et al., 1993). Experiments in vitro have shown that AFB_1 causally induces this R249S mutation (Aguilar et al., 1993) and epidemiological evidence strongly supports a role of aflatoxin exposure in its occurrence in human HCC (Chawanthayatham et al., 2017; Villar et al., 2011; Stern et al., 2001; Qi et al., 2015; Zhang et al., 2006; Lunn et al., 1997; Xie et al., 1994; Unsal et al., 1994; Szymańska et al., 2009; Kirk et al., 2005b; Shimizu et al., 1999). While the R249S mutation is found in only 3%–7% of HCCs in general (TCGA Research Network, 2017; Wellcome Trust Sanger Institute, 2017), its prevalence rises to 30%–60% in HCC cases with documented or likely AFB_1 exposure (Villar et al., 2011; Stern et al., 2001; Qi et al., 2015; Xie et al., 1994; Unsal et al., 1994; Szymańska et al., 2004, 2009; Kirk et al., 2005b; Shimizu et al., 1999; Jackson et al., 2001; Coursaget et al., 1993). Indeed, it is rarely seen in liver tumors diagnosed in Europe or North America, where aflatoxin exposure is typically very low (Unsal et al., 1994; Wong et al., 2000; Bressac et al., 1991b); exceptions are often migrants from high-exposure regions (Amaddeo et al., 2015). The emergence of this mutation as a prototypical AFB_1 hotspot can be linked to two factors: the contextual preference of AFB_1 adduction within nucleotide sequences and disruption of p53 protein function. First, a study which systematically investigated the sequence context of AFB_1 adduction found a preference for guanines in GC-rich regions, particularly when flanked by other guanines (5′-GGG-3′) or with guanine or cytosine at the 5′ position—for example, GGX, CGX (Benasutti et al., 1988). These in vitro predictions align with the observed hotspot mutation in codon 249 of human *TP53* (GGC), as well as the predominating genome-wide mutational spectra (CGC; G→T) seen in AFB_1-treated rodents (Chawanthayatham et al., 2017; Woo et al., 2011; Wattanawaraporn et al., 2012) and humans with R249S-mutant HCC (Chawanthayatham et al., 2017). Secondly, the arginine → serine missense alteration of mutant R249S p53 substantially disrupts its DNA-binding and promoter transactivation activity, leading to loss of cell cycle and apoptotic control (Gouas et al., 2009). However, its mechanism of selection in vivo (e.g., dominant negative) remains unclear and a subject of investigation.

Carcinogenicity of Aflatoxins: Evidence in Humans

IARC has conducted several systematic reviews of the available data, which most recently in 2012 reaffirmed the carcinogenicity of aflatoxins to humans (International Agency for Research on Cancer, 1987, 1993, 2002, 2012). This section will not replicate those reports, but rather highlight the validation and implementation of molecular biomarkers to establish aflatoxins' carcinogenicity.

Before the availability of biomarkers of internal and biologically effective dose, epidemiological studies relied on estimates of dietary AFB_1 exposure, acquired through food contamination surveys. However, substantial imprecision in such estimates can arise from heterogeneity in the spatial distribution of contamination within sampled food items (on the order of 10^3), errors in actual *vs.* assumed food consumption, and unappreciated temporal or geographical variation in contamination, due to unaccounted ecological factors. Nonetheless, such studies have provided circumstantial support for an association between AFB_1 intake and HCC risk. In a cohort of nearly 8000 men in Guangxi, China, HCC rates among four communities correlated with estimates of dietary aflatoxin contamination (Yeh et al., 1989). In an ecological study in high- and low-incidence regions of China, although 85% of corn samples in the high-incidence region were contaminated with AFB_1, only 5% of samples in the low-incidence community had detectable levels (Li et al., 2001). While these and other data were suggestive of the carcinogenic effects that had long been observed in animal models, the limitations of the ecological approach persist. This necessitated the development, validation, and implementation of minimally-invasive biomarkers of aflatoxin exposure, internal dose, and biologically effective dose.

Aflatoxins and Cancer Risk: Assessment with Biomarkers of Exposure

Measurement of AFB_1 or its metabolites in the urine has long been an approach for the subject-level assessment of aflatoxin exposure, continuing into the present day. Analytical techniques have advanced since early studies with radiolabeled tracers in animals (Dalezios et al., 1973; Wogan et al., 1967), and a breakthrough was the development of high-affinity monoclonal antibodies recognizing aflatoxins (Groopman et al., 1984), which could be implemented in immunoassays (Hatch et al., 1993; Zhu et al., 1987) or affinity columns for aflatoxin purification prior to analytical HPLC (Groopman et al., 1985, 1992a).

Using these approaches, studies have shown that 24-hour urinary AFM_1 excretion is highly correlated with AFB_1 dose in animals (Groopman et al., 1992a) and AFB_1 intake in humans (Zhu et al., 1987; Groopman et al., 1992b). In contrast, AFB_1 exposure does not show a linear relationship with urinary AFP_1 excretion (Groopman et al., 1992a, b) and correlations with total urinary aflatoxin excretion are inconsistent, likely due to variation in AFP_1 production (Groopman et al., 1992b, c). Thus, urinary AFM_1 is a valid biomarker of short-term ($\sim$24 hour) dietary AFB_1 exposure. With this validated tool in hand, Ross et al. were the first to report an increased risk of HCC with subject-level estimates of aflatoxin exposure (Ross et al., 1992), a finding which has subsequently been replicated several times (Hatch et al., 1993; Qian et al., 1994; Sun et al., 1999; Wu et al., 2009; Wang et al., 1996). Finally, in settings of pre-existing aflatoxin exposure, modulation of urinary AFM_1 excretion has been used as a biomarker of efficacy in chemoprevention trials (Scholl et al., 1996; Wang et al., 1999), or interventions utilizing enteric adsorbents to reduce AFB_1 uptake in the intestine (Mitchell et al., 2013).

Aflatoxins and Cancer Risk: Assessment with Biomarkers of Internal Dose

Similarly to urinary metabolites, levels of urinary AFB_1-mercapturic acid (AFB_1-NAC)—the ultimate product of AFB_1-glutathione conjugation and excretion—reflects recent aflatoxin exposures. However, unlike AFM_1 or other oxidative metabolites (AFP_1, AFQ_1), AFB_1-NAC links aflatoxin exposure with the production and detoxification of the toxic AFB_1-8,9-*exo*-epoxide (Degen et al., 1978; Moss et al., 1983, 1985). Similarly, the AFB_1-albumin (AFB_1-alb) adduct (**Fig. 3**) is formed downstream from the AFB_1-epoxide: solvolysis of the epoxide results in the AFB_1-8,9-dihydrodiol, which exists in equilibrium with AFB_1-dialdehyde (Johnson et al., 1996). This dialdehyde does not adduct DNA or participate in GST-mediated conjugation, but can react with lysine residues within proteins (Guengerich et al., 2002), notably in serum albumin (Sabbioni et al., 1987, 1990). Enhanced reduction of the dialdehyde by aldo-ketoreductases (Guengerich et al., 2001) results in lower AFB_1-alb adduct levels, but has no impact on carcinogenesis (Roebuck et al., 2009). Thus, like AFB_1-NAC, AFB_1-alb is a biomarker of internal dose, as it integrates dietary exposure with metabolic production of the mutagenic epoxide; unlike AFB_1-NAC, it does not reflect activity of the predominant phase II detoxification pathway (GST conjugation). Despite this difference, both are highly correlated with AFB_1 exposure (Sabbioni et al., 1990; Scholl et al., 1997, 2006; Wild et al., 1990, 1992) and hepatic DNA adduct formation (Scholl et al., 1997; Wild et al., 1986, 1996). Rather, the most substantial difference lies in the temporal window each provides to investigators. While urinary biomarkers reflect only the most recent exposure/dose, the half-life of albumin in circulation is $\sim$3 days in the rat, $\sim$21 days in humans, and is unaffected by AFB_1 adduction (Sabbioni et al., 2017)—meaning that previous exposures may be quantifiable for several months in humans. Additionally, this long-term indicator of internal dose represents a "smoothed" estimate of habitual aflatoxin exposure (Wild et al., 1992), while levels of dietary contamination may vary significantly (e.g., daily or between wet and dry seasons (Wild et al., 2000; Gong et al., 2004)).

Both of these biomarkers of internal dose have been used to interrogate cancer risk in humans. AFB_1-NAC was detectable in 24 of 27 HCC cases from Guangxi, China (Wang et al., 2001), while oltipraz (Wang et al., 1999) and green tea polyphenols (Tang et al., 2008) have been shown to increase urinary AFB_1-NAC excretion in secondary prevention trials. Several reports from a Taiwanese case-control study (Hatch et al., 1993) have demonstrated a significantly elevated risk of HCC with detectable (Chen et al.,

1996) or elevated (Wu et al., 2009) AFB_1-alb levels, while secondary prevention trials with enterosorbent clay (Pollock et al., 2016) and post-harvest crop processing and storage (Turner et al., 2005) have shown benefits in mitigating existing AFB_1 exposure.

Aflatoxins and Cancer Risk: Assessment with Biomarkers of Biologically Effective Dose

Spontaneous depurination of the unstable N^7-guanine adduct leads to its dose-dependent appearance in the urine of rodents (Bennett et al., 1981; Groopman et al., 1992a; Walton et al., 2001), in proportion to the initial mutagenic N^7-guanine adduct burden in the liver (Bennett et al., 1981; Groopman et al., 1992a). In humans, although direct validation of urinary N^7-guanine to hepatic adducts is not possible, urinary N^7-guanine excretion in humans very closely reflects dietary AFB_1 intake (Groopman et al., 1992b, 1992c). While also present in the urine, the urinary N^7-guanine adduct does not reflect the same 24-hour window of aflatoxin exposure as do AFM_1 and AFB_1-NAC. A single dose of AFB_1 produces an initial burden of N^7-guanine adducts, of which 30%–50% may depurinate in a biphasic decay and appear as urinary N^7-guanine over the first 48 hours (Bennett et al., 1981; Hertzog et al., 1980). Of course, AFB_1 exposure in humans is rarely in the form of a single bolus and multiple-dosing regimens in animals demonstrate that repeated exposure can result in a sustained release of N^7-guanine adducts for many days (Croy et al., 1981a). Thus, although the N^7-guanine biomarker appears in the urine and certainly reflects a narrower window of exposure (days) than does AFB_1-alb (weeks-months), it exhibits different kinetics than the other aflatoxin biomarkers measured in the urine compartment.

Ross et al. reported associations between HCC risk and subject-level aflatoxin exposure in two papers from a case-control study in China in the late 1980s (Ross et al., 1992; Qian et al., 1994). In addition to finding elevated risk with detectable urinary AFM_1, detectable urinary N^7-guanine was also associated with increased HCC risk, and to an even stronger degree: nearly fivefold in the initial report (95% CI 1.5–16.3) and more than ninefold in their follow-up paper (95% CI 2.1–11.8). Additionally, in the second report (Qian et al., 1994), the HCC risk estimates associated with detectable urinary AFM_1, AFP_1, AFB_1, or AFQ_1 all increased when detected simultaneously with the N^7-guanine adduct. Similar patterns have been reported in a Taiwanese case-control study (Yu et al., 1997). As with other aflatoxin biomarkers, N^7-guanine levels have been used in intervention and prevention trials, revealing associations with broccoli-sprout glucosinolates (Kensler et al., 2005) and reduced N^7-guanine adduct formation with the use of chlorophyllin as an orally administered enterosorbent (Egner et al., 2001).

Aflatoxins and Cancer Risk: Assessment with Biomarkers of Early Biologic Effect

The use of circulating tumor DNA as a biomarker of early carcinogenic events or tumor emergence has been of great interest for some time (Christie et al., 2016; Jahr et al., 2001). Given the high penetrance of the R249S mutation in AFB_1-induced HCC, as well as the great importance of p53 as a tumor suppressor, detection of this mutation in circulating DNA from pre-neoplastic lesions or early carcinomas became an attractive biomarker target. Kirk et al. (2000) tested plasma from Gambian controls or patients diagnosed with HCC or cirrhosis; using restriction endonuclease digestion, they found the R249S mutation in only 3/53 controls and 2/13 cirrhotic patients, but in 19/53 HCCs (36%; OR 16.4, 95% CI 3.0–90.5). Moreover, they were not able to detect the R249S biomarker in any of the 10 European controls or 50 European HCC cases. Other publications followed, with most utilizing the short oligonucleotide mass analysis (SOMA) technique (Laken et al., 1998; Qian et al., 2002). Jackson and colleagues detected the R249S mutation first in 10/25 HCC tumors from China (validated with DNA sequencing) and subsequently in 11/20 tumors from a second Chinese cohort (Jackson et al., 2001). In the second cohort, paired plasma for 6/11 mutant tumors tested positive for the circulating R249S mutation, while 0/10 plasma samples from healthy American controls did. A study in Gambian HCCs reported similar rates of tumoral R249S mutation, with greater concordance (89%) between paired tumor-plasma dyads (Szymańska et al., 2004). A later study of HCC in Qidong, initiated after aflatoxin exposure had likely begin to decrease (Chen et al., 2013), found ~60% prevalence of both tumoral and plasma R249S mutations, despite low levels of serum AFB_1-alb adducts (Szymańska et al., 2009)—thus demonstrating the ability to reveal significant past exposures not captured temporally with other aflatoxin biomarkers. Others have demonstrated relative quantitation of plasma R249S levels, enabling its use as a continuous HCC risk variable (Lleonart et al., 2005); for example, while only 2% of controls had R249S levels $>10^3$ copies per mL, 26% of HCC cases did (OR 62, 95% CI 4.7–820). Subsequent studies have found increased HCC risk with either higher relative levels of plasma R249s (Kirk et al., 2005b; Villar et al., 2012) or R249S detection (Kirk et al., 2005b; Jackson et al., 2003).

Chemical–Viral Interactions

A crucial and consistent factor impacting the role of AFB_1 in HCC risk has been the interaction between concurrent chronic HBV infection and AFB_1 exposure. This relationship has been noted since the earliest AFB_1 biomarker studies—while Ross and colleagues reported a substantially increased HCC risk overall with any detectable urinary N^7-guanine adduct (RR 4.9, 95% CI 1.5–16.3), stratification by HBV surface antigen (HBsAg) status revealed a crucial interaction (Ross et al., 1992). Detectable urinary N^7-guanine conferred no significant HCC risk in HBsAg-negative individuals (RR 1.9, 95% CI 0.5–7.5), but a 60-fold increased risk (95% CI, 6.4–561.8)—greater than multiplicative—with concurrent HBsAg-positive status. This remarkable finding was replicated in their follow-up report: while detectable urinary aflatoxin biomarkers now significantly elevated HCC risk in HBsAg-negative individuals (likely due to improved power from additional cases and controls), aflatoxin exposure in HBsAg-positive participants was still associated with a nearly 60-fold increased risk (Qian et al., 1994). While some recent studies have not returned the same magnitude of

synergy as earlier reports, this may be partly due to improved HBV vaccination in some regions (Chang et al., 2009; Sun et al., 2013; Qu et al., 2014), declining aflatoxin exposure in others (Chen et al., 2013; Sun et al., 2013), and may explain some findings of additive, rather than synergistic, interaction (Wu et al., 2009). Regardless, the existence of a chemical-viral interaction appears to hold a firm consensus, regardless of the biomarker used for estimating aflatoxin exposure (Lunn et al., 1997; Kirk et al., 2005b; Yeh et al., 1989; Wang et al., 1996). Indeed, much of the impact of aflatoxin exposure on HCC incidence must be viewed in context with HBV infection. Reduced aflatoxin exposure, rather than HBV vaccination, likely accounts for the attenuation of HCC mortality in Qidong, China over the past 30 years; however, the great majority of this effect (83%) was attributable to reducing aflatoxin exposure in HBV-infected individuals (Chen et al., 2013). Similarly, while a recent systematic review and meta-analysis reported that 17% of worldwide HCC risk could be attributed to aflatoxin, this was higher in HBV-infected (21%) than HBV-uninfected individuals (9%), with odds ratios of 73.0 (95% CI 36.0–148.3) for concurrent exposure and 6.4 (95% CI 3.7–10.9) for aflatoxin alone (Liu et al., 2012).

Several mechanisms have been proposed to explain this striking interaction between HBV and aflatoxin exposure. First, mutations in the HBV HBx gene are predictive of HCC development (Kuang et al., 2004; Muñoz et al., 2011) and HBx protein—particularly mutant HBx—physically interacts with (Gouas et al., 2010; Iyer et al., 2011) and inhibits p53 (Iyer et al., 2011), disrupting genome surveillance. Additionally, coexpression of HBx and R249S mutants has been shown to increase proliferation and aneuploidy in immortalized hepatocytes (Jiang et al., 2010). Second, HBV infection may exacerbate AFB_1-induced mutagenesis and inhibit adduct repair. HBx protein and the HBV large envelope protein have been shown to induce CYP3A4 expression, potentially leading to greater AFB_1 epoxidation and adduct formation (Niu et al., 2013; Kirby et al., 1994). Moreover, HBx appears to reduce NER through both p53-dependent and -independent mechanisms (Jia et al., 1999), with one study demonstrating specific impairment of AFB_1-adduct repair (Groisman et al., 1999). Unsurprisingly, epidemiological evidence suggests that *TP53* R249S mutations co-occur with HBx genomic integration and mutation in HCC (Ortiz-Cuaran et al., 2013; Kuang et al., 2005; Gouas et al., 2012).

Summary and Outlook

In closing, the half-century of investigation into the toxicology and molecular biology of aflatoxin exposure now represents a classic and voluminous case study in chemical carcinogenesis. Few agents have been so thoroughly characterized through basic research; fewer still provide molecular epidemiologists with as wide of an array of tools with which to investigate. While much has been revealed, work in many subfields remains ongoing—from characterization of the epigenetic impacts of AFB_1-induced hepatocarcinogenesis (Livingstone et al., 2017), to the role of AFB_1 in carcinogenesis at other organ sites (Noguier et al., 2015; Koshiol et al., 2017; Carvajal et al., 2012), to predictions of elevated and shifting aflatoxin exposures as a result of impending climate change (Medina et al., 2014; Battilani et al., 2016). Work from many investigators has identified effective, culturally competent, and cost-effective strategies for the primary and secondary prevention of aflatoxin exposure (Groopman et al., 2008). Given that the burden of aflatoxin exposure will continue to fall on many of the world's most vulnerable populations (International Agency for Research on Cancer, 2015; Wu et al., 2012), efforts to implement these strategies will likely (and rightly) become a priority.

See also: Aspirin and Cancer. Environmental Exposures and Epigenetic Perturbations. Epigenetic Therapy. Genetic and Epigenetic Deregulation of Enhancers in Cancer. Hepatocellular Carcinoma: Pathology and Genetics.

References

Abbas, H., et al., 2009. Toxin Reviews 28 (2–3), 142–153.
Aguilar, F., et al., 1993. Proceedings of the National Academy of Sciences of the United States of America 90 (18), 8586–8590.
Alekseyev, Y.O., et al., 2004. Carcinogenesis 25 (6), 1045–1051.
Amaddeo, G., et al., 2015. Gut 64 (5), 820–829.
Aoyama, T., et al., 1990. Proceedings of the National Academy of Sciences of the United States of America 87 (12), 4790–4793.
Appleton, B.S., et al., 1982. Cancer Research 42 (9), 3659–3662.
Asao, T., et al., 1963. Journal of the American Chemical Society 85 (7), 1706–1707.
Asao, T., et al., 1965. Journal of the American Chemical Society 87 (4), 882–886.
Ayres, J.L., et al., 1971. Journal of the National Cancer Institute 46, 561–564.
Bailey, E.A., et al., 1996. Proceedings of the National Academy of Sciences of the United States of America 93 (4), 1535–1539.
Bailey, G.S., et al., 1988. Carcinogenesis 9 (11), 1919–1926.
Bailey, G.S., et al., 1994. Mutation Research: Environmental Mutagenesis and Related Subjects 313 (1), 25–38.
Battilani, P., et al., 2016. Scientific Reports 6, 24328.
Bedard, L.L., et al., 2006. Cancer Letters 241 (2), 174–183.
Benasutti, M., et al., 1988. Biochemistry 27, 472–481.
Bennett, J.W., et al., 2003. Clinical Microbiology Reviews 16 (3), 497–516.
Bennett, R.A., et al., 1981. Cancer Research 41 (2), 650–654.
Blount, W.P., 1961. Turkeys 9, 52–61.
Bren, U., et al., 2007. Chemical Research in Toxicology 20 (8), 1134–1140.
Bressac, B., et al., 1991a. Nature 350, 429–431.
Bressac, B., et al., 1991b. Lancet 338 (8779), 1356–1359.

Brown, K.L., et al., 2009. Chemical Research in Toxicology 22 (5), 913–917.
Büchi, G., et al., 1967. Journal of the American Chemical Society 89 (25), 6745–6753.
Buetler, T.M., et al., 1992. Biochemical and Biophysical Research Communications 188 (2), 597–603.
Bujons, J., et al., 1995. Chemical Research in Toxicology 8 (3), 328–332.
Butler, W.H., et al., 1969. Cancer Research 29, 2206–2211.
Carnaghan, R.B., 1967. British Journal of Cancer 21 (4), 811–814.
Carvajal, M., et al., 2012. *Food Additives* & Contaminants: Part A 29 (2), 258–268.
Chang, M.H., et al., 2009. Journal of the National Cancer Institute 101 (19), 1348–1355.
Chawanthayatham, S., et al., 2015. International Journal of Cancer 136, 1254–1262.
Chawanthayatham, S., et al., 2017. Proceedings of the National Academy of Sciences of the United States of America 114 (15), E3101–E3109.
Chen, C.-J., et al., 1996. Hepatology 24 (1), 38–42.
Chen, J.-G., et al., 2013. Cancer Prevention Research 6 (10), 1038–1045.
Chen, T., et al., 2010. Environmental and Molecular Mutagenesis 51 (3), 156–163.
Chen, X., et al., 2006. Pharmacogenetics and Genomics 16, 219–227.
Cheung, K.K., et al., 1964. Nature 201 (4925), 1185–1188.
Christie, E.L., et al., 2016. Cancer Research 76 (19), 5590–5591.
Council for Agricultural Science and Technology (2003) Mycotoxins: Risks in plant, animal, and human systems council for agricultural science and technology.
Coursaget, P., et al., 1993. British Journal of Cancer 67 (6), 1395–1397.
Croy, R.G., et al., 1978. Proceedings of the National Academy of Sciences of the United States of America 75 (4), 1745–1749.
Croy, R.G., et al., 1981a. Cancer Research 41 (1), 197–203.
Croy, R.G., et al., 1981b. Journal of the National Cancer Institute 66 (4), 761–768.
Cullen, J.M., et al., 1987. Cancer Research 47, 1913–1917.
Dalezios, J.I., et al., 1971. Science 171 (3971), 584–585.
Dalezios, J.I., et al., 1973. Food and Cosmetics Toxicology 11 (4), 605–616.
Dash, B., et al., 2007. Journal of Toxicology and Environmental Health. Part A 70 (1), 58–66.
Davidson, N.E., et al., 1990. Cancer Research 50 (8), 2251–2255.
De Iongh, H., et al., 1962. Biochimica et Biophysica Acta 65, 548–551.
Degen, G., et al., 1978. Chemico-Biological Interactions 22, 239–255.
Degen, G.H., et al., 1981. Carcinogenesis 2 (4), 299–306.
Diener, U.L., et al., 1987. Annual Review of Phytopathology 25, 249–270.
Dizdaroglu, M., et al., 2008. Free Radical Biology and Medicine 45 (12), 1610–1621.
Dohnal, V., et al., 2014. Archives of Toxicology 88 (9), 1635–1644.
Doi, A.M., et al., 2002. Toxicology and Applied Pharmacology 181 (1), 48–59.
Dorner, J.W., et al., 1984. Mycopathologia 87 (1–2), 13–15.
Eaton, D.L., et al., 1988. Chemical Research in Toxicology 1 (2), 108–114.
Egner, P.A., et al., 2001. Proceedings of the National Academy of Sciences of the United States of America 98 (25), 14601–14606.
Essigmann, J.M., et al., 1977. Proceedings of the National Academy of Sciences of the United States of America 74 (5), 1870–1874.
Essigmann, J.M., et al., 1980. Cancer Research 40, 4085–4091.
Ferlay, J., et al., 2015. International Journal of Cancer 136 (5), E359–E386.
Fiala, J.L.A., et al., 2011. Toxicological Sciences 121 (1), 57–62.
Gallagher, E.P., et al., 1994. Cancer Research 54, 101–108.
Gallagher, E.P., et al., 1996. Toxicology and Applied Pharmacology 141, 595–606.
Garner, R.C., et al., 1972. Cancer Research 32, 2058–2066.
Gong, Y., et al., 2004. Environmental Health Perspectives 112 (13), 1334–1338.
Goodall, C.M., et al., 1969. International Journal of Cancer 4, 422–429.
Gopalakrishnan, S., et al., 1989. Journal of the American Chemical Society 111 (18), 7232–7239.
Gopalakrishnan, S., et al., 1990. Biochemistry 29 (46), 10438–10448.
Gouas, D.A., et al., 2009. Cancer Letters 286 (1), 29–37.
Gouas, D.A., et al., 2010. Carcinogenesis 31 (8), 1475–1482.
Gouas, D.A., et al., 2012. Carcinogenesis 33 (6), 1219–1224.
Groisman, I.J., et al., 1999. Carcinogenesis 20 (3), 479–483.
Groopman, J.D., et al., 1984. Proceedings of the National Academy of Sciences of the United States of America 81 (24), 7728–7731.
Groopman, J.D., et al., 1985. Proceedings of the National Academy of Sciences of the United States of America 82 (19), 6492–6496.
Groopman, J.D., et al., 1992a. Cancer Research 52 (2), 267–274.
Groopman, J.D., et al., 1992b. Cancer Research 52 (1), 45–52.
Groopman, J.D., et al., 1992c. Cancer Epidemiology, Biomarkers & Prevention 1 (3), 221–227.
Groopman, J.D., et al., 2008. Annual Review of Public Health 29, 187–203.
Gross-Steinmeyer, K., et al., 2010. Toxicological Sciences 116 (2), 422–432.
Guengerich, F.P., et al., 1998. Mutation Research 402 (1–2), 121–128.
Guengerich, F.P., et al., 2001. Chemical Research in Toxicology 14 (6), 727–737.
Guengerich, F.P., et al., 2002. Chemical Research in Toxicology 15 (6), 780–792.
Gurtoo, H.L., et al., 1976. Cancer Research 36 (12), 4663–4671.
Hatch, M.C., et al., 1993. International Journal of Cancer 54 (6), 931–934.
Hayes, J.D., et al., 1991. Pharmacology & Therapeutics 50, 443–472.
Hayes, J.D., et al., 1994. The Journal of Biological Chemistry 269 (32), 20707–20717.
Hertzog, P.J., et al., 1980. Carcinogenesis 1 (9), 787–793.
Hsieh, D.P.H., et al., 1984. Food and Chemical Toxicology 22 (12), 1027–1028.
Hsu, I.C., et al., 1991. Nature 350 (6317), 427–428.
Hu, R., et al., 2004. The Journal of Pharmacology and Experimental Therapeutics 310 (1), 263–271.
Ilic, Z., et al., 2010. Toxicology and Applied Pharmacology 242 (3), 241–246.
International Agency for Research on Cancer (1987) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 7:1-440.
International Agency for Research on Cancer (1993) Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins, 56.
International Agency for Research on Cancer (2002) Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, 82.

International Agency for Research on Cancer (2012) Chemical agents and related occupants, 100F.
International Agency for Research on Cancer (2015) Mycotoxin control in low- and middle-income countries, 9.
Itoh, K., et al., 1997. Biochemical and Biophysical Research Communications 236 (2), 313–322.
Iyer, R.S., et al., 1994. Journal of the American Chemical Society 116 (5), 1603–1609.
Iyer, S., et al., 2011. Molecular Carcinogenesis 50 (12), 972–980.
Jackson, P.E., et al., 2001. Cancer Research 61, 33–35.
Jackson, P.E., et al., 2003. Carcinogenesis 24 (10), 1657–1663.
Jahr, S., et al., 2001. Cancer Research 61 (4), 1659–1665.
Jia, L., et al., 1999. International Journal of Cancer 80 (6), 875–879.
Jiang, W., et al., 2010. International Journal of Cancer 127 (5), 1011–1020.
Johnson, N.M., et al., 2014. Cancer Prevention Research 7 (7), 658–665.
Johnson, W.W. *et al.* (1996) Journal of the American Chemical Society, 118 (35), 8213–8220.
Johnson, W.W., et al., 1997a. Proceedings of the National Academy of Sciences of the United States of America 94 (12), 6121–6125.
Johnson, W.W., et al., 1997b. Chemical Research in Toxicology 10 (6), 672–676.
Johnson, W.W., et al., 1997c. Biochemistry 36 (11), 3056–3060.
Jowsey, I.R., et al., 2003. Molecular Pharmacology 64 (5), 1018–1028.
Kamdem, L.K., et al., 2006. Chemical Research in Toxicology 19 (4), 577–586.
Kensler, K.H., et al., 2014. Toxicological Sciences 139 (2), 293–300.
Kensler, T.W., et al., 1985. Carcinogenesis 6 (5), 759–763.
Kensler, T.W., et al., 1987. Cancer Research 47 (16), 4271–4277.
Kensler, T.W., et al., 1997. Cancer Epidemiology, Biomarkers & Prevention 6, 603–610.
Kensler, T.W., et al., 2005. Cancer Epidemiology, Biomarkers & Prevention 14, 2605–2613.
Kensler, T.W., et al., 2007. Annual Review of Pharmacology and Toxicology 47, 89–116.
Kensler, T.W., et al., 2011. Toxicological Sciences 120 (S1), S28–S48.
Kensler, T.W., et al., 2013. Current Topics in Medicinal Chemistry 329, 163–177.
Kirby, G.M., et al., 1993. Carcinogenesis 14 (12), 2613–2620.
Kirby, G.M., et al., 1994. Molecular Carcinogenesis 11 (2), 74–80.
Kirk, G.D., et al., 2000. Journal of the National Cancer Institute 92 (2), 2–7.
Kirk, G.D., et al., 2005a. Cancer Epidemiology, Biomarkers & Prevention 14 (2), 373–379.
Kirk, G.D., et al., 2005b. Oncogene 24 (38), 5858–5867.
Klich, M.A., 2007. Molecular Plant Pathology 8 (6), 713–722.
Klich, M.A., et al., 1988. Transactions of the British Mycological Society 99 (1), 99–108.
Kobertz, W.R., et al., 1997. Proceedings of the National Academy of Sciences of the United States of America 94 (18), 9579–9584.
Koshiol, J., et al., 2017. Gastroenterology 153 (2), 488–494.
Kuang, S., et al., 2004. Proceedings of the National Academy of Sciences of the United States of America 101 (10), 3575–3580.
Kuang, S.-Y., et al., 2005. Cancer Epidemiology, Biomarkers & Prevention 14, 380–384.
Laken, S.J., et al., 1998. Nature Biotechnology 16 (13), 1352–1356.
Lancaster, M.C., et al., 1961. Nature 192 (4807), 1095–1096.
Langouët, S., et al., 1995. Cancer Research 55, 5574–5579.
Leadon, S.A., et al., 1981. Cancer Research 41, 5125–5129.
Levy, D.D., et al., 1992. Cancer Research 52 (20), 5668–5673.
Li, D., et al., 1993. Carcinogenesis 14 (2), 169–173.
Li, F.Q., et al., 2001. Journal of Agricultural and Food Chemistry 49 (8), 4122–4126.
Lin, Y.C., et al., 2014a. Carcinogenesis 35 (7), 1461–1468.
Lin, Y.C., et al., 2014b. Journal of Biological Chemistry 289 (26), 18497–18506.
Liu, Y., et al., 2010. Environmental Health Perspectives 118 (6), 818–824.
Liu, Y., et al., 2012. European Journal of Cancer 48 (14), 2125–2136.
Livingstone, M.C., et al., 2017. Molecular Carcinogenesis 9, 2382–2390.
Lleonart, M.E., et al., 2005. Cancer Epidemiology, Biomarkers & Prevention 14 (12), 2956–2962.
Loeb, L.A., et al., 1986. Annual Review of Genetics 20, 201–230.
Long, X.D., et al., 2006. Hepatology Research 36 (1), 48–55.
Long, X.D., et al., 2008. Annals of Epidemiology 18 (7), 572–578.
Long, X.-D., et al., 2013. Epidemiology 24 (5), 671–681.
Lotlikar, P.D., et al., 1980. Cancer Letters 9 (2), 143–149.
Lunn, R.M., et al., 1997. Cancer Research 57, 3471–3477.
McGlynn, K.A., et al., 1995. Proceedings of the National Academy of Sciences of the United States of America 92 (6), 2384–2387.
McWalter, G.K., et al., 2004. Journal of Nutrition 134 (12 Suppl), 3499S–3506S.
Medina, A., et al., 2014. Frontiers in Microbiology 5, 348.
Mitchell, N.J., et al., 2013. Biomarkers 18 (5), 391–398.
Monroe, D.H., et al., 1987. Toxicology and Applied Pharmacology 90, 401–409.
Monroe, D.H., et al., 1988. Toxicology and Applied Pharmacology 94 (1), 118–127.
Moss, E.J., et al., 1983. Biochemical Journal 210 (1), 227–233.
Moss, E.J., et al., 1985. Chemico-Biological Interactions 55, 139–155.
Muñoz, A., et al., 2011. Carcinogenesis 32 (6), 860–865.
Neal, G.E., et al., 1998. Toxicology and Applied Pharmacology 151 (1), 152–158.
Nesbitt, B.F., et al., 1962. Nature 195 (4846), 1062–1063.
Newberne, P.M., et al., 1969. Cancer Research 29, 236–251.
Nguyen, T., et al., 2000. The Journal of Biological Chemistry 275 (20), 15466–15473.
Niu, Y., et al., 2013. *Digestive and* Liver Disease 45 (12), 1041–1048.
Noguier, L., et al., 2015. Journal of the American Medical Association 313 (20), 26–28.
Norred, W.P., et al., 1983. Toxicology and Applied Pharmacology 70 (1), 96–104.
O'Brien, K., et al., 1983. Biochemical and Biophysical Research Communications 114 (2), 813–821.
Okajima, T., et al., 2000. Journal of Molecular Structure 532, 205–212.

Ortiz-Cuaran, S., et al., 2013. Cancer Letters 331 (1), 46–51.
Ostry, V., et al., 2017. Mycotoxin Research 33, 65–73.
Pelkonen, P., et al., 1997. Chemical Research in Toxicology 10 (1), 85–90.
Petrick, J.L., et al., 2016. International Journal of Cancer 139 (7), 1534–1545.
Pitt, J.I., et al., 2013. Food Control 32 (1), 205–215.
Pollock, B.H., et al., 2016. Food Additives & Contaminants: Part A 33 (8), 1–9.
Primiano, T., et al., 1995. Cancer Research 55 (19), 4319–4324.
Qi, L.-N., et al., 2015. Liver International 35 (3), 999–1009.
Qian, G.-S., et al., 1994. Cancer Epidemiology, Biomarkers & Prevention 3, 3–10.
Qian, G.-S., et al., 2002. Cancer Epidemiology, Biomarkers & Prevention 11, 1126–1129.
Qu, C., et al., 2014. PLOS Medicine 11 (12) e1001774.
Rahat, B., et al., 2012. Journal of Clinical and Experimental Hepatology 2 (2), 104–111.
Raj, H.G., et al., 1984. Cancer Letters 22 (2), 125–133.
Raj, H.G., et al., 1986. Cancer Letters 33 (1), 1–9.
Ramos-Gomez, M., et al., 2001. Proceedings of the National Academy of Sciences of the United States of America 98 (6), 3410–3415.
Raney, K.D., et al., 1990. Chemical Research in Toxicology 3 (3), 254–261.
Raney, K.D., et al., 1992. Chemical Research in Toxicology 5 (4), 470–478.
Rendic, S., et al., 2012. Chemical Research in Toxicology 25 (7), 1316–1383.
Roebuck, B.D., et al., 2009. Toxicological Sciences 109 (1), 41–49.
Ross, R.K., et al., 1992. Lancet 339 (8799), 943–946.
Sabbioni, G., et al., 1987. Carcinogenesis 8 (6), 819–824.
Sabbioni, G., et al., 1990. Carcinogenesis 11 (11), 2063–2066.
Sabbioni, G., et al., 2017. Chemical Research in Toxicology 30, 332–366.
Salhab, A.S., et al., 1976. Cancer Research 37 (4), 1016–1021.
Sargeant, K., et al., 1961. Nature 192 (4807), 1096–1097.
Scholl, P.F., et al., 1996. Carcinogenesis 17 (6), 1385–1388.
Scholl, P.F., et al., 1997. Chemical Research in Toxicology 10 (10), 1144–1151.
Scholl, P.F., et al., 2006. Chemical Research in Toxicology 19 (1), 44–49.
Shen, Y.-H., et al., 2014. Tumor Biology 35 (5), 4007–4015.
Shimada, T., et al., 1989. Proceedings of the National Academy of Sciences of the United States of America 86 (2), 462–465.
Shimizu, Y., et al., 1999. International Journal of Cancer 82 (2), 187–190.
Shupe, T., et al., 2004. Toxicology Letters 148 (1–2), 1–9.
Slone, D.H., et al., 1995. Pharmacogenetics 5, 224–233.
Smela, M.E., et al., 2001. Carcinogenesis 22 (4), 535–545.
Smela, M.E., et al., 2002. Proceedings of the National Academy of Sciences of the United States of America 99 (10), 6655–6660.
Smith, M.T., et al., 2016. Environmental Health Perspectives 124 (6), 713–721.
Squire, R.A., 1981. Science 214 (4523), 877–880.
Stern, M.C., et al., 2001. Cancer Epidemiology, Biomarkers & Prevention 10, 617–625.
Sun, C.-A., et al., 2001. Carcinogenesis 22 (8), 1289–1294.
Sun, Z., et al., 1999. Hepatology 30 (2), 379–383.
Sun, Z., et al., 2013. Carcinogenesis 34 (8), 1800–1805.
Suzuki, T., et al., 2015. Free Radical Biology & Medicine 88, 93–100.
Swenson, D.H., et al., 1973. Biochemical and Biophysical Research Communications 53 (4), 1260–1267.
Swenson, D.H., et al., 1974. Biochemical and Biophysical Research Communications 60 (3), 1036–1043.
Swenson, D.H., et al., 1977. Cancer Research 37, 172–181.
Szymańska, K., et al., 2004. International Journal of Cancer 110 (3), 374–379.
Szymańska, K., et al., 2009. Cancer Epidemiology, Biomarkers & Prevention 18 (5), 1638–1643.
Taguchi, K., et al., 2016. Toxicological Sciences 152 (1), 40–52.
Takahashi, Y., et al., 2002. Carcinogenesis 23 (4), 627–633.
Tang, L., et al., 2008. Carcinogenesis 29 (2), 411–417.
TCGA Research Network, 2017 [Online]. Available. http://cancergenome.nih.gov. Accessed: 10 October 2017.
Thimmulappa, R.K., et al., 2002. Cancer Research 62, 5196–5203.
Tiemersma, E.W., et al., 2001. Cancer Epidemiology, Biomarkers & Prevention 10, 785–791.
Turner, P.C., et al., 2005. Lancet 365 (9475), 1950–1956.
Ueng, Y.-F., et al., 1995. Chemical Research in Toxicology 8 (2), 218–225.
Unsal, H., et al., 1994. Proceedings of the National Academy of Sciences of the United States of America 91 (2), 822–826.
Vartanian, V., et al., 2017. Proceedings of the National Academy of Sciences of the United States of America 114 (16), 4207–4212.
Vesselinovitch, S.D., et al., 1972. Cancer Research 32, 2289–2291.
Villar, S., et al., 2011. Environmental Health Perspectives 119 (11), 1635–1640.
Villar, S., et al., 2012. PLoS One 7 (6), e37707.
Wakabayashi, N., et al., 2004. Proceedings of the National Academy of Sciences of the United States of America 101 (7), 2040–2045.
Walton, M., et al., 2001. Chemical Research in Toxicology 14, 919–926.
Wang, H., et al., 1998. Biochemistry 37 (36), 12536–12545.
Wang, J.-S., et al., 1999. Journal of the National Cancer Institute 91, 347–354.
Wang, J.-S., et al., 2001. Cancer Epidemiology, Biomarkers & Prevention 10, 143–146.
Wang, L.-Y., et al., 1996. International Journal of Cancer 67 (5), 620–625.
Wattanawaraporn, R., et al., 2012. Toxicological Sciences 128 (2), 326–333.
Wei, C.I., et al., 1985a. Food and Chemical Toxicology 23 (9), 809–819.
Wei, C.I., et al., 1985b. Food and Chemical Toxicology 23 (9), 821–825.
Wellcome Trust Sanger Institute, 2017 [Online]. Available. www.sanger.ac.uk. Accessed: 10 October 2017.
Wild, C.P., et al., 1986. Carcinogenesis 7 (6), 853–858.
Wild, C.P., et al., 1990. Cancer Research 50 (16), 245–251.
Wild, C.P., et al., 1992. Cancer Epidemiology, Biomarkers & Prevention 1 (3), 229–234.

Wild, C.P., et al., 1996. Cancer Epidemiology, Biomarkers & Prevention 5, 179–189.
Wild, C.P., et al., 2000. International Journal of Cancer 86 (1), 1–7.
Wild, C.P., et al., 2009. Cancer Letters 286 (1), 22–28.
Williams, D.E., et al., 2009. Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology 149 (2), 175–181.
Wogan, G.N., et al., 1967. Cancer Research 27 (10), 1729–1736.
Wogan, G.N., et al., 1971. Cancer Research 31, 1936–1942.
Wogan, G.N., et al., 1974a. Food and Cosmetics Toxicology 12 (5), 681–685.
Wogan, G.N., et al., 1974b. Food and Cosmetics Toxicology 12, 381–384.
Wogan, G.N., et al., 2012. Food Additives & Contaminants: Part A 29 (2), 249–257.
Wojnowski, L., et al., 2004. Pharmacogenetics 14 (10), 691–700.
Wong, J.J., et al., 1976. Proceedings of the National Academy of Sciences of the United States of America 73 (7), 2241–2244.
Wong, N., et al., 2000. Clinical Cancer Research 6, 4000–4009.
Woo, L.L., et al., 2011. Toxicological Sciences 122 (1), 38–44.
Wu, F., et al., 2012. PLoS One 7 (9) e45151.
Wu, H.-C., et al., 2009. Cancer Epidemiology, Biomarkers & Prevention 18 (3), 846–853.
Xie, J.P., et al., 1994. Cancer Research 54 (1), 281–285.
Yao, J.-G., et al., 2014. International Journal of Clinical and Experimental Pathology 7 (9), 6231–6244.
Yates, M.S., et al., 2006. Cancer Research 66 (4), 2488–2494.
Yates, M.S., et al., 2007. Molecular Cancer Therapeutics 6 (1), 154–162.
Yeh, F.-S., et al., 1989. Cancer Research 49, 2506–2509.
Yu, M.W., et al., 1997. Journal of Hepatology 27 (2), 320–330.
Zhang, Y.J., et al., 2006. International Journal of Cancer 119 (5), 985–991.
Zhu, J., et al., 1987. Cancer Research 47, 1848–1852.
van der Zijden, A.S.M., et al., 1962. Nature 195 (4846), 1060–1062.

Aging and Cancer

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Glossary

Aerobic glycolysis Refers to a metabolic phenomenon commonly observed in cancer cells that convert glucose to lactate in the presence of oxygen.

Autophagy Is a major intracellular self-degradative process that delivers cytoplasmic materials to the lysosome for degradation.

Cellular senescence Also known as replicative senescence, refers to the essentially irreversible arrest of cell proliferation (growth) that occurs when cells experience potentially oncogenic stress.

Clonal hematopoiesis Refers to a common aging-related phenomenon in which hematopoietic stem cells (HSCs) or other early blood cell progenitors carrying recurrent somatic mutations contribute to the formation of a genetically distinct subpopulation of blood cells.

Inflammasomes Are innate immune system receptors and sensors that regulate the activation of caspase-1 and induce inflammation in response to infectious microbes and molecules derived from host proteins.

Loss of heterozygosity Is a gross chromosomal event that results in loss of the entire gene and the surrounding chromosomal region.

Oxidative stress Refers to an imbalance between the production of reactive oxygen species and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants.

Progeroid syndromes Refer to a group of rare genetic disorders which mimic physiological aging, such as Werner syndrome, Hutchinson–Gilford progeria syndrome, trichothiodystrophy syndrome, and Cockayne syndrome.

Reactive oxygen species (ROS) Refer to a number of reactive molecules and free radicals derived from incomplete reaction of oxygen in mitochondria.

Warburg effect Is the enhanced conversion of glucose to lactate observed in tumor cells, even in the presence of normal levels of oxygen.

Incidence of most cancers increases over age (Fig. 1). In the United States, 50% of all malignancies occur in individuals over the age of 65, which represent 12% of the population. With the current aging rate, it is estimated that 70% of all cancers will occur in the elderly by 2030. Despite the evident age-dependent pattern of cancer incidence, the relationship between the biology of aging and cancer is far from clear. According to the widely accepted multi-step model of cancer, cancer is the end-result of various sequential mutations and successive cellular changes. Therefore, it has been debated whether aging has any causal effect on cancer independent of the prolonged exposure time to carcinogens due to aging. However, emerging evidence from studies of genetic and dietary manipulations indicates that cancer may be reduced by chronic downregulation of pro-aging pathways. On the other hand, although several mechanisms are well known to be shared between aging and cancer, the molecular and cellular hypotheses posited to

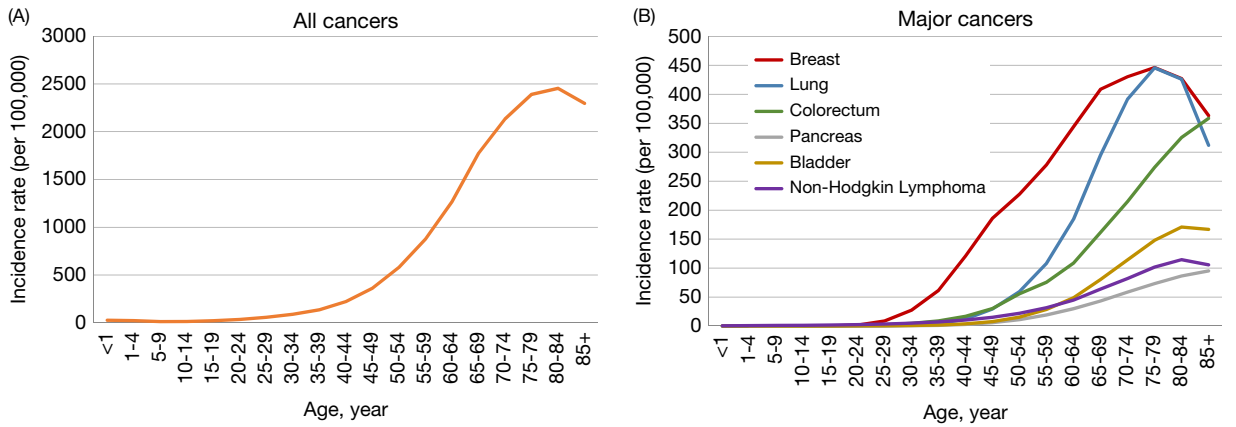

Fig. 1 The incidence of all cancers (A) and cancers of major sites (B) according to age at diagnosis. Except for gender-specific cancers, data from males and females are combined. Data are from the United States Cancer Statistics (1999–2013), Wide-ranging Online Data for Epidemiologic Research (WONDER) database (https://wonder.cdc.gov/cancer-v2013.HTML).

explain a causal relationship between the two processes remain largely untested. These controversies and unknowns are not surprising because the nascent field of geroscience is only beginning to inform oncology of the biological links between cancer and aging. Herein, I aim to summarize recent advances in aging biology that are relevant to cancer, and highlight the crucial questions that need to be addressed to not only gain a better understanding about the aging–cancer link, but also to facilitate translation of the findings from the biology of aging into the clinic for cancer prevention and treatment.

Endocrine, Genetic and Dietary Regulations of Aging Linked to Cancer

The original hypothesis of organismal longevity posits that aging is the natural result of slow, inexorable degradations in functionality of the cells, tissues, and organs of the animal. However, research over the past few decades has unveiled the surprising plasticity of aging that is at least in part regulated by genetic and nutritional signals. Hundreds of genes, particularly those involved in stress response and nutrient sensing, have been shown to modulate the aging process in model organisms. Caloric restriction has been shown to significantly increase lifespan (by up to 50%) in many species, from yeast to primates. Importantly, studies using longevity mutants and treatments have demonstrated that slowed or accelerated aging in rodents has an effect on cancer incidence, suggesting that aging process may contribute to cancer development independently of increased exposure to carcinogens due to aging.

Growth Hormone (GR)-Insulin/Insulin-Like Growth Factor (IGF-1) Signaling

Growth hormone, which is produced by the anterior pituitary, stimulates production of its secondary mediator, IGF-1, by many cell types. The intracellular signaling pathway of IGF-1 is shared by that elicited by insulin, which is produced by beta cells of the pancreatic islets in response to increased glucose concentration in the blood. The GR-insulin/IGF-1 pathway is the first identified and the most conserved aging-controlling pathway in evolution. It was first linked to life span in *Caenorhabditis elegans*, where mutations in *daf-2*, a known regulatory gene encoding an insulin/IGF-1 receptor ortholog, were found to more than double the life span of the animal. Subsequent studies identified other life-extending mutations in the phosphatidylinositol 3-kinase (PI(3)Kf)/AKT/PDK kinase cascade, a key downstream pathway of the insulin/IGF-1 signaling that regulates various cellular functions such as cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking. These mutations influence lifespan through changes in the activity of numerous transcription factors, including a FOXO family transcription factor DAF-16, the heat-shock transcription factor HSF-1, and a Nrf-like xenobiotic-response factor SKN-1. These transcription factors, in turn, upregulate or downregulate diverse genes that act cumulatively to influence lifespan.

Importantly, insulin/IGF-1 pathway and its signaling cascade, which involves PI3K, AKT, mTOR, and AMPK, have been implicated in cancer development and progression (Fig. 2). Insulin may promote cancer directly as a mitogen on susceptible cells through insulin receptors, or indirectly by decreasing IGF-1 binding proteins and thereby increasing levels of bioactive IGF-1. High levels of circulating insulin and IGF-1 have been associated with increased risk of cancer at several sites, including colorectum, endometrium, pancreas, breast, prostate, and ovary. Hyperinsulinemia and insulin resistance have been suggested as key mediators underlying the link between obesity, sedentary lifestyle, diabetes, and increased cancer risk. On the other hand, small molecules that suppress the insulin/IGF-1 pathway by targeting downstream effectors, including mTOR inhibitors (e.g., rapamycin and resveratrol) and AMPK activators (e.g., anti-diabetic drug metformin), have been shown to possess life-extending properties and are being tested as promising cancer-preventive and therapeutic agents. In fact, rapamycin has been approved by the FDA for treatment of pancreatic cancer. Metformin use have been associated with lower incidence and mortality of cancer in numerous studies. A recent phase III trial reported that low-dose administration of metformin for 1 year to patients without diabetes reduced the recurrence of colorectal adenoma, a precursor lesion for colorectal cancer. These findings provide further support for the critical role of the insulin/IGF-1 pathway in the link between aging and cancer, and open new avenues for cancer prevention and treatment.

Life-Extending/Shortening Genetic Alterations and Cancer

There is clear evidence that a single gene mutation can extend longevity in rodents and mammals. As such, studies have examined the impact on cancer development of manipulations in crucial aging-related genes. Findings indicate that compared with wild-type mice, mutant mice with disrupted GH and IGF-1 signaling are long-lived and tend to have lower cancer incidence and often longer cancer latency. For example, Ames dwarf mice, which are GH-deficient and have significantly extended lifespans due to a recessive mutation at pituitary transcription factor 1 (*Pit-1*), show a delayed occurrence of fatal neoplastic diseases and lower severity of lung adenocarcinoma compared with wild-type littermates. Similar findings have been reported in the growth hormone receptor/binding protein (GHR/BP) knockout mice, which live approximately 40%–55% longer than their normal siblings due to defective GH–IGF-1 signaling. These GHR/BP knockout mice are less frequently tumor-bearing, develop fewer different tumors, and also show a significantly lower incidence of fatal neoplastic lesions than wild-type controls.

In line with the animal evidence, humans with GHR mutations which result in GHR deficiency (GHRD) are protected against cancer. In a prospective cohort study of 99 individuals with GHRD, no cancer death was observed compared with a proportion of 20% due to cancer among all deaths in relatives without GHRD. Further in vitro studies indicated that treating human mammary epithelial cells with serum of GHRD subjects led to a spectrum of changes commonly observed in life-extending model organisms, including decreased DNA breaks, increased apoptosis, downregulation of oncogenes, and upregulation of mediators of cellular

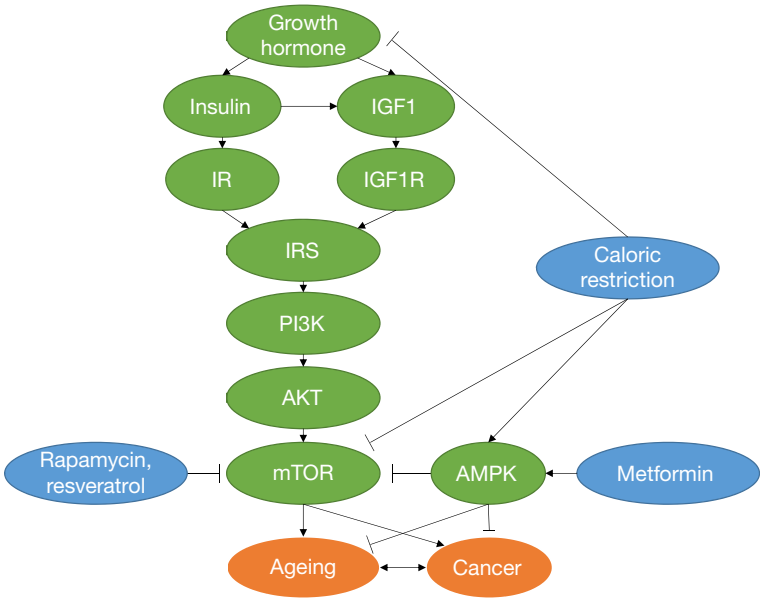

Fig. 2 Growth hormone-insulin/IGF-1 pathway in aging and cancer. Growth hormone stimulates production of IGF-1, and blocks the activity of inulin that can lead to high insulin levels which in turn increase bioactive IGF-1 levels by decreasing IGF-1 binding proteins. Insulin and IGF-1 bind to their receptors and activate, via insulin receptor substrate (IRS), the downstream signaling cascade, which involves PI3K, AKT, and mTOR, to promote aging and cancer development. On the other hand, as a conserved sensor of energy status, AMPK negatively regulates mTOR and has both life-extending and anti-tumorigenic activities. Rapamycin and resveratrol are inhibitors of mTOR and have been shown to protect against aging and carcinogenesis. Caloric restriction can inhibit mTOR signaling both directly and indirectly through activation of AMPK pathway. Metformin, which is used for treatment of diabetes, activates AMPK and inhibits cell growth in an AMPK-dependent manner. AKT, protein kinase B; AMPK, AMP-activated protein kinase; IGF1R, IGF1 receptor; IR, insulin receptor; mTOR, mechanistic target of rapamycin; PI3K, phosphoinositide 3-kinase.

protection against oxidative stress. Taken together with the aforementioned pro-cancer effect of GH-insulin/IGF-1 signaling, these findings support the overlapping effects of endocrine regulations on aging and cancer processes, and suggest that cellular changes that lead to longevity may preferentially antagonize tumor cell growth.

On the other hand, increased cancer predisposition has been observed in patients with Werner syndrome who develop a variety of signs of aging at a relatively young age due to defects in DNA repair. The cells of Werner syndrome show significant chromosomal abnormalities, increased frequency of deleterious mutations, and accumulation of DNA double-strand breaks, although the relevance of this and other progeroid syndromes to normal aging remains unresolved due, in part, to the fact that they recapitulate only some aspects of aging. The fact that disruption of DNA repair results in signs of premature aging and cancer predisposition indicates that genomic instability may be an important overlapping mechanism for aging and cancer (see section "**Genomic Instability**").

Caloric Restriction, Aging and Cancer

In addition to genetic mutations, dietary manipulations have a substantial impact on aging and cancer. The most widely studied life-extending intervention is calorific restriction, a reduction of 10%–40% in caloric intake of a nutritious diet without inducing malnutrition. Although most studies of this phenomenon have been conducted in rodents and lower animals, data accumulating from rhesus monkeys suggest that calorific restriction may also be relevant for primates, including humans. So far, three controlled intervention trials of calorific restriction have been reported in rhesus monkeys, the studies at the Wisconsin National Primate Research Center (WNPRC, $n = 76$), the National Institute on Aging (NIA, $n = 121$), and the University of Maryland Obesity, Diabetes and Aging Animal Resource Center (ODAAR, $n = 117$). The WNPRC study reported that 30% calorific restriction initiated in adult rhesus monkeys (7–14 years, average lifespan of ~27 years in captivity) lowered the incidence of aging-related deaths (13% in the intervention group vs. 37% in controls), and delayed the onset of age-associated pathologies, including the incidence of cancer, diabetes, cardiovascular disease, and brain atrophy. The ODAAR study included 8 dietary-restricted and 109 ad libitum-fed monkeys. After approximately 25 years of follow-up, control monkeys experienced a 2.6-fold increased risk of death (median survival = 25 years) than the calorically restricted monkeys of the same age (median survival = 30 years). In contrast, the NIA study did not observe any significant difference in survival between control and caloric restriction monkeys. Differences in study design, husbandry, and diet composition have been suggested to at least partly explain the discrepant findings. In particular, because the control monkeys in the NIA study were fed according to regulated portioning rather than ad libitum, they may have effectively undergone and benefited from caloric restriction. Despite the divergence, however, the NIA study also observed a markedly reduced incidence of cancer in monkeys that received caloric restriction at young age compared with controls, supporting the anti-cancer effect of caloric restriction.

Interestingly, the effects of caloric restriction are not uniform, with tumors from different tissues responding to restriction to different degrees, and a fraction of tumors originating in the same tissues showing resistance to caloric restriction. Functional studies have identified the activation status of the PI3K pathway as a molecular signature that largely predicts the sensitivity of a tumor to dietary restriction. As a downstream mediator of the insulin/IGF-1 signaling pathway, PI3K is recruited to the cell membrane, where its activity, which is antagonized by the PTEN tumor suppressor, leads to the recruitment and activation of AKT that in turn phosphorylates and regulates several targets that enhance cellular growth and inhibit apoptosis. While dietary restriction does not affect a PTEN-null mouse model of prostate and lung cancer, it significantly decreases tumor burden in a mouse model of lung cancer lacking constitutive PI3K signaling.

The benefit of caloric restriction has also been indicated in the first randomized trial in humans, the Comprehensive Assessment of the Long Term Effects of Reducing Intake of Energy (CALERIE) study. The study found that 6 months of caloric restriction improved two well-established biomarkers of longevity (reduced fasting insulin levels and body temperature), although serum dehydroepiandrosterone sulfate (DHEAS), another longevity marker, did not show slowed decline in the intervention group. These favorable effects have led to a longer-term trial, the CALERIE-2 study, which aims to test the anti-aging effect over a 2-year period of 25% caloric reduction in energy intake from baseline in nonobese adults. Feasibility of sustained caloric restriction is indicated by the fact that 82% participants completed the CALERIE-2 protocol. While no change was observed in the two primary outcomes (resting metabolic rate and core body temperature) and IGF-1 levels, a diversity of benefits were detected in support of potential anti-aging and anti-cancer effects, including persistent weight loss, reduced fat mass, increased levels of serum IGF binding protein-1, improvements in cardiometabolic risk factors (including whole-body and regional adiposity, lipid profile, blood pressure, and glucose control), adaptive decreases in daily energy expenditure, and decreases in markers of thyroid axis and inflammation (circulating triiodothyronine and tumor necrosis factor-alpha). Another two studies with 1- and 6-year caloric restriction also found no reduction in IGF-1 levels, unless it is combined with protein restriction. However, given the short duration and limited size, these trials are unable to examine the effect of caloric restriction on cancer outcomes.

Fasting and Cancer

Increasing data have suggested the potential of another dietary intervention, fasting, in cancer prevention and treatment. Different from caloric restriction, in which meal frequency is maintained, fasting is achieved by no or minimal intake of food and caloric beverages for periods that typically range from 12 h to 3 weeks. Natural experiments of religious groups that incorporate periods of fasting into their rituals indicate the benefits of fasting. For example, Muslims who fast from dawn until dusk during the month of Ramadan showed favorable physiological changes analogous to those observed in caloric restriction, including improved lipid profile, better glucose control, and decreased oxidative stress. Experimental studies have tested different fasting methods, including intermittent fasting, which typically has cycles lasting 24 h and one to a few days apart, prolonged fasting, whose cycles last two or more days and are at least 1 week apart, and very low calorie/low protein fasting-mimicking diets (FMDs), which are developed to minimize the burden of prolonged fasting. Overall, these fasting methods have been shown to retard aging and extend lifespan in rodent models, possibly by reducing IGF-1 levels and increasing insulin sensitivity and cellular stress resistance, although the magnitude of the effects on longevity depends upon the species of rodents and age at regimen initiation.

As for cancer, fasting has shown positive effects in cancer prevention and treatment. Fasting for 1 day per week delayed spontaneous tumorigenesis in p53-deficient mice, alternate day fasting in mice caused a major decline in the incidence of lymphomas, and FMD started at middle age reduced tumor incidence, delayed their onset, and caused a major reduction in the number of tumorigenic lesions. However, it has been observed that fasting-induced cell death and/or atrophy in a wide range of tissues is accompanied by a period of abnormally high cellular proliferation in these tissues, driven in part by the replenishment of growth factors during refeeding. When combined with carcinogens during refeeding, this increased proliferative activity can actually increase carcinogenesis and/or precancerous lesions in tissues including liver and colon. These findings highlight the need for an in-depth understanding of the mechanisms of action of fasting for the purpose of cancer prevention. A recent randomized controlled trial reported that compared to individuals on an unrestricted diet, those who consumed the FMD for 5 consecutive days per month for 3 months demonstrated favorable changes in a diversity of metabolic and inflammatory markers, many of which have been associated with lower cancer risk, such as lower body mass index, IGF-1, and C-reactive protein. However, because the FMD contains a generally healthy dietary pattern (plant-based, low in sugars and protein but high in unsaturated fats) beyond fasting, it is unknown whether the observed beneficial effects are driven by dietary components or fasting itself.

On the other hand, in the treatment of cancer, fasting has shown more consistent and positive effects. Prolonged fasting for 2–3 days protect normal cells while simultaneously sensitizing malignant cells to high-dose chemotherapy. This differential stress resistance (DSR) may be related to reduction of IGF-1, downregulation of proto-oncogene signals, and profound metabolic shift by fasting that promotes an anti-Warburg effect in cancer cells and results in oxidative damage and apoptosis. Multiple cycles of prolonged fasting have been shown to be as effective as chemotherapeutic agents in delaying progression of different tumors, abate the immunosuppression and mortality caused by these drugs, and augment their effectiveness in the treatment of some cancers in mice. Combination of chemotherapy and FMD enhance $CD8^+$ T cell-dependent killing of cancer cells and enrich lymphoid progenitor cells to suppress tumor progression. These data collectively indicate the potential of fasting as an adjuvant strategy in cancer chemo- and immunotherapy. It may have superior feasibility over chronic caloric restriction, because the latter generally induces weight loss and may not be feasible for cancer patients receiving chemotherapy, surgery or immunity-based treatments who are at risk for losing weight and becoming frail and cachectic.

Mechanistic Links Between Aging and Cancer

Unlike disease that typically develops within a certain time window, aging is a process lasting across the entire lifespan and starting, theoretically, immediately after birth. Therefore, from a mechanistic perspective, it is difficult to disentangle the causes and consequences of aging, especially given the possibility that the two sets may overlap (e.g., genetic damage accumulates over aging while at the same time may result in accelerated aging). It becomes even more difficult when one attempts to link to cancer, and to tease out the shared mechanisms that contribute to both aging and cancer processes from aging-induced molecular alterations that in turn affect cancer risk. However, such separation is less important from the standpoint of cancer prevention, which can be achieved by targeting any of the pathways regardless their temporal relationships with aging.

Recently, López-Otín et al. proposed nine hallmarks that are generally considered to contribute to the aging process with experimental evidence that aggravation and amelioration of these hallmarks accelerate and retard aging, respectively. Not surprisingly, all of the hallmarks are intrinsically connected to the hallmarks of cancer that were first proposed by Hanahan and Weinberg in 2000 and then updated in 2011 (Fig. 3). These connections operate at various levels of biological processes, ranging from genetic, epigenetic, to metabolic alterations, and encompassing both cell-autonomous mechanisms and changes in the intercellular communications. Such multilevel interconnections reinforce the idea that cancer and aging may represent two different manifestations of the same underlying process, namely the accumulation of cellular damage.

Herein, I summarize the major cellular and molecular hallmarks of aging that are relevant to cancer, and highlights the mechanistic connections between aging and cancer processes.

Genomic Instability

The integrity and stability of DNA are continuously challenged by exogenous physical, chemical, and biological agents, as well endogenous threats, including reactive oxygen species (ROS), DNA replication errors, and spontaneous hydrolytic reactions. To counteract DNA damage, organisms have evolved a complex network of protective mechanisms, including the DNA repair system, and mechanisms for maintaining the appropriate length and functionality of telomeres and ensuring the integrity of mitochondrial DNA. Given the massive data on the predominant role of telomere shortening and mitochondrial dysfunction in aging and cancer, they will be discussed in separate sessions in more details, and this session will be focused on nuclear DNA damage responses.

Several forms of DNA damage, including somatic mutations and chromosomal aberrations, accumulate with aging. A causal role for deficiencies of DNA repair mechanisms in aging and cancer is suggested by the development of premature aging and increased cancer risk of Werner syndrome patients who demonstrate a variety of mutations in the WRN gene that is important for the reinitiation of stalled replication forks to reduce replication errors. Experimental evidence in animal models also indicates that artificial reinforcement of nuclear DNA repair mechanisms may delay aging and suppress tumor development. Recent large-scale

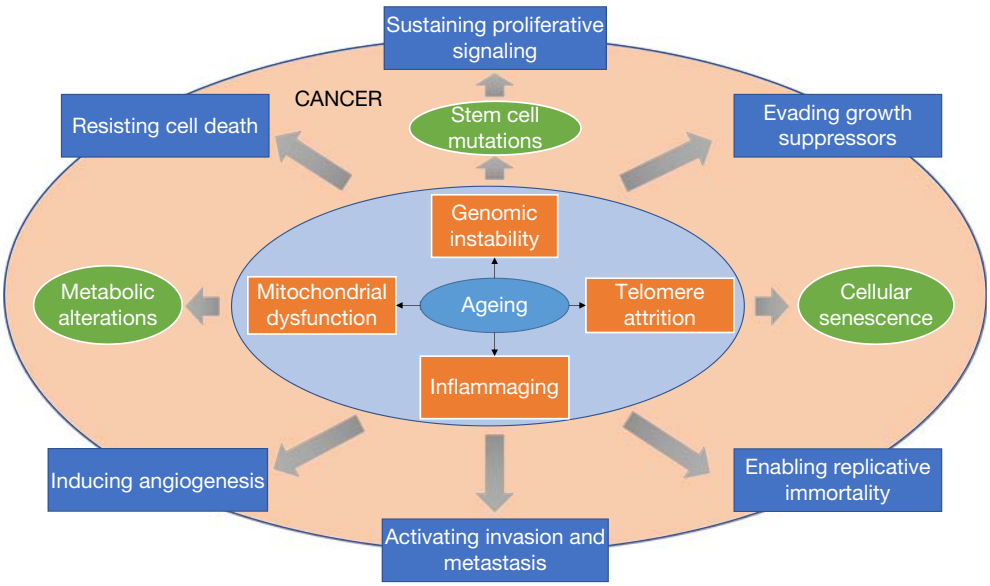

Fig. 3 Links between the major hallmarks of aging and cancer. The aging process is characterized by genomic instability, mitochondrial dysfunction, telomere attrition, inflammaging, stem cell mutations, and dysregulated nutrient sensing, which can contribute to carcinogenesis by affecting multiple hallmarks of cancer, including (1) increased production of growth factors and induction of somatic mutations to sustain chronic proliferation; (2) inhibition of tumor suppressor genes to facilitate evasion of growth suppressors; (3) attenuation of apoptosis and induction of autophagy to resist cell death; (4) alteration of telomere length and telomerase activity to enable replicative immortality of cells; and (5) disruption of tissue microenvironment to induce angiogenesis.

studies have begun to quantify the accumulation of mutations and chromosomal anomalies in human: one study using whole-exome sequencing data from blood DNA of 17,182 individuals found that detectable somatic mutations were very rare in persons younger than 40 years of age, but rose appreciably in frequency with age (9.5% among persons 70–79 years, and 18.4% among those of 90–108 years). Furthermore, the study found that persons with detectable mutations had a markedly increased risk of hematologic cancer. Similarly, a comprehensive assessment of somatic mutations in various tumor types found a strong positive correlation between the number of mutations and age of diagnosis for a wide range of solid tumors that are characterized by certain mutational signatures with prominence of C > T substitutions at NpCpG trinucleotides. These data together support the possibility that mutations may alter key genes and transcriptional pathways, resulting in dysfunctional cells that, if not eliminated by apoptosis or senescence, may jeopardize tissue and organismal homeostasis, therefore leading to accelerated aging and higher cancer risk. However, to what extent and through what mechanisms accumulation of mutations contributes to the aging process and drives the evolution of normal cells into cancer cells still remains an open question. Recent data point to a critical role of the decline in functional capacity and genetic integrity of adult tissue stem cells in the impairment in the tissue maintenance and the increase in cancer formation during aging.

Aging is associated with clonal dominance of mutant stem and progenitor cells. For example, the number of somatic mutations in hematopoietic stem cells increases exponentially with age, contributing to their clonal expansion, a phenomenon termed "clonal hematopoiesis." Importantly, most such clonal expansions appear to involve "driver mutations," which increase the selective growth advantage of the cells containing them, and thus represent an early event in the development of hematologic cancers. Similar aging-related accumulation of clonal crypt-dominance has also been reported in intestinal stem cells (ISCs) harboring chromosomal gains and losses. A recent study uncovered two mechanisms of genome instability in adult *Drosophila* ISC: first, frequent loss of heterozygosity (LOH) driven by mitotic homologous recombination results in genetic mosaicism, a phenomenon of the occurrence of more than one genetically diverse cell population in an organism arising from a single fertilized egg, which may contribute to tumor formation by facilitating expansion of positively selected variant clones with greater fitness; second, somatic deletion of DNA sequences and large chromosomal rearrangements led to spontaneous inactivation of the X-linked tumor suppressor *Notch* that in turn drove neoplasia in 10% of aged wild-type males. In addition to these cell-intrinsic mechanisms, there is increasing evidence that aging-associated defects in the cell-extrinsic factors, including the stem cell niches, systemic environment, and immune surveillance, may also affect the selection of mutant stem cells during aging, which can be potentially modifiable by environmental exposures, such as diet.

Linking the genetic and stem cell models of cancer, recent studies proposed that accumulation of random mutations through stem cell divisions, the so-called "bad luck," is a major determinant of the variation in cancer risk across tissues, on the basis of the observed high correlation between the number of stem cell divisions of a given tissue and the lifetime risk of cancer in that tissue. While these studies present a novel, quantitative assessment of the potential stochastic effects of DNA replication on cancer development, the proposed hypothesis does not appear to be consistent with the empirical data on genome-wide mutation patterns in human adult stem cells (ASCs). A recent study sequenced clonal organoid cultures derived from primary multipotent cells of human donors, and observed that the annual mutation rate in ASCs was in the same range for all assessed tissues, despite the markedly dissimilar cancer incidence in these tissues. For example, cancer incidence in the colon is approximately 10 times higher than that in the small intestine, but ASCs from both tissues accumulated about 36 mutations per year with very similar mutation spectra. Another limitation of the "bad luck" hypothesis is that it fails to account for potential modification in the number and replication rate of stem cells by host and extrinsic factors. For example, tall persons tend to have higher number of stem cells and height has been linked to increased risk of cancer in various tissues. Therefore, the estimated stochastic effects may be at least partly attributable to height, which is a trait determined by both genetic and early-life exposures. Finally, the hypothesis proposed in the original study did not clearly distinguish the variation in cancer risk across tissues versus the cancer risk variability across individuals/populations, which are two completely different concepts, thereby creating confusion and leading some to question the preventability of cancer.

In summary, there is extensive evidence that genetic instability is a shared mechanism for aging and cancer. Growing data implicate the particularly important role of DNA damage in stem cells in this mechanism. However, it remains to be determined how genetic mutations in stem and other cells drive aging and carcinogenesis, and whether and to what extent these pathways may be modified to slow aging and reduce cancer risk.

Telomere

Telomeres are repetitive sequences at the ends of chromosomes that protect the end of the chromosome from deterioration or from fusion with neighboring chromosomes. Telomeres shorten with cell division and limit proliferative capacity of in vitro-cultured cells, by activating DNA damage response pathways that cause cells to undergo irreversible growth arrest, the so-called replicative or cellular senescence or Hayflick limit. A causal role for telomere shortening in organismal aging has been established in genetically modified animal models that exhibited decreased or increased lifespans with shortened or lengthened telomeres, respectively. In contrast, the role of telomere length in cancer development has been inconclusive, with supporting evidence for both pro- and anti-cancer effects of telomere shortening.

On the one hand, cellular senescence and apoptosis induced by telomere shortening have been shown to act as a tumor suppressor mechanism by limiting the proliferation of preneoplastic cells and blocking their further malignant transformation. Furthermore, human cancer cells have been found to acquire mechanisms to maintain telomeres, either through expressing high levels of telomerase, a specialized DNA polymerase that elongates telomeres, or through alternative lengthening of telomeres

(ALT) by homologous recombination between telomeres. Mice deficient in telomerase activity show resistance to cancer induced by a variety of genetic defects or carcinogenic treatments. On the other hand, telomere shortening and the resulting telomere dysfunction have been suggested to contribute to cancer susceptibility by increasing the risk of chromosomal aberrations caused by breakage-fusion-bridge cycles, and initiating clonal drift in stem cell pools and promoting clonal dominance of mutant stem cells during tissue aging. Moreover, in addition to suppressing tumorigenesis, replicative senescence may facilitate cancer development by rendering more fertile grounds in old tissues. Studies have shown that senescent cells can disrupt the tissue microenvironment and promote tumorigenesis through production of proinflammatory cytokines, extracellular matrix components, vascular endothelial growth factor, and other cancer-promoting factors.

In parallel with the mixed mechanistic data, epidemiologic studies have also yielded inconsistent findings for the relationship between leukocyte telomere length and cancer incidence. Early retrospective case-control studies generally reported a positive association between telomere shortening and cancer risk. However, this association may, at least in part, be due to reverse causation, where shorten telomere may be a consequence rather than cause of the presence of malignant disease, because leukocyte DNA was obtained from cancer cases after diagnosis. In support of this explanation, prospective studies tended to report weak or null associations of longer telomeres with higher overall and site-specific risk of cancer, with some exceptions. These contradictory findings may also result from confounding effect, because shorter leukocyte telomeres are correlated with numerous cancer risk factors, including older age, male sex, smoking, higher body mass index, and physical inactivity. These complex confounding patterns pose great challenge for observational studies to elucidate the causality of telomere length in cancer development. To overcome this difficulty, a recent study applied the Mendelian randomization approach to study the effect of genetically determined telomere length on incidence of various noncommunicable diseases using summary data from published large-scale GWAS studies. Because of the independent assortment of alleles during gamete formation, which is analogous to the randomized assignment in intervention trials, the Mendelian randomization approach ensures that the association of genetic variants with disease outcome is unconfounded by other common factors. Sixteen single nucleotide polymorphisms corresponding to 10 independent genomic regions were used as the instrumental variables that collectively account for 2%–3% of the variation in leukocyte length. Genetically increased telomere length was associated with a modestly increased risk of overall cancer, and a positive association was found for 9 of 22 primary cancers, suggesting the predominance of tumor-suppressing activities of telomere shortening. However, the magnitudes of the association were found to be highly variable across cancer types with approximately sixfold difference. The associations tended to be stronger for cancers arising from the tissue sites that have lower rates of stem cell division, such as glioma, neuroblastoma, and serous ovarian cancer, whereas for other cancer sites with high tissue turnover rates the associations were largely null (e.g., colorectal and pancreatic cancer). This variation may explain the mixed findings in previous studies when overall cancer risk is the outcome, and mirrors the aforementioned pleiotropic effects on cancer risk of telomere shortening and associated cellular senescence, suggesting the importance of tissue-dependent mechanisms underlying the telomere-cancer relationship.

In summary, in contrast with the well-established role of telomere shortening in aging, the relationship of telomere length with cancer risk is complex and remains controversial. Telomere shortening may possess both cancer-suppressive and predisposing effects, and the collective impact on cancer development is probably tissue-specific, as a function of the regeneration rate of the tissue and the contribution of cell-extrinsic factors to tumorigenesis in that tissue.

Mitochondrial Dysfunction

Mitochondria are complex organelles that perform many roles beyond energy production, including the generation of reactive oxygen species (ROS), redox molecules and metabolites, regulation of cell signaling and cell death, and biosynthetic metabolism. Many of these functions are critical regulators of aging and cancer. The importance of mitochondrial dysfunction in aging is indicated by the mitochondrial free radical theory, which proposes that increased production of ROS resulting from the progressive mitochondrial dysfunction that occurs with aging causes further mitochondrial deterioration and global cellular damage, resulting in overall loss of organismal fitness over time. However, intense re-evaluation of this hypothesis over the past two decades has led to revelation of a neutral or even positive effect of ROS on aging. For example, increased ROS in the mitochondria may prolong lifespan in yeast and *C. elegans*. Genetic manipulations in mice that increase mitochondrial ROS and oxidative damage do not accelerate aging, and mice with increased antioxidant defenses do not present an extended lifespan. Moreover, ROS are shown to be required to increase replicative lifespan of human fibroblasts under hypoxia. In support of these observations, mechanistic studies have indicated that ROS can facilitates cellular adaptation to endogenous and exogenous stimuli of stress through multiple physiological mechanisms, including increase in adaptation to hypoxia by activation of hypoxia-inducible factor (HIF), regulation of autophagy by induction of autophagic cell death, maintenance of normal innate and adaptive immune functions, and regulation of stem cell differentiation. Therefore, ROS have been regarded as a stress-elicited survival signal that can activate compensatory homeostatic responses. Recognition of the dual function of ROS in promoting both cell damage and cell adaptation significantly expands the original mitochondrial free radical theory of aging and suggests that the balance of the ROS levels in different environmental conditions may be crucial for organismal survival.

Similar complex picture exists for the relationship between ROS and cancer development. Historically, high levels of ROS were believed to promote cancer development and metastasis by increasing oxidation of macro-molecules, such as lipids, proteins, and DNA; inducing genomic instability; regulating oncogenic signaling pathways; and promoting inflammatory responses. This led to great enthusiasm in testing the chemopreventive effect of dietary antioxidants in several large clinical trials. However, these studies have produced inconsistent results, and some studies indicated that antioxidants might even increase lung cancer risk among

smokers. Based on the insufficient evidence regarding the balance of benefits and harms for antioxidant multivitamins, the U.S. Preventive Services Task Force recommends against their use for the prevention of cardiovascular disease and cancer. Similar to the dual effect in aging, ROS in moderate concentrations may be essential for the body's stress defense system to clear damaged cells, including those that are precancerous and cancerous, and protect against cancer development. Recent data indicate that enhanced ROS detoxification may mediate the pro-tumor activity of oncogenes such as K-RAS and B-RAF by activation of NRF2-mediated transcription of endogenous antioxidant genes. Moreover, dietary supplementation with antioxidants such as *N*-acetyl-cysteine and vitamin E markedly increased tumor progression by reducing P53 expression in mouse models of lung cancer, and promoted metastasis in mice with endogenous malignant melanoma. These findings suggest the importance of the balance of ROS and the endogenous antioxidant system, and warn against the use of antioxidant supplements that may disrupt this balance among high-risk individuals who have either precancerous lesions or established tumors.

In addition to oxidative stress regulation, mitochondrial dysfunction may contribute to tumorigenesis through other mechanisms. As a central hub for both catabolic and anabolic metabolism, mitochondria drive metabolic reprogramming of cancer cells to support macromolecule synthesis and bioenergetic demand through multiple mechanisms, including limiting its respirational uptake of pyruvate to support aerobic glycolysis during Warburg metabolism, mitochondrial localization of the tumor suppressor sirtuin to promote consumption of glutamine for nucleotide and amino acid synthesis, and production of citrate to support lipogenesis. Furthermore, metabolites generated by mitochondrial metabolic pathways, the so-called oncometabolites, can promote tumor initiation and growth by influencing both nuclear gene transcription via chromatin modification and cytosolic signaling pathways. In addition to oncometabolites, other components released from mitochondria, such as cytochrome *c*, may influence cell behavior through altering the pro- and anti-apoptotic balance. Finally, due to the extremely dynamic morphology, mitochondria may promote K-Ras-dependent cellular transformation, and cancer cell growth and migration through the balance between fusion and fission events.

Given the complex role of mitochondria in cancer, studies have attempted to examine how abnormalities in mitochondrial DNA may impact tumorigenesis. Mitochondria contain multiple copies of a circular 16kB genome that are maternally inherited and encode the electron transport chain machineries, mitochondrial rRNAs, and tRNAs. Somatic mutations in mitochondrial DNA may induce mitochondrial dysfunction that contributes to cancer development through multiple pathways, including regulation of apoptosis in aging cells and some cancer cells, activation of mitochondria-to-nucleus retrograde signaling to modulate the expression of nuclear genes involved in metabolic reprogramming, and facilitation of cells to adapt to altered environments, and development of resistance to chemotherapeutic agents, and promotion of metastatic properties of cancer cells. Although numerous epidemiologic studies have linked germline and somatic mutations in mitochondrial DNA to cancer risk, the results remain inconsistent. Differences in mtDNA copy number are also implicated in tumorigenesis. However, both an increase and a decrease in copy numbers have been associated with increased risk of cancer, even for the same type of cancer. These controversial findings may not be surprising given the multiplicity of mitochondrial genomes, which allows for the coexistence of mutant and wild-type genomes within the same cell, a phenomenon known as "heteroplasmy."

In summary, the original mitochondrial free radical theory of aging is now considered to be too simplistic. ROS possess a diversity of physiological activities in organismal homeostasis. Besides ROS, mitochondrial dysfunction may influence tumorigenesis through alterations in several pathways related to their bioenergetic, biosynthetic and signaling functions. Despite these data, however, human data relating mitochondrial DNA mutations and copy number changes to cancer risk remains inconclusive. Further large-scale studies are needed, especially by accounting for potential metabolic heterogeneity across tumor subtypes.

Inflammaging

In addition to cell-autonomous alterations, aging also involves a global reduction in the capacity to cope with a variety of stressors and a concomitant progressive increase in proinflammatory status, which is referred to as "inflammaging." Inflammaging may result from various causes, including the accumulation of proinflammatory tissue damage, the failure of the immune system to effectively clear pathogens and dysfunctional host cells (known as "immunosenescence"), increased production of proinflammatory cytokines by senescent cells (see "Telomere"), enhanced activation of NF-κB signaling pathway by persistent stress, and a decline in autophagic capacity that impairs cellular housekeeping. These alterations stimulate intracellular danger-sensing multiprotein platforms called inflammasomes, which subsequently activate inflammatory pathways and contribute to the pathogenesis of numerous aging-related diseases, including cancer. In fact, chronic, low-grade inflammation has been established as an enabling characteristic that contributes to multiple hallmark capabilities of cancer by supplying bioactive molecules to the tumor microenvironment, including growth factors that sustain cell proliferation, survival factors that limit cell death, proangiogenic factors, and extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis.

Despite these well-established mechanistic links, the influence of age-acquired immunodeficiency on the incidence of cancer remains an open question. Compared to younger individuals, older persons have markedly lower frequency of naive $CD8^+$ T cells and a slightly lower frequency of naïve $CD4^+$ T cells in the peripheral blood, possibly due to the conversion of naïve cells to memory cells over a lifetime of exposures to immune challenges. However, the functional consequences for carcinogenesis of this age-associated decline in circulating immune cells remain to be determined. On the other hand, differences between older and younger people for other immune compartments, such as B cells, NK cells, and dendritic cells, are subtler. Furthermore, the malignancies associated with profound immunodeficiency, such as in patients with AIDS or organ transplantation, are mostly

virus-induced (e.g., lymphoma, Kaposi's sarcoma, and leukemia) and not the common malignancies that inflict the elderly population. In addition, if it is true that aging-associated immunosenescence reduces the ability of T cells to recognize and control tumor growth, immunotherapy would be expected to be less effective in older patients. However, existing, albeit still limited, evidence indicates similar response rate in the elderly and younger patients in the treatment of advanced melanoma with anti-CTLA-4 antibodies.

In summary, although substantial data support the role of aging in chronic inflammation and the tumor-promoting effect of inflammation, it remains unresolved whether and how functional decline in the immune system during the aging process may affect carcinogenesis. Addressing this question has great implications for cancer prevention and treatment in the elderly population, especially in the context of the recent demonstration that cancer immunotherapy extends survival of patients with advanced cancers.

Prospective Vision

Studies of genetic and dietary manipulations indicate that alterations in the aging process may influence cancer development independently of changes in duration of exposure to carcinogenic factors. These influences can be broadly categorized into the seed effect, which is characterized by the increased susceptibility of aging cells to carcinogens (e.g., due to genomic instability and metabolic dysfunction), and the soil effect, which is mediated by systemic and micronevironmental conditions favoring tumor development (e.g., immunosenescence and tumor-permissive inflammation). However, the detailed biological underpinnings of the interplay between aging and cancer are intrinsically complex and remain largely unknown. Some aging processes may possess both pro- and anti-cancer activities, possibly in a tissue-specific manner. Because most of the available evidence is based on animal models, further human studies are urgently needed to better understand the collective impacts of the aging process on cancer development in a specific tissue. These studies may employ either novel high-throughput sequencing technologies or new interventional approaches to examine the functional consequences of aging-related alterations on carcinogenesis. In addition to mechanistic investigations, more translational research is also needed to address how findings from the biology of aging can be translated into effective strategies for cancer prevention and treatment. As the aging population is leading to an unprecedented increase in cancer burden across the world, understanding the links between aging and cancer is more important than ever. Systematic, in-depth research in this area will undoubtedly lead to improved knowledge about the fundamental biology of cancer and development of novel preventive and therapeutic strategies.

See also: Multiple Myeloma: Pathology and Genetics.

Further Reading

Benz, C.C., Yau, C., 2008. Aging, oxidative stress and cancer: Paradigms in parallax. Nature Reviews. Cancer 8, 875–879.
Blasco, M.A., 2005. Telomeres and human disease: Aging, cancer and beyond. Nature Reviews. Genetics 6, 611–622.
de Magalhaes, J.P., 2013. How aging processes influence cancer. Nature Reviews. Cancer 13, 357–365.
Fernandez, L.C., Torres, M., Real, F.X., 2016. Somatic mosaicism: On the road to cancer. Nature Reviews. Cancer 16, 43–55.
Johnson, S.C., Rabinovitch, P.S., Kaeberlein, M., 2013. mTOR is a key modulator of aging and age-related disease. Nature 493, 338–345.
Junnila, R.K., List, E.O., Berryman, D.E., et al., 2013. The GH/IGF-1 axis in aging and longevity. Nature Reviews. Endocrinology 9, 366–376.
Kenyon, C.J., 2010. The genetics of aging. Nature 464, 504–512.
Longo, V.D., Mattson, M.P., 2014. Fasting: Molecular mechanisms and clinical applications. Cell Metabolism 19, 181–192.
Lopez-Otin, C., Blasco, M.A., Partridge, L., et al., 2013. The hallmarks of aging. Cell 153, 1194–1217.
Pavlova, N.N., Thompson, C.B., 2016. The emerging hallmarks of cancer metabolism. Cell Metabolism 23, 27–47.
Salminen, A., Kaarniranta, K., Kauppinen, A., 2012. Inflammaging: Disturbed interplay between autophagy and inflammasomes. Aging (Albany NY) 4, 166–175.
Sena, L.A., Chandel, N.S., 2012. Physiological roles of mitochondrial reactive oxygen species. Molecular Cell 48, 158–167.
Taylor, R.W., Turnbull, D.M., 2005. Mitochondrial DNA mutations in human disease. Nature Reviews. Genetics 6, 389–402.
Vyas, S., Zaganjor, E., Haigis, M.C., 2016. Mitochondria and cancer. Cell 166, 555–566.

Anal Cancer: Pathology and Genetics

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Nomenclature

AIN Anal intraepithelial neoplasia
AJCC American Joint Committee on Cancer
Apocrine Type of gland in anal mucosa
ASIN Anal squamous intraepithelial carcinoma
CDKN2A Cyclin-dependent kinase inhibitor 2A, gene encoding p16 protein
CDX2 Gene encoding homeobox protein CDX-2
Eccrine Type of gland in anal mucosa
EGFR Epidermal growth factor receptor
GCDFP15 Gross cystic disease fluid protein 15
GIST Gastrointestinal stromal tumor
HER2 Human epidermal growth factor receptor 2
HIV Human immunodeficiency virus
HPV-16 Human papilloma virus type 16
HSIL High grade squamous in situ lesion
KIT Proto-oncogene c-Kit
Koilocytic HPV induced squamous epithelial abnormality
LSIL Low grade squamous in situ lesion
Morphotype Histological type of a neoplastic lesion
MRP1 Multidrug resistance-associated protein 1
PD-L1 Programmed death-ligand 1
PI3K/ATK/mTOR pathway Intracellular signaling pathway important in regulating the cell cycle
SOX 2 Sex determining region Y-box 2, gene encoding a transcription factor
TP53 Gene encoding p53, tumor associated gene

Definitions

The complex anatomy and histology of the anal canal, which comprises of several distinct zones, has led to different terminologies used in different disciplines. The term "anal canal" has been in use for more than a century but the definition anatomists use (the structure between the dentate line and the perianal skin) is not used by surgeons (for whom the definition is the structure between the rectum and the perianal skin, which makes it to extend about 2 cm. above the dentate line). In a clinical context the latter definition is mostly used, which results in an anal canal with three zones: an upper part covered by rectal mucosa, a transitional zone covered by a simple "transitional" epithelium and the longest part down to the perianal skin, covered by a non-keratinizing squamous epithelium. Intestinal type adenocarcinomas in the upper zone are to be considered as rectal carcinomas and staged as such. Tumors of the anal mucosa proper can be glandular, transitional or squamous and are all staged as anal cancers. Cancers in the perianal skin (squamous cell carcinoma, Bowen's disease, basal cell carcinoma, Paget's disease) are to be considered as skin cancers and staged as such.

Anal Squamous Cell Carcinoma

Burden

Anal cancers are uncommon and represent around 2% of large bowel malignancies. The incidence in the general population is estimated at between 0.8 and 1.4 cases per 100,000 people-years. Life time anal cancer risk is approximately 0.2%. The incidence has doubled in the last 25 years. The most frequent tumor of the anal canal is squamous cell carcinoma (SCC), the incidence of which is increasing significantly, notably in high risk groups such as males with male sexual partners, immunodeficient patients due to HIV or to immunosuppressive therapy and women with a history of HPV associated cervical squamous intra-epithelial lesions. The incidence in these high risk groups goes up to 35 cases per 100,000 people-years (males with male sexual partners) and even to more than 100 cases per 100,000 people-years (HIV infected patients).

Risk Factors

For the development of anal cancer multiple risk factors have been identified: HPV infection (notably with HPV-16), immunosuppression, long term use of steroid drugs, anal sexual intercourse, HIV infection and smoking. Anal squamous cell carcinoma develops from a premalignant precursor lesion, anal intraepithelial neoplasia (AIN).

Pathology

Anal intraepithelial neoplasia

The terminology of this condition has been somewhat confusing. The WHO classification of 2010 proposes the term anal squamous intraepithelial carcinoma (ASIN). The American Joint Committee on Cancer (AJCC) has recommended using low grade squamous in situ lesion (LSIL) and high grade squamous in situ lesion (HSIL), in analogy to the terminology used for in situ neoplastic lesions of the uterine cervix. Oncogenic HPV genotypes (16, 18, 31, 33) are found in 90% of AIN and play a major etiological role. Immunosuppressed patients (HIV infected patients and immunosuppressed organ transplant recipients) are at high risk for HPV infection and AIN development because of incapacity of the immune system to clear the HPV infection. Ultimately, the oncogenic HPV genome is integrated into the genome of premalignant cells and AIN and the lesion then progresses to squamous cell carcinoma. The HPV oncoprotein E7 binds to retinoblastoma protein (pRb), which makes the transcriptional factor E2F constitutionally active. This induces overexpression of CDKN2A (also known as p16), a cyclin-dependent kinase involved in regulation of the cell cycle. Expression of p16 is frequently used as surrogate marker for HPV infection.

AIN are characterized by squamous epithelium with varying degrees of architectural and cytonuclear atypia (Fig. 1). Architectural atypia includes loss of stratification. Keratinization may be found and superficial cells often show koilocytic changes. Cytonuclear atypia includes nuclear pleomorphism and hyperchromasia as well as increased mitotic activity, often even high up in the epithelium. AIN is graded as AIN-L (synonymous with LSIL) or AIN-H (synonymous with HSIL). When in doubt immunohistochemical staining for CDKN2A (p16) is recommended as AIN-H is almost invariably CDKN2A positive.

Bowenoid papulosis is a peculiar multifocal form of AIN. Clinicians recognize BP as multiple, small, well-demarcated, gray-brown, red, pink, or skin-colored papillomatous papules in the perianal area (but they also occur on the glans penis or foreskin in males and in the vulva in females). The disease almost always has a benign course in spite of the histopathologic features of high grade AIN. As in AIN, oncogenic HPV types (mostly HPV 16) are responsible for the development of this condition. Rare cases of Bowenoid papulosis have been reported to progress to squamous cell carcinoma. Given its similarity to AIN, it is primarily considered as a clinical entity and the diagnostic term is discouraged for histopathological classification.

Anal squamous cell carcinoma

Anal squamous cell carcinoma may present as a bleeding, painful mass. More rarely a change in bowel habits is the presenting symptom. On examination, a raised or even polypoid mass may be seen but some lesions are flat. Examination of inguinal lymph nodes is important, as these are the sites of potential metastases. Lymph node metastases are associated with decreased success of local treatment, risk of disease progression and poor prognosis.

Most anal carcinomas are SCC's. In the past several morphological subtypes were recognized (keratinizing, large cell non-keratinizing, small-cell non-keratinizing, basaloid). It has become clear, however, that subtypes have no real clinical significance

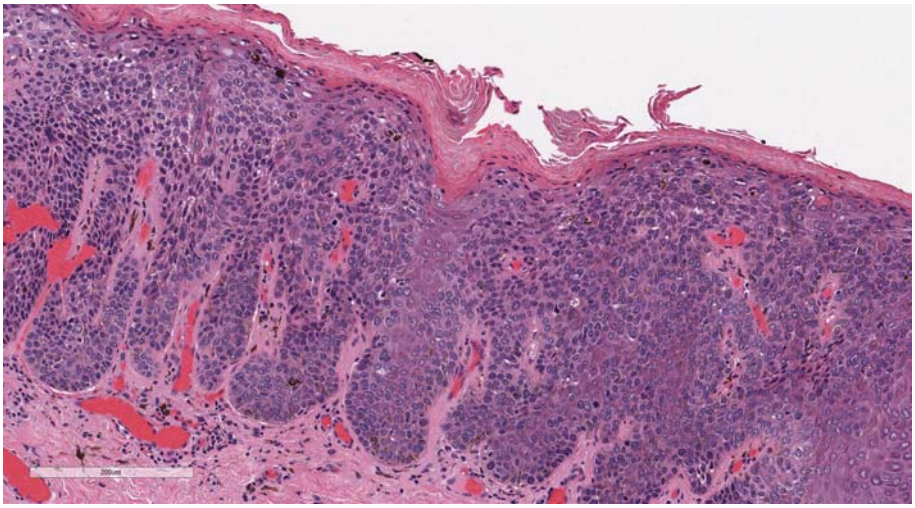

Fig. 1 High grade anal intraepithelial neoplasia (AIN 3). Note irregular epithelial architecture and cytonuclear atypia throughout the epithelium. Epithelial surface is slightly parakeratotic.

and moreover most anal SCC show several different morphotypes. As a result, presently all are diagnosed as SCC. The only variant with different characteristics is verrucous carcinoma, which will be discussed separately.

Histologically different patterns may be encountered. When fields of large eosinophilic cells are seen with distinct cell borders and variable nuclear atypia, associated with keratinization in the center of the field, this is recognized as keratinizing SCC. Depending on the degree of cytonuclear atypia the carcinoma can be graded as well, moderately or poorly differentiated (Fig. 2A). Poorly differentiated SCC may present more spindle shaped highly atypical cells. Rather often, anal SCC will contain circumscribed fields of smaller basophilic cells with relatively little cytoplasm and with palisading arrangement of the cell nuclei at the border of these fields (Fig. 2B). For this morphology the term basaloid has been introduced (Fig. 2C). For carcinomas in which this morphotype dominates the term basaloid carcinoma has been used (but also the misnomer "cloacogenic"). As the pure morphotype is rare and tumors with such morphology do not behave differently from those with conventional keratinizing morphology, they should be diagnosed as SCC with as further characterization "basaloid type." This has to be differentiated from peri-anal basal cell carcinoma which shows overlapping morphological characteristics. Histological differentiation is facilitated by immunohistochemical staining for CDKN2A (also known as p16) or SOX2, which are both almost invariably expressed in BSCC but not in basal cell carcinoma. CDKN2A is a marker for infection with HPV.

Molecular Pathology and Genetics

In more than 80% of anal SCC HPV DNA can be detected. In anogenital condylomata acuminata this is usually HPV-6 or -11 but these subtypes do not carry any risk for the development of cancer. Cancer risk is most frequently associated with infection with HPV-16 and -18. Anal SCC often shows loss of function p53. This can be caused by deletion of the TP53 locus on chromosome 17p or by a *TP53* point-mutation. More typically in anal SCC loss of p53 function is caused by sequestering of p53 by the HPV E6 protein. HPV E7 protein binds to pRb, the protein encoded by the Rb gene. The combination of p53 and pRb loss leads to inability of the cell to respond to DNA damage by cell cycle arrest and apoptosis. As a result, lesional cells continue to accumulate gene mutations, which contributes to progression of the lesion.

As anal SCC is rather chemotherapy resistant, several studies have addressed the question which mechanisms might be responsible. It was found that most anal SCC express multi-drug resistance-associated protein 1 (MRP1), which is associated with resistance to chemotherapy due to drug efflux. Furthermore, expression has been found of excision repair cross-complementing gene 1, which may confer resistance to platinum-based chemotherapy, and of thymidylate synthase, which is leads to fluoropyrimidine resistance. Several studies have explored signaling pathways involved in anal SCC in search for plausible targets for targeted therapy. In most anal SCC, immunohistochemistry has shown EGFR to be expressed, which suggests that anti-EGFR therapy might be considered even though this has proven a poor marker for response to anti-EGFR therapy. The limited experience with this approach to anal SCC treatment has provided encouraging results, however. Dysregulation of the PI3K/ATK/mTOR pathway has been identified in more than half of anal squamous cell carcinomas. AKT has been found activated in anal SCC and studies in mouse models suggest that mTOR is also involved. Both would provide a plausible therapy target. Finally, in a small subset of anal SCC HER2 amplification was found, which suggests that HER2-targeting agents might be considered as treatment.

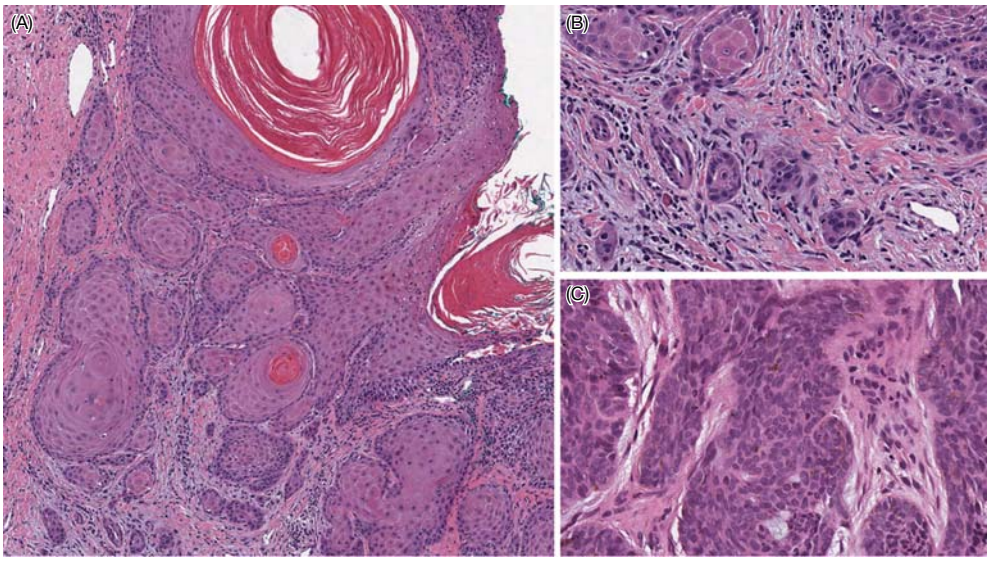

Fig. 2 Characteristic histology of squamous cell carcinoma and basaloid carcinoma. (A) Well differentiated squamous cell carcinoma. Note keratin "plugs" as evidence of squamous differentiation. (B) Higher magnification of invasion front of (A). Note small groups of cells invading into the surrounding mesenchymal stroma. (C) Area of basaloid differentiation, characterized by the presence of compact well circumscribed nests of cells with basophilic nuclei and little cytoplasm.

Microenvironment Including Immune Response

Few studies have explored the host response to anal SCC. In a recent study expression of PD-L1 was found in about half of a cohort of anal SCC, independent from HIV status. This suggests that checkpoint blocking immunotherapy might be considered, even in HIV positive patients. First results with such treatment are encouraging.

Staging and Grading

T-stage is determined mostly by size, 5 cm being the threshold between T stages 2 and 3. In T-stage 4 the tumor has invaded adjacent organs. Lymph node status has major prognostic implications. Anal SCC proper (location in the anal canal above the dentate line) metastasize to lymph nodes along the pelvic and ultimately femoral veins. Perianal squamous cell carcinoma first metastasizes to inguinal lymph nodes. Abundant lymph node metastases may inverse lymph flow, resulting in retrograde patterns of metastases also involving inguinal lymph nodes in cases of anal carcinoma. Tumor grade is conventional in well, moderately well and poorly differentiated. Grading is not optimally reproducible and prognostic significance is limited.

Prognostic and Predictive Biomarkers

Tumor budding and the presence of tumor infiltrating lymphocytes has been found of prognostic significance. Positive HIV status tends to be associated with poor prognosis. Prognostic significance has been reported for expression of p53 and cyclinA.

Verrucous Carcinoma

Definition

Verrucous carcinoma is also known as giant condyloma acuminatum or Buschke–Löwenstein tumor. It is a highly differentiated squamous cell carcinoma with a verrucous architecture, cytologically bland and blunt, pushing margins. Verrucous carcinomas grow slowly and have a very low tendency to metastasize. However, they can be locally destructive of underlying and adjacent tissues. Transformation to a more malignant form of squamous cell carcinoma occurs in about half the cases when the lesion is incompletely resected, but over a prolonged period of time (about 5 years).

Burden

Verrucous carcinoma occurs at any age but most frequently between the age of 30 and 60 and slightly more frequently in males than in females.

Risk Factors

Epidemiological studies have associated sexual promiscuity, poor personal hygiene, immune deficiency associated with HIV infection or immunosuppressive treatment, prolonged steroid use, diabetes and alcohol abuse with an increased risk for developing verrucous carcinoma. The disease is caused by HPV infection, mostly types 6 and 11.

Pathology

Verrucous carcinomas are large, slowly growing, exophytic cauliflower-like lesions in the anus or perianal region (Fig. 3). Histology shows acanthosis and papillomatosis but bland cytological characteristics. The epithelium shows orderly maturation and mostly only mild dysplasia. Mitotic activity is confined to the basal layer of the epithelium (Fig. 3, inset). Characteristically, invasion is pushing and blunt, and vascular or perineural invasion are absent. The lesions are low grade by definition. When a usual squamous cell carcinoma has developed, this is graded separately. Verrucous carcinoma does not metastasize unless usual squamous cell carcinoma has developed.

Staging

Verrucous carcinoma is staged as anal squamous cell carcinoma.

Extramammary Paget's Disease

Definition

Extramammary Paget's disease (EMPD) is an intraepidermal adenocarcinoma occurring in areas rich in apocrine glands. In most cases the lesion is entirely intra-epidermal but occasionally extension into the underlying dermis is seen. Perianal Paget's disease

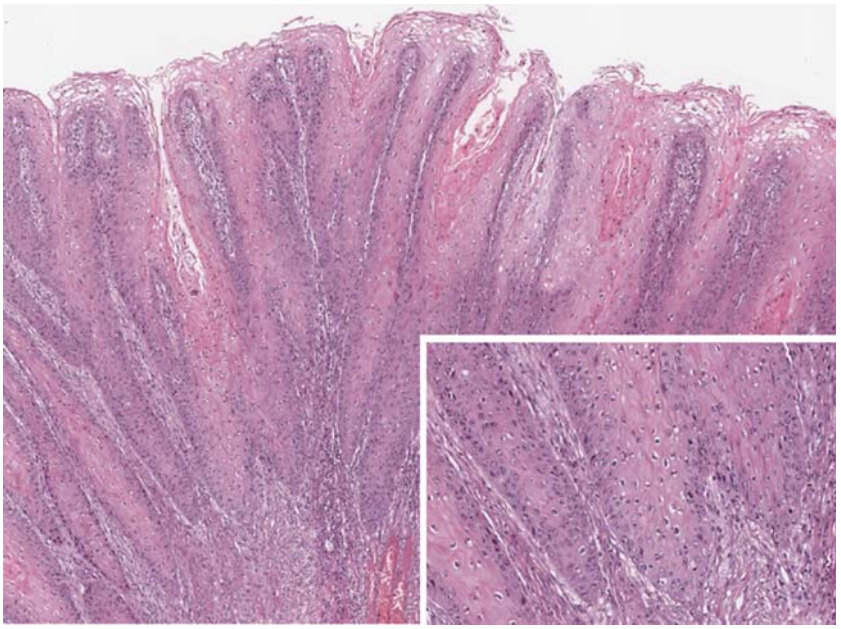

Fig. 3 Histology of a verrucous carcinoma. Insert: high magnification of the basal layers. Note bland nuclear morphology and lack of invasive growth.

is called primary when it is not associated with an underlying anal or rectal adenocarcinoma. Secondary Paget-like extension can occur in cases of distal rectal or anal adenocarcinomas.

Burden

EMPD generally occurs between the ages of 50 and 80 years, most frequently in Caucasians and more in females than in males (female/male ratio 1.4:1). EMPD occurs in the (*peri*)anal region but also in the vulva, axillae, on the penis or scrotum, on eyelids, around the umbilicus and in groins.

Risk Factors

The pathogenesis of EMPD is controversial. Most likely it develops from neoplastic changes in apocrine and eccrine skin adnexa, but often such changes are not found. Alternatively, EMPD might develop from pluripotent squamous epithelial stem cells within the epidermis, or so-called Toker cells, clear cells which are found in areolar or vulvar epidermis.

Pathology

EMPD can be primary or secondary. EMPD (or intraepidermal EMPD) is primary when neoplastic cells are found exclusively in the epidermis. The cells, however, have the capacity to become invasive into the dermis, gain access to lymph vessels and metastasize to lymph nodes and distant organs. EMPD is called secondary, when neoplastic cells have spread into the anal mucosa from an adenocarcinoma in an underlying gland or from a primary cancer in the genitourinary or gastrointestinal tract. EMPD cells have characteristic morphological features: they are large with abundant pale cytoplasm and vesicular pleomorphic nuclei. Infiltration is typically by single cells or small cell clusters (Fig. 4). In case of an underlying adenocarcinoma, deeper layers of the lesion may show glandular structures. The differential diagnosis with anal melanoma, underlying visceral carcinoma or squamous cell carcinoma in situ can be difficult. In such cases immunohistochemical markers are useful. Cells in primary EMPD often express cytokeratin 7 but not cytokeratin 20. They tend to be GCDFP15 positive but CDX2 negative. In contrast, secondary EMPD is often cytokeratin 7 negative but cytokeratin 20 positive while GCDFP15 negative. They also express CDX2.

Anal Melanoma

Definition

Primary anorectal melanoma is very rare; metastasis of a primary melanoma elsewhere to the digestive tract is much more common. The diagnosis is made by exclusion. The anus is the most common site in the digestive tract and most arise at or close to the dentate line.

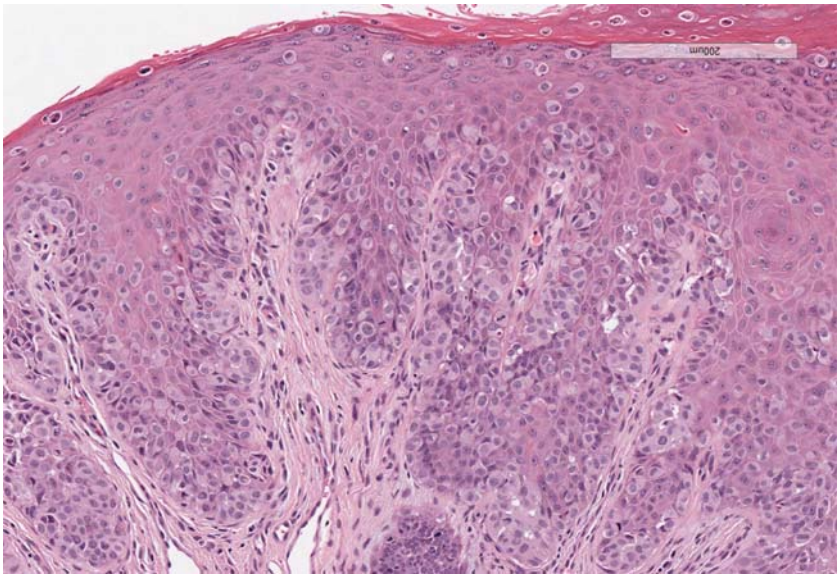

Fig. 4 Extramammary Paget's disease. Note the presence of individual cells and cell nests with slightly paler cytoplasm, mounting almost up to the surface in a slightly parakeratotic squamous epithelium.

Burden

Anorectal malignant melanoma accounts for 1% to 3% of all anal tumors and 0.3% of all melanoma. Most occur in the 5th to 6th decade in Caucasian patients with an equal frequency in men and women. After cutaneous and ocular melanoma, anorectal melanoma is the third most common melanoma location.

Risk Factors

Etiology and predisposing conditions are unknown.

Pathology

Anal melanoma is most commonly found at or close to the dentate line. Less than half of the tumors is pigmented. When small, anal melanoma presents as sessile papules. Most cases, however, are diagnosed at an advanced stage and then appear as bulky masses deeply infiltrating in underlying tissues.

Microscopically anal melanoma is composed of sheets and clusters of cells with large pleomorphic nuclei (Fig. 5) with prominent nucleoli and occasionally nuclear pseudo-inclusions. Most often the tumor cells appear epithelioid, but spindle cells also

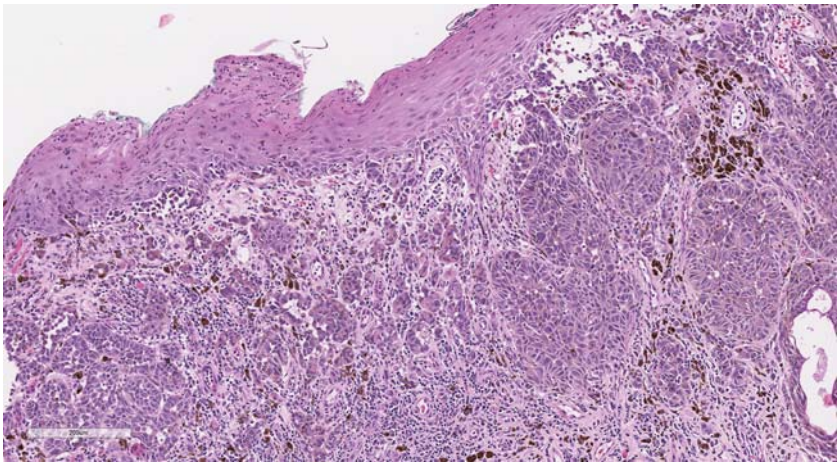

Fig. 5 Anal melanoma. Nests of pleomorphic largely amelanotic tumor cells diffusely proliferate in the subepithelial stroma and focally invade the squamous epithelial mucosa. The pigmented cells are melanin laden macrophages.

occur. Often pagetoid spread of tumor cells in the overlying epithelium is noted. Wide surgical resection is necessary as anal melanoma tends to infiltrate adjacent structure profusely. Regional lymph node metastases (including to inguinal lymph nodes) are frequent.

Amelanotic melanoma can be difficult to differentiate from gastro-intestinal stromal tumor (GIST). Both tumors express c-Kit and immunohistochemical staining for CD117 may lead to confusion.

Molecular Pathology and Genetics

Melanomas often harbor *KIT* or *BRAF* mutations and respond to tyrosine kinase inhibitors such as imatinib or BRAF inhibitors such as vemurafenib.

Further Reading

Bernardi, M.P., Ngan, S.Y., Michael, M., Lynch, A.C., Heriot, A.G., Ramsay, R.G., Phillips, W.A., 2015. Molecular biology of anal squamous cell carcinoma: Implications for future research and clinical intervention. The Lancet Oncology 16, e611–21.

Bucchi, D., Stracci, F., Buonora, N., Masanotti, G., 2016. Human papillomavirus and gastrointestinal cancer: A review. World Journal of Gastroenterology 22, 7415–7430.

Graham, R.P., Arnold, C.A., Naini, B.V., Lam-Himlin, D.M., 2016. Basaloid squamous cell carcinoma of the anus revisited. The American Journal of Surgical Pathology 40, 354–360.

Hoff, P.M., Coudry, R., Moniz, C.M., 2017. Pathology of anal cancer. Surgical Oncology Clinics of North America 26, 57–71.

Jones, C.M., Goh, V., Sebag-Montefiore, D., Gilbert, D.C., 2017. Biomarkers in anal cancer: From biological understanding to stratified treatment. British Journal of Cancer 116, 156–162.

Martin, D., Rödel, F., Balermpas, P., Rödel, C., Fokas, E., 2017. The immune microenvironment and HPV in anal cancer: Rationale to complement chemoradiation with immunotherapy. Biochimica et Biophysica Acta 1868, 221–230.

Messick, C.A., Rodriguez-Bigas, M.A., 2017. Anal dysplasia. Surgical Oncology Clinics of North America 26, 33–43.

Roma, A.A., Liu, X., Patil, D.T., Xie, H., Allende, D., 2017. Proposed terminology for anal squamous lesions: Its application and interobserver agreement among pathologists in academic and community hospitals. American Journal of Clinical Pathology 148, 81–90.

Yang, E.J., Kong, C.S., Longacre, T.A., 2017. Vulvar and anal intraepithelial neoplasia: Terminology, diagnosis, and ancillary studies. Advances in Anatomic Pathology 24, 136–150.

Animal Models of Cancer: What We Can Learn From Mice

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Glossary

Allograft Transplantation of organ or tissues from one individual to another of the same species with a different genotype. For example, transplantation of mouse cell lines in recipient mice.

Cre/LoxP system Cre/LoxP system used to spatiotemporal control the activation of gene modifications by implementing enzymes (Cre recombinases) able to mediate recombination of DNA sequences flanked by Loxp sites, previously introduced in the germline of a desired organism.

Engraftment Successful incorporation of transplanted material into the body of the recipient organism.

Immunosurveillance A process by which the immune system of immunocompetent individuals is able to initially detects and suppress neoplastic cells.

Translational oncology A process by which basic knowledge of oncological processes is used to achieve clinically relevant advancements.

Transposons A DNA sequence able to change its position in the genome. Therefore, transposable elements can create gene modifications by their insertion in or near genes.

Tumor microenvironment Environment created by the interaction between tumoral cells and their niche composed of immune cells, stromal cells, and normal cells.

Xenograft Transplantation of organ or tissues from one individual to another of different species. For example, transplantation of human cell lines or tumor material in recipient mice.

Introduction: The Mouse as Experimental Model

A range of animal models are being exploited to experimentally address fundamental biological processes and the choice of which species is most suitable depends on the type of biological question. For instance, the genetics of pattern formation is studied primarily in Drosophila, neural system development in *C. elegans* and physiology in rats whereas experiments addressing complex cognitive function are performed in monkeys. The mouse serves as the most preferred species for studying mammalian development, and (human) diseases with emphasis on genetic diseases that are either inherited or somatically acquired as is almost always the case for cancer. The mouse has several features making it an ideal experimental model: it is a relatively cheap and very efficient breeding mammal with large litters and short gestation time; its (embryonal) development, physiology and metabolism shares many similarities with humans. Initially, in the prerecombinant DNA era, cancer modeling "in vivo" involved the exposure of mice and other rodents to cancer-causing agents such as chemical compounds, radiation and pathogens such as viruses. On this basis, mouse models have been established for many tumor types. This approach has been taken to test the carcinogenic nature of all kinds of environmental agents. With the advent of sophisticated gene modification technologies, the use of genetically engineered mouse models (GEMMs) started to increasingly dominate the cancer modeling arena permitting the fundamental study of causality at the molecular level. An enormous body of knowledge on cancer related genes has been acquired from studies using GEMMs and this expertise is now employed for building accurate models of human cancers to study the onset and progression of cancer in vivo and for the preclinical evaluation of therapeutic intervention protocols.

We provide here a comprehensive yet selective description of the various preclinical mouse models, outlining their suitability for possible applications and their drawbacks.

Modeling Cancer in the Mouse

Both in vitro cell line testing and mouse modeling are being used to facilitate the process of anticancer drug discovery. Without doubt, in vivo tumor models constitute the best tool when it comes to comprehensively mimic the genetics, the morphology, and ultimately the intimate crosstalk between cancer cells and their local and systemic environment including immune cell infiltrations. However, they have a range of drawbacks as well and the different systems have their own specific advantages and disadvantages (Table 1). Cell line tests in vitro and mouse models are complementary and both needed, depending on the question to be answered. That being said, in this article we will focus on mouse models. Currently, there are different mouse

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Table 1 Characteristics of cancer-related mouse models

	External agent induced models	*Cell transplantation models*	*PDX*	*GEMMs*	*Transplanted organoids models*
Pros	• De novo tumor development • Multistep process of tumorigenesis • Immunocompetent mice • Can be combined with GEMMs	• Low cost and synchronous tumor growth • Rapid preclinical evaluation of drug candidates • Easy monitoring	• Bypass the potential artifacts of in vitro cultures • Orthotopic PDX models more predictive of human therapeutic response • Extensively characterized tumor collections • Coclinical trials to guide treatment decisions	• De novo tumor development • Multistep process of tumorigenesis • Genetic lesions can be locally and temporally controlled • Synchronous tumor growth • Immunocompetent mice	• Rapid preclinical evaluation of drug candidates • Reproducible tumor growth • Multistep process of tumorigenesis • Can be made for almost every tumor type • Synchronous tumor growth • Immunocompetent host (if from mouse)
Cons	• Not all forms of cancer are recapitulated • Latency and penetrance not homogeneous in the cohort	• Mutation load of most established mouse cell lines is limited • Immune compromised host for human cell lines • Risk of genetic and phenotypic drift and clonal selection during vitro culturing	• Severely immune compromised recipient • Variable engraftment rates • Undergo selective pressure, diverging from the primary tumor after multiple passages • Logistics • High costs	• Time consuming strategies for stem cells manipulation • Time consuming backcrossing to the desired background • High costs	• Immune compromised host for human organoids • Culture reagents expensive • Heterogeneity might be lost.

model categories that are being used in different areas of cancer research (Fig. 1). We conveniently divide them in the following five categories:

1. External agent-induced tumor models.
2. Cell line-derived mouse cancer models (allograft, xenograft).
3. Patient-derived xenografts (PDX).
4. Genetically engineered mouse models (GEMMs).
5. Transplantable organoids models.

External Agent-Induced Models

Cancer is a genetic disease. However, the contribution of inherited genetic lesions to the development of the majority of cancers is relatively modest and most cancer-causing mutations are somatically acquired through spontaneous events or environmental exposure. Carcinogen exposure has been linked to higher risk of developing cancer already for over 200 years when the incidence of nasal polyps was found higher in users of sniff and scrotal cancer in chimney sweepers exposed to polycyclic aromatic hydrocarbons (PAHs). Reducing the exposure to PAHs diminished the cancer risk. Other noteworthy examples are the association of lung cancer to tobacco consumption, bladder cancer to exposure to aromatic amines and mesothelioma to asbestos. Therefore, cancer is for a substantial part an environmental disease connected to the exposure to compounds that can induce DNA mutations.

For this reason, external agent-induced mouse cancer models are valuable for mimicking some human tumors, particularly those that are caused by environmental exposure. Several distinct carcinogens can be used to induce tumors through a defined multistep process. Pioneer studies have been carried out by locally applying ultraviolet light to develop skin tumors such as squamous cell carcinoma. Another well-established protocol encompasses the combination of the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA), also known as the initiator, and 12-O-tetradecaboylphorbol-13-acetate (TPA), known as the promoter for causing papillomas which may evolve into squamous cell carcinoma (Fig. 1A). This treatment recapitulates the multistep process of tumorigenesis, and the topical application of the carcinogen followed by the promoter enables the formation of local tumors which can be easily monitored. There are other efficient procedures not only to induce skin tumors, but also breast (by a combination of progesterone receptor stimulation and DMBA) and colon cancer (combining the proinflammatory agent dextran sodium sulfate, DSS, and the DNA damaging agent azoxymethane, AOM), as well as lung cancer (urethane). Unlike skin tumors, the other tumor types are more difficult to monitor and require micro-CT or ultrasound imaging.

Since 1970, these models have been used by the US National Cancer Institute to test the carcinogenic potential of many compounds in the so-called Carcinogenesis Bioassay Program, this in order to assess safe exposure levels. Mouse models of chemical carcinogenesis are currently used in experimental cancer research, as well as translational research in particular for chemoprevention or early detection of cancer.

A number of inbred mouse strains spontaneously develop cancer at high incidence or following environmental exposure. They have been used to study the properties of spontaneous cancers, to identify cancer genes and to assess the effects of carcinogens and drugs. However, these models only develop a limited subset of tumors, show variable penetrance and latency, and do not accurately reflect the common types of human cancers.

More recently, chemical carcinogens have been combined with GEMMs which allow study of the complex interaction between genotype and environmental exposure to cancer development. This approach can yield new insights in areas of cancer research that have been gaining more attention over the last 10 years such as the epigenetic basis of cancer, cancer prevention, the systems biology of cancer, and the effects of inflammation and the immune system on cancer development (Table 2).

Cell Line-Derived Mouse Models

From 1950 onwards, researchers have used human cancer cell lines. In spite of their limitation in mimicking the complexity of human tumors, they represent a very useful tool for biochemical analysis and a wide variety of screening approaches. Evidently, cell lines are more convenient to define the critical drivers of transformation and evaluate the therapeutic efficacy of candidate anticancer agents (e.g., EGFR inhibitors). A logical next step is to assess to what extent the in vitro behavior of tumor cells has its corollary in vivo.

Cell line transplantation models exploit in vitro established tumor cell lines. Low cost, and synchronous tumor growth make cell lines attractive for the rapid identification and validation of cancer-related genes and preclinical evaluation of drug candidates. Consequently, cell line transplantation models serve as the work horse in both basic and translational oncology. Eighty-two percentage of articles published in 2016 that include the use of mouse cancer models are based on cell line transplantation according to Gengenbacher and colleagues in a recent analysis published in Nature Reviews in 2017.

Transplantable cell lines can be established from mouse (allograft) or human (xenograft) tumors. Xenograft studies have to be performed in immunodeficient mouse strains, such as athymic nude mice, severe combined immunodeficiency (SCID), non-obese diabetic (NOD)-SCID, and recombination-activating gene 2 (*R*ag2)-common-gamma chain knockout mice and combinations thereof. The more severely immunocompromised the host, the higher the probability that human tumor cells will efficiently engraft.

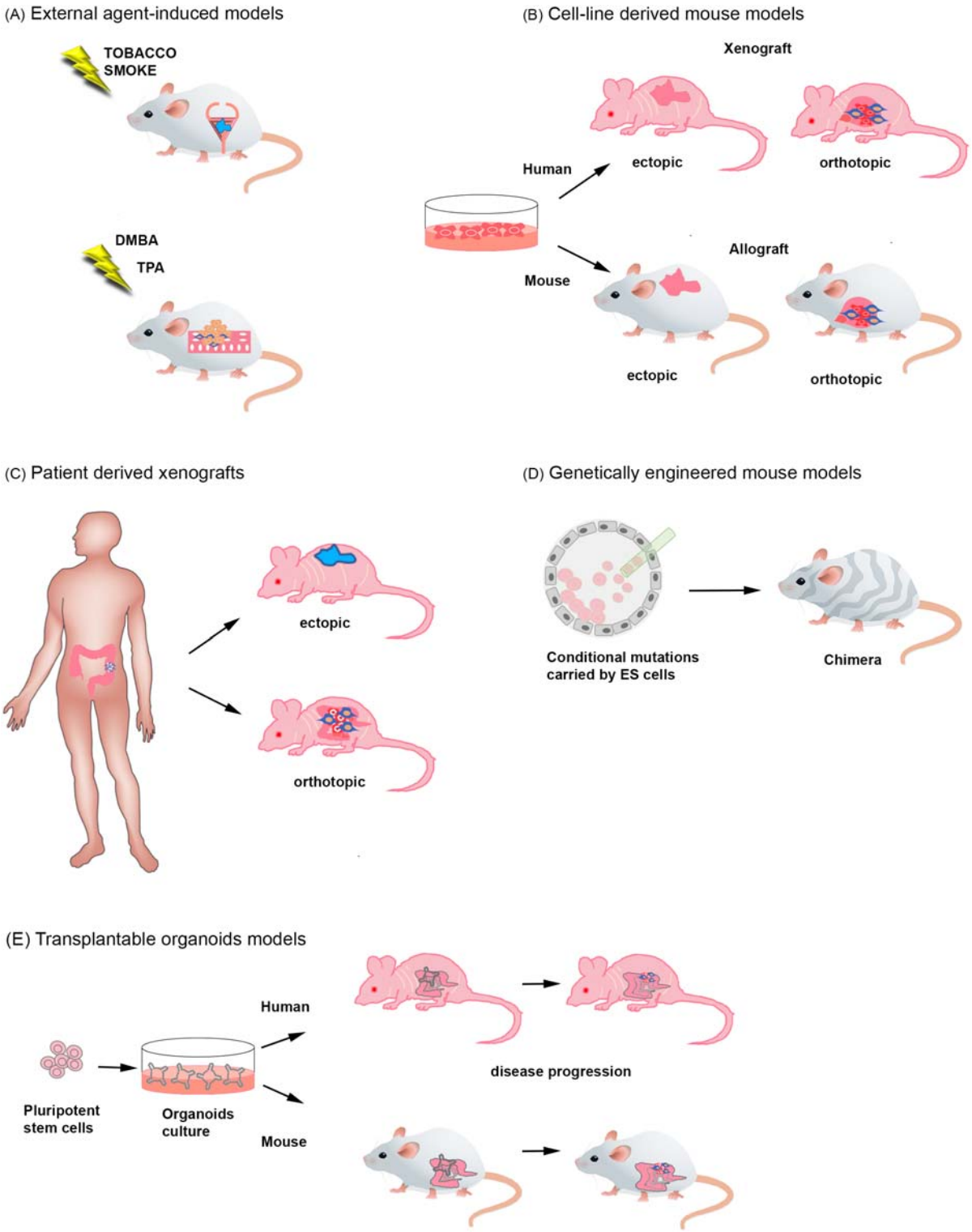

Fig. 1 Categories of cancer-related mouse models. (A) Cancer can be induced in mice by exposure to external agents such as carcinogens contained in tobacco smoke or DMBA/TPA which can cause bladder and skin tumors respectively. (B) Cell lines of human origin (xenograft) can be transplanted either ectopically (different site of original tumor) or orthotopically (same site of original tumor) into immunocompromised mice. Mouse cell lines (allograft) can be transplanted into immunocompetent mice either ectopically or orthotopically. (C) Patient derived xenografts are directly transplanted into recipient immunocompromised mice either ectopically or orthotopically, without undergoing in vitro culturing. (D) Genetical engineered mouse models are generated by in vitro modification of embryonic stem (ES) cells that are subsequently injected into the blastocyst of recipient mice to generate chimeric mice. If the mutant ES cells contribute to the germline of chimeric mice, the genetic modification will be stably transmitted to the progeny. (E) Three-dimensional organoids of either human or mouse origin can be established in vitro by culturing pluripotent stem cells or tumor initiating cells. Human organoids can only be propagated into immunocompromised mice (either ectopically or orthotopically), whereas mouse organoids can also be transplanted into immunocompetent hosts. In both cases it is possible to follow the multistep process of tumorigenesis, from the formation of initial lesions, to disease progression (metastasis).

Table 2 The utility of mouse models on different areas of cancer research

	External agent induced models	*Cell transplantation models*	*PDX*	*GEMMs*	*Transplanted organoids models*
Prevention	• Testing agents that reduce DNA damage • Testing inhibitory strategies of tumor outgrowth	• Testing inhibitors of colonization (e.g., upon IV injection)	• Not suitable	• Testing inhibitory strategies of tumor outgrowth	• Testing inhibitors of colonization
Drug development	• Not suitable	• For validation	• Limited combinations (coclinical trials, Avatar)	• For validation	• Limited combination (coclinical, Avatar)
Metastasis	• Primary tumor too difficult to resect due to multifocal growth • Latency too long for spontaneous metastasis • Asynchronous formation in multiple organs • Few models develop spontaneous metastasis • No certain correlation genotype-phenotype	• Primary tumor is easy to remove because implanted in one site • Orthotopic models have a higher incidence of metastasis • Lung and liver metastasis over-represented in subcutaneous or tail vein injection tumor models • Highly metastatic variant can be selected	• Can be directly derived from patient metastasis • If not directly derived from metastasis, primary tumor is easy to remove because implanted in one site • Orthotopic models have a higher incidence of metastasis • In ectopic models, metastatic spread is affected by the way of injection • No possible to study interaction with Immune system unless using humanized mice	• Primary tumor too difficult to resect due to multifocal growth • Latency too long for spontaneous metastasis • Asynchronous formation in multiple organs • Few models develop spontaneous metastasis	• Suitable for some tumor types

Mouse or human cell lines can be inoculated ectopically (in a tissue different from the original primary tumor site), orthotopically (the cognate location of the original tumor), or systemically (via intravenous injection) to facilitate colonization of multiple sites. Orthotopic models are best suited to mimic the interaction of tumor cells with their natural microenvironment (Fig. 1B).

Mouse allograft may be superior to human xenograft models because of species compatibility and option to use syngeneic mice with an intact immune system allowing studies of the in vivo interaction between tumor cells and the immune system. However, the collection of mouse cell lines is relatively modest compared to the panel of human cell lines. Many of these cell lines were established from tumors induced by chemical mutagenesis.

Fortunately, the number of cell lines derived from tumors induced in GEMMs is steadily expanding. In contrast to many of the human tumor cell lines, they harbor relatively few mutations. Since immunogenicity appears to correlate with mutational load these cell lines are less suitable for immunotherapeutic studies. However, this can be overcome by equipping these cell lines with defined antigens that are recognized as foreign by the immune system.

Human xenograft cell lines might be more predictive of response to therapeutic compounds specifically targeting tumor cells. Although cost-effective and convenient to propagate and utilize in xenograft experiments, these long-term propagated cell lines suffer from the way they have been derived. The long-term culturing of cells often results in the selection of clones that have a high proliferation rate and are adapted to artificial culture conditions. This selection often profoundly affects their phenotypic characteristics, causes further genetic drift and skews their response to many anticancer agents, one of the reasons why cell lines and allo- or xenograft models are not very reliable predictors for drug responses in humans.

To overcome this limitation, researchers are increasingly switching to organoid models that can be propagated more stably in vitro under specific culture condition or to PDX models. In the latter case, the tumor is excised from the patient and immediately implanted into a recipient mouse. Thereby, the artificial culturing step and in vitro selection is eliminated (see following section). A further refinement constitutes humanized mouse models that are engineered such that (parts of) a human immune system can be cografted in the mouse and its interaction with the xenografted tumor assessed.

Patient-Derived Xenografts

Patient-derived xenografts (PDXs) are obtained by transplantation of human tumor material directly into recipient immune-deficient mice (Fig. 1C). Hence, compared to cell line-derived mouse models, they bypass the potential artifacts of in vitro culture.

In order to establish a PDX model, efficient engraftment of primary tumor tissues is needed. This requires an even more severely immunocompromised recipient than used for most cell line transplantation studies. NOD-SCID mice with IL-2Rγ mutations (NSG or NOG mice) have enhanced immunodeficiency and permit engraftment of most human cancers.

Once they have given rise to a sizable tumor, tumor cells or tumor fragments are transplanted into secondary recipients for further expansion. The expanded tumors can then be harvested and cryopreserved or transplanted into tertiary recipients for specific studies. Tumor transplantation routes are similar as described above with orthotopic implantation usually being the most challenging. However, the advantage of the latter is that the natural local interaction of the implanted tumor with its microenvironment is preserved. Therefore, orthotopic PDX models are believed to be more predictive of human response to therapeutic intervention.

Currently, there are many collections of PDXs covering a range of human tumor types. They are often well characterized in terms of genomic, transcriptomic, and proteomic features, metastatic behavior, treatment response to a variety of standard treatments and experimental therapeutics.

Several studies have shown that PDXs largely retain the biological characteristics, the genetic mutations, the gene expression profile, the histopathology, and the therapeutic response of the original tumor. Remarkable overlap between PDXs and patients' response to anticancer drugs has been shown in, for example, in colorectal and pancreatic adenocarcinoma PDXs models, whose response to cetuximab and gemcitabine, respectively, closely resembled those of the corresponding patients enrolled in clinical trials.

This has led to an increased application of PDXs in personalized medicine, where so called "Avatar PDX models" are employed in coclinical trials to guide treatment decisions for patients from which they were derived.

Despite these advantages of PDX models, sometimes poor engraftment rates, slow growth, and high costs as well as logistic difficulties have limited their application as recorded over 2016, at least in academia. According to Gengenbacher and colleagues, PDX models were employed in only 7% of cancer-related studies in 2016.

In addition, a recent study argues against the common belief that PDXs model are better than cell-line transplantation models in terms of genomic stability. According to this study PDXs models are exposed to very different selection pressures as compared to the primary tumors in the donor patient: some copy number alterations which are barely detectable in human tumors were found to be selected for whereas prominent lesions were irreversibly lost. Moreover, PDX models share a number of drawbacks with human cell line-derived models. They cannot be xenografted into immunocompetent mice and PDXs as such are therefore not suitable to study the role of the immune system in immunosurveillance which is believed a critical component of tumor–host interactions.

Oncoimmunology research generally has been performed in mouse models lacking human cells, defining an area of cancer research where the use of human material was limited. However, the recent development of Humanized PDX models offers new inroads for studying tumor–host interactions.

Humanized PDXs are based on the coengraftment of patient tumor with hematopoietic stem cells (HPSC) from the same patient. Successful coengraftment depends on the mouse strain used and the isolation of HPSC (usually CD34 expressing cells). Mouse strains which better support the engraftment of the human immune system are NSG and NOG mice. These mice strains

can be further optimized to support growth of human innate immune effector cells through the replacement of four endogenous mouse cytokines, important for innate immune cell development with their human counterparts (e.g., interleukin-3 (IL-3), granulocyte–macrophage colony-stimulating factor (GM-CSF), M-CSF and thrombopoietin). Variations on this theme constitutes the cografting of tumor cells and human B or T cells tuned to recognize specific antigens or epitopes expressed or presented by the tumor cells.

GEMMs as a Cancer Model

Genetic modification technologies

In the 1970s of last century recombinant DNA technology caused a revolution in biological science. This made it possible to molecularly characterize the genetic events underlying the development of cancer both in humans and in (experimental) animals. For example, the role of viruses involved in the onset of cancers in different species such as the mouse and chicken, could now be studied at the genetic level and a substantial range of oncogenes were identified through this route. Moreover, human oncogenes were uncovered by the molecular analysis of genetic events such as translocations in hematopoietic malignancies. In all these cases solid validation of the oncogenic capacity of these genes required their introduction and expression in an in vivo system, that is, a transgenic animal, preferably a mammal. To achieve this, the initial approach was to genetically manipulate embryos in an early as possible stage. The mouse had been widely used in embryological research for a long time and isolation, culture and handling of mouse preimplantation embryos and their subsequent transfer into fosters were already common practice. Therefore, the first attempts to genetically manipulate the mammalian genome were performed in the mouse. The first successful experiment introducing foreign DNA into all cells of a mouse was reported in 1971 even before recombinant DNA technology was available. In this experiment SV40 DNA isolated from viral suspensions was injected into the cavity of mouse blastocysts, which resulted in life-born pups carrying the SV40 DNA in their genome. A few years later retroviral infection of preimplantation murine embryos was described as an efficient method for the introduction of foreign genetic information into the genome of the mouse. However, the value of these approaches to analyze the biological significance of the transferred genetic information, for example, in validating the candidacy of identified oncogenes in vivo, was limited because the genes embedded in proviral genomes integrated in preimplantation stages were in most cases not expressed due to methylation triggered by their proviral backbone.

The most effective and relatively straightforward method to generate transgenic mice was reported in 1981 and was based on the direct injection of purified DNA molecules in the pronucleus of zygotes that were subsequently transferred into the oviduct of pseudopregnant fosters (**Fig. 2**A). Usually, multiple copies of the injected DNA are incorporated randomly in the host genome and transmitted to all daughter cells and consequently present in all cells of the developing animal. This method yielded very high success rates and remained for a long period the standard for the production of transgenic mice, although for some of the most-used laboratory mouse strains (e.g., C57Bl6) its efficiency was rather low. This was mainly due to limited responses to super-ovulation procedures and poor in vitro development of the injected zygotes. Furthermore, proper tissue specific expression of transgene constructs was often a matter of trial and error and the tissue-specific expression levels could vary substantially between independent transgenic mice depending on copy number and integration site. This variation can also be quite informative as it permits assessment of the expression level on the phenotype.

Overexpression of putative oncogenes has been observed in many human tumors and the oncogenicity of many of these has been validated in transgenic mice. Subsequently, such strains were shown to be instrumental in identifying new collaborating oncogenes in screening approaches using insertional mutagenesis with replication competent murine leukemia viruses. Also, the effects of the combinations of oncogenes could be easily studied in vivo by crossbreeding mice overexpressing these oncogenes.

Whereas transgenesis enables study of the effects of overexpression of individual genes in an intact animal, the normal function of genes cannot be properly addressed by this approach. For this, gene inactivation in a living animal is necessary. While the number of transgenic mouse strains did rapidly grow during the 1980s, at the same time researchers in embryology put quite some effort on the isolation of embryonic stem cells from the inner cell mass (ICM) of blastocysts and on the conditions to culture and manipulate these cells with the aim to retain their totipotency. An essential criterion for this is that ES cells introduced into developing preimplantation embryos contribute to the germ line of the resulting chimeric mouse (**Fig. 1**D and **2**B). These efforts finally resulted in robust procedures enabling the generation of ES cell clones carrying targeted modifications in defined endogenous genes. The basis of these procedures is homologous recombination of DNA fragments carrying a desired modification with the cognate gene of interest in ES cells resulting in the desired modification in the target gene. Homologous recombination in ES cells is very efficient and this ensures that targeted insertion is much more frequent than random insertion facilitating the identification of correctly modified ES cell clones. Initially, the application of these procedures focused on the simple inactivation of endogenous genes in ES cells, which were subsequently used to produce chimeric mice that transmitted the knockout gene to their offspring. In the area of cancer research knockout strains have been invaluable for the validation of tumor suppressor genes and for assessment of their normal functions.

Genetic modification via the ES cell route is much more labor-intense and time-consuming than transgenesis via pronuclear injection. However, since all the steps in the procedure including the check for the desired genetic alterations can be performed in vitro, only those ES cells that contain the desired genetic changes will be used to generate a mutant mouse strain and thus, the modifications in the ultimate animal are exactly known which is not the case in transgenic animals generated via pronuclear injection.

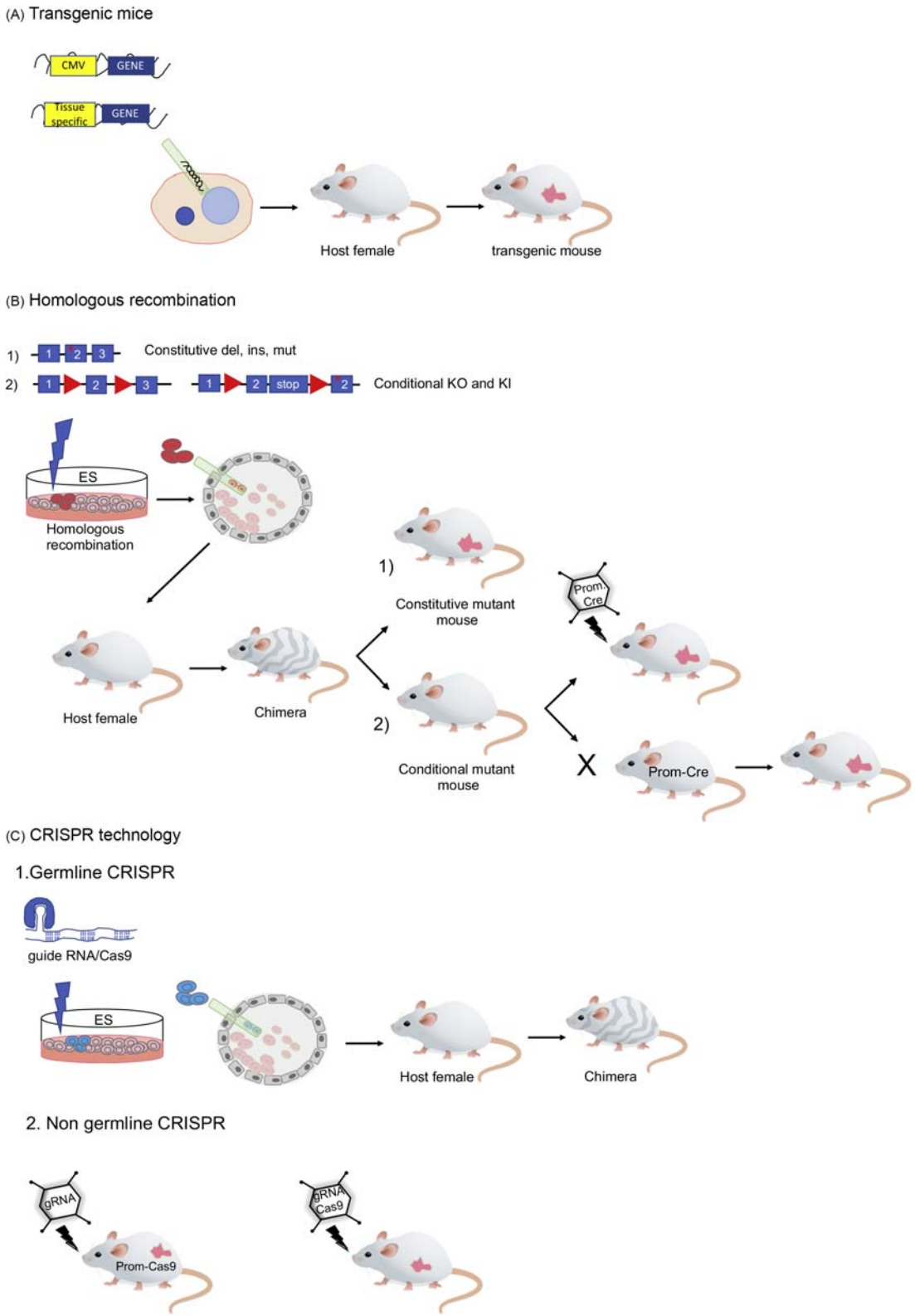

Fig. 2 Approaches to generate cancer mouse models by genetic modifications. (A) Transgenic mice are generated by introducing purified DNA (plasmid) carrying the transgene, into the male pronucleus of a fertilized egg. This approach can only be used to over-express gene product. The transgene can be constitutively expressed (CMV promoter) or can be driven by a tissue specific promoter. (B) Knock out of tumor suppressor genes or knock in of mutation in oncogenes can be achieved by specifically targeting the homologous genes in ES cells. Targeting vector carrying either constitutive deletion, insertion or mutations (1) or conditional modification (e.g., by using Cre/LoxP system) (2), flanked by arms of homology with the endogenous gene locus, to be electroporated into ES cells in order to modify the genome by homologous recombination. Mutant ES cell clones are subsequently injected into the blastocyst of pseudopregnant females. If the mutant ES cells contribute to the germline of the derived chimeric mice, the mutation will be stably transmitted to the progeny. If the mutation introduced is conditional, its activation can be can be spatiotemporally controlled by injection of recombinant viruses carrying Cre recombinase under specific promoters. The activation of the mutation can also be controlled by crossing the mouse model with other mice carrying Cre recombinase under the control of tissue specific promoters. In case the Cre is fused to a hormone binding domain additional temporal control is possible by administering the corresponding hormone.

In addition, the ES cell route allows the introduction of more complicated modifications in a single ES cell clone before generating animals from that specific clone. This is especially relevant when multiple manipulation steps are needed, for example, in case of creating conditional mutant genes that are required for the local and temporal induction of gene modifications (Fig. 2B).

Conditional gene modification, both activating and inactivating, constitutes a significant advance in the modeling of human cancer development in mice. Along with the control over the activation of gene mutations, a range of reporter systems were developed to visualize, either histochemically or via in vivo imaging, the cells in which the gene mutation had materialized (see below). This enabled the imaging of tumor progression in live animals and also permitted measuring the response to therapeutic intervention.

ES cell manipulation also enables transgenesis in a much more controlled fashion, for example, by targeting gene constructs to endogenous regulatory sequences such as promoters to impose authentic tissue-specific expression. These include oncogenes and constructs encoding reporters for lineage tracing and or recombinases instrumental for effectuating the conditional genetic mutations.

However, despite the enormous progress that has been made over the last decades, the ES cell technology also had its limitations. First, competent ES cells could be isolated only from a limited number of mouse strains (primarily 129) and these strains do not belong to the mouse strains most used in research. In many cases the chimeric mice generated from the modified ES cell clones were bred to the standard mouse strains. As a consequence, the experimental cohorts consisted of mice with variable genetic backgrounds, which can complicate interpretation of the results especially in the case of cancer research. To a large extent this problem can be solved by repeated backcrossing to the desired background, but this procedure is expensive and time consuming and even then, a subset of the genes contributed by the ES cell background will remain part of the backcross line due to linkage with the modified locus or loci. However, recent advances in the culture conditions have made it possible to isolate, culture and modify germline-competent ES cells from virtually any background. As a result, now experiments can be performed in ES cells from any desired strain. Moreover, ES cells can be derived from existing (compound) mouse (cancer) models in order to introduce additional modifications and analyze their effects using the original mouse model as reference. This procedure can tremendously speed up assessing the effects of additional genetic lesions in mice with the same background. As a short cut, using a series of differently modified ES cell clones with an identical genetic background, highly chimeric mice can be generated that directly serve as experimental cohorts which makes study of the effects of additional mutations even faster.

Recently, new revolutionizing genome-editing technologies have become available that are based on engineered nucleases or "gene scissors" such as zinc finger nucleases (ZFN), the transcription activator-like effector nucleases (TALEN) and the CRISPR guided Cas nucleases to precisely target endogenous sequences (Fig. 2C). They offer an accurate, inexpensive and efficient way of modifying genes in living cells including ES cells and even zygotes. The engineering includes inactivation, replacing or inserting distinct DNA fragment but also controlled activation and inactivation as well as introducing chromatin modifications. ZFNs and TALENs are chimeric nucleases composed of programmable, sequence-specific DNA-binding modules linked to a nonspecific DNA cleavage domain. CRISPR/Cas nuclease specificity is based on RNA-guided (gRNA) DNA endonucleases. From a practical standpoint, CRISPR/Cas is now by far the most preferred system as each ZFN or TALEN nuclease must be constructed de novo, whereas the specificity of the Cas9 nuclease component is determined by a small guide RNA that can be easily adapted. In addition, CRSPR/Cas is very flexible as it allows the modification of multiple endogenous loci at the same time. Importantly, it also can be applied somatically. Especially for modeling cancer in vivo this is of significant importance. The set-up of proper experimental cohorts can be simplified by applying CRSPR/Cas gene editing through viral or other gene transfer methods directly into somatic tissues of mice from any background strain (Fig. 2C).

Transplanted Organoid Models

First created at the Hubrecht Institute by Hans Clevers and his team in 2009, organoids represent 3D in vitro cultures of tissue stem cells that retain their identity by the use of specific growth media. In order to obtain these 3D cultures, they isolated fragments of the small intestine enriched for pluripotent stem cells and cultures those cells in matrigel supplemented with the growth factors also supplied by their natural niche. This enabled the cells to self-organize and recapitulate the original tissue organization resulting in miniguts in a dish. Organoids can be generated from both mice and humans and can be used to study developmental processes but also to understand the molecular basis of many diseases, including cancer (Fig. 1E).

In 2013, a biobank of genetically diverse human tumor organoids for use in preclinical drug testing has been established by Clever's team and his colleague Johannes Bos, in collaboration with scientists from the Hubrecht Institute, University Medical Center Utrecht, Netherlands Cancer Institute and Wellcome Trust Sanger Institute.

Organoid cultures are established from patients' tumor samples and used to test the response to single drugs or drug combinations. In this way also the underlying mechanisms of drug sensitivity or drug resistance can be explored.

Genome-editing tools like the CRISPR/Cas system, can be used in organoids to identify genes that have a causal role in the development of distinct tumors, that are critical for their survival, or instrumental in resistance to specific drug regimens. Organoid cultures appear relatively genetically stable over time.

Another major advantage is that organoids cultures can be made from almost any tumor, including the ones for which cell lines are lacking, such as prostate, and pancreas. Moreover, they can be used in xenograft studies. Neoplastic organoids have been orthotopically transplanted in mice, fully mimicking the tumorigenic process by forming early-grade neoplasms that progress to locally invasive and metastatic carcinomas.

Transplanted organoids can be used for genomic and proteomic profiling, and for revealing genes and pathways altered during tumor progression.

Recently, a protocol to swiftly create a mouse model of CRC by organoid transplantation into an immunocompetent mouse recipient has been described. The model recapitulates the entire adenoma–adenocarcinoma–metastasis axis in vivo and shows that Wnt pathway activation is crucial for tumor progression. The study also shows preliminary results how to generate a rapid transplantable model from patient-derived human CRC organoids.

Establishing organoids cultures and engrafting them is a routine once protocols for their generation and maintenance have been established. However, the costs of cell culture reagents is high and can therefore be a limiting factor.

The Utility of Mouse Models for Different Areas of Cancer Research

Metastasis

Although metastasis is the major cause of cancer-related deaths, accounting for 90% of cases, studies with preclinical models are still primarily focusing on primary tumor growth.

Metastatic dissemination appears more difficult to model. However, understanding in detail the factors driving metastatic spread and gaining insight into newly acquired vulnerabilities of metastasizing cells is important for designing better intervention strategies. Mouse models in which the whole sequence of events—acquiring migratory capacity, intravasation of tumor cells into the circulation, extravasation into the distal organ, and colonization of the foreign site—can be effectively studied are therefore receiving increasing attention.

GEMMs are the eligible cancer model when it comes to mimic the early stages of tumor initiation and progression, but only a subset gives rise to metastatic growth as observed in humans. The lifespan of GEMM tumor models is often limited by multifocal tumor development, and therefore provide less opportunity for lesions to give rise to metastases. Resection of multiple primary tumors in order to enable the growth of metastasis is in many cases not possible. Limiting the number of target cells in which the oncogenic lesions are induced, for example, by using low titer viral vector infections carrying Cre or Cas9 is one way to increase the probability of inducing fewer primary lesions and metastasis to occur. Nevertheless, there are several GEMMs that spontaneously develop metastasis and have been used as model system of metastasis; this is the case for a breast mouse model in which *Her2* is expressed under the control of Mouse Mammary Tumor Virus (MMTV) and a model of pancreatic islet carcinoma in which SV40 T antigen (Tag) is expressed under the control of the rat insulin promoter (RIP–Tag). These models develop metastasis to lung or other organs. The same holds for small cell lung cancer models that show metastasis to multiple organs.

In the case of pancreatic islet carcinoma, antiangiogenic therapy caused regression of the primary tumor but in parallel elicited progression to a higher malignancy state facilitating the growth of distant metastasis. Besides the relatively few GEMMs that spontaneously develop metastasis, other approaches involving non-autochthonous models have been proven suitable to dissect the process of metastasis. Both cell line- and tumor-xenotransplantation models have been successfully used for this. Despite the disadvantages of transplantation models, tumors can be grafted into a single site, making it relatively easy to surgically remove the primary tumors, allowing the monitoring of the metastatic growth of cells spread to distant sites.

In this regard, orthotopic transplantation models appear the better approach because they have a higher incidence of metastasis compared to ectopic models. Moreover, cells from the primary tumors then can follow their natural routes to spread to distant organs, better mimicking what likely happens in human. Subcutaneous or tail vein injection tumor models are still used but metastatic spread is strongly skewed by this way of inoculation leading to overrepresentation of metastatic growth in lung and liver. Metastatic models have been made more effective by selection of cell variants with a higher metastatic potential as compared to the parental cell lines they originated from. Genetic and/or expression profile differences between the variants and the parental lines then can point to the genes that facilitate metastasis.

Breast and melanoma cell lines either from human patients or GEMMs, are relatively simple to orthotopically transplant compared to cell lines from cancer types, such as lung or prostate. The metastatic process can be best monitored by introducing reporter constructs such as luciferase into the cells. Highly metastatic variants of melanoma cell lines have been derived from the WM-239 human melanoma through in vivo selection involving orthotopic grafting of primary tumor, followed by its resection and isolation of lung metastasis several months later. The resulting highly metastatic variant appeared able to cause metastasis in 4 weeks compared to 4–6 months for the parental cell line. The same approach has also been used to isolate highly metastatic variants of the MDA-MB-231 breast cancer cell line.

Considering mouse models currently available to study cancer, metastatic models using a combination of orthotopic tumor transplantation and surgical resection of the primary tumor, offer the best perspectives as they permit study of all phases of the metastatic process.

Drug Development

Next to their use in the study of tumor development and progression, mouse models play an important role in drug validation as they are "the" preclinical "in vivo" intermediate between "in vitro" testing and clinical trials.

Since 1995, prescreening of new agents was primarily performed by using three cell lines: MCF-7 breast cancer, NCI-H460 large-cell lung cancer and SF268 glioblastoma cancer. Only if a drug would inhibit the proliferation of at least one of these cell lines, it

would proceed to be tested on a panel of 60 cancer cell lines representative of 9 different cancer subtypes. This panel has been used to test 70,000 compounds, including a range of chemotherapeutic agents.

The advances in our insights into the molecular mechanisms driving tumor initiation and progression promoted a shift from random testing to more focused strategies targeting tumor drivers found to be activated in the tumor cells under study. This has resulted in remarkable clinical responses in tumors such as chronic myelogenous leukemia (using inhibitors of BCR-ABL), melanoma (using inhibitors of $BRAF^{V600E}$), and lung adenocarcinomas carrying mutations in EGFR (using EGFR inhibitors).

Nevertheless, long-term clinical remissions for investigational drugs remain rare. The average number of anticancer drugs approved by the US Food and Drug Administration (FDA) per year has declined since 1990s. There is clearly a divergence between R&D costs and the approval of new drugs. Many of the drugs fail during evaluation in clinical trials. An important reason behind this failure is the poor predictive value of standard in vivo preclinical models. As discussed above, xenotransplantation of human cell lines is the simplest procedure to perform drug screenings but not the most realistic one due to the fact that established cell lines are in general not robust predictors of response. Nevertheless, drug screenings using a collection of cell lines combined with xenograft models for a single type of cancer, adequately representing its diversity, is a rational and useful first approach. However, a follow up with more sophisticated testing in GEMMs can have substantial added value as it might bring out tumor features that have been lost in the cell culture adapted, often fast dividing cells used to identify the specific drug sensitivity whereas those "lost features" might be critical for its response to the drug in question.

The suitability of GEMM tumor models for drug screening will depend on a number of parameters: (1) mice should carry a corresponding set of mutations that drives the cognate human tumor and express these in the appropriate target cell; (2) The histopathology should be as similar as possible; (3) tumor development should preferentially proceed through the same, or similar, "preneoplastic" stages.

Even if all these criteria are met: a mouse model remains a model. In this regard PDX models are more suitable than GEMMs when it comes to examine therapeutic responses to drugs (see section "**Patient-Derived Xenografts**" for more details). Nevertheless, the use of GEMMs has increased exponentially in the last decade, thanks to new genome editing technologies (see section "**GEMMs as a Cancer Model**" for more details). Collectively, murine models are critical for drug development, but to the testing trajectory really useful a rational approach is required beginning with target discovery and drug response measurements which can be performed in in vitro, confirmed by genetic approaches in GEMMs, followed by toxicology and pharmacology studies conducted in simple cell-line transplantation models, progressing with PDXs for target validation, and establishing treatments regimen.

One of the advantages of mouse models is that the underlying mechanisms resulting in drug resistance of tumors can be more easily studied. One route to identify drug resistance related mechanisms using "in vivo" material takes advantage of the comparison of resistant tumors with the nonresistant primary tumors. This comparison, which might include WGS, expression and chromatin profiling, might reveal putative resistance genes that can be validated in the same model. Drug resistance mechanisms can also be tackled in GEMMs using the extensive amatory of genetic tools. Screening experiments with shRNA and CRISPR/Cas libraries on tumor cells treated with drugs targeting specific pathway have clearly shown the utility of this concept "in vitro". Screening strategies for drug resistance directly in "in vivo" models can be based on activation of transposon systems, shRNA or CRISPR/Cas9 libraries as used "in vitro". Screening experiments in vitro and in vivo have shown benefits of strategies based on drug combinations targeting multiple pathways or multiple targets in what is considered the "same" pathway, this all depending on the specific tumor subtype and resistance mechanisms that were identified. Also, the specific drug scheduling can be a critical parameter for efficacy. Rigorous "in vivo" validation entails next to the use of transplanted cell lines, tumors in GEMMs in which the cognate pathways are mutated, organoid-based tumors and/or PDXs. This knowledge can be subsequently used to design hopefully more effective clinical trials.

Prevention

Cancer prevention is not only intended as a series of actions taken in order to limit the exposure to environmental agents which have been shown to have a causal role in cancer (primary prevention) but also to limit cancer cell outgrowth or minimize the adverse consequences that result from exposure to tumor-inducing agents. This requires a precise understanding of the genetic and epigenetic factors that promote cancer, and how environmental components such as diet, hormones and drugs affect them (secondary prevention).

Mouse models are particularly suited to investigate aspects of cancer prevention that are difficult to study in humans, such as identifying factors or interventions that prevent the outgrowth of preneoplastic and early tumor lesions. Genetic context and environmental conditions can be precisely defined in mice, which is difficult to accomplish in humans. Mouse model suitable for prevention studies should meet a number of criteria:

1. Tumors should originate de novo in their natural microenvironment and in the context of an intact immune system, which applies to GEMMs, some nongermline GEMMs and carcinogen-induced models, but excludes most non-autochthonous models.
2. Tumor development should reproduce the stages of disease progression from preneoplastic to more malignant lesions; again, this criterion is only met by autochthonous models.
3. Genetic and/or environmental factors employed to cause the cancer should have relevance for the human cancers. For example, loss of function of *Pten* represents the driver genetic alteration of prostate cancer in both human and many GEMMs. Similarly, carcinogens from tobacco smoke are responsible for bladder cancer in both human and mouse models.

4. The histological and molecular features of mouse tumors should resemble their human counterparts; again, this has been recently demonstrated for bladder cancer. Additionally, cells-of-origin should be the same in human and mouse; tumor penetrance should be high in the cohort of mice and latency not excessively long.

There are successful studies performed with GEMMs and carcinogen induced models that have proven the value of cancer prevention models. Examples are experiments carried out in mouse models of colon cancer, in which loss of function mutations introduced in the APC gene either by chemical exposure or by gene manipulation, enabled investigation of chemopreventive agents, such as COX inhibitors. GEMMs can be instrumental for the identification of early detection biomarkers considering the easy access to body fluids and tumors from the earliest stages of cancer initiation to the latest stages of cancer progression. Indeed, putative biomarkers for early detection of ovarian, colon and lung cancer have been already identified by using GEMMs.

Ultimately, a combination of GEMMs, outbred strains and carcinogen induced models will be of great help in understanding the interaction between environmental exposure, the genetic background, and specific oncogenic lesions in the initiation and progression of cancer.

Distinct Cancer-Related Questions (Basic and Translational Medicine Related)/Applications

Identification of Cancer Genes and Their Contribution to Cancer Initiation and Progression

The developments of high-throughput genome sequencing and subsequent analyses using advanced bioinformatic tools have made it possible to profile entire cancer genomes and identify recurrent amplified or repressed gene loci in cancer. The often high complexity of human cancer genomes and the concomitant large number of genetic alterations can make it hard to distinguish "driver" from the "passenger" mutations. Even if recurrent in a subset of patients, a distinct genetic alteration might turn out to be only secondary to tumor formation or occur very late in the multistep process of tumorigenesis thereby contributing only to a limited extent to tumor development. Hence, targeting such alterations will unlikely result in a robust clinical response unless these mutations have created new vulnerabilities or represent drivers of convergent tumor evolution. Here, mouse models can swiftly provide answers (**Table 3**). They enable the validation of candidate cancer genes; they also allow the discovery of new genes and pathway involved in tumorigenesis. In this way the contribution of defined genetic lesions to the process of initiation and progression of human cancer can be assesses.

The "in vivo" identification of (new) driver mutations can be based on several strategies: (1) chemical mutagenesis (e.g., using DMBA) followed by deep sequencing of independent tumors enabling the identification of recurrent mutations indicative of their causality. (2) Infection with replication-competent retroviruses leading to enhanced expression of cancer promoting genes at the site of proviral integration. However, the use of retroviruses has been limited to a small number of tissues with the hematopoietic system and mammary tissue as the most pronounced examples. (3) Activation of transposons, for example, Piggy Bag and Sleeping Beauty, which integrate more or less randomly into the host genome. Transposons have been modified to enable both activation and inactivation of genes near or at the site of integration. As such the use of transposons can enable the identification of tumor driver genes by genome analysis similar to the retroviral tagging approach. To apply this "in vivo", mice need to be transgenically equipped with inducible transposon systems whereas for retroviral infection wildtype mice can be used. However, the transposon system can be spatiotemporally activated (e.g., by using Cre-ERT/LoxP and Dox systems), enabling identification of drivers in cancers arising in many different tissues. (4) Currently, genome wide CRISPR/Cas gene editing (see above) is being widely used "in vitro" in defined cancer cell lines to find genes involved in transformation, migration, metastatic capacity, drug response etc. In principle these screening strategies could also be applied to find new driver mutations "in vivo". However, since tumor development requires multiple modifications in the same cell, CRISPR/Cas screens using retroviral libraries will be challenging in a wildtype background since the likelihood of tagging multiple relevant genes in one cell is low. In a way CRISPR/Cas screens in wildtype mice might resemble chemical carcinogenesis except that it permits cell-type specific targeting which is impossible with carcinogens.

Table 3 The utility of mouse models on different cancer related applications

	External agent induced models	*Cell transplantation models*	*PDX*	*GEMMs*	*Transplanted organoids models*
Cancer gene identification	• Suitable	• Suitable	• Suitable	• Suitable	• Suitable if target is known
Allele variation	• Suitable	• Not suitable	• Not suitable	• Suitable	• Not suitable
Cell of origin and/or tumor propagating cell	• Not suitable	• Can be used to define tumor propagation capability of specific isolated cell populations	• Not suitable	• Lineage tracing experiments require previous genetic manipulation to introduce Cre/Lox sites	• Suitable for identifying tumor initiating cells
Immune therapy	• Suitable to test immunosurveillance	• Useful in syngeneic setting	• Not suitable unless humanized systems are applied	• Suitable if engineered with appropriate neoantigens	• Not suitable unless humanized systems are applied

Transposon systems are also attractive for identifying tumor progression events by including these "mutagens" in established tumor models. This can lead to accelerated primary tumor development, metastatic spread as well as drug resistance, the latter when inapplied a therapeutic intervention setting.

An attractive bioinformatic-oriented strategy to identify new drivers is based on the molecular comparison of murine cancers generated by combinations of gene modifications with their human counterparts in which the same genes are affected. This comparison includes deep sequencing analyses, expression profiling and the characterization of copy number variation and epigenetic landscape changes in syntenic regions. These comparisons have provided candidate genes that could be subsequently validated in the relevant mouse model (e.g., Nfib and p73 in SCLC).

Allele Variation: Predisposition

Most mouse tumor models have been generated, by whatever technology, in a single genetic background. After validation, subsequent studies of tumorigenesis are usually performed in the same mouse strain. However, it has been known for a long time that the genetic background can strongly influence tumor development. Taking advantage of the many available mouse strains (e.g., mice of the Collaborative Cross) it is possible to identify tumor susceptibility loci and the responsible gene(s). Subsequent cognate human cancers carrying the same somatic driver mutations can be analyzed for the presence of susceptibility conferring alleles of the same gene(s) (**Table 3**).

Cell of Origin and Tumor Propagating Cells

Although the combination of mutations is critical for the tumor phenotype, increasing evidence also points to the cell-of-origin as an important factor in determining tumor characteristics; moreover, the cell-of-origin and the resulting cancer initiating cell can play an important role not only in tumor initiation but also in tumor maintenance and metastasis. Since tumor initiating cell might also be more resistant to treatment their eradication by specific targeted therapies is crucial to avoid tumor relapse.

Cells in a tissue have very different capabilities to give rise to tumors. Some cells, even if targeted by a specific tumor-associated set of mutations will just be resistant to transformation. Moreover, tissue cell subpopulations capable to generate a tumor, will likely produce neoplasms with unique morphology, location, grade of differentiation and hence aggressiveness. Genetic lineage-tracing experiments concomitant with the conditional expression of oncogenes and/or the deletion of tumor suppressor genes in defined cell lineages of mouse models, have been instrumental in defining the cell-of-origin of different human tumors. To achieve the activation of genetic lesions in a subset of cells, Cre or other recombinases are targeted to the desired subset of cells by topical delivery of viral vectors equipped with cell-type-specific promoters driving a recombinase (or Cas9) or by activation of an inducible Cre/Cas9 inserted in a distinct genetic locus with defined expression characteristics (see section "Genetic modification technologies").

Before designing lineage-tracing experiments, it is important to know the hierarchy of stem and progenitor cells in a given tissue, their location and marker profile. Genetic lineage-tracing experiments conducted in wildtype mice carrying a conditional reporter gene (β-galactosidase or a GFP) whose expression can be activated in a particular cell lineage using the aforementioned tools, have greatly contributed to our understanding of the cellular hierarchy of normal tissues under physiological conditions.

Using cell-type specific (in)activation of oncogenes and tumor suppressor genes the cell-of-origin of tumors can be assessed. For example, neuroendocrine cells in lung are the most effective cell lineage to initiate SCLC upon *p53* and *Rb* deletion as shown by Sutherland et al. by using intratracheal delivery of cell-type restricted adenoviral vectors carrying Cre recombinase driven from different promoters, specific for different lung cell subpopulations. The same approach has been used to define the cell-of-origin of lung adenocarcinoma (LADC) and lung squamous cell carcinoma (LSCC). Interestingly, in $Sox2^{Col1A1\,+/-}$;$Pten^{fl/fl}$;$Cdkn2ab^{fl/fl}$ mice, LSCC could be initiated by the same set of genetic lesions in different subsets of lung cells, although with a different efficiency. Accordingly, tumor arising from different cells had different locations.

The tumor propagating cell might retain markers of the cell-of-origin not shared by the bulk of the tumor cells. Tumors can be collected from specific GEMMs or directly from cancer patients and the desired subsets of cells isolated using cell surface markers or fluorescence reporters by activated cell sorting (**Table 3**) . Once sorted, subsets of cells are transplanted into immunocompromised mice. If the cell fraction is able to cause tumor growth and recapitulates the original tumor morphology in serial transplantations, it is considered to harbor the tumor-initiating cell. The ultimate proof can be provided by showing that a single cell has this capacity (as shown for breast cancer and leukemia).

For example, only brain tumor cells positive for the CD133 cell surface marker, were capable of tumor initiation in a severely immunodeficient NOD-SCID mouse graft model. The tumor which was a phenocopy of the patient's tumor, could be serially transplanted, whereas injection of different dilutions of cells negative for CD133 did not cause tumor growth.

Similarly, cells isolated from LSCC of $Lkb1^{fl/fl}$;$Pten^{fl/fl}$ mice positive for $SCA1^{+}NGFR^{+}$ were enriched for tumor-propagating capacity and could be serially transplanted in orthotopic assays carried out in immunocompromised mice.

Immunotherapy (Tumor–Host Interaction; Microenvironment)

Escaping from immune surveillance is now considered as a critical feature for tumor development. Indeed, the immune system plays a role in detecting and eliminating tumor cells from the host. However, there are still many unanswered questions regarding the interaction between the innate and adaptive immune system and tumors arising in an immunocompetent host. Tumors more

likely develop when the innate and/or adaptive immune response is impaired or repressed. This hypothesis has been tested in a variety of mouse models that were deficient in one or more components of the innate or adaptive immune system. For example, the elimination of interferon (IFN)-γ gene resulted in an increased incidence and growth of spontaneous and chemically-induced tumors. However, immune cells can also support tumor growth.

Syngeneic allograft models are used the most in immune-oncology research (Table 3). The immune system can directly influence preclinical tumor monitoring that utilizes reporter genes such as GFP or Luciferase as the reporter proteins are can be recognized as foreign. To make mice tolerant to these possibly immunogenic proteins, "glowing head" mice have been used, in which low levels of GFP or Luciferase are locally expressed by using a transgene expressed in the anterior pituitary gland where these proteins cause immune tolerance because of their expression early in life. As compared to GEMMs, allografts have the advantage to be a simple low-cost model. Furthermore, tumor growth is largely synchronous permitting the investigator to easily follow immune responses over time. However, they often lack specific immunophenotypes such as chronic inflammation which is very often seen in human cancers. Moreover, they mostly arise in a site different from the original tumor (usually subcutaneously) which lacks the natural microenvironment of the original tumor. In spite of these limitations, transplantable models have been critical for three revolutionary discoveries of cancer immunotherapy: (1) the utility of Immune checkpoint blockers (ICBs) to enhance an immune response against tumor cells; (2) the contribution of immunogenic cell death (ICD) to most of the currently used anticancer chemotherapeutic agents (CT26 colon carcinomas respond much more efficiently to chemotherapy when grafted in BALB/c mice with an intact immune system).

Consequently, anticancer therapies using a combination of ICD inducers and ICBs are more effective.

For studying the natural interplay between tumor–host, carcinogen-induced tumor models are more realistic because they carry many mutations, are genetically more diverse and heterogeneous, and tumors arise at their natural site. Carcinogens-induced tumor models can also be used to assess the contribution of the immune system to conventional therapies. Unfortunately, such experiments are cumbersome because tumors take considerable time to establish and can be quite heterogeneous, making it difficult to interpret the results. GEMMs accurately reflect human tumors and are genetically defined but as is the case for carcinogens-induced tumor models, the tumor latency can also be quite long, and monitoring can be challenging dependent on the tumor site. Furthermore, they carry few new mutations. Therefore, these tumors will unlikely express any neoantigens the immune system might react to. It required additional genetic manipulations in order to express defined neoantigens in the tumor. Although much can be learned from autochthonous mouse models, at the same time there remain ample differences between mouse and human and therefore it is important to also aim for humanized models so that the features unique to the human immune system can be studied.

Summary/Prospective Vision

Mouse tumor models have come a long way. The capacity to genetically manipulate the genomes of the mouse have not only resulted in cancer models, it has also provided insight in the function of genes critical for development and maintenance of mammalian organism. Next to their significance for the overwhelming body of fundamental knowledge on cancer development mouse models have been essential for elucidating how the immune system works. With the currently exploding interest in its role in cancer this has not always been appreciated but is now probably their most relevant contribution for the cancer field. This has only been possible by transgenic and knockout studies in the mouse in which the function of genes involved in immune responses was assessed. Undoubtedly, mouse models will remain invaluable to further evaluate the many the modulators that influence immune responses. Mouse cancer models mimicking the cognate human tumors have been similarly instrumental for our understanding of the role of oncogenes and tumor suppressor genes and many other modulating factors that influence tumor development. With the current armature of genetic tools, one can easily generate a wide diversity of mouse models carrying multiple distinct lesions thus permitting to mimic human tumors very closely. Organoids, PDXs and Humanized mouse models will gain in importance and create the conditions in which these models become more predictive with regard to treatment responses. Moreover, the mouse is the system of choice to explore tumor susceptibility genes that only start to have sizable effects when combined with distinct somatic lesions that occur by accident or as a result of environmental exposures. Finally, mouse models in which we can monitor early lesions will be invaluable to develop early detection and rational primary and secondary prevention measures. Therefore, mouse models will be around for the foreseeable future and continue to make contributions to our knowledge that cannot be acquired otherwise.

See also: Nonsmall-Cell Cancers of the Lung: Pathology and Genetics. TP53.

Further Reading

Aparicio, S., Hidalgo, M., Kung, A.L., 2015. Examining the utility of patient-derived xenograft mouse models. Nature Reviews Cancer 15, 311–316.
Dow, L.E., Fisher, J., O'Rourke, K.P., et al., 2015. Inducible in vivo genome editing with CRISPR-Cas9. Nature Biotechnology 33, 390–394.
Francia, G., Cruz-Munoz, W., Man, S., Xu, P., Kerbel, R.S., 2011. Mouse models of advanced spontaneous metastasis for experimental therapeutics. Nature Reviews Cancer 11, 135–141.

Gengenbacher, N., Singhal, M., Augustin, H.G., 2017. Preclinical mouse solid tumour models: Status quo, challenges and perspectives. Nature Reviews Cancer 17, 751–765.
Gould, S.E., Junttila, M.R., de Sauvage, F.J., 2015. Translational value of mouse models in oncology drug development. Nature Medicine 21, 431–439.
Le Magnen, C., Dutta, A., Abate-Shen, C., 2016. Optimizing mouse models for precision cancer prevention. Nature Reviews Cancer 16, 187–196.
O'Rourke, K.P., Loizou, E., Livshits, G., et al., 2017. Transplantation of engineered organoids enables rapid generation of metastatic mouse models of colorectal cancer. Nature Biotechnology 35, 577–582.
Sharma, S.V., Haber, D.A., Settleman, J., 2010. Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. Nature Reviews Cancer 10, 241–253.
Zitvogel, L., Pitt, J.M., Daillère, R., Smyth, M.J., Kroemer, G., 2016. Mouse models in oncoimmunology. Nature Reviews Cancer 16, 759–773.

Anoikis

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What Is Anoikis?

Anoikis is the name given to the induction of apoptosis in cells upon loss of attachment to the extracellular matrix (ECM) and neighboring cells. The relationship between cell adhesion and apoptosis was first elucidated and defined by Steven M. Frisch and Hunter Francis in 1994. In their finding, cell death upon detachment was observed in epithelial cell lines but not in fibroblasts. This phenomenon was then introduced as 'anoikis,' a term derived from the Greek words that mean homelessness. Anoikis plays a fundamental role in preventing inappropriate translocation and attachment of cells, which can lead to abnormal growth in an ectopic environment. Anoikis is largely regulated by interaction with the ECM and nearby cells, although some cellular stress events such as autophagy and hypoxia are also known to be involved in some circumstances.

An example of how anoikis is required for normal cellular function can be seen in the turnover of enterocytes. Enterocytes are epithelial cells lining the small intestine, responsible for the absorption of nutrients in the digestive system. These cells have a short life span of approximately 3–5 days, and they are constantly renewed from a pool of precursor cells, migrating towards the apex of villus in the small intestine and eventually dying. As such, enterocyte cell death has been demonstrated to take place through the induction of anoikis when they are detached from the ECM of the villus and lose signaling from the cell adhesion molecules (CAM).

ECM is a mesh of various molecules, such as proteoglycans and proteins, that serve as a scaffold to accommodate cell localization and attachment. The composition of ECM shows variation across tissue types, which determines their roles and cell type specificity for adhesion. Components of the ECM are known to be produced and excreted into the ECM by several types of cells, mainly fibroblasts. Examples of such components are collagen, fibronectin, hyaluronic acid, and laminin. Each of these molecules is capable of various functions, such as assisting cell attachment, ligating numerous receptors, and even storing growth factors within the ECM.

In addition to providing structural support, ECM also participates in wound healing through active remodeling of its structure, which is mediated by fibroblasts. Epithelial-to-mesenchymal transition (EMT) is also induced as a result, enabling repopulation of cells to replace the injured tissue. While this process is strictly regulated in normal cells, cancer cells often abuse the dynamic nature of ECM to induce EMT to gain a more aggressive phenotype during metastasis. As such, the preservation of normal functions and integrity of the ECM is essential in maintaining proper tissue homeostasis.

Aberrantly induced anoikis has been shown to be responsible for various diseases, such as aneurysm and chronic vascular diseases. Normally in these situations, it is not the properties of the cells themselves that causes anoikis, but rather the changes in the ECM, such as degradation or modification of ECM proteins by proteases. For example, plasmin mediated proteolysis of fibronectin in the ECM was observed to cause smooth cell anoikis, whereas methylglyoxal mediated modification of type IV collagen was found to cause endothelial cell anoikis. In the absence of survival signaling from binding to these ECM ligands, cell detachment ensues and anoikis is subsequently initiated. While such diseases occur due to the gratuitous activation of anoikis, the biggest threat posed by anoikis malfunction happens when it is suppressed during carcinogenesis, contributing to invasion and metastasis of cancer cells.

The interaction between cells and the ECM is facilitated by the focal adhesion. Focal adhesion is an intricate assembly made up of integrins, a type of CAM, and various adaptor proteins. The function of focal adhesion is in forming the connection between ligands found in the ECM and the actin cytoskeleton. Furthermore, the intracellular portion of the focal adhesion also associates with various kinases, such as the focal adhesion kinase (FAK), to initiate signaling pathways. As such, focal adhesion serves as the bridge between the extracellular signals and the intracellular events related to cell attachment, motility, and apoptosis.

Like cell–ECM interaction that provides signals inhibiting anoikis, cell–cell interaction also generates similar signaling, occurring through another CAM known as cadherin. A notable member of the cadherin family pertinent to anoikis is E-cadherin, which can be found expressed in epithelial cells. This receptor is ligated by other E-cadherins from neighboring cells and its activation is necessary for various survival signaling pathways. Additionally, E-cadherin is also connected to the actin filaments through α-catenin, providing a link between cell–cell interaction and intracellular cytoskeletal dynamics.

When cells are detached and anoikis is induced, cell death takes place through the apoptotic pathways. This means that anoikis could occur through both intrinsic and extrinsic apoptotic pathways. Loss of attachment causes changes in the cytoskeletal dynamics, initiating the intrinsic pathway to cause permeabilization of outer mitochondrial membrane through the actions of apoptotic regulators. Meanwhile, similar activation of extrinsic pathway can also occur during anoikis through the upregulation of ligands for the tumor necrosis factor receptor (TNFR) family. Regardless of the route taken, both intrinsic and extrinsic apoptotic pathways lead to the eventual activation of caspases, which causes cell death. Additionally, caspase-independent cell death has also been established as a possibility in anoikis through a mitochondrial protein, Bit1. Taken together, the apoptotic regulators involved in these pathways are important players in anoikis execution. However, it is the upstream signaling factors related to cell adhesion that gives anoikis its additional distinction from the basic apoptosis process, which will be discussed in the next subtopic.

Regulation of Anoikis

Signaling pathways and major players governing anoikis sensitivity and resistance have been well documented over the years. As previously mentioned, the information regarding cellular attachment to the ECM and neighboring cells are relayed by a number of cell surface proteins. Since the anoikis network connects the cytoskeletal system with the apoptotic machinery, various signaling pathways associated with survival and apoptosis have been shown to function downstream of receptors and integrins.

One of such receptors is the epidermal growth factor receptor (EGFR). EGFR belongs to the ErbB receptor family and is often found mutated to be constitutively active in cancer. In an active homodimeric or heterodimeric form when ligated, EGFR transduces signals to initiate key pathways regulating anoikis, such as the MAPK/ERK and PI3K/Akt pathways. The initiation of these pathways results in activation of downstream effectors promoting survival and inhibiting anoikis. Additionally, activation of MAP/ERK signaling pathway by EGFR also results in the phosphorylation and degradation of the proapoptotic protein Bim, further enhancing the anti-anoikis effect of activated EGFR. Due to the prevalent nature of EGFR in preventing cancer cell apoptosis, inhibition of EGFR as a therapeutic approach has shown success in clinical settings, such as through the use of erlotinib, a receptor tyrosine kinase inhibitor. Interestingly, cancer cells have shown the ability to overcome such inhibition through the formation of an EGFR heterodimer with insulin growth factor 1 receptor (IGF1R), conferring drug resistance and enabling cell survival. IGF1R is another cell surface receptor well known to suppress anoikis. As a hormone receptor, IGF1R plays a crucial role in body growth and development. As for its regulation of anoikis, ligation of IGF1R prevents anoikis from being induced mainly through the activation of PI3K/Akt pathway.

Findings from a large number of studies on anchorage-independent growth provide a consensus on the major role played by MAPK/ERK and PI3K/Akt pathways in preventing anoikis. Conforming to that, tropomysin receptor kinase B (TrkB) adds to the list of cell surface receptors regulating anoikis through both pathways. Studies across multiple cancer cell lines have proven TrkB to not only suppress anoikis by activating these pathways, but also induce epithelial-to-mesenchymal transition (EMT) to further reinforce anoikis resistance in cancer cells. Despite playing an important role in human neural tissues, tropomysin receptor kinase (Trk) family was first identified to be oncogenic in colon cancer few decades ago. However, it was only recently that the Trk family gained necessary attention as a promising target in cancer therapy, thanks to emerging studies revealing the prominent involvement of Trk gene fusions in various cancer types. As such, an inhibitor targeting abnormally expressed Trk receptors named larotrectinib is currently in clinical trial with positive response so far.

In addition to kinase receptors, it is worth emphasizing the regulation of anoikis by another type of receptor, the E-cadherin. E-cadherin is a well-established transmembrane glycoprotein that mediates cell–cell interaction, enabling cell adhesion. E-cadherin has been found to interact with β-catenin to suppress the Wnt/β-catenin pathway and subsequently the transcription of various genes promoting EMT and anoikis resistance. As such, inactivation or functional loss of E-cadherin is often correlated with increased plasticity of cancer cells due to the acquisition of a mesenchymal phenotype. Arguably, E-cadherin is the most important transmembrane receptor when it comes to maintaining anoikis sensitivity, and its role goes beyond just regulating the Wnt/β-catenin pathway, as is better explained in the next subtopic.

While E-cadherin is responsible for cell–cell interaction, integrins are receptors responsible for cell–ECM interaction. Integrins exist on the cell surface as heterodimers, with varying combination of one α- and one β-subunits. As a result, a total of 24 combinations of integrin pairs can occur, and each of these combinations can be found in a varying distribution across different cell types with unique ligand specificity. Some components of the ECM, such as collagen and fibronectin, act as ligands and activate the integrins upon binding. Accordingly, activated integrins transduce signals from the ECM to the cytoplasm, controlling a wide array of cellular events including anoikis. Among the ways this is achieved is through the activation of FAK/Src pathway and other effectors such as integrin linked kinase (ILK) and transcriptional factors STAT3 and c-Myc, causing the activation of PI3K/Akt pathway and inhibition of anoikis.

Lastly, the topic of anoikis related signaling pathways will not be complete without the mention of Hippo pathway. Hippo pathway gained prominence when it was discovered to play a critical role in determining and retaining organ sizes. This pathway is activated by a complex network of transmembrane proteins, which results in the initiation of a kinase cascade. Cell detachment and even cytoskeletal perturbation have been found to cause the activation of Hippo pathway in normal cells, and consequently, anoikis. The notable key component of this pathway relevant to anoikis is the Yes-associated protein (YAP), whose function is inhibited through phosphorylation and sequestration by Hippo pathway kinases and various other regulators such as E-cadherin and α-catenin. When no longer inhibited, the oncogenic transcriptional regulator YAP is activated and promotes the expression of genes related to proliferation and anoikis resistance, making Hippo pathway an important regulatory system for maintaining anoikis.

New studies on anoikis regulation continue to shed light on other signaling pathways and novel proteins requiring further investigation. Regardless, the aforementioned receptors and pathways make up a significant part of the signaling cascades that converge to form a multifaceted network regulating anoikis (**Fig. 1**).

Interplay between EMT and Anoikis in Cancer

EMT is characterized by the loss of epithelial markers, such as E-cadherin and α-catenin, and gain of mesenchymal markers, such as N-cadherin, vimentin, and fibronectin. Functional loss of epithelial markers results in the dedifferentiation of cells, enabling them

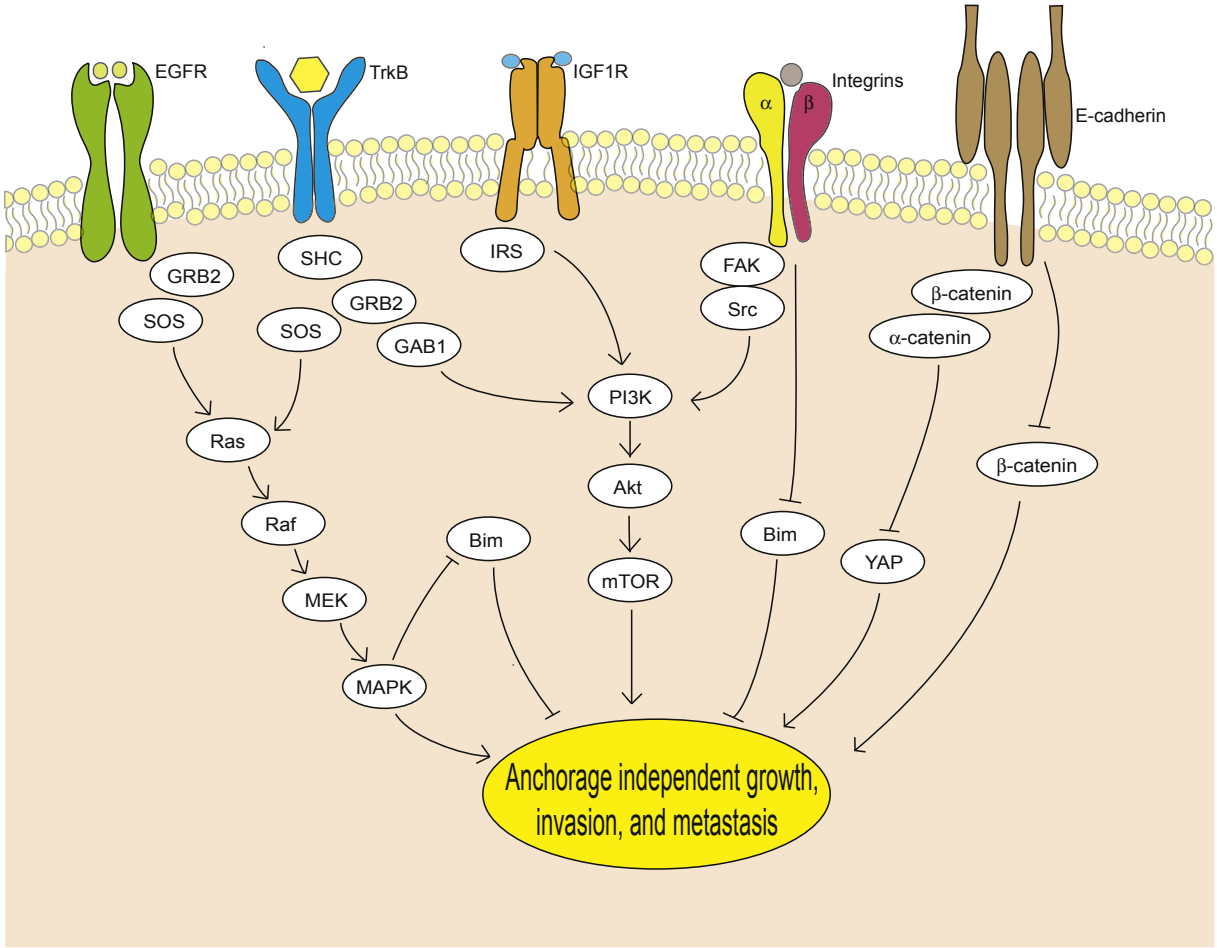

Fig. 1 An overview of signaling pathways regulating anoikis. Major pathways involved are the PI3K/Akt, MAPK/ERK, and Hippo pathways, composing the core of survival signaling originating from receptors mediating cell–ECM and cell–cell interactions. Examples of such receptors with validated roles in regulating anoikis are EGFR, TrkB, IGF1R, integrins, and E-cadherin.

to adopt a mesenchymal phenotype marked by increased cell motility and invasiveness. EMT is a reversible process that plays a pivotal role during embryogenesis and wound healing. This is potentially followed by the reversal of EMT, which is aptly termed mesenchymal-to-epithelial transition (MET). Consequently, the cells can undergo differentiation to return to their former epithelial state.

As expected, this dynamic process requires the cells to overcome various regulatory checkpoints such as anoikis, which would otherwise restrict movement and changes in the differentiated epithelial cells. In cancer cells, EMT similarly occurs during tumorigenesis as they begin to metastasize. Cancer cells also undergo MET, which seeds the growth of secondary tumors and colonization of distant organs, as the disseminated cells reassume their original state. Regardless of the use of the word epithelial, this process is not exclusive to epithelial cancer, as similar genetic signature for EMT has been noted in other cancer types such as sarcoma.

Despite the various mutations accumulated during tumor progression, cancer cells still need to overcome anoikis to begin the process of metastasis. As such, EMT enables yet another way for cancer cells to acquire anoikis resistance. This ability of EMT to repress anoikis lies in the regulation of major receptors mediating cell–cell interaction. In normal cells, these receptors provide pro-survival signaling when in correct attachment with the neighboring cells. Alteration of these receptors enables the bypass of this regulatory system and essentially, anoikis. To understand the tight interconnection between EMT and anoikis, one must first look at the important role played by E-cadherin, whose removal marks the beginning of EMT.

In addition to facilitating cell–cell contact through its extracellular domain, E-cadherin also regulates various signaling pathways through its cytoplasmic domain, which is largely responsible for its role in suppressing EMT. As previously mentioned, one such pathway is induced by β-catenin. B-catenin, which interacts with the cytoplasmic domain of E-cadherin, is kept sequestered away from the nucleus. In the absence of E-cadherin, the Wnt/β-catenin signaling pathway is activated, resulting in the translocation of β-catenin into the nucleus and activation of mesenchymal markers such as Snail, Twist, and ZEB1, endowing cancer cells with mesenchymal motility. Another protein that is regulated by E-cadherin is PTEN, a well-established tumor suppressor protein often found inactivated in cancer. Through the activation of E-cadherin, PTEN expression is upregulated and kept stable, allowing it to

suppress the pro-survival PI3K/Akt pathway signaling pathway by dephosphorylating PIP_3, a positive regulator of this pathway. Ankyrin-G also joins the list of E-cadherin interacting proteins, which was shown to activate a tumor suppressor protein promoting anoikis called P14ARF through inhibition of its transcriptional repressor TBX2. However, the anoikis inducing nature of P14ARF has been put into question, as a study recently unraveled the paradoxical nature of P14ARF inhibiting anoikis, suggesting that it may play a cancer type dependent role. These are just some examples of how E-cadherin positively regulates anoikis, highlighting the necessity of its removal during tumorigenesis.

Activation of EMT can also occur through TGF-β signaling pathway, during which numerous transcriptional repressors of E-cadherin are upregulated, such as Twist, ZEB1, ZEB2, Snail, and Slug, resulting in the abrogation of E-cadherin expression. Concomitantly, transient E-cadherin silencing has also shown to upregulate Twist and ZEB1 expression, suggesting that there exists a feedback loop between these transcription factors and E-cadherin. In addition to repressing E-cadherin expression, some of these transcription factors have also shown to change integrins expression in cancer cells, such as Snail and ZEB2, thus inducing EMT from multiple angles.

Next, in view of the important role played by integrins in mediating cell–ECM interactions, it comes as no surprise that cancer cells alter the integrin repertoire on the cell surface to favor metastasis inducing signaling pathways. Such changes in integrin expression in common cancer types have been listed and elegantly reviewed by Koistinen and Heino. In one of their examples, a study found breast adenocarcinoma to have significant downregulation of integrin $\alpha2\beta1$ compared to normal mammary epithelial cells. In a subsequent study, $\alpha2\beta1$ when ectopically expressed was able to diminish the malignancy of breast cancer cells by reducing its motility and invasiveness. This does not necessarily define it as a tumor suppressor across cancer types, as oncogenic function of $\alpha2\beta1$ has been exhibited in other cancer types as well.

As for an example of integrin upregulated in cancer, $\alpha5\beta1$ is of particular interest in the context of EMT and anoikis. $\alpha5\beta1$ is often found highly expressed, such as in prostate cancer and glioma, and correlated with poor prognosis in cancer patients. Experimental studies have uncovered the specific mechanisms by which these integrins can affect EMT, which starts with the ligation of $\alpha5\beta1$. This leads to the activation of FAK/Src and ILK pathways, both of which result in a cascade of signaling pathways repressing E-cadherin and activating EMT promoting transcription factors. Additionally, one of the products of these transcription factors, fibronectin, is produced and excreted into the ECM. Fibronectin, an extracellular mesenchymal marker, can act as a ligand to activate similar motility related integrins, fueling a feedback loop and further augmenting the integrin mediated pro-EMT signaling pathways.

Through the suppression of E-cadherin and manipulation of integrins during EMT, cancer cells are able to proceed with initiation of metastasis and become more invasive. This is also aided by mesenchymal markers which allow the restructuring of cytoskeletal dynamics to enable increased proliferation and motility. In further clarifying the intricate cross talk between EMT and anoikis, a study was performed to elucidate the role of several genes known to be upregulated during EMT in an ovarian cancer cell line. Interestingly, loss of function studies on each of these genes revealed that they did not have significant effect on anoikis regulation, with a notable exception of SYDE1, a Rho family GTPase.

Transient silencing of SYDE1 enabled both the recovery of E-cadherin expression and an increase in anoikis sensitivity, although the role played by SYDE1 in regulating anoikis remains elusive due to the limited number of studies investigating its function. Even more curious was the finding that ZEB1 silencing increased the expression of E-cadherin without any effect on anoikis. In keeping with that, other ZEB1 overexpression studies showed downregulation of E-cadherin but without an increase in anoikis resistance. Taken together, these suggest that although EMT is tightly linked to anoikis, varying the expression of E-cadherin alone is insufficient to cause changes in anoikis sensitivity.

In brief, inhibition of EMT has been consistently linked with increased anoikis sensitivity in a wide array of cancer types. As EMT precedes metastasis, inhibiting EMT as a therapeutic approach has been extensively studied. Such studies have yielded potential therapeutic molecules capable of suppressing EMT and inducing anoikis in various cancer cell lines. In addition, promising EMT targeting candidates have also been advanced to the clinical trial stages. Unfortunately, despite being advanced from development stages, they are yet to translate into successful clinical applications as many of these drugs fail to show efficacy in cancer patients.

Regulation of Anoikis by Autophagy in Cancer

Considering how anoikis involves the activation of apoptotic pathways, it is inevitable for a bevy of cellular processes related to cell survival and death to be affected by anoikis as well. As it has been previously established, anoikis occurs upon loss of ECM attachment. However, removal from the ECM does not result in imminent cell death, as studies have shown that another process, namely autophagy, is capable of interfering prior to initiation of anoikis. In this scenario, autophagy delays the activation of anoikis, providing the detached cells a small window of opportunity to promptly recover proper attachment with the ECM and neighboring cells.

Autophagy is a process in which cytosolic component are degraded as a tradeoff to enable survival in the event of cellular stress. Although there are different types of ways autophagy is achieved, the eventual outcomes are usually controlled degradation and turnover of cellular proteins and organelles, which increase the survival rate. The connection between anoikis and autophagy is primarily modulated by regulators of both processes, which link the cytoskeleton to the intrinsic apoptotic pathway. Two BH3-only family members crucial for anoikis, Bim and Bmf, are apoptotic activators notable for regulating autophagy. In their interaction with the cytoskeletal complex in normal attached cells, both proteins are found in an inactivated form, with Bim being associated

with dynein light chain 1 (DLC1), and Bmf with dynein light chain 2 (DLC2) of the cellular microtubule. This association enables Bim and Bmf to sequester Beclin-1, a pro-autophagy regulator and prevent its functional activation. Ultimately, cell detachment from the ECM translates to disturbance of the cytoskeletal architecture, which in turn releases Beclin-1 from this inhibition and enables the initiation of autophagy.

Unsurprisingly, the short gap that occurs after detachment afforded by autophagy is effectively exploited by cancer cells to continuously survive while metastasizing. Indeed, studies have shown that inhibiting autophagy limits cancer cells survival in suspension, whereas inducing autophagy results in increased survival and resistance towards anoikis. The complexity of how cancer cells achieve this is evident from findings of numerous studies, each highlighting unique key players and signaling pathways. Among the identified mechanisms are the activations of protein kinase A (PKA), transcriptional factor ATF4, HGF/c-Met pathway, and autophagy regulators such as ATG3.

In addition, cancer cells are also known to manipulate cellular stress as another method of utilizing autophagy to evade anoikis. For example, PERK, a transmembrane protein kinase found in the endoplasmic reticulum, is responsible for mediating stress response by suppressing protein translation. As an oncogene, PERK has found to be upregulated in a variety of cancer types and inhibition of PERK has shown to impair aggressive cancer phenotypes. As for its specific role in impeding anoikis, PERK was shown to promote cell survival after ECM detachment by inducing autophagy regulators through the activation of AMP-activated protein kinase (AMPK). Taken together, these studies suggest that cancers cells take advantage of the readily available loophole of autophagy to escape the anoikis barrier and become metastatic.

Nevertheless, the nature of autophagy is not as linear as being cytoprotectant in cancer cells during tumorigenesis. Studies have also brought to light the contrasting role played by autophagy in cancer cells, in which activation of autophagy was found to have a tumor suppressive role instead. One of the early studies investigating the function of Beclin-1 found it to be downregulated in breast cancer cells. When overexpressed, Beclin-1 was able to inhibit tumorigenesis in vitro and in vivo, through the activation of autophagy and subsequently apoptosis. Other studies corroborated the tumor suppressive function of autophagy. Separate studies on breast cancer cells revealed autophagy to promote cell death through the inhibition of IP3R and the degradation of Notch1 intracellular domain. Furthermore, several compounds have been shown to induce autophagy and apoptosis concurrently, such as fluvastatin in lung cancer metastasis and genistein in pancreatic cancer cells.

Autophagic cell death in cancer cells is also apparent in studies on several FDA-approved anticancer drugs. For example, tamoxifen treatment was found to involve the activation of autophagy in inducing cell death in luminal and triple-negative breast cancer, whereas gemcitabine was found to potentiate cell death through autophagy in pancreatic cancer. Interestingly, a study recently uncovered that autophagy induced by gemcitabine can also play an opposing role of promoting survival in bladder cancer cells through the activation of nuclear protein HMGB1, once again stressing the convoluted relationship between autophagy and cell death. Although anoikis induced cell death was also found to be upregulated in some of these instances in which autophagy promoted cell death, number of similar examples is limited, presumably due to the emphasis of such studies being mostly on cancer cell proliferation and tumor regression, and not on anoikis resistance. As such, it remains unclear if targeting the anoikis-autophagy interaction will work as an effective strategy in treating cancer, as more studies are needed to delineate the context dependent role played by autophagy.

Hypoxia and Anoikis

The highly proliferative nature of cancer cells presents a challenge in terms of oxygen delivery, which often serves as a limiting factor for tumor size. Cancer cells overcome this restriction by forming additional blood vessels through the process of angiogenesis. In the event of reduced oxygen availability, also known as hypoxia, hypoxia-inducible factors (HIF) are activated, which promote the expression of genes enabling adaptation to the stress. HIF, such as HIF-1, are heterodimeric transcription factors whose expression is kept low by the molecular oxygen sensor PHD2.

Although hypoxia in cancer mainly concerns cell embedded within the tumor, activation of HIF has results that extend beyond angiogenesis, as it can also negatively regulate anoikis. First of such regulation is by the induction of EMT through several mechanisms, such as activation of TGF-β signaling pathway and upregulation of EMT transcription factors. The link between hypoxia and EMT is by the virtue of HIF's regenerative role in stimulating wound repair. However, this can be opportunistically appropriated by cancer cells to increase motility and escape the hypoxic environment. Consequently, anoikis resistance is also concurrently acquired due to the induction of EMT. In addition, hypoxia is also correlated with autophagy, where autophagy inducers such as BNIP3 have been shown to be upregulated upon the activation of HIF-1α, a subunit of HIF-1. The outcome of this is the involvement of autophagy during hypoxia in cancer cells, which can play a protective role against anoikis.

Interestingly, hypoxia can be triggered even in the absence of oxygen deprivation through alternate methods. For example, constitutive activation of CAM such as EGFR and another ErbB family receptor, ErbB2, can stabilize and elevate HIF-1 expression, which otherwise would be immediately degraded in normoxic conditions. Since such receptors are often upregulated during anchorage-independent growth, activation of HIF provides another pathway for cancer cells to evade apoptosis while in suspension. Conversely, silencing of HIF-1 has shown to inhibit anti-anoikis signaling pathways such as PI3K/Akt pathway, further confirming the negative regulation of anoikis by HIF. Taken together, the connection between HIF and anoikis demonstrates how even cellular stress such as lack of oxygen can be exploited to benefit the growth and survival of cancer cells through the suppression of anoikis.

Regulation of Anoikis in Cancer by Non-coding RNA

Non-coding RNA (ncRNA) refers to sequence of transcribed RNA that is not translated into protein. Although some are regarded as 'junk RNA,' the highly abundant ncRNAs have shown to be functionally prolific. Next, we will look at how anoikis is regulated by some of these ncRNAs, namely microRNA (miRNA), long non-coding RNA (lncRNA), and small nucleolar RNA (snoRNA).

miRNA

Recent studies continue to reveal the complexity of anoikis regulation by elucidating more novel ways cancer cells regulate anoikis related genes. This brings us to miRNA, a short strand of RNA molecules approximately 22 nucleotides in size. The miRNAs regulate gene expression post-transcriptionally, which means that it interacts with mRNA causing either its degradation or translational impairment. The interaction between a miRNA and its target mRNA depends on sequence complementarity between both RNA molecules. Unlike in plant cells where nearly perfect complementarity is needed, miRNAs in mammalian cells only require a minimum base pairing at its seed region spanning 6 nucleotides with the 3′ UTR of the mRNA.

miRNA mediated gene silencing is based on RNA interference. A miRNA begins as a transcript of pri-miRNA in the nucleus, which gets processed into pre-miRNA before being exported to the cytoplasm. There, the final processing is carried out by the enzyme Dicer, producing a mature miRNA. In the cytoplasm, a miRNA will get loaded into an RNA Induced Silencing Complex (RISC). This enzyme complex containing the catalytic protein argonaute is directed to a target mRNA, which binds to the complementary miRNA. Consequently, the mRNA will be either cleaved or unavailable for translation. Regardless, the outcome of this miRNA-mRNA interaction is the downregulation of the miRNA target gene.

Dysregulation of miRNAs have been well observed not only in cancer, but also in numerous other diseases such as atherosclerosis and cystic fibrosis. In cancer, various miRNAs have been established to play clear oncogenic and tumor suppressive roles, although many of them have cancer specific functions. It is not uncommon for miRNAs to play oncogenic role in one cancer type and an opposing tumor suppressive role in another. With that in mind, miRNAs that regulate key cancer phenotypes such as anoikis, EMT, and autophagy have been extensively studied and reviewed.

Interestingly, some miRNAs have consistently shown up in studies investigating such processes, such as the miR-200 family. This miRNA group, consisting of miR-200a, miR-200b, miR-200c, miR-141, and miR-429, has shown definite roles as tumor suppressive miRNAs by regulating both EMT and anoikis in a variety of cancer types. From such miRNAs, certain pathways have been found to be frequently targeted through various target genes, which are the PI3K/Akt, Wnt/β-catenin, and TGF-β signaling pathways, thus underlining the significance of these pathways in regulating anoikis and EMT.

In addition to regulating itself with miRNAs, cancer cells are also able to utilize miRNAs to control other cells. This is owing to its ability to produce exosomes, the purpose of which is to either dispose tumor suppressive miRNAs or influence neighboring cells with oncogenic miRNAs. However, there are more evidences to support the latter function than the former. Through exosomal excretion, cancer cells are able to transfer oncogenic miRNAs extracellularly, in addition to proteins required for miRNA processing, through exosomes which then get absorbed by nearby cells. Once absorbed, these exosomal miRNAs can carry out their usual function in a new cell, despite originating elsewhere. This is especially useful for cancer cells that have acquired resistance to anoikis, as they are now able to impart similar characteristic to adjacent anoikis sensitive cells through exosomal miRNAs. Indeed, exosomes sourced from cancer cells have been shown to induce anoikis resistance among even normal cells upon exposure.

As previously established, since only a short stretch of complementary sequence is needed in a target mRNA, a single miRNA can target and downregulate multiple genes while numerous miRNAs can target a single gene, which creates a complicated network of post-transcriptional regulation. Herein lies miRNA's advantage for therapeutics, as it is capable of regulating multiple signaling pathways at once, making it more forceful compared to using a target specific siRNA. However, it is important to note that there are limited circumstances when the use of miRNA based therapeutics has definite advantages in clinical application. In the context of anoikis resistance, potent miRNAs must first be found, whose manipulation will result in little to no side effects to normal cells, as is the case for any ideal anticancer drug. This will require extensive studies to determine the specific roles played by the chosen miRNAs, which is complicated due to their confounding functional characteristics. This is due to the contradictory role miRNAs play in different cell types and thus will require extensive studies to elucidate and narrow down potential miRNA candidates.

On the other hand, miRNA based prognostic is less ambiguous in nature and deserves more attention for development. Clear correlation has been demonstrated between serum level of oncogenic miRNAs conferring anoikis resistance and predicted outcome for cancer patients. As such, miRNA based prognostics can be developed through the monitoring of changes in serum miRNA throughout cancer treatment. Since this prognostic method is noninvasive with immediate results as compared to other biopsies, pairing it with existing treatment regimens will allow evaluation and prediction of the effectiveness of such treatments earlier in individual patients. This will also enable necessary treatment modifications to be implemented sooner due to the relative ease of miRNA based prognostics. For this to be practical, however, probable miRNA biomarkers need to be examined and established for specific cancer types. Personalized medicine still has far to go, and miRNA based therapeutics, and prognostics has a lot to offer to push it forward.

lncRNA

Another type of ncRNA known to control anoikis is lncRNA. lncRNA is characterized by a size of more than 200 nucleotides and is significantly more tissue specific when compared to mRNA. Initially dismissed as non-functional, lncRNAs are now found to control

various protein expression machineries through a range of approaches, including epigenetic mechanisms such as methylation of DNA and modification of histone. In addition, lncRNAs are also able to control gene expression at both transcriptional and post-transcriptional levels. This makes lncRNAs highly versatile by modulating gene expression at numerous stages, unlike miRNAs that only perform post-transcriptional gene regulation. Interestingly, recent studies on lncRNA have unearthed another novel translational role for lncRNAs that were previously thought to be non-coding, as a small number of them were found to encode for functional micropeptides. As such, the broad functions played by lncRNA have made it a subject of increasing importance in studying dysregulation of genes in cancer cells.

Naturally, with diverse mode of action and abundant expression, lncRNAs have been shown to regulate anoikis in various cancer types. Studies have revealed several prominent lncRNAs such as the metastasis associated lung adenocarcinoma transcript 1 (MALAT1), HOX transcript antisense RNA (HOTAIR), and highly upregulated in liver cancer (HULC) with validated roles in suppressing anoikis in cancer. MALAT1 and HOTAIR were found to modulate anoikis sensitivity through the downregulation of their target genes, while HULC has been shown to inhibit anoikis and induce protective autophagy in various cancer cell lines. These are just few examples, for there are many other lncRNAs that have also been implicated in regulating related processes such as invasion and metastasis, highlighting its compelling role in dysregulation of genes in cancer cells.

In addition, lncRNA is also demonstrated to interact with miRNA, which further complicates lncRNAs' regulatory network. This relationship between lncRNA and miRNA can take place through one of two ways. The first interaction is by the binding of lncRNAs to complementary miRNAs, which can cause either their degradation or simply the inhibition of the miRNAs' function. In this case, lncRNA acts as a negative regulator of miRNA. Another interaction is one in which the lncRNA acts as a source of miRNA, wherein functional miRNAs can be spliced out of the lncRNAs. As such, sequestering or inhibiting tumor suppressive miRNA through the regulation of associated lncRNAs can also effectively serve as a strategy to evade anoikis in cancer cells.

Despite its abundance, lncRNA is only now being extensively studied and understood due to its complex nature. So far, numerous lncRNAs have been experimentally identified to be correlated with specific cancer types, acting as either oncogenes or tumor suppressor genes. As such, it can be expected that future studies may continue to unravel more novel ways lncRNAs regulate anoikis in cancer cells and how it can be beneficial for cancer treatments.

snoRNA

Similar to other ncRNAs, snoRNAs have also been found dysregulated in cancer and implicated in the regulation of anoikis. It is approximately 60–300 nt in size and can be categorized into two types, which are the box C/D and box H/ACA snoRNAs. This classification is based on the presence of conserved sequence motifs in the snoRNA and its respective functions. Box C/D snoRNAs carry out methylation whereas Box H/ACA carry out pseudouridylation of ribosomal RNA (rRNA). Although snoRNA's function is mainly associated with modification of rRNA, it has also been found performing other roles such as regulation of mRNA by interacting with snRNA. Interestingly, snoRNA has also been described to act as miRNA (sno-miRNA), albeit in relatively limited circumstances.

While recent studies have uncovered an increasing number of snoRNA that are dysregulated in cancer cells, specific role of such snoRNAs' in regulating anoikis remains largely unknown as of now. This does not imply that snoRNAs play a minimal role in regulating anoikis, as many of these studies have found connection between snoRNA expression and anoikis related pathways such as the PI3K/Akt pathway to influence cancer cell proliferation and similar processes.

As such, the most prominent snoRNA known to regulate anoikis is small nucleolar RNA 42 (SNORA42), a box H/ACA snoRNA. SNORA42 was initially identified as an oncogene when it was found to be highly expressed in non-small cell lung cancer (NSCLC). Since then, more recent studies conducted in NSCLC and colorectal cancer (CRC) revealed poor prognosis among patients expressing high level of SNORA42. Meanwhile, overexpression studies in CRC showed that SNORA42 was able to increase anoikis resistance. There are several suggestions to explain the function of SNORA42 as an oncogene, such as through upregulation of stem cell genes and inhibition of p53-dependent apoptosis. However, this is still being investigated and the exact mechanism has yet to be determined. With studies revealing the increasingly important roles of snoRNAs, future findings may reveal more snoRNAs like SNORA42 and how manipulation of such ncRNAs may prove beneficial in cancer therapeutics.

Circulating Tumor Cells and Anoikis

So far, we have seen the molecular perspective of how anoikis is regulated and how various cellular mechanisms can intervene to potentiate anoikis resistance in cancer cells, generating potent seeds for metastasis. Next, we will look at what happens when these modifications are acquired as the cancer cells enter the blood stream to form circulating tumor cells (CTCs).

CTCs are anoikis resistant cancer cells that have escaped the original tumor formation site and entered the circulatory system. Naturally, entering the circulatory system exposes the cancer cells to selective pressure, allowing only those with resistance to anoikis to remain and survive as CTCs. Although many CTCs are cells that have undergone EMT and intravasation into the blood vessels, some of these cells may have detached merely due to inflammation or sheer force. As such, CTCs do not inherently possess the competency to seed a secondary tumor right away. In fact, the formation of a secondary tumor by CTCs has been acknowledged to be an improbable event and a bottleneck step in metastasis. While anoikis resistance enables CTCs survival in suspension, stem cell characteristics are further needed for the final stages of metastasis, which brings us to cancer stem cells (CSCs).

As implied by its name, CSCs are cancer cells that portray similar characteristics as stem cells, such as self-renewal and ability to form tumorspheres. These cells can further undergo changes such as EMT, endowing them with even more plasticity to become important drivers for metastasis. Not surprisingly, CSCs make up a large subset of CTCs. Indeed, numerous studies on metastasis have proven CTCs to be highly heterogeneous, with subgroups of varying degree of pathogenicity. The interplay between CTCs and CSCs suggests that CTCs may play an important role in survival in suspension and invasiveness, whereas CSCs may be responsible for the establishment of the metastases upon extravasation.

Examination of spheroid cultures in vitro revealed a constitutive activation of EGFR to transduce MAPK/ERK signaling pathway among others, providing protection from anoikis in the absence of ECM. Furthermore, CTCs analysis also revealed increase in mesenchymal markers and decrease in epithelial markers, consistent with the findings that CTCs undergo EMT in addition to being anoikis resistant. Such changes are warranted as CTCs attempt to survive in an anchorage-independent environment within the circulatory system. As such, CTCs presents a major problem for cancer patients as conventional radiotherapy does not work against these cells. To make things worse, chemotherapy is not as effective either as CTCs in suspension do not rapidly replicate, as the emphasis is placed on survival instead.

These issues necessitate individual attention to CTCs, which may reveal vital information for effective cancer treatments that otherwise would have gone overlooked. To illustrate this point, consider the following scenario of gastric cancer treatment and the molecular classification of the tumor cells. Recent studies have shown the existence of CTCs exhibiting HER2 positive phenotype in gastric cancer patients, despite the primary tumor biopsy showing HER2 negative phenotype. Preliminary findings showed that these patients benefited from combined treatment using trastuzumab, a cancer drug targeting HER2 overexpressing cancer cells. As such, it is indisputable that this potential treatment strategy would not have been discovered if not for the analysis of CTC population.

Even though platforms exist for the isolation and enrichment of CTCs for analytical purpose, sporadic molecular characteristic of CTCs and difficulties in obtaining enough CTCs from liquid biopsy hinder its application as prognostic biomarkers. Furthermore, while a long list of markers has been identified to classify CTCs, it is not exhaustive and continues to be updated and changed thanks to recent studies revealing more details on the heterogeneity of CTCs. Presently, there are many strategies for detection of CTCs based on sizes or molecular features, though they are mostly either time consuming or insufficiently sensitive to analyze the whole representative of CTCs. Additionally, indirect approximation of CTCs through the analysis of serum miRNAs have also been demonstrated and suggested as an alternative to CTCs detection. Regardless, the increased attention that CTCs have gotten over the past few years have resulted in emergence of newer methods requiring shorter time and lesser samples, paving way for another option for personalized cancer treatment. If such newer methods can be reliably accomplished, characterization of CTCs derived from patients will definitely contribute to better decision making in treating cancer metastasis.

Tackling Anoikis Resistance in Treating Cancer

Understandably, the pivotal role anoikis resistance plays in enabling CTCs' existence and survival has made targeting anoikis network in cancer treatment a necessary approach. In addition to existing cancer drugs, many new compounds have been experimentally shown to sensitize cancer cells to anoikis and reduce its malignancy. The following are examples of such compounds (Table 1).

Table 1 Summary of example compounds known to regulate anoikis in preclinical and clinical stages

Type of drug	*Name*	*Mechanism for regulating anoikis*
Inhibitors	Dasatinib	Suppression of SFK
	Erlotinib	Inhibition of EGFR
	Lapatinib	Inhibition of EGFR
	Sorafenib	Inhibition of MAP/ERK pathway
Repurposed drugs	Disulfiram	Activation of calpain and increase of intracellular zinc level
	Metformin	Activation of AMPK
Antibiotics	Anisomycin	Inhibition of apoptosis regulator FLIP
	Rapamycin	Inhibition of PI3K/Akt pathway
	Renieramycin M	Inhibition of PI3K/Akt and MAP/ERK pathways
	Salinomycin	Suppression of STAT3
miRNA based drug	MRG106	Inhibition of pro-EMT miR-155
Natural compounds	*Morus alba* leaf lectin	Inhibition of FAK
	Sambucus sieboldiana lectin	Suppression of MGAT5 mediated anoikis resistance
	Apigenin	Suppression of FAK and MAP/ERK pathway
	Moscatilin	Inhibition of PI3K/Akt and MAP/ERK pathways
	Geraniin	Inhibition of TGF-β signaling pathway
	Gigantol	Inhibition of PI3K/Akt and MAP/ERK pathways

Inhibitors

Owing to the myriad of enzymes involved downstream of receptors regulating anoikis, it should come as no surprise that many FDA-approved cancer drugs belong to the inhibitor class compound and work very well in abrogating anoikis resistance in cancer cells. Examples of inhibitors that reduced anchorage-independent growth in cancer cell cultures are dasatinib, erlotinib, lapatinib, and sorafenib. Dasatinib and sorafenib are multi-kinase inhibitors that have shown the ability to induce anoikis by suppressing Src family kinases (SFK) and MAP/ERK signaling pathway respectively. Meanwhile, erlotinib and lapatinib similarly promote anoikis but by inhibiting the EGFR instead. Intriguingly, some of these drugs have also shown increased efficacy when used in combination, such as lapatinib and sorafenib in human glioblastoma, by activating various cell death mechanisms including both extrinsic and intrinsic apoptotic pathways.

Repurposed Drugs

While new drugs continue to be developed, existing drugs for other conditions are finding new niche in cancer treatment. As these drugs are already FDA approved in terms of safety for human use, they face significantly less hurdle in re-entering the market as anticancer drugs. One of such drugs that has shown efficacy in treating anoikis resistance in cancer cells is disulfiram (DSF). Used to treat a completely unrelated indication of alcohol dependence, DSF has also shown potency as an anticancer agent. Remarkably, epidemiology survey has shown reduced occurrence of breast and prostate cancer within a Danish DSF user sample population. The ability of DSF to induce anoikis in cancer cells has been attributed to activation of calpain in a copper dependent manner. In addition, DSF has also been experimentally shown to act as an ionophore, increasing the intracellular level of zinc to sensitize breast cancer cells to anoikis. This isn't surprising, given that the value of zinc as an anticancer agent has been well studied, especially as a promoter of anoikis through the inhibition of PI3K/Akt pathway.

Another similar drug with pro-anoikis property is metformin. Metformin is a type 2 diabetes medication that has been found to have an increasing number of potential uses, including against cancer. Several studies investigating the anticancer effect mediated by metformin found evidence that it induces anoikis in numerous cancer cell lines mainly through the activation of AMPK. These studies have also shown that metformin can work against cancer cells both by itself and as an adjuvant. Similar to DSF, metformin use has also been associated with overall reduced incidence of various types of cancer, highlighting its latent benefits and the necessity to repurpose such drugs in the battle against cancer.

Antibiotics

In line with the discussion on repurposed drugs, it is worth noting another group of compounds, antibiotics, that has shown efficacy in promoting anoikis among cancer cells. Various antibiotics have exhibited success in treating anoikis resistance in cancer cells, such as anisomycin, rapamycin, renieramycin M, and salinomycin. The ways these antibiotics are able to induce anoikis are numerous, such as impeding the formation of tumorspheres, suppressing pro-survival signaling pathways, and inhibiting anoikis suppressor proteins. Despite demonstrating promising results in vitro, studies on antibiotics use to treat cancer have been mostly preclinical, although a small scale clinical study has been carried out with salinomycin. Presently, salinomycin has been subjected to extensive studies in the context of cancer treatment, with specific emphasis on the underlying mechanisms enabling its effects against cancer cells. In fact, a proprietary formulation of salinomycin (VS-507) which worked against CSCs by targeting the Wnt pathway was under development by Verastem Inc. in 2012. However, no updates have since been heard about VS-507, and it is assumed to no longer be in development. Notably, salinomycin's toxicity primarily affected cancer cells with stem cell properties. As such, the practicality of developing antibiotic based drugs as a standalone appears questionable for now. Use of antibiotics as chemotherapeutic drugs for cancer is fascinating, especially considering that antibiotics are often deemed necessary for patients undergoing chemotherapy due to their weakened immune system and predisposition to infections. Although antibiotics use has been reported to have some success in improving cancer patients' survival, whether this is due to staving off infections or the therapeutic effect on cancer cells or a combination of both is yet to be established.

miRNAs

Many miRNAs regulating anoikis have been well studied and characterized. However, passing the preclinical stages appears to be the biggest barrier, as only a handful of miRNAs are currently in the drug development pipeline. As of now, the most prominent miRNA based therapeutic is miravirsen, a miR-122 inhibitor developed for treatment of Hepatitis C by Santaris Pharma. Interestingly, miravirsen is not the only experimental drug to use this strategy, as RG-101 from Regulus Therapeutics works similarly by targeting miR-122 in the liver, but with a structural difference in the inhibitor. How this difference translates to different safety and efficacy in human application is yet to be conclusively determined, as both drugs are currently in different stages of clinical trial, each with its own pros and cons. Meanwhile, development of miRNA based cancer therapeutics is not as fortunate, as MRX34, a mimic of tumor suppressor miR-34 in Phase 1 was recently halted after five immune-related patient deaths were reported. Although MRX34 is not the only anticancer miRNA based drug in the development pipeline, it was the first miRNA mimic to advance into Phase 1 and showed promising results prior to these serious adverse events, causing a huge setback for such drugs. Regardless, there are still few miRNA based cancer drugs in clinical stages, among which is MRG106. An antagonist of miR-155, MRG106 can be

considered to be significant in promoting anoikis in cancer. MRG106 was developed for cutaneous T-cell lymphoma and targets miR-155, a known oncogenic miRNA which suppresses anoikis by inducing EMT. Depending on its success in clinical trials, we may see more of such miRNA based drugs in the future, as there is a long list of miRNAs with potential for becoming anticancer drugs.

Natural Compounds

Natural compounds and their derivatives represent an emerging class of potential cancer drug candidates, whose popularity is credited to the relatively low toxicity towards normal cells, easy availability, and low cost. Originally prevalent for traditional uses, various plants around the world are now being realized as sources harboring valuable natural compounds with therapeutic potential. Indeed, many FDA-approved drugs such as vinblastine and paclitaxel are derivatives that exist today thanks to the discoveries of potent anticancer property of natural compounds found in plants. That being the case, many new natural compounds have been revealed to promote anoikis in cancer cells. Lectin from *Morus alba* leaf and *Sambucus sieboldiana*, apigenin, moscatilin, geraniin, and gigantol are just some examples of natural compounds that were recently discovered to induce anoikis in vitro in different cancer types. While more research is needed before these discoveries can be translated into viable anticancer drugs, the very abundance of such compounds increases the probability of similar compounds being found and developed past the preclinical stages.

Conclusion

The critical role played by anoikis in maintenance of tissue homeostasis makes it an important barrier to overcome among cancer cells attempting to metastasize. We have seen how resistance to anoikis is often acquired through various approaches, such as through manipulation of receptors regulating anoikis and dysregulation of various types of ncRNAs. Accordingly, anoikis resistance poses a big threat to conventional treatment, as they endow cancer cells with unique properties distinguishing them from the original tumor. Indeed, adoption of anoikis resistance is usually correlated with poor prognosis in patients regardless of cancer types. As such, targeting anoikis resistant cancer cells plays a key role in determining whether a cancer treatment will be effective, as we have seen how CTCs can be markedly different from majority of cancer cell population in a tumor.

Studies on anoikis manipulation in cancer cells have begun to gain momentum and more novel ways cancer cells can be made sensitive to anoikis are being revealed. Unless specifically targeted, these anoikis resistant cells may continue to fuel metastasis or remain dormant in circulation during treatment, possibly resulting in recurrence in cancer patients. As cancer therapeutics gradually incorporates a more personalized strategy, it is imperative that anoikis resistance is taken into consideration for a wholesome approach in treating cancer.

Further Reading

Desouza, M., Gunning, P.W., Stehn, J.R., 2012. The actin cytoskeleton as a sensor and mediator of apoptosis. BioArchitecture 2 (3), 75–87.
Frisch, S.M., Ruoslahti, E., 1997. Integrins and anoikis. Current Opinion in Cell Biology 9 (5), 701–706.
Frisch, S.M., Screaton, R.A., 2001. Anoikis mechanisms. Current Opinion in Cell Biology 13 (5), 555–562.
Glick, D., Barth, S., Macleod, K.F., 2010. Autophagy: cellular and molecular mechanisms. The Journal of Pathology 221 (1), 3–12.
Kim, Y.N., Koo, K.H., Sung, J.Y., Yun, U.J., Kim, H., 2012. Anoikis resistance: an essential prerequisite for tumor metastasis. International Journal of Cell Biology 2012.
Koistinen, P., Heino, J., 2000-2013. Integrins in cancer cell invasion. In: Madame Curie bioscience database. Landes Bioscience, Austin, TX (Internet).
Lu, P., Weaver, V.M., Werb, Z., 2012. The extracellular matrix: a dynamic niche in cancer progression. The Journal of Cell Biology 196 (4), 395–406.
Majmundar, A.J., Wong, W.J., Simon, M.C., 2010. Hypoxia-inducible factors and the response to hypoxic stress. Molecular Cell 40 (2), 294–309.
Malagobadan, S., Nagoor, N.H., 2015. Evaluation of microRNAs regulating anoikis pathways and its therapeutic potential. BioMed Research International 2015.
Thiery, J.P., 2002. Epithelial-mesenchymal transitions in tumour progression. Nature Reviews. Cancer 2 (6), 442–454.
Vachon, P.H., 2011. Integrin signaling, cell survival, and anoikis: distinctions, differences, and differentiation. Journal of Signal Transduction 2011.

Aspirin and Cancer

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Abbreviations

CAPP2 Colorectal Adenoma/Carcinoma Prevention Programme 2
CRC Colorectal cancer
CVD Cardiovascular disease
GI Gastrointestinal
MI Myocardial Infraction
RCTs Randomized Controlled Trials
USPSTF U.S. Preventive Services Task Force
WHS Women's Health Study

Historical Perspective

Aspirin is a drug that has reinvented itself a few times over the 20th and early 21st century. Although medicinal acetylsalicylic acid (ASA), that is Aspirin was first synthesized at Bayer AG in 1897, (Mahdi et al., 2006; Sneader, 2000) salicylates from the bark of willow tree have been used for their medicinal purposes for millennia before that. Felix Hoffmann is widely credited for the synthesis of ASA; however, the historical account is controversial with Arthur Eichengrün's claim that Hoffmann did so under Eichengrün's instructions. (Sneader, 2000; Eichengrün, 1949) For the first several decades of its use, Aspirin was used for its analgesic, antipyretic, and anti-inflammatory properties. Early reports of Aspirin's effect on myocardial infarction started appearing in medical literature in 1950s and 1960s, and soon cardiovascular trials, beginning with the UK Medical Research Council I (MRC-I) trial were launched (Elwood, 2006; Miner and Hoffhines, 2007). The mechanism of Aspirin action for all these effects however remained elusive for 70 odd years until John Vane (Vane and Botting, 2003; Vane, 1971) demonstrated in 1971 that Aspirin inhibits prostaglandin synthesis and he was jointly awarded Nobel Prize in 1982 for this important contribution. The mechanistic understanding of Aspirin's antiplatelet effects combined with the positive results (Lewis et al., 1983; Elwood et al., 1974) of randomized controlled trials (RCTs) led to increasing use of Aspirin as an antithrombotic agent in prevention of cardiovascular disease (CVD) from 1980s (Elwood, 2006; Miner and Hoffhines, 2007). With this widened use and new clinical trials (Steering Committee of the Physicians' Health Study Research G, 1988; Steering Committee of the Physicians' Health Study Research Group, 1989) came the first hints of Aspirin's beneficial effects on cancer and precancerous lesions (Kune et al., 1988; Gann et al., 1993; Logan et al., 1993; Rosenberg et al., 1991; Thun et al., 1991, 1992, 1993; Suh et al., 1993). Aspirin's effects on cancer incidence and mortality have subsequently been a subject of a very large number of observational studies and analyses from randomized trials (Algra and Rothwell, 2012; Bosetti et al., 2012; Cuzick et al., 2009, 2015; Flossmann and Rothwell, 2007; Rothwell et al., 2010, 2011, 2012a; Thorat, 2016; Thorat and Cuzick, 2013). These data collectively confirm that Aspirin has beneficial effects in reducing incidence and mortality of certain cancers (Cuzick et al., 2009, 2015; Thorat, 2016; Thorat and Cuzick, 2013).

Evidence for Effects on Cancer

Peculiarities of Aspirin's Effects on Cancer

A very large body of evidence now supports Aspirin's beneficial effect on cancer and this evidence is discussed below in detail. It is, however, important to recognize specific features or peculiarities of Aspirin's effects on cancer (**Box 1**).

Aspirin's effects on cancer are site-specific and the evidence for Aspirin's benefit is most clear and consistent for three gastrointestinal (GI) cancers, namely colorectal cancer (CRC), esophageal cancer, and gastric cancer. The magnitude of benefit is also the largest for these three GI cancers. The evidence for Aspirin's effect is somewhat less clear for the cancers of the breast, prostate, and lung; the magnitude of benefit is also much smaller than that observed for GI cancers (Cuzick et al., 2015; Thorat, 2016; Thorat and Cuzick, 2013). Although some studies report effects on other major cancer types including hepatocellular (Petrick et al., 2015; Sahasrabuddhe et al., 2012), pancreatic, endometrial (Burn et al., 2011; Zhang et al., 2016a), ovarian (Zhang et al., 2016b; Trabert et al., 2014; Baandrup et al., 2013; Ni et al., 2013), and hematopoietic tumors, this limited evidence is not consistent across study types and does not show unequivocally clear effect (Rothwell et al., 2011, 2012a; Cook et al., 2013; Jacobs et al., 2012).

Box 1 Peculiarities of Aspirin's effects on cancer

- **Site-specificity:** The effects of large magnitude seen on three GI cancers (colorectal, esophageal, and gastric); smaller effects on breast, prostate, and lung cancers.
- **Duration of use:** Effects are clearer in those who have used Aspirin for 5 years or more.
- **Lag in beneficial effects:** Effects become apparent only after 3 (for cancer incidence) to 5 years (for cancer mortality).
- **Carry-over of beneficial effects:** Effects then continue even after use is stopped; effects on mortality continue for up to at least 20 years as observed in trials with long-term follow-up, effects on incidence continue for at least up to 5 years.
- **Mechanisms of action:** The magnitude of effect on cancer mortality is larger than that on incidence suggesting that the beneficial effects on cancer mortality are mediated through different/additional mechanisms of action.

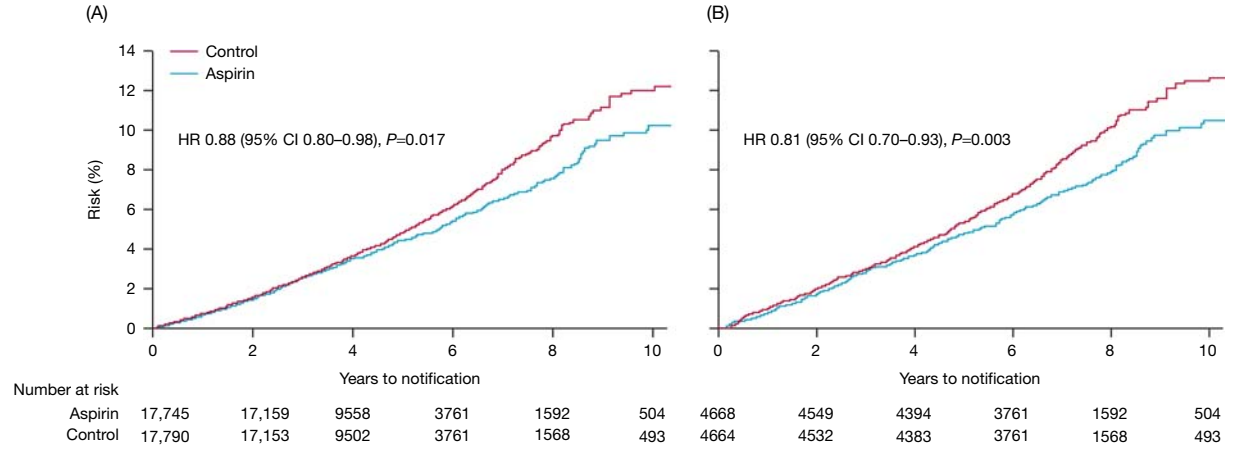
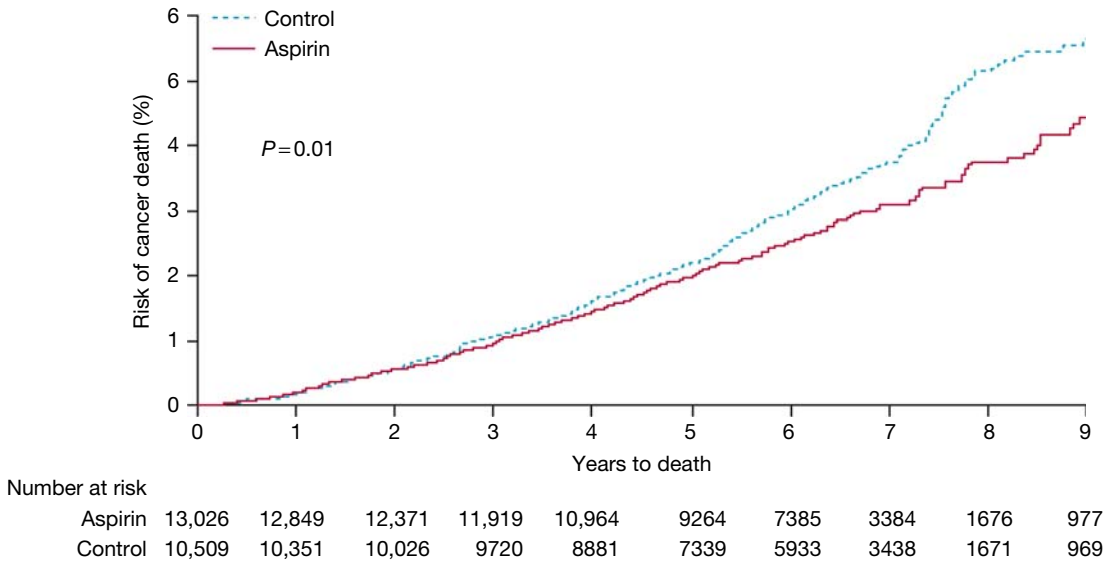


Fig. 1 Aspirin's effects are clearer in those who have used it for 5 years or more—Hazard ratio (HR) of 0.88 in all trials versus HR of 0.81 in trials with scheduled treatment duration of at least 5 years. Pooled analysis of effect of allocation to Aspirin on incidence of cancer during six randomized trials of daily low-dose (75–100 mg daily) Aspirin versus placebo in primary prevention of vascular events. (A) All patients. (B) All patients with scheduled duration of trial treatment of at least 5 years. HR = hazard ratio. Reprinted from: Rothwell, P.M., Price, J.F., Fowkes, F.G., Zanchetti, A., Roncaglioni, M.C., Tognoni, G., et al. (2012) Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: Analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* **379**(9826), 1602–1612. Copyright (2012), with permission from Elsevier.

The duration of Aspirin use is important as the beneficial effects are much clearer with at least 5 years of Aspirin use (**Fig. 1**). The pooled analyses of clinical trials by Rothwell et al. (2011) show that the effect sizes are larger for the trials where scheduled treatment duration was 5 years or longer. Beneficial effects of Aspirin with a much shorter duration of 2 years of use have been reported in the CAPP2 trial, a randomized trial in Lynch syndrome carriers (Burn et al., 2011). The CAPP2 trial reported a 63% reduction in CRC incidence among those completing 2 years of treatment (Burn et al., 2011). However, this was a trial in high-risk individuals taking higher than standard doses of Aspirin (600 mg/day). The current body of evidence suggests that for an average-risk individual taking low-dose Aspirin, at least 5 years of use is necessary for modification of their cancer risk.

It is now also clear from the pooled analyses of a large number of clinical trials performed by Rothwell et al. (2011, 2012a) that Aspirin's beneficial effects on cancer are not apparent immediately and there is a lag of approximately 3 years for its effects on cancer incidence to become apparent and a lag of approximately 5 years for its effects on cancer deaths to become apparent (**Fig. 2**). The lag period for Aspirin's effects on cancer deaths can be observed from the lack of separation of Kaplan–Meier survival plots for first 5 years in **Fig. 2**. In the Women's Health Study (WHS), which used low-dose Aspirin (100 mg /day) on alternate days, the beneficial effects on CRC incidence were clearly observed only in the post-trial follow-up period as these emerged after 10 years (Cook et al., 2013). It is therefore unsurprising that the meta-analyses (Seshasai et al., 2012; Sutcliffe et al., 2013) with a short overall follow-up of 5–6 years have failed to observe a significant effect of Aspirin on cancer deaths and all-cause deaths. The apparent lack of effect with short follow-up was clearly demonstrated in analyses by Rothwell et al. (2011, 2012a), the Kaplan–Meier survival plots overlap each other in the first panel in **Fig. 3**.

Similarly, the beneficial effects continue for several years after cessation of Aspirin use (**Fig. 3**). This carry-over period lasts for at least 5 years for effects on cancer incidence and at least 10 years for effects on cancer deaths. In the pooled analyses by Rothwell et al. (2011, 2012a), the effects on cancer deaths remained constant until the end of follow-up period of at least 20 years when treatment duration in all these trials was < 10 years. **Fig. 3** shows that the Kaplan–Meier survival plots remain separated at 20 years in the trials



Number at risk

Aspirin	13,026	12,849	12,371	11,919	10,964	9264	7385	3384	1676	977
Control	10,509	10,351	10,026	9720	8881	7339	5933	3438	1671	969

Fig. 2 Lag in Aspirin's effect on cancer deaths—the separation of Kaplan–Meier survival plots starts at 5 years of follow-up. Effect of allocation to Aspirin versus control on risk of death due to cancer during the trial treatment periods in a pooled analysis of the 23,535 patients in seven trials. Reprinted from: Rothwell, P.M., Fowkes, F.G., Belch, J.F., Ogawa, H., Warlow, C.P., Meade, T.W. (2011). Effect of daily aspirin on long-term risk of death due to cancer: Analysis of individual patient data from randomised trials. *Lancet* **377**(9759), 31–41. Copyright (2011), with permission from Elsevier.

with long-term follow-up. In the WHS (Cook et al., 2013), the effects on CRC appeared only in the posttreatment period, but continued for the entire posttrial follow-up period with the total median follow-up being 18 years.

The overall evidence from observational studies, randomized trials, and meta-analyses of these studies (summarized in **Table 1** for major cancer sites) shows that the beneficial effects of Aspirin on overall cancer mortality are larger in magnitude than effects on cancer incidence (Cuzick et al., 2015; Rothwell et al., 2011, 2012a,b; Thorat and Cuzick, 2013). This suggests that in addition to modifying the process of carcinogenesis to reduce cancer incidence, Aspirin also affects other processes like cancer progression and cancer dissemination or metastasis. Therefore, at least two different, and possibly several mechanisms of action drive Aspirin's effect on cancer (discussed later).

Aspirin's Effects on Different Cancers

Colorectal cancer

A very large body of evidence shows that regular Aspirin use is associated with a reduction in CRC incidence and mortality (Thun et al., 1993; Algra and Rothwell, 2012; Bosetti et al., 2012; Rothwell et al., 2010, 2011, 2012a; Burn et al., 2011; Cook et al., 2013; Jacobs et al., 2012; Chan et al., 2007a, 2008; Ratnasinghe et al., 2004; Rothwell, 2013; Cao et al., 2016; Chubak et al., 2015). Pooled results of case-control studies (Algra and Rothwell, 2012; Bosetti et al., 2012) show almost 40% reduction in CRC incidence whereas the pooled analyses of cohort studies show a smaller effect size with approximately 20% reduction (**Table 1**). The overall reduction in CRC incidence observed in pooled analyses of randomized trials (Rothwell et al., 2010) is approximately 25%, with 38% reduction in trials with scheduled treatment duration of 5 years or more. In the WHS (Cook et al., 2005, 2013), no effect was observed for the first 10 years of the trial, and 42% reduction in CRC incidence was observed in the posttrial period with 100 mg alternate day Aspirin use.

The pooled analyses of cohort studies (Thun et al., 1993; Jacobs et al., 2012; Ratnasinghe et al., 2004) show that the magnitude of Aspirin's effect on CRC death is larger than that on CRC incidence with approximately 35% reduction in CRC deaths observed. The pooled analyses of RCTs also report similarly larger effect on CRC deaths, with almost 50% reduction observed in trials with scheduled treatment duration of 5 years or more (Rothwell et al., 2010). A semi-quantitative summary of Aspirin's effects on CRC incidence and deaths is given in **Table 2**.

Larger reductions have also been observed in individuals at high risk of colorectal cancer such as those with Lynch syndrome (Burn et al., 2011). A 63% reduction in CRC incidence was observed among those completing 2 years of 600 mg daily Aspirin in the CAPP2 trial (Burn et al., 2011). Similarly, reduction in the colorectal adenoma risk or recurrence risk of adenoma with Aspirin use has been observed (Cole et al., 2009; Veettil et al., 2017; Zhao et al., 2016).

Esophageal cancer

Pooled analyses of case-control studies (Algra and Rothwell, 2012; Bosetti et al., 2012) show almost 45% reduction in the incidence of esophageal cancer with Aspirin use whereas approximately 25% reduction is observed in cohort studies (**Table 1**). The effects do

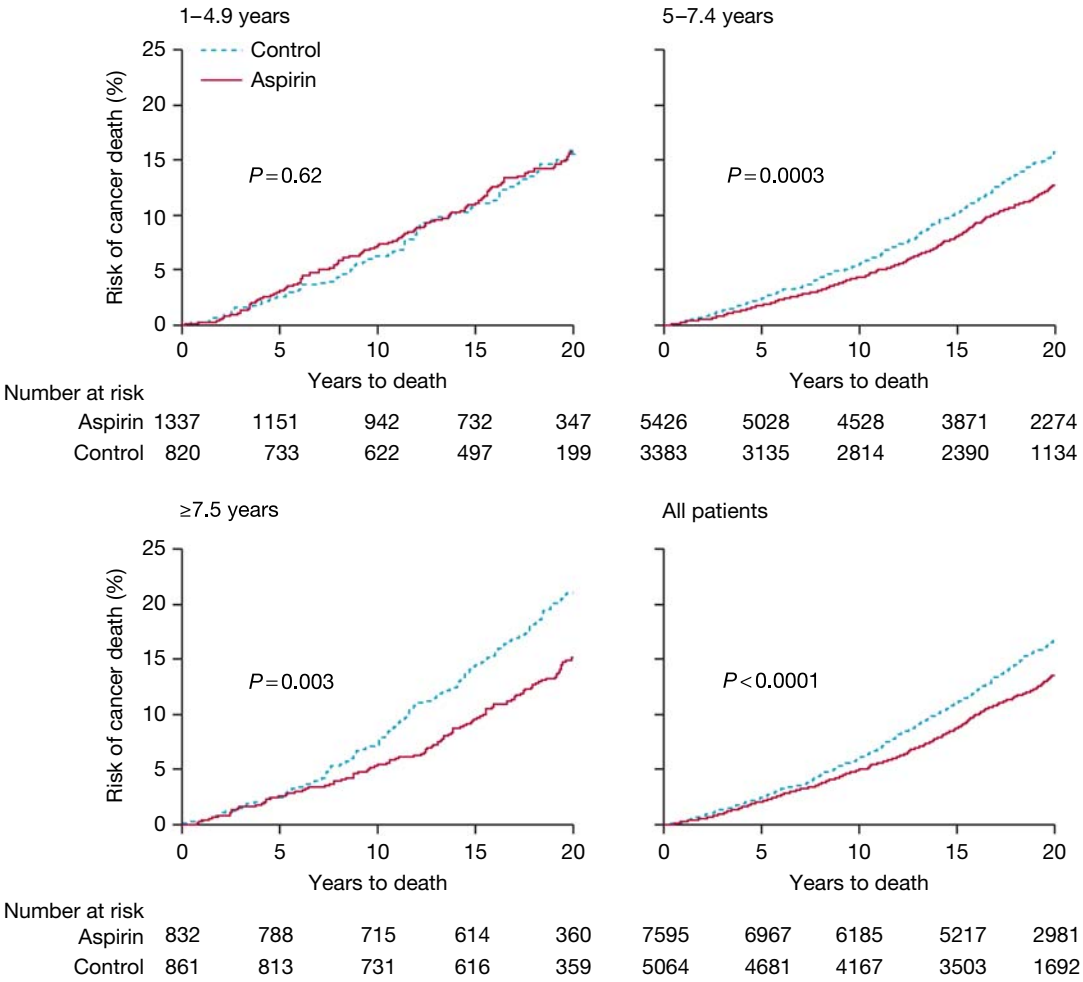


Fig. 3 Carry-over of Aspirin's beneficial effect on cancer deaths—the Kaplan–Meier survival plots remain separated at 20 years of follow-up. Effect of allocation to Aspirin versus control on 20-year risk of death due to any solid cancer stratified by scheduled duration of trial treatment in three trials with long-term follow-up. Continuous variable interaction, $P = 0{\cdot}01$. Reprinted from: Rothwell, P.M., Fowkes, F.G., Belch, J.F., Ogawa, H., Warlow, C.P., Meade, T.W. (2011). Effect of daily aspirin on long-term risk of death due to cancer: Analysis of individual patient data from randomised trials. *Lancet* **377**(9759), 31–41. Copyright (2011), with permission from Elsevier.

Table 1 Relative risks of Aspirin use on the incidence and mortality of major cancers from recent overviews and major studies

	Cancer incidence			*Cancer mortality*		
	No. of studies and source	No. of cases	Relative risk (95% CI)	No. of studies and source	No. of cases	Relative risk (95% CI)
Colorectal cancer						
Case control	15[a]	21,414	0.63 (0.56–0.70)	1[e]	433	0.72 (0.56–0.92)
	22[b]	17,231	0.61 (0.55–0.67)			
Cohort	15[a]	16,105	0.82 (0.75–0.89)	2[f]	1124	0.68 (0.56–0.83)
	8[b]	2955	0.78 (0.71–0.84)	1*[g]	149	0.64 (0.42–0.98)
RCT	3[c]	196	0.75 (0.56–0.97)	3[c]	130	0.61 (0.43–0.87)
	3*[c]	135	0·62 (0·43–0·94)	3*[c]	91	0·48 (0·30–0·77)
Esophageal cancer						
Case control	7[a]	1075	0.54 (0.44–0.67)	–	–	–
	9[b]	2307	0.58 (0.44–0.76)			
Cohort	4[a]	1118	0.73 (0.51–1.07)	2[f]	194	0.56 (0.35–0.91)
	1[b]	102	0.78 (0.42–1.44)	1*[g]	45	0.61 (0.30–1.23)
RCT	–	–	–	3[h]	62	0.42 (0.25–0.71)
Stomach cancer						

Table 1 Relative risks of Aspirin use on the incidence and mortality of major cancers from recent overviews and major studies—cont'd

		Cancer incidence			*Cancer mortality*	
Case control	7[a]	2411	0.60 (0.44–0.82)	–	–	–
	8[b]	3000	0.61 (0.40–0.93)			
Cohort	6[a]	2108	0.77 (0.58–1.04)	2[f]	314	0.59 (0.40–0.86)
	1[b]	184	0.49 (0.22–1.12)	1*[g]	39	0.36 (0.15–0.88)
RCT	–	–	–	3[h]	71	0.69 (0.43–1.10)
Pancreatic cancer						
Case control	3[a]	1406	0.82 (0.68–1.00)	–	–	–
	5[b]	1619	1.02 (0.83–1.26)			
Cohort	7[a]	6471	0.95 (0.85–1.05)	2[j]	4655	0.97 (0.86–1.09)
	3[b]	2415	1.00 (0.79–1.27)	1*[g]	186	1.03 (0.73–1.46)
RCT	–	–	–	3[h]	77	0.81 (0.51–1.26)
Lung cancer						
Case control	5[a]	4863	0.73 (0.55–0.98)	1[e]	979	0.88 (0.73–1.05)
	12[b]	11,683	0.84 (0.66–1.08)			
Cohort	15[a]	11,356	0.98 (0.92–1.05)	2[k]	410†	0.97 (0.83–1.14)
	5[b]	1856	1.07 (0.96–1.19)	1*[g]	462	1.04 (0.84–1.29)
RCT	–	–	–	3[h]	326	0.71 (0.58–0.89)
Prostate cancer						
Case control	9[a]	5795	0.87 (0.74–1.02)	–	–	–
	8[b]	7857	0.86 (0.69–1.08)			
Cohort	15[a]	31,657	0.91 (0.85–0.97)	1[m]	580‡	0.84 (0.69–1.02)
	5[b]	3865	0.93 (0.86–1.01)	1*[g]	43	0.57 (0.28–1.15)
RCT	–	–	–	3[h]	210	0.81 (0.61–1.06)
Breast cancer						
Case control	10[a]	25,835	0.83 (0.76–0.91)	1[e]	864	0.95 (0.80–1.13)
	12[b]	22,046	0.81 (0.72–0.93)			
Cohort	22[a]	27,091	0.93 (0.87–1.00)	2[f]	131†	0.86 (0.65–1.15)
	9[b]	7713	0.88 (0.82–0.93)	1*[g]	32	0.28 (0.06–1.20)
RCT	1[d]	1230	0.98 (0.87–1.09)	—[b]	23	1.17 (0.50–2.71)

Several studies appear in more than one overview.

Number of cases are the number of events, either cancer diagnoses or cancer deaths.

Relative risks for >5 years daily use are also given where available (*).

[a]From Bosetti, C., Rosato, V., Gallus, S., Cuzick, J. and La Vecchia, C. (2012) Aspirin and cancer risk: A quantitative review to 2011. *Annals of Oncology* **23**(6), 1403–1415.

[b]From Algra, A.M. and Rothwell, P.M. (2012). Effects of regular aspirin on long-term cancer incidence and metastasis: A systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncology* **13**(5), 518–527 (Based on maximum Aspirin use data).

[c]From Rothwell, P.M., Wilson, M., Elwin, C.E., Norrving, B., Algra, A., Warlow, C.P., et al. (2010). Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* **376**(9754), 1741–1750.

[d]From Cook, N.R., Lee, I.M., Gaziano, J.M., Gordon, D., Ridker, P.M., Manson, J.E., et al. (2005). Low-dose aspirin in the primary prevention of cancer: The Women's Health Study: A randomized controlled trial. *JAMA* **294**(1), 47–55.

[e]From Chan, A.T., Manson, J.E., Feskanich, D., Stampfer, M.J. and Colditz, G.A., Fuchs, C.S. (2007). Long-term Aspirin use and mortality in women. *Archives of Internal Medicine* **167**(6), 562–572 (Women only Nested Case-control study, current users versus never users).

[f]Pooled risk ratios from Ratnasinghe, L.D., Graubard, B.I., Kahle, L., Tangrea, J.A., Taylor, P.R. and Hawk, E. (2004). Aspirin use and mortality from cancer in a prospective cohort study. *Anticancer Research* **24**(5B), 3177-3184 and Thun, M.J., Namboodiri, M.M., Calle, E.E., Flanders, W.D. and Heath, C.W., Jr. (1993). Aspirin use and risk of fatal cancer. *Cancer Research* **53**(6), 1322–1327.

[g]From Jacobs, E.J., Newton, C.C., Gapstur, S.M. and Thun, M.J. (2012). Daily aspirin use and cancer mortality in a large US cohort. *Journal of the National Cancer Institute* **104**(16), 1208-1217.

[h]From Rothwell et al.(Rothwell, P.M., Fowkes, F.G., Belch, J.F., Ogawa, H., Warlow, C.P. and Meade, T.W. (2011). Effect of daily aspirin on long-term risk of death due to cancer: Analysis of individual patient data from randomised trials. *Lancet* **377**(9759), 31–41.

[j]Pooled risk ratios from Ratnasinghe, L.D., Graubard, B.I., Kahle, L., Tangrea, J.A., Taylor, P.R. and Hawk, E. (2004). Aspirin use and mortality from cancer in a prospective cohort study. *Anticancer Research* **24**(5B), 3177–3184 and Jacobs, E.J., Connell, C.J., Rodriguez, C., Patel, A.V., Calle, E.E. and Thun, M.J. (2004). Aspirin use and pancreatic cancer mortality in a large United States cohort. *Journal of the National Cancer Institute* **96**(7), 524–528.

[k]Pooled risk ratios from Ratnasinghe, L.D., Graubard, B.I., Kahle, L., Tangrea, J.A., Taylor, P.R. and Hawk, E. (2004). Aspirin use and mortality from cancer in a prospective cohort study. *Anticancer Research* **24**(5B), 3177-3184 and Thun, M.J., Namboodiri, M.M., Calle, E.E., Flanders, W.D. and Heath, C.W., Jr. (1993). Aspirin use and risk of fatal cancer. *Cancer Research* **53**(6), 1322–1327 reported all respiratory cancer deaths as one group, which have been approximated as lung cancer deaths.

[m]From Dhillon, P.K., Kenfield, S.A., Stampfer, M.J. and Giovannucci, E.L. (2011). Long-term aspirin use and the risk of total, high-grade, regionally advanced and lethal prostate cancer in a prospective cohort of health professionals, 1988-2006. *International Journal of Cancer* **128**(10), 2444–2452.

†Number of deaths (lung cancer or breast cancer) not reported in Cancer Prevention Study II, Thun, M.J., Namboodiri, M.M., Calle, E.E., Flanders, W.D. and Heath, C.W., Jr. (1993). Aspirin use and risk of fatal cancer. *Cancer Research* **53**(6), 1322–1327.

‡Number of lethal prostate cancers, that is any metastatic prostate cancer or prostate cancer death.

Table 2 A semi-quantitative summary of site-specific effects of Aspirin on cancer incidence and mortality (reductions in percentages)

	Incidence	*Mortality*
Cancer site	*Reduction*	*Reduction*
Colorectal cancer	30%–35%	35%–40%
Esophageal cancer	25%–30%	45%–50%
Gastric cancer	25%–30%	30%–35%
Lung cancer	0%–5%	10%–15%
Breast cancer	5%–10%	0%–5%
Prostate cancer	5%–10%	10%–15%

not differ by histology; similar reductions have been reported for squamous cell esophageal cancers (39%) and adenocarcinomas (36%) including gastric cardia (Bosetti et al., 2012). The benefit of Aspirin in reducing esophageal cancer deaths has been approximately 40% in cohort studies and almost 60% in RCTs (Rothwell et al., 2011), although the latter is based on a relatively smaller number of events.

Gastric cancer

Similarly larger effect sizes in case-control studies than cohort studies (Algra and Rothwell, 2012; Bosetti et al., 2012), a reduction of approximately 40% and 25% respectively, have also been seen for incidence of gastric cancer (**Table 1**). More recent meta-analyses also demonstrate similar significant reductions (Huang et al., 2017). Nonsignificant 31% reduction in gastric cancer deaths ($P = 0.11$), mostly contributed by a delayed (after 10 years) reduction of 58% ($P = 0.007$), has been observed in RCTs (Rothwell et al., 2011). Cohort studies show a 41% reduction in gastric cancer mortality (Thun et al., 1993; Ratnasinghe et al., 2004).

Breast cancer

No effect on breast cancer incidence was seen in the first analysis of WHS (Cook et al., 2005), or after a longer 17.5-year follow-up (Cook et al., 2013). However, approximately 18% reduction in breast cancer incidence in case-control studies and approximately 10% reduction in cohort studies (Algra and Rothwell, 2012; Bosetti et al., 2012) has been reported (**Table 1**). More recent meta-analyses of cohort studies (Lu et al., 2017), case-control and cohort studies (Zhong et al., 2015a), and all types of studies including RCTs (Luo et al., 2012) show a reduction in breast cancer incidence to the tune of 10%. Similar small reductions in breast cancer mortality of 5% in a case-control study (Chan et al., 2007a) and 14% in cohort studies have also been observed (Thun et al., 1993; Ratnasinghe et al., 2004). Meta-analyses of observational studies (Zhong et al., 2015b) showed almost 20% reduction in breast cancer mortality in sensitivity analyses carried out to control heterogeneity. Results from RCTs (Algra and Rothwell, 2012) are unreliable due to a small number of events ($n = 23$).

Lung cancer

Pooled analyses of case-control studies show approximately 20% reduction in the incidence of lung cancer, but no reduction was observed in cohort studies (Algra and Rothwell, 2012; Bosetti et al., 2012). More recent meta-analyses (Hochmuth et al., 2016; Xu et al., 2012; Jiang et al., 2015) report similar reductions; however, a meta-analysis by McCormack and colleagues reported benefit only in men (McCormack et al., 2011), a finding also reported by Jiang et al. (2015).

Similar trends for the reduction in lung cancer mortality, that is approximately 10% reduction (nonsignificant) in case-control studies and no overall effect in cohort studies (Thun et al., 1993; Jacobs et al., 2012; Ratnasinghe et al., 2004) have been reported. However, data from the first and second National Health and Nutrition Examination Study studies (Ratnasinghe et al., 2004) show a significant 31% reduction in lung cancer deaths among men and no effect in women. Similar reduction of 29% has also been reported in pooled analyses of RCTs (Rothwell et al., 2011).

Prostate cancer

The effect of Aspirin on prostate cancer incidence is also small like that on breast cancer incidence. Several meta-analyses have now pooled data from various observational studies and RCTs to generate estimates of Aspirin's effect on prostate cancer incidence, incidence of advanced prostate cancer, and prostate cancer deaths; these have been summarized in **Table 3**. In general, case-control studies report a larger reduction in prostate cancer incidence, as compared with cohort studies (Algra and Rothwell, 2012; Bosetti et al., 2012) and an overall reduction of 5%–10% in prostate cancer incidence in all users is reported in observational studies. A larger benefit, a reduction of 8%–18%, is seen with Aspirin use of 5 years or more and a larger effect is also seen on advanced prostate cancer, incidence of which is reduced by 12%–19%. The reductions observed in RCTs are larger, above 20%, but statistically not significant. Prostate cancer deaths are reduced by 11%–12% in observational studies and by almost 20% in RCTs; the latter benefit is however statistically not significant.

Table 3 Summary of meta-analyses evaluating effects of Aspirin on prostate cancer incidence and mortality

Author	*Year*	*Studies included*			*Total prostate cancer incidence*	
		Case control (n)	Cohort (n)	RCTs (n)	All users	Longer duration
Algra and Rothwell (2012)	2012	13	8		0.94 (0.82–1·08)	0.86 (0.69–1.08)
Bosetti et al. (2014)	2014	9	16		0.91 (0.86–0.96)	0.92 (0.83–1.01)
Huang et al. (2014)	2014	14	10		0.90 (0.85–0.95)	0.82 (0.72–0.93)
Wang et al. (2014)	2014	11	13		0.95 (0.93–0.98)	
Liu et al. (2014)	2014	18	13		0.92 (0.87–0.97)	0.88 (0.79–0.99)
Algra and Rothwell (2012)	2012			6	0.77 (0.59–1.01)	0.79 (0.59–1.05)
					Advanced prostate cancer incidence	
		Case-control (n)	Cohort (n)	RCTs (n)	All users	Longer duration
Bosetti et al. (2014)	2014	9	16		0.88 (0.82–0.95)	
Huang et al. (2014)	2014	14	10		0.86 (0.78–0.95)	
Liu et al. (2014)	2014	18	13		0.81 (0.73–0.89)	
Rothwell et al. (2012b)	2012			5	0.64 (0.27–1.53)[a]	
					Prostate cancer deaths	
		Case control (n)	Cohort (n)	RCTs (n)	All users	Longer duration
Liu et al. (2014)	2014	2	6		0.86 (0.78–0.96)	
Elwood et al. (2016)	2016	2	6		0.89 (0.79–0.99)	
Rothwell et al. (2011)	2011			7	0.70 (0.29–1.73)	
Rothwell et al. (2011)	2011			3		0.81 (0.61–1.06)

[a]Metastatic prostate cancer; Risk estimates are relative risks (RR) for meta-analyses of observation studies and Hazard ratios (HR) for meta-analyses of RCTs.

Aspirin as an Adjuvant Treatment

The potential role of Aspirin in cancer treatment has been discussed in detail elsewhere (Langley et al., 2011) and more recent studies provide further evidence to support evaluation of Aspirin as an adjuvant treatment. It is clear from the larger effect sizes of Aspirin's benefit on cancer deaths than cancer incidence that it prevents cancer progression or metastasis after the cancer has developed. Aspirin's effects in reducing cancer recurrence risk have particularly been reported for breast cancer and CRC. A meta-analysis of observational studies (Huang et al., 2015) reported improved breast cancer survival with postdiagnostic Aspirin use (Holmes et al., 2010). A recent cohort study (Cronin-Fenton et al., 2016) based on the Danish Breast Cancer Cooperative Group registry however reported that only prediagnostic Aspirin use reduced the risk of recurrence. Prediagnosis (Coghill et al., 2011) or postdiagnosis (Bastiaannet et al., 2012; McCowan et al., 2013; Goh et al., 2014) Aspirin use is associated with an improved CRC survival (Ng et al., 2015), particularly in CRC with *PIK3CA* mutation (Liao et al., 2012; Domingo et al., 2013) or COX-2 overexpression (Chan et al., 2007b) or wild-type BRAF tumors (Frouws et al., 2017), although lack of such association has also been reported (Murphy et al., 2017). Several trials (**Table 4**) are currently investigating the adjuvant role of Aspirin in treatment of cancers, for example, ASCOLT trial (Ali et al., 2011) in CRC, the ABC trial (Chen et al., 2017) in breast cancer and ADD-Aspirin trial (Langley et al., 2011; Coyle et al., 2016) in a range of cancers including colorectal, gastro-esophageal, breast, and prostate cancer. Several other trials are being planned, for example, AIDA and SAKK 41/13 trials in CRC, latter in patients with *PIK3CA* mutation.

Aspirin as Preventive Therapy in General Population

Benefit–Harm Analyses

Aspirin has now been used for secondary prevention of CVD for >3 decades but its role in primary prevention of CVD has remained controversial (Baigent et al., 2009; Preventive Services, 2009). However, data from pooled analyses of trials by Rothwell et al. (2011, 2012a) demonstrated that the main significant benefit of Aspirin in general population is reduction in cancer deaths and not reduction in vascular deaths. Therefore, it is necessary to consider Aspirin's effects on all diseases including cancer and CVD when considering its role as preventive therapy in general population. We carried out first such comprehensive analyses (Cuzick et al., 2015) using actual population level data (Thorat and Cuzick, 2015) to model Aspirin's benefits and harms. Instead of numerically equating minor (e.g., nosebleed) and major (e.g., hemorrhagic stroke) harms, these analyses, for the first time took severity of harms into account (Cuzick et al., 2015). Furthermore, as another first, these analyses also employed a modeling approach (similar to a Markov model) that took in to account peculiarities of Aspirin's effects like lag and carryover of benefits (Thorat, 2016) (**Fig. 4**).

Our benefit–harm analyses (Cuzick et al., 2015) show a net benefit for average-risk individuals aged 50–65 years taking Aspirin for 10 years. In such individuals, there would be a relative reduction of between 7% (women) and 9% (men) in the number of cancer, myocardial infarction, or stroke events over a 15-year period and an overall 4% relative reduction in all deaths over a 20-year period; the main benefits being driven through reduction in cancer incidence (**Fig. 5**) and cancer deaths (**Fig. 6**). These

Table 4 Ongoing trials of Aspirin

Trial name	*Comparison*	*Sample size*	*Participants*
Target population: general population			
ASPREE (NCT01038583)	100 mg Aspirin versus Placebo	19,000	People over 65 years of age
ARRIVE (NCT00501059)	100 mg Aspirin versus Placebo	12,551	Individuals with 10-year CHD risk of approx. 10%–20%
ACCEPT-D (ISRCTN48110081)	100 mg Aspirin versus None	5170	Individuals with type one or type two diabetes with no clinical evidence of vascular disease and receiving statins—Simvastatin (starting dose 20 mg/d)
ASCEND (NCT00135226)	2 × 2 Factorial design A. 100 mg Aspirin versus placebo B. 1 g of omega-3-Ethyl Esters versus placebo	15,480	Patients with diabetes who are not known to have occlusive arterial disease
Target population: High-risk individuals			
CaPP3 (NCT02497820)	600 mg versus 300 mg versus 100 mg of enteric coated Aspirin	3000	Carriers of a germline pathological mismatch repair gene defect, Lynch Syndrome
AspECT (NCT00357682)	2 × 2 Factorial design (A) 300 mg Aspirin versus none (B) 20 versus 80 mg Esomeprazole	2513	Patients with Barrett's metaplasia
Target population: Cancer patients – adjuvant setting			
ADD-Aspirin (ISRCTN74358648)	Aspirin 300 mg versus 100 mg versus placebo	Total: 9920 Breast (3100), Colorectal (2600), Gastro-esophageal (2100), Prostate (2120)	Adjuvant treatment after primary therapy in nonmetastatic breast, colorectal*, gastro-esophageal, and prostate cancers
ASCOLT (NCT00565708)	200 mg Aspirin versus Placebo	1200	Patients with Dukes' C or high risk Dukes' B colorectal cancer.
APREMEC (NCT02607072)	Aspirin 200 mg versus 100 mg versus placebo	3000	Stages I–III colorectal cancer completely removed with R0 resection margin
ASPIRIN (NCT02301286)	80 mg Aspirin versus Placebo	1588	Stage II and III colon cancer patients of 45 years of age and older
ALASCCA (NCT02647099)	160 mg Aspirin versus Placebo	816 Patients screened – 3900	Stages I–III colorectal cancer with *PIK3CA* mutation (exon 9 and 20) or with mutations in other PI3K pathway genes PIK3CA (other than exon 9 and 20), PIK3R1 or PTEN
SAKK 41/13 (NCT02467582)	100 mg Aspirin versus Placebo	185	Stages II–III completely resected (R0) colon cancer with *PIK3CA* mutation (exon 9 and 20); rectal cancer excluded
The ABC Trial (NCT02927249)	300 mg Aspirin versus Placebo	2936	Early stage node-positive HER2 negative breast cancer patients
Target population: cancer patients—primary therapy setting			
PROVENT (ISRCTN91422391)	3 × 2 Factorial design (A) Aspirin 300 mg versus 100 mg versus placebo (B) Vitamin D3: 4000 IU/d versus placebo	Feasibility phase	Men on active surveillance for prostate cancer

*Includes fully resected metastatic colorectal cancer.

main analyses assumed beneficial effects on six cancers discussed above; however, sensitivity analyses assuming effects on only three GI cancers (esophageal, gastric, and colorectal) or CRC alone [Supplement in Cuzick et al. (2015)] also show net benefit with Aspirin use. The absolute benefit of Aspirin in an individual is dependent on sex and age at which Aspirin use is started since the baseline risks differ by age and sex. These analyses (Cuzick et al., 2015) show that 1 death would be prevented if 47 men aged 65 or 213 women aged 50 took Aspirin for 10 years, a much larger absolute benefit in older men since the baseline cancer and CVD risks are much higher in older men as compared with younger women. We also estimated that the net benefit could be further improved by reducing the risk of some of the harms like peptic ulcer or GI bleeding by almost a third through eradication of *Helicobacter pylori* infection (Thorat and Cuzick, 2015) which is still substantially prevalent in older cohorts of men and women in the developed world.

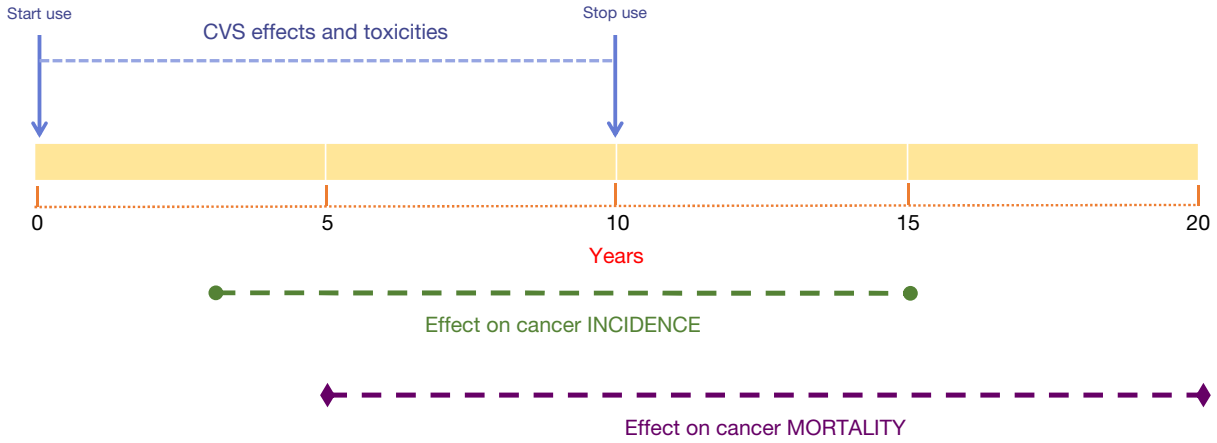


Fig. 4 Aspirin's effects in general population—Modeling to account for lag and carryover effects.

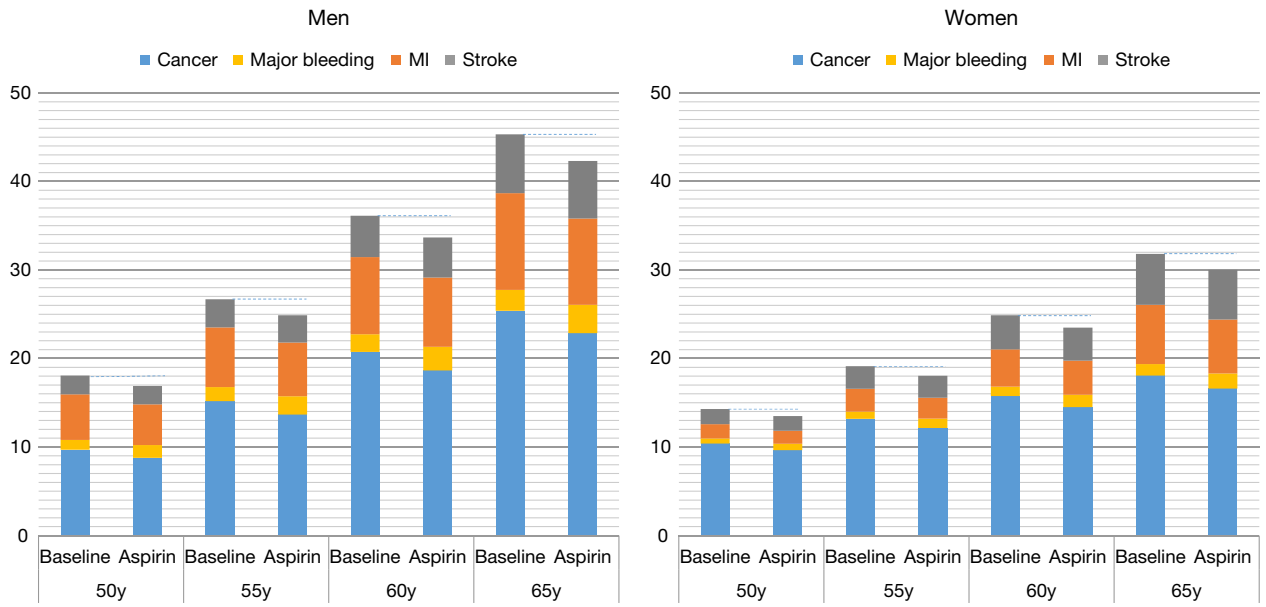


Fig. 5 Aspirin's effects in general population—reduction in incidence of major events over a 15-year period with 10 years of Aspirin use. *Y*-axis—events in a proportion (%) of individuals with or without Aspirin; *MI*—Myocardial infarction.

Health Economics

Full economic evaluation of Aspirin as a preventive therapy in general population remains to be done. Our benefit–harm analyses (Cuzick et al., 2015) assuming effects on only three GI cancers (esophageal, gastric, and colorectal) show that if everyone in the UK aged 50–64 (UK population year 2013) took Aspirin for 10 years, every year, approximately 5900 GI cancers and 4000 heart attacks or strokes will be prevented whereas approximately 2800 extra major bleeding events will be caused. Aspirin will also prevent approximately 3500 deaths every year. Aspirin, being a generic medicine, is available at a low cost of £3/per year per person (Joint Formulary Committee, 2016). Using economic costs of cancer and CVD reported in the British Heart Foundation report (British Heart Foundation, 2013), we estimate that such Aspirin use will result in a net healthcare cost savings of at least £76 million every year. Bupa report (Macdonald, 2011) estimated the average per cancer treatment cost to be £30,000, which is expected to increase to £40,000 by year 2021 (Choices, 2011). Calculations using these higher per cancer costs show that Aspirin use will potentially save the UK National Health Service up to £157 million every year and these annual savings could increase to £216 million by the year 2021. A thorough economic evaluation of Aspirin as a preventive therapy in general population is a research priority but these estimates demonstrate that with ever increasing cancer care costs in the healthcare systems in the developed world, Aspirin has a potential to be a highly cost-effective preventive intervention in the general population.

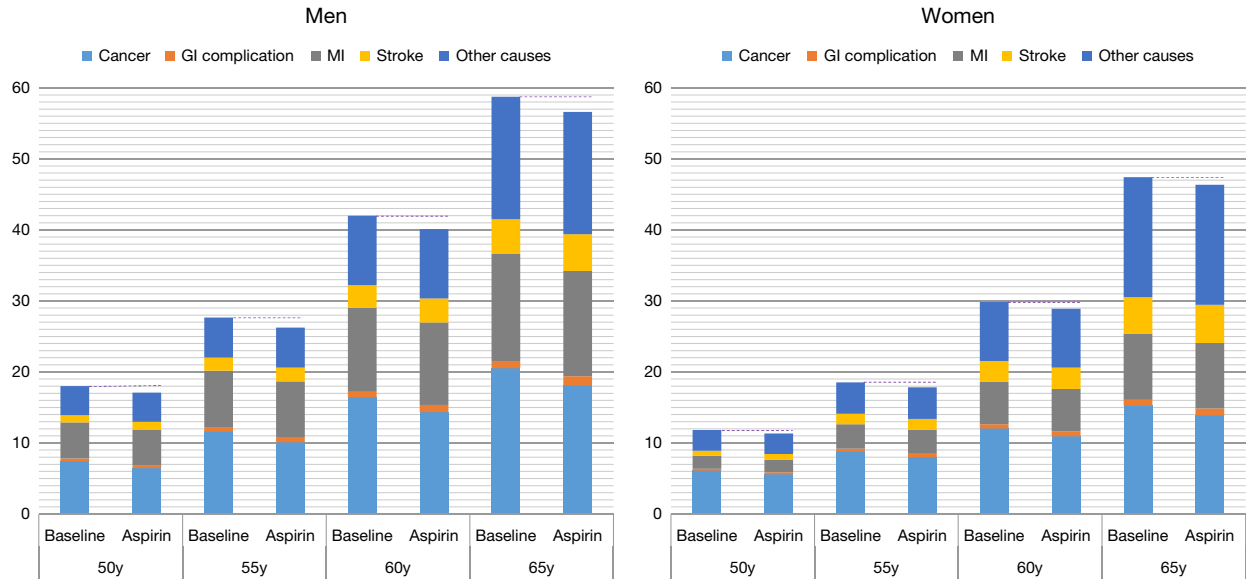


Fig. 6 Aspirin's effects in general population—reduction in deaths over a 20-year period with 10 years of Aspirin use. *Y*-axis—events in a proportion (%) of individuals with or without Aspirin; *MI*—Myocardial infarction; *GI*—Gastrointestinal.

Guidelines

Uncertainty regarding Aspirin's benefits for the primary prevention of CVD (Baigent et al., 2009), and oligo-dimensional approach (Thorat, 2016) taken by various guideline producing organizations resulted in divergent recommendations (Vandvik et al., 2012; Force, 2007, 2009; Perk et al., 2012). However, after our comprehensive analyses, the U.S. Preventive Services Task Force (USPSTF) also adopted a similar approach and resultantly, their recommendations (Bibbins-Domingo and Force, 2016) changed in favor of Aspirin use as a preventive therapy. The USPSTF now recommends initiating low-dose Aspirin use for the primary prevention of CVD and CRC in adults aged 50–59 years who have a 10% or greater 10-year CVD risk (Bibbins-Domingo and Force, 2016). There are currently no recommendations regarding Aspirin as an adjuvant therapy in cancer as it is a subject of active investigation.

GI Cancer Prevention—Aspirin and Screening

A recent systematic review by Emilsson and colleagues (2017) reported that Aspirin was more effective than Fecal Occult Blood Test (FOBT) screening [RR 0.36; 95% predictive interval (PrI) 0.22–0.59] and flexible sigmoidoscopy (FlexiSig) screening (RR 0.37; 95% PrI 0.22–0.62) in preventing new diagnosis of cancer or death from cancer in the proximal colon. Overall, Aspirin was equally effective as FOBT and FlexiSig screening in reducing CRC incidence and mortality. Authors concluded that a randomized comparative effectiveness trial of Aspirin versus screening is warranted (Emilsson et al., 2017). However, FlexiSig, although highly effective for CRC screening, has poor sensitivity for proximal colon lesions (Lin et al., 2016). Aspirin, on the other hand, has much greater effect on incidence of proximal CRC, and mortality from proximal CRC (Rothwell et al., 2010). Therefore, FlexiSig screening and Aspirin as a preventive therapy could prove complementary to each other. Furthermore, an observational study (Brenner et al., 2010) has shown that Aspirin increases sensitivity of Fecal Immunochemical Test. Therefore, even this method of CRC screening and Aspirin could prove complementary to each other in preventing CRC mortality. In summary, instead of evaluating comparative effectiveness of Aspirin versus screening as Emilsson and colleagues (2017) suggest, research to evaluate combined impact of these two methods of CRC prevention and research to facilitate adoption of such combined approach is required.

Mechanisms of Aspirin Action

Aspirin's effects on CVD and cancer are very clear from epidemiological data; however, the mechanisms underpinning these effects barring the antiplatelet mechanism are far from clear. Aspirin's pharmacological effects range from antiplatelet action at low doses to antiinflammatory action at high doses and several new mechanisms of action continue to be discovered.

Prostanoid Synthesis

Enzyme Prostaglandin H Synthase (PGHS) or Cyclooxygenase metabolizes arachidonic acid to synthesize prostanoids which are tissue-specific lipid compounds involved in signaling. Prostanoids include Prostaglandin (PG) D_2, E_2, $F_{2\alpha}$, Thromboxane A_2

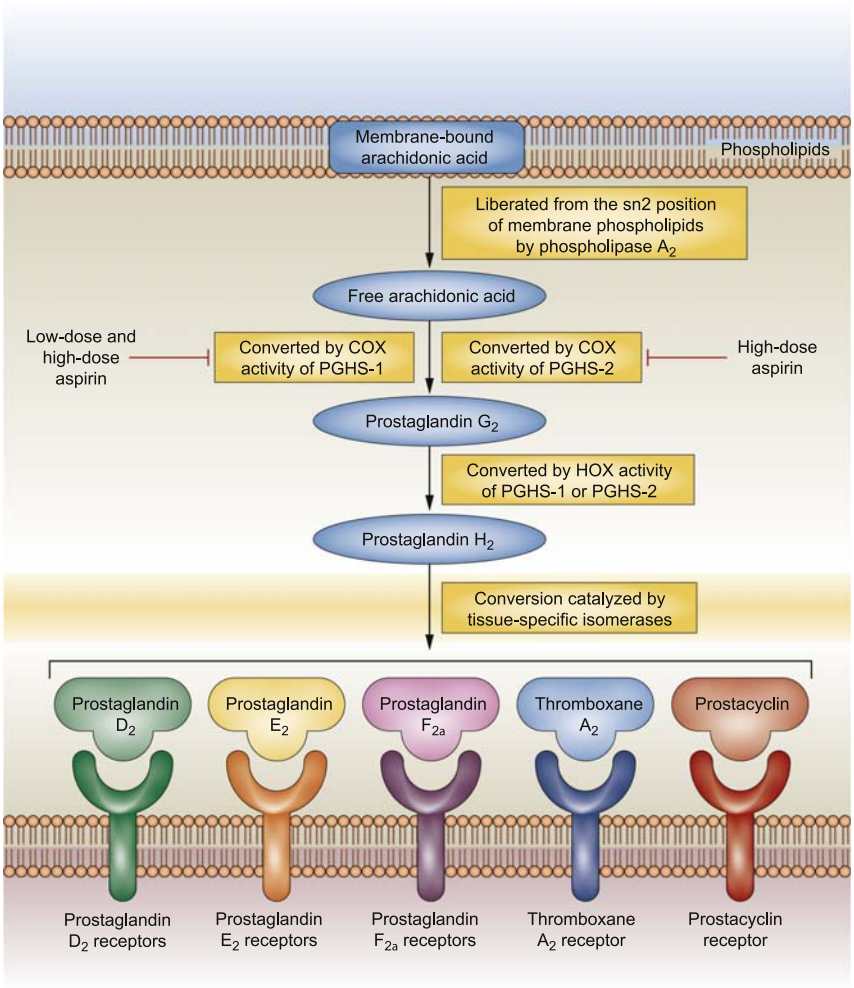


Fig. 7 Prostanoid synthesis. Reprinted from: Thun, M.J., Jacobs, E.J., Patrono, C. (2012). The role of aspirin in cancer prevention. *Nature Reviews. Clinical Oncology* **9**(5), 259-267. Copyright (2012), with permission from Macmillan Publishers Limited.

(TXA_2), and prostacyclin (PGI_2). PGHS has two major isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues while COX-2 expression is tissue-specific, for example vascular endothelium, brain, and kidney (Fig. 7).

Aspirin irreversibly inactivates both COX-1 and COX-2. However, the level of inhibition of activity is dependent on bioavailability of Aspirin in the target tissue and de novo enzyme synthesis that leads to recovery of COX activity (Pedersen and FitzGerald, 1984).

Antiplatelet Action

Mature platelets only express COX-1. Platelet COX-1 is involved in synthesis of TXA_2, which has a central role in platelet aggregation. Vascular endothelium, on the other hand, expresses COX-2 involved in PGI_2 synthesis (Patrono et al., 2005). Aspirin inhibits both COX-1 in platelets and COX-2 in vascular endothelium. While enzymatic activity in vascular endothelium (COX-2) recovers through de novo enzyme synthesis, COX-1 activity in platelets does not recover since mature platelets lack nuclei and therefore cannot synthesize COX-1 enzyme (Pedersen and FitzGerald, 1984). Furthermore, platelets also pass through portal circulation where Aspirin concentration is much higher than that in systemic circulation (Zhou et al., 2011). The net result is inactivation of platelets in systemic circulation even at low doses of Aspirin whereas vascular endothelial function continues as normal at low doses. Therefore, doses as low as 30 mg Aspirin per day have very transient and minimal effects on nonplatelet targets but result in significant cardio-protective antiplatelet effects.

Antiinflammatory Action

Aspirin has a very short half-life of 15–20 min as it is metabolized rapidly during its first-pass through liver and also by plasma esterases (Zhou et al., 2011). As a result, achieving and maintaining sustained COX-1 and COX-2 enzyme inhibition in nucleated

cells that are capable of de novo enzyme synthesis requires frequent dosing (3–4 times per day) and a large cumulative dose (around 2000 mg per day). At these doses, Aspirin has effect on nucleated cells driving inflammatory response resulting in its antiinflammatory action. Sustained inhibition of prostanoid synthesis is necessary for this action, but several other mechanisms including inhibition of protein kinase IkB kinase β (IKKβ) in the NF-kB pathway (Yuan et al., 2001) and Aspirin-triggered 15-epi-lipoxins (Serhan, 2005) are also involved.

Analgesic Action

Analgesic effects of Aspirin appear to be dose-dependent (McQuay and Moore, 2007) and these are also thought to be mediated through several mechanisms (Smith, 2012).

Anticancer Action

Aspirin has a larger effect on cancer deaths than that on cancer incidence. This means that apart from carcinogenesis, it also affects cancer progression and metastasis. While the effects of Aspirin on cancer are clear, the mechanisms are not clear yet. The most accepted among several hypotheses is that anticancer action is mediated through COX-2 inhibition (Chan et al., 2012; Thun et al., 2002). This hypothesis however cannot explain unequivocal anticancer effects of Aspirin that occur at low dose (Rothwell et al., 2010, 2011, 2012a). On the other hand, as proposed by Thun and colleagues (2012), Aspirin may prevent platelet-mediated COX-2 induction and downstream signaling at the site of intestinal mucosal injury and hence increase apoptosis, reduce proliferation and angiogenesis. While this hypothesis of abrogation of platelet-mediated COX-2 induction preventing carcinogenesis remains to be proven, a recent study by Patrignani and colleagues (2017) has demonstrated that low-dose Aspirin is capable of acetylating COX-1 enzyme in colorectal mucosa and thereby reducing PGE_2 synthesis as well as phosphorylation of S6 despite of low bioavailability due to rapid first-pass metabolism. Therefore, it is possible that Aspirin acts through both COX-1 and COX-2 mediated pathways in preventing carcinogenesis at low doses. Furthermore, low-dose Aspirin's ($\leq$100 mg/day) antiplatelet effect may reduce direct interaction of platelets with cancer cells, the so-called platelet chaperoning (Rothwell et al., 2012b), and thus prevent metastasis (Gasic et al., 1968, 1972). These mechanisms are compatible with low-dose Aspirin's effects in preventing carcinogenesis as well as cancer metastasis.

Several other mechanisms of Aspirin action have been proposed, although these require higher drug concentrations than those achieved by low-dose Aspirin (Shaw and Cantley, 2012). Additional potential mechanisms of Aspirin action, many previously listed by us (Thorat and Cuzick, 2013), include inhibition of NF-kB (Chan et al., 2007b), induction of polyamine catabolism (Hubner et al., 2008), inhibition of mTOR signaling (Din et al., 2012) and activation of AMP-activated protein kinase (Din et al., 2012; Hawley et al., 2012), effects on PI3-kinase pathway (Liao et al., 2012), particularly in *PIK3CA*-mutants (Zumwalt et al., 2017) or its cross-talk with COX-2 (Kaur and Sanyal, 2010; Uddin et al., 2010) and induction of apoptosis through other pathways (Dikshit et al., 2006; Lu et al., 2008). Although Aspirin's anticancer effects at a low dose are clear, the relationship between Aspirin's dose and its anticancer effects is not clearly understood (discussed below). It is possible that different mechanisms are activated at different doses and higher Aspirin doses may confer greater anticancer benefit.

Ongoing Trials

Several trials (**Table 4**) are already underway or due to start evaluating Aspirin in general population, or high-risk individuals or as a cancer treatment. >50,000 individuals have been recruited in the trials in general population and these are expected to report their first results by year 2020. Approximately 8000 high-risk individuals will also have been recruited in Aspirin trials over next few years. Approximately 20,000 cancer patients will also enter Aspirin trials in the next few years. Data from these approximately 80,000 Aspirin trial participants will help us understand Aspirin's effects and mechanisms in a much better manner; however, a word of caution is due at this stage. Aspirin's effects on cancer are not apparent for at least first few years and therefore, early trial reports with short follow-up need to be carefully interpreted. Furthermore, all trials should be followed up for a sufficiently long period, preferably for at least 10 years after cessation of treatment.

Research Priorities

We have previously identified duration of Aspirin use and optimum dose as important research questions (Cuzick et al., 2015; Thorat and Cuzick, 2013, 2015) for most effective use of Aspirin for prophylaxis in general population.

Aspirin needs to be consumed for at least 5 years to derive anticancer benefit and the benefits continue for several years after cessation of Aspirin use. While it seems prudent to stop Aspirin use by age of 70 years to minimize bleeding harms which rise sharply after 70 (Thorat and Cuzick, 2015), the optimum duration of Aspirin use and upper age of stopping is not clear and therefore an important research question.

As mentioned above, the relationship between Aspirin dose and anticancer effect is also not clear. An indirect comparison of RCTs shows that increasing dose does not confer a greater reduction in cancers (Rothwell et al., 2010) or adenomas (Baron

et al., 2003). However, observational studies (Chan et al., 2008; Friis et al., 2003) have reported that reductions in advanced adenomas are greater with the higher dose (Cole et al., 2009) and significant effects of Aspirin with doses above 300 mg. Furthermore, CAPP2 trial used 600 mg Aspirin dose in high-risk individuals and reported a much larger effect on CRC than in other RCTs (Burn et al., 2011). CAPP3 trial (**Table 4**) is hence evaluating different Aspirin doses, but in high-risk individuals. Since it is possible that different mechanisms get activated at different dose levels of Aspirin, a particular dose may confer greatest anticancer benefit. The linearity of relationship between Aspirin's harms and dose is also not clearly established (Thorat and Cuzick, 2015). Therefore, identifying optimum dose of Aspirin at which its net benefit is the highest is an important research priority.

All the ongoing trials in general population setting are using low dose of Aspirin and none of these is evaluating duration of Aspirin use. Furthermore, all of the trials except ASPREE are in individuals at a risk higher than average. Therefore, dose and duration of Aspirin use in average-risk general population still remain important research questions that need to be addressed in a trial in an average-risk general population.

Lastly, mechanisms underpinning Aspirin benefit are poorly understood and a greater understanding of these mechanisms will not only allow us to use this drug in a more effective manner but also lead to the development of additional cancer therapy options. Bio-specimens from the participants in ongoing and future trials will help greatly in this quest. The need for effective use of bio-specimens from Aspirin trials and long-term follow-up of these trials cannot be overemphasized.

Conclusions

Aspirin has a range of beneficial effects on cancer and it reduces incidence and mortality from three GI cancers, namely colorectal, esophageal, and stomach as well as from lung, breast, and prostate cancer although the effects of these non-GI cancers are less clear and the magnitude of benefit is much smaller. Aspirin's effects on cancer are not immediately apparent but continue for several years after cessation of use. Aspirin, taken as preventive therapy for at least 5 years by men and women aged 50–70 years from developed world, will provide net health benefits primarily its effects on cancer. Aspirin also has a potential role as an adjuvant treatment in cancer and this is currently being evaluated in several trials. Further research to determine optimum dose and duration of Aspirin use and to elucidate its mechanisms of action is needed.

See also: Chemoprevention Trials.

References

Algra, A.M., Rothwell, P.M., 2012. Effects of regular aspirin on long-term cancer incidence and metastasis: A systematic comparison of evidence from observational studies versus randomised trials. The Lancet Oncology 13 (5), 518–527.

Ali, R., Toh, H.C., Chia, W.K., 2011. The utility of aspirin in dukes C and high risk dukes B colorectal cancer—The ASCOLT study: Study protocol for a randomized controlled trial. Trials 12, 261.

Baandrup, L., Faber, M.T., Christensen, J., Jensen, A., Andersen, K.K., Friis, S., et al., 2013. Nonsteroidal anti-inflammatory drugs and risk of ovarian cancer: Systematic review and meta-analysis of observational studies. Acta Obstetricia et Gynecologica Scandinavica 92 (3), 245–255.

Baigent, C., Blackwell, L., Collins, R., Emberson, J., Godwin, J., Peto, R., et al., 2009. Aspirin in the primary and secondary prevention of vascular disease: Collaborative meta-analysis of individual participant data from randomised trials. Lancet 373 (9678), 1849–1860.

Baron, J.A., Cole, B.F., Sandler, R.S., Haile, R.W., Ahnen, D., Bresalier, R., et al., 2003. A randomized trial of aspirin to prevent colorectal adenomas. The New England Journal of Medicine 348 (10), 891–899.

Bastiaannet, E., Sampieri, K., Dekkers, O.M., de Craen, A.J., van Herk-Sukel, M.P., Lemmens, V., et al., 2012. Use of aspirin postdiagnosis improves survival for colon cancer patients. British Journal of Cancer 106 (9), 1564–1570.

Bibbins-Domingo, K., Force, U.S.P.S.T., 2016. Aspirin use for the primary prevention of cardiovascular disease and colorectal cancer: U.S. preventive services task force recommendation statement. Annals of Internal Medicine 164 (12), 836–845.

Bosetti, C., Rosato, V., Gallus, S., Cuzick, J., La Vecchia, C., 2012. Aspirin and cancer risk: A quantitative review to 2011. Annals of Oncology 23 (6), 1403–1415.

Bosetti, C., Rosato, V., Gallus, S., La Vecchia, C., 2014. Aspirin and prostate cancer prevention. Recent Results in Cancer Research 202, 93–100.

Brenner, H., Tao, S., Haug, U., 2010. Low-dose aspirin use and performance of immunochemical fecal occult blood tests. Journal of the American Medical Association 304 (22), 2513–2520.

British Heart Foundation, 2013. Economic costs of physical inactivity. British Heart Foundation national Centre (BHFNC) for Physical Activity and Health, Loughborough University, Loughborough.

Burn, J., Gerdes, A.-M., Macrae, F., Mecklin, J.-P., Moeslein, G., Olschwang, S., et al., 2011. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: An analysis from the CAPP2 randomised controlled trial. The Lancet 378 (9809), 2081–2087.

Cao, Y., Nishihara, R., Wu, K., Wang, M., Ogino, S., Willett, W.C., et al., 2016. Population-wide impact of long-term use of aspirin and the risk for cancer. JAMA Oncology 2 (6), 762–769.

Chan, A.T., Manson, J.E., Feskanich, D., Stampfer, M.J., Colditz, G.A., Fuchs, C.S., 2007a. Long-term aspirin use and mortality in women. Archives of Internal Medicine 167 (6), 562–572.

Chan, A.T., Ogino, S., Fuchs, C.S., 2007b. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. The New England Journal of Medicine 356 (21), 2131–2142.

Chan, A.T., Giovannucci, E.L., Meyerhardt, J.A., Schernhammer, E.S., Wu, K., Fuchs, C.S., 2008. Aspirin dose and duration of use and risk of colorectal cancer in men. Gastroenterology 134 (1), 21–28.

Chan, A.T., Arber, N., Burn, J., Chia, W.K., Elwood, P., Hull, M.A., et al., 2012. Aspirin in the chemoprevention of colorectal neoplasia: An overview. Cancer Prevention Research 5 (2), 164–178.

Chen, W.Y., Winer, E.P., Barry, W.T., Hudis, C.A., Openshaw, T.H., Visvanathan, K., et al., 2017. ABC trial (A011502): A randomized phase III double blinded placebo controlled trial of aspirin as adjuvant therapy for node positive breast cancer. Journal of Clinical Oncology 35 (15_suppl), TPS586-TPS.

NHS Choices. Cancer survival rates threatened by rising cost2011 04/08/2017. Available from: http://www.nhs.uk/news/2011/12December/Pages/cancer-treatment-cost-may-increase.aspx.

Chubak, J., Kamineni, A., Buist, D.S.M., Anderson, M.L., Whitlock, E.P., 2015. Aspirin Use for the Prevention of Colorectal Cancer: An Updated Systematic Evidence Review for the US Preventive Services Task Force. U.S. Preventive Services Task Force Evidence Syntheses, formerly Systematic Evidence Reviews, Rockville (MD).

Coghill, A.E., Newcomb, P.A., Campbell, P.T., Burnett-Hartman, A.N., Adams, S.V., Poole, E.M., et al., 2011. Prediagnostic non-steroidal anti-inflammatory drug use and survival after diagnosis of colorectal cancer. Gut 60 (4), 491–498.

Cole, B.F., Logan, R.F., Halabi, S., Benamouzig, R., Sandler, R.S., Grainge, M.J., et al., 2009. Aspirin for the chemoprevention of colorectal adenomas: Meta-analysis of the randomized trials. Journal of the National Cancer Institute 101 (4), 256–266.

Cook, N.R., Lee, I.M., Gaziano, J.M., Gordon, D., Ridker, P.M., Manson, J.E., et al., 2005. Low-dose aspirin in the primary prevention of cancer: The Women's health study: A randomized controlled trial. Journal of the American Medical Association 294 (1), 47–55.

Cook, N.R., Lee, I.M., Zhang, S.M., Moorthy, M.V., Buring, J.E., 2013. Alternate-day, low-dose aspirin and cancer risk: Long-term observational follow-up of a randomized trial. Annals of Internal Medicine 159 (2), 77–85.

Coyle, C., Cafferty, F.H., Rowley, S., MacKenzie, M., Berkman, L., Gupta, S., et al., 2016. ADD-ASPIRIN: A phase III, double-blind, placebo controlled, randomised trial assessing the effects of aspirin on disease recurrence and survival after primary therapy in common non-metastatic solid tumours. Contemporary Clinical Trials 51, 56–64.

Cronin-Fenton, D.P., Heide-Jorgensen, U., Ahern, T.P., Lash, T.L., Christiansen, P., Ejlertsen, B., et al., 2016. Low-dose aspirin, nonsteroidal anti-inflammatory drugs, selective COX-2 inhibitors and breast cancer recurrence. Epidemiology 27 (4), 586–593.

Cuzick, J., Otto, F., Baron, J.A., Brown, P.H., Burn, J., Greenwald, P., et al., 2009. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: An international consensus statement. The Lancet Oncology 10 (5), 501–507.

Cuzick, J., Thorat, M.A., Bosetti, C., Brown, P.H., Burn, J., Cook, N.R., et al., 2015. Estimates of benefits and harms of prophylactic use of aspirin in the general population. Annals of Oncology 26 (1), 47–57.

Dikshit, P., Chatterjee, M., Goswami, A., Mishra, A., Jana, N.R., 2006. Aspirin induces apoptosis through the inhibition of proteasome function. The Journal of Biological Chemistry 281 (39), 29228–29235.

Din, F.V., Valanciute, A., Houde, V.P., Zibrova, D., Green, K.A., Sakamoto, K., et al., 2012. Aspirin inhibits mTOR signaling, activates AMP-activated protein kinase, and induces autophagy in colorectal cancer cells. Gastroenterology 142 (7), 1504–1515 e3.

Domingo, E., Church, D.N., Sieber, O., Ramamoorthy, R., Yanagisawa, Y., Johnstone, E., et al., 2013. Evaluation of PIK3CA mutation as a predictor of benefit from nonsteroidal anti-inflammatory drug therapy in colorectal cancer. Journal of Clinical Oncology 31 (34), 4297–4305.

Eichengrün, A., 1949. Jahre aspirin. Die Pharmazie 4, 582–584.

Elwood, P., 2006. The first randomized trial of aspirin for heart attack and the advent of systematic overviews of trials. Journal of the Royal Society of Medicine 99 (11), 586–588.

Elwood, P.C., Cochrane, A.L., Burr, M.L., Sweetnam, P.M., Williams, G., Welsby, E., et al., 1974. A randomized controlled trial of acetyl salicylic acid in the secondary prevention of mortality from myocardial infarction. British Medical Journal 1 (5905), 436–440.

Elwood, P.C., Morgan, G., Pickering, J.E., Galante, J., Weightman, A.L., Morris, D., et al., 2016. Aspirin in the treatment of cancer: Reductions in metastatic spread and in mortality: A systematic review and meta-analyses of published studies. PLoS One 11 (4), e0152402.

Emilsson, L., Holme, O., Bretthauer, M., Cook, N.R., Buring, J.E., Loberg, M., et al., 2017. Systematic review with meta-analysis: The comparative effectiveness of aspirin vs. screening for colorectal cancer prevention. Alimentary Pharmacology & Therapeutics 45 (2), 193–204.

Flossmann, E., Rothwell, P.M., 2007. Effect of aspirin on long-term risk of colorectal cancer: Consistent evidence from randomised and observational studies. Lancet 369 (9573), 1603–1613.

Force, U.S.P.S.T., 2007. Routine aspirin or nonsteroidal anti-inflammatory drugs for the primary prevention of colorectal cancer: U.S. preventive services task force recommendation statement. Annals of Internal Medicine 146 (5), 361–364.

Force, U.S.P.S.T., 2009. Aspirin for the prevention of cardiovascular disease: U.S. preventive services task force recommendation statement. Annals of Internal Medicine 150 (6), 396–404.

Friis, S., Sorensen, H.T., McLaughlin, J.K., Johnsen, S.P., Blot, W.J., Olsen, J.H., 2003. A population-based cohort study of the risk of colorectal and other cancers among users of low-dose aspirin. British Journal of Cancer 88 (5), 684–688.

Frouws, M.A., Reimers, M.S., Swets, M., Bastiaannet, E., Prinse, B., van Eijk, R., et al., 2017. The influence of BRAF and KRAS mutation status on the association between aspirin use and survival after colon cancer diagnosis. PLoS One 12 (1), e0170775.

Gann, P.H., Manson, J.E., Glynn, R.J., Buring, J.E., Hennekens, C.H., 1993. Low-dose aspirin and incidence of colorectal tumors in a randomized trial. Journal of the National Cancer Institute 85 (15), 1220–1224.

Gasic, G.J., Gasic, T.B., Stewart, C.C., 1968. Antimetastatic effects associated with platelet reduction. Proceedings of the National Academy of Sciences of the United States of America 61 (1), 46–52.

Gasic, G.J., Gasic, T.B., Murphy, S., 1972. Anti-metastatic effect of aspirin. Lancet 2 (7783), 932–933.

Goh, C.H., Leong, W.Q., Chew, M.H., Pan, Y.S., Tony, L.K., Chew, L., et al., 2014. Post-operative aspirin use and colorectal cancer-specific survival in patients with stage I-III colorectal cancer. Anticancer Research 34 (12), 7407–7414.

Hawley, S.A., Fullerton, M.D., Ross, F.A., Schertzer, J.D., Chevtzoff, C., Walker, K.J., et al., 2012. The ancient drug salicylate directly activates AMP-activated protein kinase. Science 336 (6083), 918–922.

Hochmuth, F., Jochem, M., Schlattmann, P., 2016. Meta-analysis of aspirin use and risk of lung cancer shows notable results. European Journal of Cancer Prevention 25 (4), 259–268.

Holmes, M.D., Chen, W.Y., Li, L., Hertzmark, E., Spiegelman, D., Hankinson, S.E., 2010. Aspirin intake and survival after breast cancer. Journal of Clinical Oncology 28 (9), 1467–1472.

Huang, T.B., Yan, Y., Guo, Z.F., Zhang, X.L., Liu, H., Geng, J., et al., 2014. Aspirin use and the risk of prostate cancer: A meta-analysis of 24 epidemiologic studies. International Urology and Nephrology 46 (9), 1715–1728.

Huang, X.Z., Gao, P., Sun, J.X., Song, Y.X., Tsai, C.C., Liu, J., et al., 2015. Aspirin and nonsteroidal anti-inflammatory drugs after but not before diagnosis are associated with improved breast cancer survival: A meta-analysis. Cancer Causes & Control 26 (4), 589–600.

Huang, X.Z., Chen, Y., Wu, J., Zhang, X., CC, W., Zhang, C.Y., et al., 2017. Aspirin and non-steroidal anti-inflammatory drugs use reduce gastric cancer risk: A dose-response meta-analysis. Oncotarget 8 (3), 4781–4795.

Hubner, R.A., Muir, K.R., Liu, J.F., Logan, R.F., Grainge, M.J., Houlston, R.S., et al., 2008. Ornithine decarboxylase G316A genotype is prognostic for colorectal adenoma recurrence and predicts efficacy of aspirin chemoprevention. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research 14 (8), 2303–2309.

Jacobs, E.J., Newton, C.C., Gapstur, S.M., Thun, M.J., 2012. Daily aspirin use and cancer mortality in a large US cohort. Journal of the National Cancer Institute 104 (16), 1208–1217.

Jiang, H.Y., Huang, T.B., Xu, L., Yu, J., Wu, Y., Geng, J., et al., 2015. Aspirin use and lung cancer risk: A possible relationship? Evidence from an updated meta-analysis. PLoS One 10 (4), e0122962.

Joint Formulary Committee, 2016. British National Formulary (BNF) 71: Pharmaceutical Press. British Medical Association and the Royal Pharmaceutical Society, London, UK.

Kaur, J., Sanyal, S.N., 2010. Pl3-kinase/Wnt association mediates COX-2/PGE(2) pathway to inhibit apoptosis in early stages of colon carcinogenesis: chemoprevention by diclofenac. Tumour Biology 31 (6), 623–631.
Kune, G.A., Kune, S., Watson, L.F., 1988. Colorectal cancer risk, chronic illnesses, operations, and medications: Case control results from the Melbourne colorectal cancer study. Cancer Research 48 (15), 4399–4404.
Langley, R.E., Burdett, S., Tierney, J.F., Cafferty, F., Parmar, M.K., Venning, G., 2011. Aspirin and cancer: Has aspirin been overlooked as an adjuvant therapy? British Journal of Cancer 105 (8), 1107–1113.
Lewis Jr., H.D., Davis, J.W., Archibald, D.G., Steinke, W.E., Smitherman, T.C., Doherty 3rd, J.E., et al., 1983. Protective effects of aspirin against acute myocardial infarction and death in men with unstable angina. Results of a veterans administration cooperative study. The New England Journal of Medicine 309 (7), 396–403.
Liao, X., Lochhead, P., Nishihara, R., Morikawa, T., Kuchiba, A., Yamauchi, M., et al., 2012. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. The New England Journal of Medicine 367 (17), 1596–1606.
Lin, J.S., Piper, M.A., Perdue, L.A., Rutter, C.M., Webber, E.M., O'Connor, E., et al., 2016. Screening for colorectal cancer: Updated evidence report and systematic review for the US preventive services task force. Journal of the American Medical Association 315 (23), 2576–2594.
Liu, Y., Chen, J.Q., Xie, L., Wang, J., Li, T., He, Y., et al., 2014. Effect of aspirin and other non-steroidal anti-inflammatory drugs on prostate cancer incidence and mortality: A systematic review and meta-analysis. BMC Medicine 12, 55.
Logan, R.F., Little, J., Hawtin, P.G., Hardcastle, J.D., 1993. Effect of aspirin and non-steroidal anti-inflammatory drugs on colorectal adenomas: Case-control study of subjects participating in the Nottingham faecal occult blood screening programme. BMJ 307 (6899), 285–289.
Lu, M., Strohecker, A., Chen, F., Kwan, T., Bosman, J., Jordan, V.C., et al., 2008. Aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing survivin levels. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research 14 (10), 3168–3176.
Lu, L., Shi, L., Zeng, J., Wen, Z., 2017. Aspirin as a potential modality for the chemoprevention of breast cancer: A dose-response meta-analysis of cohort studies from 857,831 participants. Oncotarget 8 (25), 40389–40401.
Luo, T., Yan, H.M., He, P., Luo, Y., Yang, Y.F., Zheng, H., 2012. Aspirin use and breast cancer risk: A meta-analysis. Breast Cancer Research and Treatment 131 (2), 581–587.
Macdonald, N.-J., 2011. Bupa: Cancer diagnosis and treatment—A 2021 projection. Bupa Health and Wellbeing.
Mahdi, J.G., Mahdi, A.J., Mahdi, A.J., Bowen, I.D., 2006. The historical analysis of aspirin discovery, its relation to the willow tree and antiproliferative and anticancer potential. Cell Proliferation 39 (2), 147–155.
McCormack, V.A., Hung, R.J., Brenner, D.R., Bickeboller, H., Rosenberger, A., Muscat, J.E., et al., 2011. Aspirin and NSAID use and lung cancer risk: A pooled analysis in the international lung cancer consortium (ILCCO). Cancer Causes & Control 22 (12), 1709–1720.
McCowan, C., Munro, A.J., Donnan, P.T., Steele, R.J., 2013. Use of aspirin post-diagnosis in a cohort of patients with colorectal cancer and its association with all-cause and colorectal cancer specific mortality. European Journal of Cancer 49 (5), 1049–1057.
McQuay, H.J., Moore, R.A., 2007. Dose-response in direct comparisons of different doses of aspirin, ibuprofen and paracetamol (acetaminophen) in analgesic studies. British Journal of Clinical Pharmacology 63 (3), 271–278.
Miner, J., Hoffhines, A., 2007. The discovery of aspirin's antithrombotic effects. Texas Heart Institute Journal 34 (2), 179–186.
Murphy, C., Turner, N., Wong, H.L., Sinnathamby, M., Tie, J., Lee, B., et al., 2017. Examining the impact of regular aspirin use and PIK3CA mutations on survival in stage 2 colon cancer. Internal Medicine Journal 47 (1), 88–98.
Ng, K., Meyerhardt, J.A., Chan, A.T., Sato, K., Chan, J.A., Niedzwiecki, D., et al., 2015. Aspirin and COX-2 inhibitor use in patients with stage III colon cancer. Journal of the National Cancer Institute 107 (1), 345.
Ni, X., Ma, J., Zhao, Y., Wang, Y., Wang, S., 2013. Meta-analysis on the association between non-steroidal anti-inflammatory drug use and ovarian cancer. British Journal of Clinical Pharmacology 75 (1), 26–35.
Patrignani, P., Sacco, A., Sostres, C., Bruno, A., Dovizio, M., Piazuelo, E., et al., 2017. Low-dose aspirin acetylates cyclooxygenase-1 in human colorectal mucosa: Implications for the chemoprevention of colorectal cancer. Clinical Pharmacology and Therapeutics. https://doi.org/10.1002/cpt.639.
Patrono, C., Garcia Rodriguez, L.A., Landolfi, R., Baigent, C., 2005. Low-dose aspirin for the prevention of atherothrombosis. The New England Journal of Medicine 353 (22), 2373–2383.
Pedersen, A.K., FitzGerald, G.A., 1984. Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase. The New England Journal of Medicine 311 (19), 1206–1211.
Perk, J., De Backer, G., Gohlke, H., Graham, I., Reiner, Z., Verschuren, M., et al., 2012. European guidelines on cardiovascular disease prevention in clinical practice (version 2012). The fifth joint task force of the European Society of Cardiology and Other Societies on cardiovascular disease prevention in clinical practice (constituted by representatives of nine societies and by invited experts). European Heart Journal 33 (13), 1635–1701.
Petrick, J.L., Sahasrabuddhe, V.V., Chan, A.T., Alavanja, M.C., Beane-Freeman, L.E., Buring, J.E., et al., 2015. NSAID use and risk of hepatocellular carcinoma and intrahepatic cholangiocarcinoma: The liver cancer pooling project. Cancer Prevention Research 8 (12), 1156–1162.
Preventive Services, U.S., 2009. Task force (USPSTF). Aspirin for the prevention of cardiovascular disease: U.S. preventive services task force recommendation statement. Annals of Internal Medicine 150 (6), 396–404.
Ratnasinghe, L.D., Graubard, B.I., Kahle, L., Tangrea, J.A., Taylor, P.R., Hawk, E., 2004. Aspirin use and mortality from cancer in a prospective cohort study. Anticancer Research 24 (5B), 3177–3184.
Rosenberg, L., Palmer, J.R., Zauber, A.G., Warshauer, M.E., Stolley, P.D., Shapiro, S., 1991. A hypothesis: Nonsteroidal anti-inflammatory drugs reduce the incidence of large-bowel cancer. Journal of the National Cancer Institute 83 (5), 355–358.
Rothwell, P.M., 2013. Alternate-day, low-dose aspirin and cancer risk. Annals of Internal Medicine 159 (2), 148–150.
Rothwell, P.M., Wilson, M., Elwin, C.E., Norrving, B., Algra, A., Warlow, C.P., et al., 2010. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet 376 (9754), 1741–1750.
Rothwell, P.M., Fowkes, F.G., Belch, J.F., Ogawa, H., Warlow, C.P., Meade, T.W., 2011. Effect of daily aspirin on long-term risk of death due to cancer: Analysis of individual patient data from randomised trials. Lancet 377 (9759), 31–41.
Rothwell, P.M., Price, J.F., Fowkes, F.G., Zanchetti, A., Roncaglioni, M.C., Tognoni, G., et al., 2012a. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: Analysis of the time course of risks and benefits in 51 randomised controlled trials. Lancet 379 (9826), 1602–1612.
Rothwell, P.M., Wilson, M., Price, J.F., Belch, J.F., Meade, T.W., Mehta, Z., 2012b. Effect of daily aspirin on risk of cancer metastasis: A study of incident cancers during randomised controlled trials. Lancet 379 (9826), 1591–1601.
Sahasrabuddhe, V.V., Gunja, M.Z., Graubard, B.I., Trabert, B., Schwartz, L.M., Park, Y., et al., 2012. Nonsteroidal anti-inflammatory drug use, chronic liver disease, and hepatocellular carcinoma. Journal of the National Cancer Institute 104 (23), 1808–1814.
Serhan, C.N., 2005. Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. Prostaglandins, Leukotrienes, and Essential Fatty Acids 73 (3–4), 141–162.
Seshasai, S.R., Wijesuriya, S., Sivakumaran, R., Nethercott, S., Erqou, S., Sattar, N., et al., 2012. Effect of aspirin on vascular and nonvascular outcomes: Meta-analysis of randomized controlled trials. Archives of Internal Medicine 172 (3), 209–216.
Shaw, R.J., Cantley, L.C., 2012. Cell biology. Ancient sensor for ancient drug. Science 336 (6083), 813–814.
Smith, H.S., 2012. Aspirin-inspired analgesia: Old drug, new mechanism, sans cox? Pain Physician 15 (4), E359–61.
Sneader, W., 2000. The discovery of aspirin: A reappraisal. BMJ 321 (7276), 1591–1594.

Steering Committee of the Physicians' Health Study Research G, 1988SteeringCommitteeofthePhysiciansHealthStudyResearch. Preliminary report: Findings from the aspirin component of the ongoing Physicians' health study. The New England Journal of Medicine 318 (4), 262–264.
Steering Committee of the Physicians' Health Study Research Group, 1989SteeringCommitteeofthePhysiciansHealthStudyResearch. Final report on the aspirin component of the ongoing Physicians' health study. The New England Journal of Medicine 321 (3), 129–135.
Suh, O., Mettlin, C., Petrelli, N.J., 1993. Aspirin use, cancer, and polyps of the large bowel. Cancer 72 (4), 1171–1177.
Sutcliffe, P., Connock, M., Gurung, T., Freeman, K., Johnson, S., Kandala, N.B., et al., 2013. Aspirin for prophylactic use in the primary prevention of cardiovascular disease and cancer: A systematic review and overview of reviews. Health Technology Assessment 17 (43), 1–253.
Thorat, M.A., 2016. Individualised benefit-harm balance of aspirin as primary prevention measure—A good proof-of-concept, but could have been better. BMC Medicine 14 (1), 101.
Thorat, M.A., Cuzick, J., 2013. Role of aspirin in cancer prevention. Current Oncology Reports 15 (6), 533–540.
Thorat, M.A., Cuzick, J., 2015. Prophylactic use of aspirin: Systematic review of harms and approaches to mitigation in the general population. European Journal of Epidemiology 30 (1), 5–18.
Thun, M.J., Namboodiri, M.M., Heath Jr., C.W., 1991. Aspirin use and reduced risk of fatal colon cancer. The New England Journal of Medicine 325 (23), 1593–1596.
Thun, M.J., Calle, E.E., Namboodiri, M.M., Flanders, W.D., Coates, R.J., Byers, T., et al., 1992. Risk factors for fatal colon cancer in a large prospective study. Journal of the National Cancer Institute 84 (19), 1491–1500.
Thun, M.J., Namboodiri, M.M., Calle, E.E., Flanders, W.D., Heath Jr., C.W., 1993. Aspirin use and risk of fatal cancer. Cancer Research 53 (6), 1322–1327.
Thun, M.J., Henley, S.J., Patrono, C., 2002. Nonsteroidal anti-inflammatory drugs as anticancer agents: Mechanistic, pharmacologic, and clinical issues. Journal of the National Cancer Institute 94 (4), 252–266.
Thun, M.J., Jacobs, E.J., Patrono, C., 2012. The role of aspirin in cancer prevention. Nature Reviews. Clinical Oncology 9 (5), 259–267.
Trabert, B., Ness, R.B., Lo-Ciganic, W.H., Murphy, M.A., Goode, E.L., Poole, E.M., et al., 2014. Aspirin, nonaspirin nonsteroidal anti-inflammatory drug, and acetaminophen use and risk of invasive epithelial ovarian cancer: A pooled analysis in the ovarian cancer association consortium. Journal of the National Cancer Institute 106 (2), djt431.
Uddin, S., Ahmed, M., Hussain, A., Assad, L., Al-Dayel, F., Bavi, P., et al., 2010. Cyclooxygenase-2 inhibition inhibits PI3K/AKT kinase activity in epithelial ovarian cancer. International Journal of Cancer 126 (2), 382–394.
Vandvik, P.O., Lincoff, A.M., Gore, J.M., Gutterman, D.D., Sonnenberg, F.A., Alonso-Coello, P., et al., 2012. Primary and secondary prevention of cardiovascular disease: Antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest 141 (2 Suppl) e637S-68S.
Vane, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature: New Biology 231 (25), 232–235.
Vane, J.R., Botting, R.M., 2003. The mechanism of action of aspirin. Thrombosis Research 110 (5–6), 255–258.
Veettil, S.K., Teerawattanapong, N., Ching, S.M., Lim, K.G., Saokaew, S., Phisalprapa, P., et al., 2017. Effects of chemopreventive agents on the incidence of recurrent colorectal adenomas: A systematic review with network meta-analysis of randomized controlled trials. OncoTargets and Therapy 10, 2689–2700.
Wang, X., Lin, Y.W., Wu, J., Zhu, Y., XL, X., Xu, X., et al., 2014. Meta-analysis of nonsteroidal anti-inflammatory drug intake and prostate cancer risk. World Journal of Surgical Oncology 12, 304.
Xu, J., Yin, Z., Gao, W., Liu, L., Wang, R., Huang, P., et al., 2012. Meta-analysis on the association between nonsteroidal anti-inflammatory drug use and lung cancer risk. Clinical Lung Cancer 13 (1), 44–51.
Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z.W., Karin, M., et al., 2001. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 293 (5535), 1673–1677.
Zhang, D., Bai, B., Xi, Y., Zhao, Y., 2016a. Can aspirin reduce the risk of endometrial cancer?: A systematic review and meta-analysis of observational studies. International Journal of Gynecological Cancer 26 (6), 1111–1120.
Zhang, D., Bai, B., Xi, Y., Wang, T., Zhao, Y., 2016b. Is aspirin use associated with a decreased risk of ovarian cancer? A systematic review and meta-analysis of observational studies with dose-response analysis. Gynecologic Oncology 142 (2), 368–377.
Zhao, T.Y., Tu, J., Wang, Y., Cheng, D.W., Gao, X.K., Luo, H., et al., 2016. The efficacy of aspirin in preventing the recurrence of colorectal adenoma: A renewed meta-analysis of randomized trials. Asian Pacific Journal of Cancer Prevention 17 (5), 2711–2717.
Zhong, S., Chen, L., Zhang, X., Yu, D., Tang, J., Zhao, J., 2015a. Aspirin use and risk of breast cancer: Systematic review and meta-analysis of observational studies. Cancer Epidemiology, Biomarkers & Prevention 24 (11), 1645–1655.
Zhong, S., Zhang, X., Chen, L., Ma, T., Tang, J., Zhao, J., 2015b. Association between aspirin use and mortality in breast cancer patients: A meta-analysis of observational studies. Breast Cancer Research and Treatment 150 (1), 199–207.
Zhou, G., Marathe, G.K., Willard, B., McIntyre, T.M., 2011. Intracellular erythrocyte platelet-activating factor acetylhydrolase I inactivates aspirin in blood. The Journal of Biological Chemistry 286 (40), 34820–34829.
Zumwalt, T.J., Wodarz, D., Komarova, N.L., Toden, S., Turner, J., Cardenas, J., et al., 2017. Aspirin-induced chemoprevention and response kinetics are enhanced by PIK3CA mutations in colorectal cancer cells. Cancer Prevention Research 10 (3), 208–218.

Further Reading

Chubak, J., Whitlock, E.P., Williams, S.B., Kamineni, A., Burda, B.U., Buist, D.S., et al., 2016. Aspirin for the prevention of cancer incidence and mortality: Systematic evidence reviews for the U.S. preventive services task force. Annals of Internal Medicine 164 (12), 814–825.
Dhillon, P.K., Kenfield, S.A., Stampfer, M.J., Giovannucci, E.L., 2011. Long-term aspirin use and the risk of total, high-grade, regionally advanced and lethal prostate cancer in a prospective cohort of health professionals, 1988-2006. International Journal of Cancer 128 (10), 2444–2452.
Jacobs, E.J., Connell, C.J., Rodriguez, C., Patel, A.V., Calle, E.E., Thun, M.J., 2004. Aspirin use and pancreatic cancer mortality in a large United States cohort. Journal of the National Cancer Institute 96 (7), 524–528.
Patrignani, P., Patrono, C., 2016. Aspirin and cancer. Journal of the American College of Cardiology 68 (9), 967–976.
Puhan, M.A., Yu, T., Stegeman, I., Varadhan, R., Singh, S., Boyd, C.M., 2015. Benefit-harm analysis and charts for individualized and preference-sensitive prevention: Example of low dose aspirin for primary prevention of cardiovascular disease and cancer. BMC Medicine 13, 250.

Relevant Websites

https://www.lumc.nl/org/atcg/ - Aspirin Trialist Collaborative Group
http://www.benefit-harm-balance.com/ - Benefit Harm chart for low dose aspirin for the primary prevention of cardiovascular disease and cancer

Ataxia Telangiectasia Syndrome☆

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Glossary

Ataxia The loss of muscular coordination.
Autosomal Any chromosome except X and Y.
Homozygote An individual carrying two of the same allele (one from each parent) in a particular loci.
Linear energy transfer (LET) The energy absorbed by the medium through which an ionizing particle is traveling per unit length of the track of the particle, usually expressed in keV μ^{-1}.
Recessive The allele phenotype that is obscured in a heterozygote individual by the dominant allele.
Telangiectasia The dilatation of peripheral blood vessels.
Tumor suppressor A gene product preventing the transformation of normal cells to tumor cells.

Clinical Aspects

Clinical Symptoms

AT disease was first reported by Syllaba and Hellner in 1928 and described by Boder& Sedgwick later on. Its prevalence is not restricted to any geographical region or ethnic group (Hoche et al., 2012); having an incidence rate of 1–2.5 in 100 000 (Palau and Espinos, 2006). Two symptoms, cerebellar ataxia and oculocutaneous telangiectasia, are considered the minimal criteria for a positive diagnosis of the ataxia telangiectasia disease. The ataxia, due to primary neuronal degeneration, progresses steadily and typically results in the patient's confinement to a wheelchair by early adolescence. Telangiectasia in or around the eyes usually develops between 3 and 6 years of age and later spreads in a symmetrical pattern to the eyelids, face, ears, and neck, and in particular sunlight-exposed and friction areas of the skin. Apart from neurodegeneration and vasodilations (ocular and dermal telangiectasia), AT shows a number of other clinical manifestations like pulmonary infections, predisposition to cancer- lymphoma or leukemia, insulin-resistant diabetes, immunodeficiency, radiation sensitivity, sterility (gonadal dysfunction), mild hepatitis, growth retardation and premature aging (Ambrose and Gatti, 2013). The high secretion of alpha-fetoprotein is a hallmark of AT disease, often used as one of the diagnostic parameter to confirm the disease. The life span of AT patients is less than 20–30 years though an exception was reported for a 48 years old AT patient harboring ATM deleterious variants that were not null mutations (Davis et al., 2013). The major AT clinical conditions are described below (**Figure 1**).

Neurodegeneration

Neurodegeneration is the most prominent feature of the AT disease that manifest as early as 6 to 18 months of age and progress steadily over time (McKinnon, 2012). Children with AT learn to walk at a normal age but start showing truncal swaying, abnormal gait, muscle hypotonia and dyssynergia early on. All these symptoms lead to patients' dependency on a wheel chair before the end of the first decade of life. Based on the various neuropathological anomalies, AT can be classified as a primary neuronal degeneration. Primary neuronal degeneration is a progressive disease characterized by the premature death of neurons in the absence of pathological evidence of a specific cause. The neurological abnormalities in AT include progressive cerebellar ataxia, dysarthric (slurring) speech, movements of the extremities, peculiar eye movements called oculomotor dyspraxia, hyporeflexia or areflexia, and apparent arrest of cognitive development. The uncontrolled jerk movements and tremors become evident at later stage (after 12 years of age) (Carrier and Fornace, 2002) (McKinnon, 2012). At the autopsy, a marked atrophy of the cerebellar cortex primarily due to degeneration of the Purkinje and granular cells and, to a lesser extent, of basket cells is also observed. Eventually, the posterior and lateral columns of the spinal cord degenerate and the anterior horn cells are lost. There are exceptions to this phenotype since in some cases of AT, patients showed atypical slow progression lacking the characteristic early onset of an abnormal gait (Carrillo et al., 2009). Those milder forms of AT are often associated with ATM mutations that retain some low levels of ATM protein kinase activity and consequently functionality.

☆*Change History:* April 2015. Parekh and Carrier updated the text in the cellular and molecular sections to reflect the newly described ATM functions in the cytosol and they added the new ATM targets associated with neurodegeneration. Moreover the use of human neuronal stem cells to study the molecular mechanisms of neurodegeneration in AT are presented in the Future Directions. A revised Figure 2 and a new Figure 3 have been added to reflect these changes. This article is an update of Palak R. Parekh, France Carrier, Ataxia Telangiectasia Syndrome, In Encyclopedia of Cancer (Second Edition) edited by Joseph R. Bertino, Academic Press, 2002, Pages 153–164.

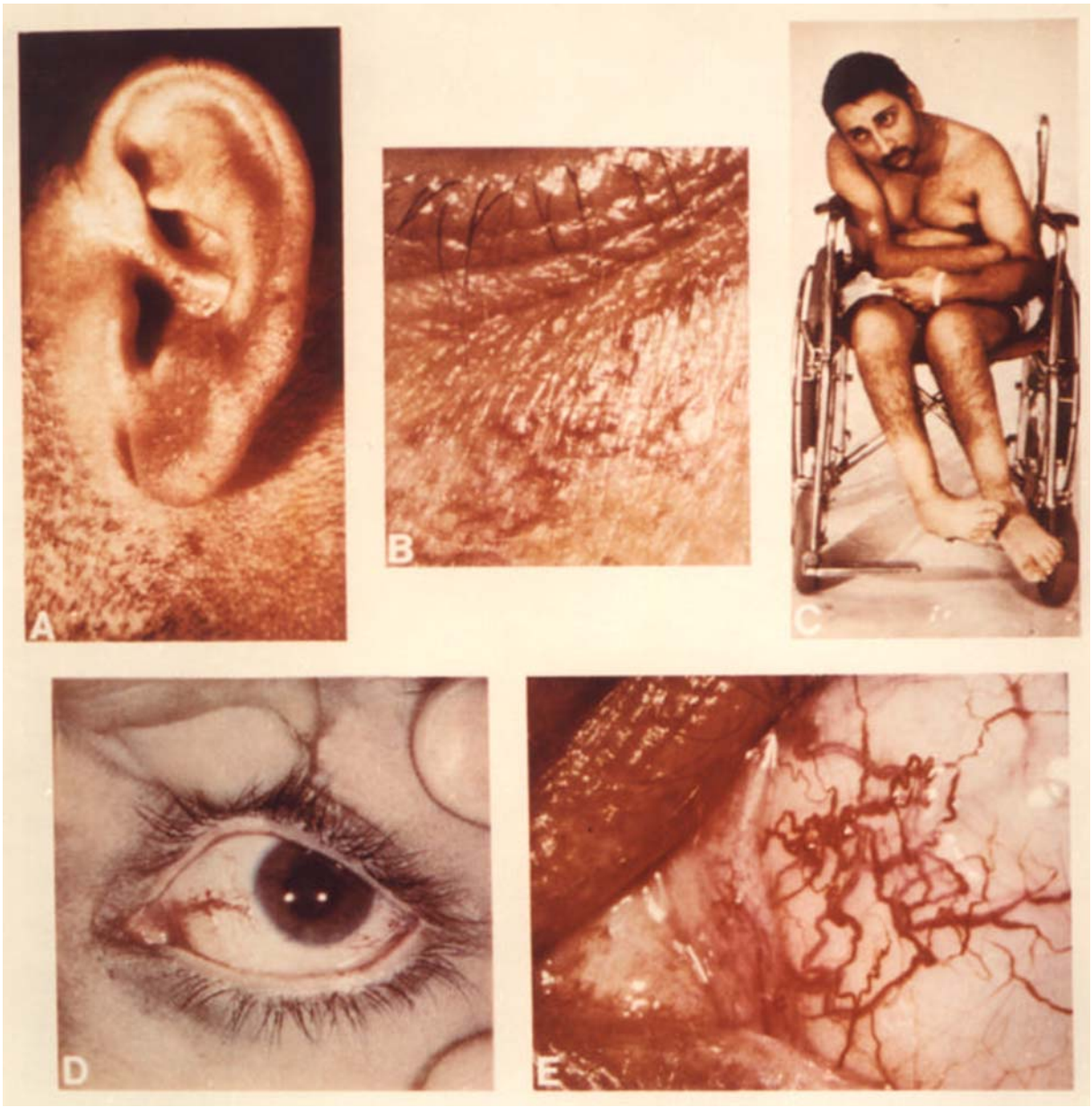

Figure 1 (A) Pinna of a 22-year-old patient showing many fine telangiectasia vessels. (B) Telangiectasia of the malar area and lower lid in another patient. (C) The 22-year-old patient, who is unable to walk unaided. (D, E) His eye shows interpalpebral telangiectasia.

Cancer predisposition

The risk of developing malignancy in AT patients is about 1200-fold greater than that of an age-matched control population. The incidence of new cancer from age 10 on is estimated to be 1 in 10 patients each year. Cancer is therefore observed several years after the initial neurologic disorders have started to take their toll on the AT patients. The vast majority of the AT malignancies, more than 85%, are acute lymphocytic lymphoma or leukemia. Other cancer predispositions have also been noticed in at least 38% of the patients; carcinomas of the breast, stomach, liver, pancreas, ovary, oral cavity, and salivary gland have been reported. Early studies have suggested an increased cancer predisposition in AT heterozygotes. The cancer most often associated with single gene carriers of this disease is breast cancer. Carriers of ATM mutations have a 2–7-fold increased risk of developing breast cancer. The increased risk is presumably associated with loss of heterozygosity, sporadic loss of the normal ATM allele resulting in either total loss of or a large reduction in functional ATM protein. The risk of AT heterozygotes to develop breast cancer has been calculated at 11% by age 50 and at 30% by age 70. Since heterozygote cells in culture also show some sensitivity to radiation, it is possible that doses of radiation too small to produce any cancer risk in noncarriers induce cancer in heterozygotes.

Immunodeficiency & lung disease

The most consistent abnormality of the AT immune system is that invariably the thymus fails to develop normally, resembling an embryonic stage of development, and sometimes is even totally absent. Defects in both cellular or humoral immune system can occur. Because of immunodeficiency, frequent sinopulmonary infections are common mainly due to ataxia and swallowing difficulties. Severe recurrent lung infections are often the major cause of death in the affected patients. Recent studies with improved diagnosis of the lungs without using radiation, have found that whether AT patients show pulmonary infection symptoms or not, they have abnormal lungs (Montella et al., 2013). Immunodeficiency also leads to non-Hodgkin lymphoma. The basic reason for these above varied patho-physiological condition is disturbed B and T cells homeostasis (Driessen et al., 2013). Humoral immunodeficiencies are found in most patients: 80% have IgG_2 deficiency, 60% have IgA deficiency, and approximately 50% have IgE deficiency. Despite the frequency of these humoral immunodeficiencies, deficiencies of IgM, IgG_1, and IgG_3 are uncommon. As will be discussed below, the majority of the AT cancers are acute lymphocytic lymphoma or leukemia, but it is interesting that acute or chronic myeloid leukemia are completely absent. The combined immunodeficiency in AT patients is reminiscent of the phenotypes found in a mutant mouse model named severe combined immunodeficient mutation (SCID). Cells from these mice are deficient in a recombination process utilized in both DNA double-strand break repair and lymphoid variable (diversity)-joining [V(D)J] recombination. The resulting phenotype is therefore manifested in both cellular hypersensitivity to ionizing radiation and a lack of B- and T-cell immunity. The DNA-dependent kinase p350, a strong candidate for the murine SCID defect, is, however, normal in SV40-transformed AT cells (GM 5849). This indicates that even though AT and the SCID mice share similar phenotypes, the affected mechanisms that regulate them are most likely different.

Tissue radiosensitization

A universal characteristic of all AT patients is their hypersensitivity to radiation. The marked hypersensitivity is seen in both patients and cells derived from their normal tissue. Sensitivity of AT fibroblasts to ionizing radiation was first investigated in the mid-1970s due to reported fatal radiation burns in AT patients receiving radiotherapy for malignancies. Other severe reactions caused by the administration of standard radiotherapy to AT patients include ulcerative dermatitis, severe esophagatis, dysphagia, and deep tissue necrosis. It is estimated that the 1- to 9-mGy dose of radiation received in a diagnostic exposure is sufficient to increase considerably the risk of breast cancer in AT heterozygote women while a dose of 100–200 mGy is required to see a similar effect in normal individuals.

Genetics and frequency

Ataxia telangiectasia is a rare disease with a birth frequency any where between 1 in 80 000 to 1 in 300 000. The gene frequency of the AT allele has been estimated at 0.007 based on the assumption that AT is due to a mutation in both alleles of the AT gene. People that are heterozygote for the AT gene have been estimated at 1.4% of the U.S. white population. The genetics of this disease is fairly homogenous; linkage studies of 176 families from various parts of the world localize the major AT locus to the region between S1819(A4) and S1818(A2) at chromosome 11q22.3 (Gatti et al., 1988) in all but 7 families. In addition to the United States, cases of ataxia telangiectasia have been reported throughout Europe, Japan, China, Africa, the Middle East, India, and South and Central America. The frequency of the disease in the U.S. black population is similar to the proportion of black individuals in the general population, but the cancer rates in black AT patients in the United States are considerably higher than those in white patients. The differences between the two ethnic groups might reflect differences between the AT alleles or other genetic or environmental factors.

Cellular Aspects

Cellular Radiosensitization

The basis for AT cells radiosensitivity is still not completely understood but may be due to a slower rate of repair of DNA double strand breaks or to the continued existence of residual breaks (see **Repair Parameters** below). Although there is no gross defect in DNA repair of DSBs in AT cells, residual breaks persist even longer than in cells deficient for Non-Homologous End joining repair (Lavin, 2008). The chromatin environment rather than the nature of the DNA lesions seems to underlie the capacity to repair the small fraction of breaks remaining in heterochromatin. In fact, ATM can phosphorylate the transcriptional corepressor Kruppel-associated box (KRAB)-associated protein-1 (KAP1) which facilitates entry of the DNA-repair machinery into heterochromatin. The absence of a functional ATM could thus reduce the capacity to repair a fraction of DNA DSBs and increase radiosensitivity. Nonetheless, in Purkinje cells, a primary target for neurodegeneration in AT, the chromatin is mostly of the euchromatic type while granule neurons are more heterochromatic (Kriaucionis and Heintz, 2009). Accessibility of the DNA-repair machinery to DNA DSBs is thus probably not sufficient to carry the radiosensitivity phenomenon. Radiosensitivity in AT cells is exhibited by both a loss of colony formation ability and a higher frequency of chromosomal damage within individual cells. Although several abnormalities have been reported in cells from obligate AT heterozygotes, no clear effect on the capacity to stop DNA synthesis following irradiation (radiation-resistant DNA synthesis) has been demonstrated so far. Several studies indicate that radiosensitivity levels measured in AT heterozygotes by cell survival, chromosome aberrations, production of micronuclei, or flow cytometry are intermediate in between that of controls and AT homozygotes. However, others have pointed out that when larger scale blind evaluations were performed, sufficient overlap between heterozygotes and normal cells was obtained so that no assays could really distinguish between the two groups. Radiation sensitivity is defined by the steepness of a slope obtained from a survival curve when cells are

exposed to graded doses of IR. The slope is proportional to $1/D_0$, where D_0 is the dose required to reduce cell survival to 37%. Normal human fibroblasts have typical D_0 values ranging from 1.2 to 1.4 Gy. AT cells, on the other hand, have D_0 values around 0.7 Gy. At 10% survival (D_{10}), AT cells are about twice as sensitive as normal cells. AT cells are also hypersensitive to certain radiometric chemicals, inhibitors of DNA topoisomerases, and restriction endonucleases. Some variability exists between the different AT cell lines, but AT cells in general are sensitive to compounds that induce DNA strand breaks most probably via free radical attack. The hypersensitivity of AT cells to the X-ray type of DNA-damaging agent is specific since AT cells are not hypersensitive to 254 nm UV radiation. However, damage from densely ionizing radiation [α-particles, high linear energy transfer (LET)], which causes more extensive local damage to DNA, is less effective on AT cells, relative to normal, than damage from sparsely ionizing radiation (X-rays, low LET). Radiosensitivity of AT cell lines is considerably greater than that of cell lines from other neurodegenerative diseases such as Huntington and Alzheimer diseases. This is even more clearly illustrated with cells from AT heterozygotes that have no clinical evidence of neurological abnormalities but are more radiosensitive than cells from patients with other radiosensitive diseases demonstrating significant neuronal degeneration.

Cell Cycle

Exposure of normal mammalian cells to DNA-damaging agents, particularly IR, results in transient inhibition or delay of their progression through the cell cycle at a number of different checkpoints, including G_1, S, and G_2. It is thought that these delays allow cells time to repair their DNA and avoid the passage of defective genetic information to the next generation. The importance of these checkpoints for the maintenance of cellular integrity is obvious in that the G_1/S checkpoint precedes DNA replication and that the G_2/M checkpoint precedes the segregation of chromosomes. It is now well established that a wild-type phenotype for the tumor suppressor p53 is necessary to observe a normal G_1 delay following IR. The involvement of p53 in such a critical point in the life of a cell is another indication of the importance of these delays since p53 mutations or mutations in cellular pathways regulating p53 functions are found in the majority of human cancer cells. Anomalies in the progression of the AT cells through the cell cycle following exposure to IR have been described in virtually all of these cell cycle checkpoints. The first observation of a defect in the AT cell cycle checkpoints described their inability to stop DNA synthesis following irradiation and most likely referred to the S phase checkpoint since the DNA synthesis was measured 40 min after irradiation. The radioresistance to DNA synthesis allows the AT cells to proceed through S phase unchecked which results in a reduced mitotic delay and various cellular aberrations. However, increasing evidence suggests that the inability to stop the DNA synthesis cannot be responsible for the hypersensitivity as it was first thought. The two phenomena, radioresistant DNA synthesis and sensitivity to cell killing are dissociated in cell hybrids between AT lines and a normal line. In AT heterozygote, neither the radioresistant DNA synthesis nor the shortened mitotic delay is observed. Unlike the arrest in G_1 after IR, the IR-induced S phase arrest seems to be independent of p53.

The correlation between p53 and a normal G_1 arrest is also reflected in the increased levels of the p53 protein. In AT homozygous, the G_1 delay and the normal increased p53 levels are not observed following a 1-hr exposure to 2 Gy of IR. The abnormal G_2 block in AT cells following ionizing radiation is more complex since although cells already in G_2 fail to arrest at the G_2-M checkpoint, cells irradiated in the G_1/S phase arrest when they reach G_2. This observation may reflect differences in the type of DNA damage that is produced in G_2 versus G_1. Furthermore, there may be distinct protein complexes (defective in AT) involved in processing and/or repair of one type of DNA lesion versus another. A correlation has been suggested between the number of cells arrested in G_2 and radiosensitivity, but in AT it seems that the number of cells arrested in G_2 correlates with an adverse prognostic indicator. The isolation of the AT gene has allowed a better understanding of the molecular basis of the defective checkpoints. This is described in more detail below (Section **Molecular Aspects**).

Cytogenetics

Two types of basal (uninduced) cytogenetic abnormalities are seen in cells from AT patients: (i) an excess of 'spontaneous' chromosome breaks (e.g., gaps, fragments) and rearrangements distributed randomly among the chromosomes, and (ii) clonal abnormalities usually involving chromosome 7 or 14. The incidence of these spontaneous changes varies among different patients and also within the same patient as a function of donor age and cell types, that is, fibroblasts versus lymphoblast. The chromosomal translocations and inversions almost exclusively involve these six breakpoints: three T-cell receptor complexes, 7q35, 7q14, and 14q11, and three B-cell receptor complexes, 22q12, 14q32, and 2p12. All these sites are sites of gene rearrangements, implicating perhaps a common recombinase. These specific translocations are also observed in normal lymphocytes but at a lower frequency. Chromosomal aberrations also occur in fibroblasts but they are nonspecific and almost never involve the six sites mentioned earlier. The AT gene(s) site, 11q23 (Gatti et al., 1988), is never compromised in the chromosomal breakpoints, suggesting that this type of aberration is a consequence rather than a cause of the AT phenotype. Abnormalities in chromosome 14 are also associated with Burkitt lymphoma, Hodgkins disease, and various leukemia, indicating that this chromosome may contain a locus predisposing to lymphoproliferative malignancies. The proximity of the chromosomes 7 and 14 to some immunoglobin genes might be an indication that cytogenetic anomalies play a role in the immunodeficiency observed in AT. The type of aberrations produced in AT lines by radiation differs from that in control lines. In the mitosis following irradiation in the G_0 early G_1 stage of the cell cycle, AT cells show both chromosome and chromatid aberrations while only chromosomal aberrations are observed in normal lines. The reason for this particular response is still unclear, but it implies that some single-strand breaks persist for excessive periods in AT.

Repair Parameters

The increased sensitivity of AT to ionizing radiation derives most likely from an inability to recover from DNA breakage in the normal manner. Subtle defects in the ability to process DNA breaks such as accuracy of strand rejoining and persisting residual breaks may affect the ability to recover from DNA breakage. Conflicting reports in the literature have been published on the capacity of AT cells to repair their damaged DNA. This early confusion may have resulted from the differences in the sensitivity of the various techniques used. For example, cytogenetic experiments utilizing comparison of the breakage and rejoining of prematurely condensed chromosomes (pcc) indicated that after a dose of 6.0 Gy, both AT and normal cells had the same initial frequency of breaks and the same rate for rejoining of the breaks, but the fraction of breaks that did not rejoin was five to six times greater in the AT cells. It should be noted that the frequency of such events is orders of magnitude less than IR-induced DNA strand breaks. Fornace and Little, and subsequently others, using filter elution assays from the early 1980s, found no convincing differences between AT and normal lines in the ability to repair DNA strand breaks. Another technique, the pulse field gel electrophoresis, which detects double-stranded breaks in genomic DNA after low doses of γ-irradiation, indicated that the residual amount of unrepaired double-strand breaks in normal cells was 1.4% while it was 5.2 and 2.1% in AT cells and AT heterozygotes, respectively. There is also evidence of misrejoining of double-strand breaks in damaged plasmids. Interestingly, the cellular aberrations found in the next mitosis following the treatment of AT cells in G_1 with IR or electroporated restriction enzymes commonly involve only one chromatid. This suggests that AT cells have a defect in the repair of double strand breaks. However, the levels or activity of repair enzymes such as 5,6 dihydroxydihydrothymine, apurinic-site specific endonuclease, and ADP-ribose transferase are normal in AT. It seems therefore that a search for a specific DNA repair defect might not be the most likely way to find an explanation for the AT phenotype. In fact, it has been suggested that AT main defects might reside in the signaling of DNA repair. In this regard, phosphorylation of several key regulators of DNA repair has been described in an AT dependent manner (see Section **Molecular Aspects**). In addition, as mentioned above, the chromatin environment and the accessibility of the repair machinery to the heterochromatin rather than the nature of the DNA lesions seem to underlie the capacity of AT cells to repair residual DNA breaks.

Molecular Aspects

The AT Gene

Cloning of the AT gene in 1995 opened the door to numerous molecular studies that had been awaited for quite some times. Because roughly 97% of tested AT families link to chromosome 11, the concept that a common multimeric molecule, with different subunits of the molecule being affected in different patients, could lead to the indistinguishable AT phenotype became plausible. Early linkage analysis clearly localized the AT gene to a 3 cM region of chromosome 11q22-23. Subsequently, a more precise analysis involving 176 AT families localized the AT gene(s) within a 500-kb interval flanked by S1819 and S1818. There are several functional genes that have already been mapped to chromosome 11q22-23; many of these genes have remained together for over 80 million years of evolution. There is also a number of diseases associated with this region, one of which, tuberous sclerosis, confers radiation hypersensitivity. Long-range cloning of the AT locus ultimately led to the isolation of a major transcript of approximately 13 kb predicting a product of 3056 amino acids. The carboxy terminal region of this protein shows considerable similarity to several yeast, *Drosophila*, and mammalian phosphatidylinositol-3′ (PI-3) kinases that are involved in mitogenic signal transduction, meiotic recombination, cell cycle control, or DNA damage detection. The pleiotropic nature of the AT phenotype is therefore well served by this protein. This gene product, named ATM (AT mutated), is a serine/threonine protein kinase sharing the substrate recognition motif Ser/Thr-Gln-Glu of the phosphatidylinositol 3-kinase-like (PIKK) family of protein kinases. The ATM protein is predominantly found in the nucleus of proliferating cells but a variable amount (~10-20%) has also been observed in the cytoplasmic fraction of differentiated cells such as cerebellar neurons. ATM probably interacts with other proteins to facilitate its entry into the nucleus since there are no obvious nuclear localization signals in ATM primary sequence. ATM could thus possibly play different roles depending on its presence or absence in a given cellular compartment. ATM kinase activity is rapidly enhanced following treatment with agents that cause DNA double strand breaks (DSB) but is insensitive to other types of DNA damaging agents such as UV radiation and alkylating agents. It is now believed that the initial signal triggering ATM activation following IR is probably relaxation of higher order chromatin structure following DSB. In the absence of cellular stress, ATM exists as an inactive non-covalently associated dimer but converts to an active monomer following autophosphorylation on Ser 1981 upon DNA damage in the presence of Mre11/Rad50/Nbs1 (MRN) complex. A number of other phosphorylation sites and additional post-translational modifications such as acetylation have also been reported. However, ATM can also be activated by cellular stress in the absence of DSB such as hypoxia and brief exposure to oxidative stress. These recent findings support a broader role for ATM in not only responding to genotoxic stress but also maintaining cellular homeostasis (Shiloh and Ziv, 2013). Several laboratories using slightly different strategies have developed *ATM* knockout mice. All animals exhibit most of the noncentral nervous system features such as growth retardation, mild neurological dysfunction, male and female infertility, immunodeficiency, radiosensitivity, and predilection for thymic lymphoma. Nonetheless, none of the animals reproduced the neurodegeneration changes observed in AT patients, even though one laboratory showed evidence of neuron degeneration in the cerebellar cortex of 2-month-old *ATM* −/− mice. Mutations of the *ATM* gene, mainly characterized by premature truncation of the protein product, have been found in all AT patients regardless of the phenotype severity. This indicates that *ATM* is probably the sole gene responsible for the AT disorder. Most AT patients are

compound heterozygotes and have two different *ATM* mutations. More than 600 mutations have been described so far; a current list of *ATM* mutation can be found on the internet (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ATM).

The ATM Substrates

ATM substrates that could be linked to neurodegeneration have been elusive until recently. A new study by (Chan et al., 2014) demonstrated that the transcription factor myocyte enhancer factor 2D (MEF2D), which plays a critical role in promoting survival of cerebellar granule cells, is phosphorylated by ATM in response to DNA damage. Phosphorylated MEF2D activates the pro-survival Bcl-xL gene. This offers an alternative explanation for the role of ATM in neurodegeneration since it is estimated that only about 10% of DSB repair are mediated by ATM. Nonetheless, phosphorylation of MEF2D by ATM has only been confirmed in Atm knockout mice and not in AT-patients yet. However, given that ATM kinase is activated by agents that cause DSB, there are numerous reports on ATM substrates involved in (1) cell cycle checkpoint (2) stress response, or (3) DSB repair, all aspects of the cellular response to genotoxic stress. Specific substrates involved in these cellular functions are described below.

Cell cycle checkpoint

Key regulators of all phases of the cell cycle have been identified as ATM substrates. The tumor suppressor p53 was the first cell cycle regulator identified and is among the best characterized substrates. In normal human hematopoietic and other cells, increases in the levels of the tumor suppressor p53 correlate with a transient G_1 arrest after irradiation. Cells that are mutant for p53 show no G_1 arrest but retain the G_2 arrest found typically after irradiation. In AT cells, the G_1 arrest and the p53 response is delayed. The suboptimal p53 activation is also reflected in the lower induction levels of the proteins Gadd45, MDM2, and $p21^{WAF1/CIP1}$, which are encoded by three p53 downstream effector genes. Moreover, the DNA-binding activity of p53 is also abnormal following IR in AT, and failure to inhibit cyclin/cdk complexes by IR, an activity associated with $p21^{WAF1/CIP1}$ induction, has also been reported. However, the defective p53 activation might be specific to IR since induction of p53 by other DNA damaging agents appeared relatively normal in AT. Disruption of p53 function in normal fibroblasts (**Figure. 2**) results in increased spontaneous recombination rates and loss of the p53-dependent G_1/S cell cycle checkpoint. These two phenomena, also present in AT, indicate that an abnormal p53 response might contribute to the AT phenotype. In addition to these effects, a role for p53 in the mediation of the molecular abnormalities in AT has been demonstrated by insertion of mutant p53 protein, or viral protein known to degrade p53, in AT fibroblasts. Disruption of functional p53 in AT, results in increased resistance to streptonigrin and IR due to suppression of the apoptotic response. ATM regulates p53 activation and stabilization by a number of different mechanisms. First, ATM phosphorylates p53 directly on Ser15 and other residues and increases its transcriptional activity. Second, ATM activates the checkpoint kinase 2 (chk2) by phosphorylating it on Thr68, which allows it to phosphorylate p53 on Ser20 and increases p53 stability by interfering with binding to the mouse double minutes 2 (MDM2) protein. MDM2 is the main mediator of the proteoasome-mediated degradation of p53, where it functions as the E3 ligase in p53 ubiquitination process. A third mechanism used by ATM to regulate p53 action is to phosphorylate MDM2 directly on Ser395, which prevents p53 export out of the nucleus, a necessary step in p53

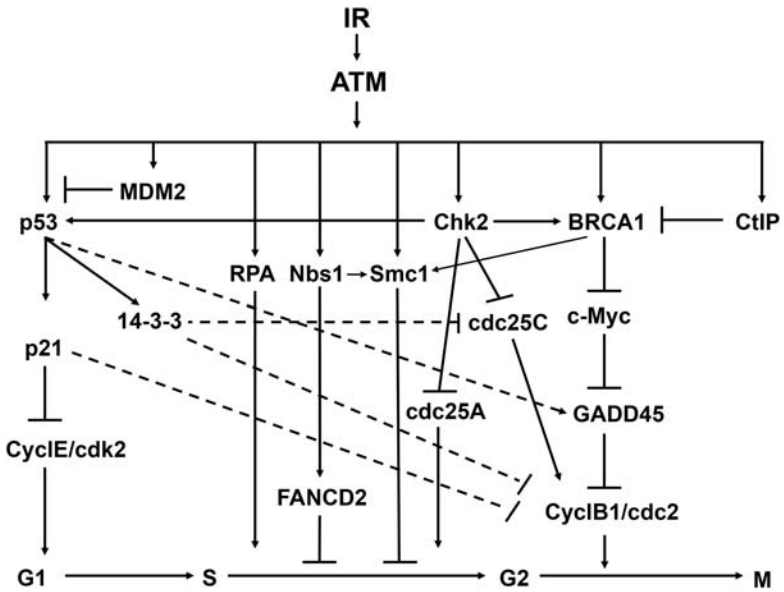

Figure 2 The AT gene product normally plays a role in the activation of several cellular responses following ionizing radiation. ATM activates key regulators of every phase of the cell cycle. Positive regulations are indicated by arrows while negative regulations are indicated by blunt arrows. Dashed lines indicate G2 regulation mediated by p53 when cells enter G2 with damaged DNA or are arrested in S phase (see details in the text).

degradation. Several other indirect mechanisms including other post translation modification such as acetylation, dephosphosphorylation and deubiquitylation were recently reviewed (Shiloh and Ziv, 2013).

Activation of chk2 by ATM provides also a mechanism for ATM's control of the G_2/M checkpoint. The activated chk2 can phosphorylate and inactivate the cdc25C phosphatase. This phosphatase is responsible for dephosphorylation and activation of cdc2, the kinase controlling entry into mitosis. ATM can also regulate the G_2/M checkpoint through the breast cancer susceptibility gene 1 (BRCA1). Similar to p53 regulation, ATM regulation of BRCA1 also involves several mechanisms. First, ATM phosphorylates BRCA1 directly on Ser1423 and Ser1525, chk2 in response to ATM phophorylates BRCA1 on another serine, Ser988, and, finally, ATM phosphorylates and inactivates CtIP, a negative regulator of BRCA1. The precise mechanism by which BRCA1 regulates the G_2/M checkpoint is still not completely understood, but current findings indicate a role as co-repressor. BRCA1 does not physically interact with DNA but rather associates with the proto-oncogene c-Myc and represses its transactivation. Among the different genes regulated by c-Myc is the growth arrest and DNA damage inducible gene *GADD45*.The expression of *GADD45* can be repressed by c-Myc through a C/EBP element within the *GADD45* promoter. Gadd45 binds to cdc2 and disrupts the cyclinB1/cdc2 complex, which results in the inhibition of the kinase activity and prevents cell cycle progression beyond G_2. By phosphorylating BRCA1, ATM may thus control the G_2/M checkpoint by allowing BRCA1 to relieve the transcriptional repression imposed by c-Myc on the *GADD45* promoter. ATM can also control G_2 arrest through p53 when cells enter G_2 with damaged DNA or are arrested in S phase. Under these conditions, p53 inhibits cdc2 simultaneously by three transcriptional targets, GADD45, p21, and 14-3-3 sigma. The p21 protein may contribute to G_2 arrest by several mechanisms including direct binding to CyclinB1/cdc2 and inhibition of CAK, the kinase phosphorylating cdc2. The 14-3-3 protein is a direct substrate of p53 and binds to Cyclin B1/cdc2 as well as Cdc25 and sequesters them in the cytoplasm.

The role of ATM in the regulation of the S-phase checkpoint is complex and involves the phosphorylation of several key regulators. Hyperphosphorylation of the replication protein A (RPA) following IR is reduced and delayed in AT cells. RPA is a three-subunit protein complex involved in the initiation and elongation stages of DNA replication and in repair. It seems that in AT cells, the underphosphorylation of RPA sub-unit, p34, is not due to the absence of RPA kinases but rather to a slower phosphorylation rate. This delay might be mediated by another kinase sharing similar substrate specificity such as DNA-PK or the ATM-related kinase ATR. On the other hand, the delay might be caused by reduced accessibility of RPA to repair sites. This might imply that there is a defect in AT chromatin structure. The evidences supporting this possibility are discussed below (repair parameters). Whether phosphorylation of RPA by ATM in response to IR is required for the S phase checkpoint is still not clear since over-expression of the ATM kinase domain is sufficient to restore the radioresistant DNA synthesis (RDS) defect without correcting the RPA phosphorylation status. Another important substrate of ATM for the S-phase checkpoint is the Nbs1 protein also known as nibrin or p95. Nbs1 is part of a large nuclease complex also containing Mre11 and Rad50 that is involved in repair of DSB generated at stalled replication forks. The nbs1 gene is mutated in the Nijmegen breakage syndrome (NBS), a syndrome sharing a variety of phenotypic abnormalities with ATM. Nbs1 is phosphorylated on Ser278, Ser343, Ser397, and Ser615 by ATM. Mutation of either Ser278 or Ser343 is sufficient to abrogate an S-phase checkpoint induced by IR. ATM binds directly to Nbs1; this interaction is enhanced upon DNA damage but ATM phosphorylation of Nbs1 does not affect Nbs1 binding to Mre11. However, ATM, the NBS proteins, and BRCA1 are part of a super complex named BASC (BRCA1-associated genome surveillance complex) that is thought to function as a sensor for DNA damage. Phosphorylated Nbs1 acts as an adaptor for the ATM-dependent phosphorylation of other substrates such as the structural maintenance of chromosomes protein Smc1. ATM phosphorylates Smc1 on Ser957 and Ser966 *in vivo* and *in vitro* following IR. Optimal phosphorylation of Smc1 by ATM requires Nbs1 and BRCA1, but the reasons for this requirement are not clearly understood yet. It is possible that Nbs1 and BRAC1 are used as scaffold proteins to enhance ATM activity on Smc1. Mutations of the Smc1 protein at the ATM phosphorylation sites are sufficient to abrogate the IR-induced S-phase cell cycle checkpoint. As mentioned above, phosphorylation of chk2 by ATM provides a mechanism for ATM to control both G_1 and G_2 checkpoint. Phosphorylation of chk2 by ATM can also control the S-phase checkpoint through phosphorylation of the cdc25A phosphatase. Chk2 phosphosrylates Ser123 on cdc25A in response to IR in an ATM dependent manner. Phosphorylation of cdc25A triggers its degradation, which prevents dephosphorylation of cdk2 and arrest the cells in S phase. ATM can also control the S phase checkpoint through direct phosphorylation of the Fanconi Anaemia complementation group D2 (FANCD2) protein on Ser222. Moreover, biallelic disruption of *FANCD2* results in IR hypersensitivity.

Stress response

Another class of substrates that are phosphorylated by ATM includes several important stress responsive proteins. ATM phosphorylates the non-receptor nuclear tyrosine kinase c-Abl on Ser465 in response to IR. This phosphorylation activates c-Abl which then phosphorylates its substrates such as the C-terminal repeated domain (CTD) of RNA polymerase II and the Jun N-terminal kinase (JNK), among others. JNK is a member of a subgroup of stress activated kinases known as the MAPKs that are activated by several cellular stresses including DNA damage. JNK stimulates the transcriptional activity of the AP-1 transcription factors such as c-*jun*. In AT cells, activation of the JNK pathway by IR is defective, but activation by UV radiation and protein synthesis inhibitors is intact. The inhibitor proteins known as IκB are also phosphorylated by ATM. IκB interacts with the transcription factor NFκB and prevent it from translocating to the nucleus by masking NFκB nuclear localization domain. Phosphorylation of IκB leads to its degradation and release of the NFκB to the nucleus where it can transactivate its downstream effector genes. NFκB is involved in the regulation of immunoglobulin genes and is a member of a gene family involved in lymphomagenesis. Moreover, NFκB is constitutively expressed at high levels in neurons, suggesting a possible link to central nervous symptoms in AT. The molecular mechanisms underlying ATM mediated IκB degradation are currently emerging as a complex interdependency between ATM activation and IKK, the kinase

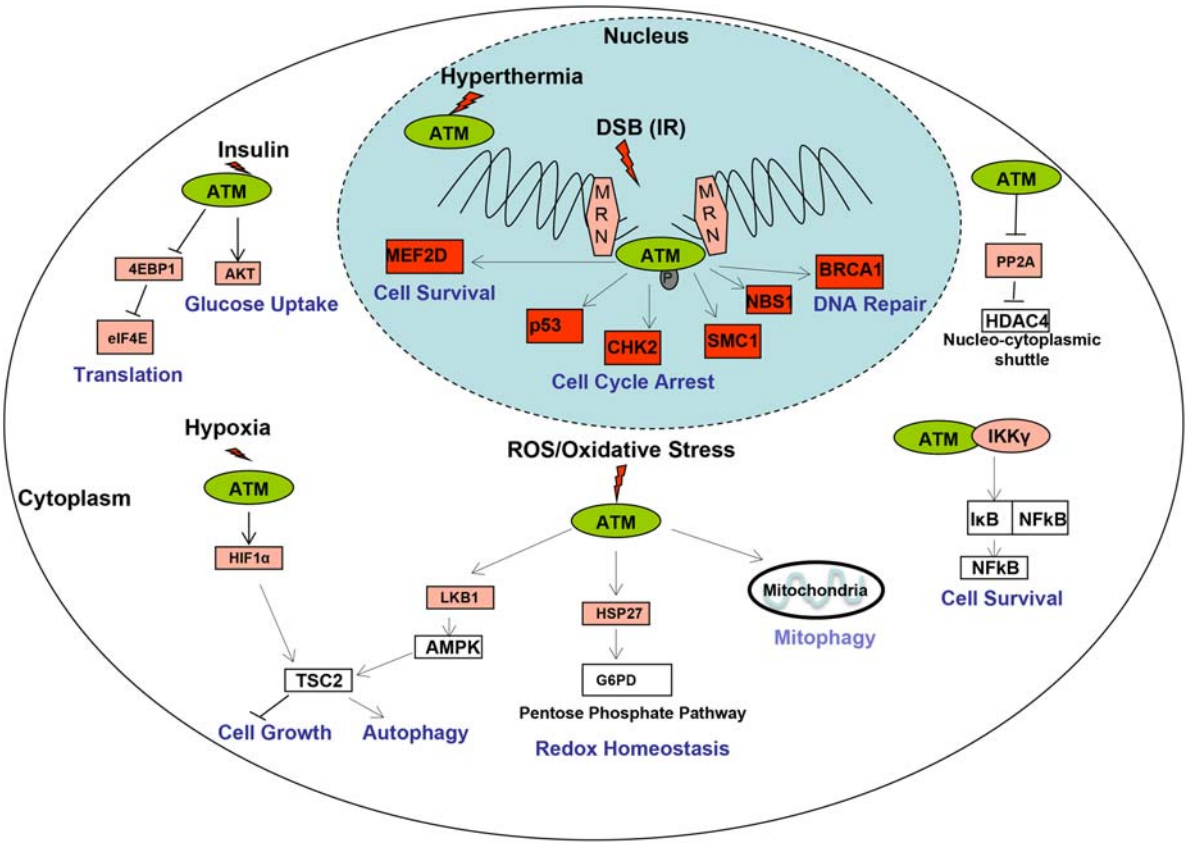

Figure 3 ATM; a master regulator of cellular homeostasis. Involvement of ATM inregulating cellular response to a variety of stimuli- DNA double strand break (DSB), inducing irradiation or chemicals, oxidative stress, hypoxia, hyperthermia, metabolic, signaling (Insulin). ATM acts as a major signal transducer in response to DSB formation, in the nucleus, leading to cell cycle arrest, DNA repair or apoptosis. In the cytoplasm it, acts predominantly on maintaining metabolic, oxidative and growth homeostasis.

regulating IkB degradation. In response to genotoxic stress, ATM autophosphorylation on Ser1981 results on phosphorylation of IKKγ, triggering a sumoylation-for-ubiquitination exchange on IKKγ. The ubiquitinated IKKγ-ATM complex is then exported from the nucleus to the cytosol where ATM associates with and causes the activation of IKK (Figure 3).

DSB repair

In addition to the Nbs1 protein, ATM also participates in the activation of other important proteins involved in DSB repair. The RAD51 protein is an indirect substrate of ATM; it is phosphorylated by c-Abl in an ATM dependent manner following IR exposure. Phosphorylation of RAD51 increases the interaction of RAD51 with RAD52, a complex mediating homologous recombinational repair (McKinnon, 2012).

Defect in Recombination and Chromatin Modification

A defect(s) in some form of DNA recombination in AT has been suggested based on the presence of spontaneously occurring chromosome translocations involving breakpoints in T-cell receptor (TCR) genes. Interestingly, the 70-fold increased frequency of TCR hybrid genes in AT lymphocytes does not affect the productivity nor the structure of the recombined TCR. This suggested that unlike the case with scid mice, the recombination process in AT cells is qualitatively normal. Using recombination vectors it has been shown that spontaneous intrachromosomal recombination rates are 30–200 times higher in AT fibroblasts line compared to normal cells, whereas extrachromosomal recombination frequencies are near normal. This indicates that the abnormality is specific to the AT chromosomal integrity and is not related to viral or plasmid DNA. The hyperrecombination rate is also particular to the AT phenotype and is not a consequence of defective DNA repair since cells from xeroderma pigmentosum patients, which are defective in excision repair, do not show abnormal recombination rates. The increased recombination rate is therefore an integral component of the AT phenotype and might contribute to genetic instability and an increased risk of cancer.

The defects in recombination may be linked to an ATM direct interaction with DNA and chromatin. ATM binds to meiotic chromosomes and is part of the recombination nodules, the structures thought to mediate chromatid exchanges. Fragmentation of meiotic chromosomes occurs at these sites in ATM deficient mice and may explain the infertility of these animals. A defective

chromatin structure in AT has been suggested as a possible reason for the higher rate of conversion of DNA double-strand breaks into chromosomes breaks. This was suggested based on apparent alterations of nucleosomal periodicity near the telomere chromatin. Moreover, a defective chromatin structure was also postulated to explain the inability of AT cells to stop DNA synthesis following exposure to IR. It is presumed that the defective chromatin structure could reduce accessibility of RPA to repair sites. The fact that in ATM deficient mice RPA remains attached to the ends of abnormal chromosomes fragments formed at the sites of the recombination nodules supports this possibility. There are several lines of evidence indicating that ATM could modulate the chromatin structure. First, transient dephosphorylation of histone H1, but not histone H3, following IR is ATM-dependent. Dephosphorylation of histone H1 is believed to increase chromatin decondensation. The exact pathways leading to H1 dephosphorylation have not been elucidated yet. Second, ATM rapidly interacts with the histone deacetylase HDAC1, both *in vivo* and *in vitro*. The amount of HDAC1 activity associated with ATM increases after IR. Histones deacetylation also decreases chromatin decondensation, which in turn affects radiosensitivity (Carrier, 2013). A third line of evidence comes from ATM capacity to phosphorylate the histone H2A variant H2AX in response to DSB. Phosphorylation of H2AX at Ser139 is rapid (within 1–3 min) and specific to the sites of DNA damage. In yeast, H2AX phosphorylation causes chromatin decondensation, while in mammals it mediates the recruitment of repair or damage signaling factors such as BRCA1, Nbs1, RAD50, and RAD51 to the sites of DNA damage. H2AX phosphorylation following IR is severely compromised in *ATM* −/− cells but phosphorylation of H2AX can be restored by ectopic expression of ATM in these cells. In fact, ATM mutations associated with different AT phenotypes have been linked to diverse degrees of chromatin condensation and reorganization with the more severe phenotype presenting the most decondensed chromatin and consequently alteration of gene expression.

Abnormal Apoptosis

The control of the cell number in each lineage is determined by a balance between cell proliferation and cell death. The process regulating cell proliferation is highly regulated with numerous checks and balances. The regulation of cell death is now appearing to be as complex as cell proliferation. Differentiated cells have the ability to carry out their own death through the activation of an internal suicide program called apoptosis. The apoptotic process is usually turned on to eliminate cells that have developed improperly, have been produced in excess, or have sustained irreparable damage to their DNA. Apoptotic cell death is different from necrotic cell death which is a pathological form of death resulting from acute cellular injury. The difference between these two forms of death can be observed morphologically. Cells dying from necrosis will swell rapidly and lyse which will result in the leakage of cytoplasmic contents and the induction of an inflammatory response. An apoptotic cell undergoes a controlled autodigestion with the maintenance of the plasma membrane integrity and therefore no inflammatory response. In mouse, ATM is essential for IR-induced apoptosis in the developing nervous system. ATM-mediated apoptosis in the nervous system also required p53 and the pro-apoptotic effector Bax. The apoptotic response in ATM null mice is defective but the precise role of ATM in apoptosis is still debated. The conflicting results often reported in the literature may be associated with the cell types, the different apoptotic stimuli, or even the cellular states (proliferative vs quiescent) of the cells used by the investigators. Interestingly, AT fibroblasts are unusually sensitive to drugs that also produce internucleosomal DNA cleavage, characteristic of apoptosis. Widespread apoptosis was detectable in four A-T fibroblast lines, AT22IJE, AT4BVI, AT5BIVA, and AT2SFSV but not in two control lines. Apoptosis began 24 hr after exposure to X-rays or streptonigrin (radiomimetic agent) and peaked 72 to 96 hr following treatment. No apoptotic differences were detected between AT and control fibroblasts following exposure to 30 J m^{-2} of UV irradiation. Streptonigrin also induced widespread apoptotis in AT lymphoblasts but not in control lymphoblasts. The interval between exposure of cells to apoptotic agents and the induction of apoptosis can be short, being detected in some instances as soon as 4 hr after exposure. The significant delay in AT to trigger the apoptosis machinery might be due to a defect in the same pathway responsible for the loss of G_1 delay since p53 is required for radiation-induced apoptosis. Current evidence, however, suggests that c-Abl may rather link ATM to p73, a member of the growing p53-like family that is not induced by DNA damage but plays a role in damage-induced apoptosis. In response to ATM phosphorylation, c-Abl phosphorylates and activates p73. As mentioned above, one of the AT gene domains shares homology with PI-3 kinase. PI-3 kinase is required for the prevention of apoptosis in rat pheochromocytoma cells by nerve growth factor. This facet of the AT gene might correlate with the increased nerve cell death in AT and the increased apoptosis in cultured AT cells exposed to DNA-damaging agents.

Future Directions

Since the cloning of the ATM gene, tremendous efforts have been directed at delineating ATM functions in cell cycle regulation, sensing of DNA damage, and initiation of DNA repair mechanisms. The predominantly nuclear localization of the protein is in good agreement with these important cellular functions. However, it is now becoming evident that ATM probably plays other important roles in cellular homeostasis that may be mediated by the small pool of cytoplasmic protein (Shiloh and Ziv, 2013). For example, ATM could react with Reactive Oxygen Species (ROS) in the cytosol and affect the redox state of a cell. Several lines of evidence are supporting a role for ATM in the oxidative stress response. For instance, increased levels of ROS have been detected in the cerebellum of *ATM* deficient mice. The absence of *ATM* may thus result in oxidative damage and cause the degeneration of cerebellar neurons. In fact, ATM has now been described as a redox sensor in addition to its role as a sensor of DSBs. Recent reports indicate that ATM can be oxidized and activated by the formation of covalent dimers through disulfide bonds between two

monomers. This activation process is different and independent of the MRN/DNA complex (Guo et al., 2010). In addition, other evidences have demonstrated a role for ATM in the production of gluthathione by the pentose phosphate pathway (PPP) and the maintenance of redox homeostasis. ATM helps in glucose 6-phosphate dehydrogenase activation through phosphorylation of heat shock protein 27 which in the end produces NADPH (Cosentino et al., 2011) (Ambrose and Gatti, 2013).

The variety of symptoms and the presence of ATM in the cytoplasm indicate that certain ATM substrates are likely to be involved in mechanisms other than the ones related to the DNA damage response. Substrates found in the cytosol, such as the translational regulatory protein 4E-BP-1, are good examples of this possibility. ATM phosphorylates this regulatory protein in response to insulin stimulation. There is also evidence that ATM may be linked to growth factor mediated pathways such as the insulin-like growth factor-1 receptor (IGF-1R). Transcriptional expression of IGF-1R appears to be ATM-dependent. Evidence is now accumulating to suggest that ATM is actually involved in the development of insulin resistance through down regulation of AKT activity. Cytoplasmic ATM is not only a major upstream activator of AKT in response to insulin but can also regulate the transport of GLUT-4 to the surface of cells and modulate insulin-mediated glucose uptake (Halaby et al., 2008). This function could explain the insulin resistance condition in some AT patients.

The most challenging AT symptom that remains to be alleviated is progressive neurodegeneration. Although a number of factors have been implicated in the neurodegenerative phenotype of AT, the unavailability of a human model system able to recapitulate the neurological disease has prevented the identification of a causative factor. In order to compensate for this deficiency, human neuronal stem cells (hNSCs) have been used to gain insight into the molecular mechanisms of neurodegeneration in AT. hNSCs can be induced to differentiate into the three major central nervous system (CNS) cell types, neurons, astrocytes and oligodendrocytes. Upon differentiation, ATM-proficient and ATM-deficient ihNSCs (immortalized with v-myc) yield a similar number of neurons expressing the MAP2 and β-Tubulin III markers but ATM deficient ihNSCs consistently yield attenuated levels of neurons exhibiting a GABAergic phenotype (Carlessi et al., 2013). This finding is consistent with pathological and clinical findings showing a GABA deficiency in the cerebellum of AT patients, and amelioration of the ataxia manifestation by treatment with a GABA-analog. Neurodegeneration can also be studied with human somatic cells that are reprogrammed into induced pluripotent stem cells (iPSCs). Human iPSCs re-programmed from AT fibroblasts and capable of generating a variety of functional neurons have been generated. In spite of numerous advantages, these stem cell models have obvious limitations including the inability to generate Purkinje neurons, a primary target underlying the ataxia phenotype in AT. Nonetheless, stem cells are more and more in demand and under study specifically for regenerative medicine purpose and particularly for neurological disorders. Transplantation of neuronal stem cells in the brain of AT patients will have to await further investigations on the techniques to be used and a better understanding of possible unintended side effects since transplantation of fetal neuronal stem cells in the brain of one AT patient resulted in uncontrolled growth in his spinal cord and brain (Amariglio et al., 2009).

As mentioned above, clues regarding ATM participation in preventing neurodegeneration may be provided by the cytoplasmic localization of a fraction of ATM in normal neurons. In addition to its role as a redox sensor ATM can also interact with the synaptic proteins VAMP2 and Synapsin-1 in the cytoplasm of neurons. Moreover, neurodegeneration in AT has been linked to the translocation of the histone deacetylase HDAC4 from the cytoplasm to the nucleus with consequent suppression of neuronal gene expression. To remain in the nucleus HDAC4 must be dephosphorylated by the protein phosphatase 2A (PP2A), which is down regulated by ATM phosphorylation. As a consequence, PP2A activity is enhanced in AT cells and contributes to neurodegeneration. It thus appears that the neurodegenerative phenotype in AT is linked to, at least in part, an impaired ATM cytoplasmic activity that is distinct from its nuclear activity. ATM could also affect cerebellar degeneration through regulation of Ligase III levels and mitochondria homeostasis in the brain (Sharma et al., 2014). Reduced levels of Ligase III impede mitochondria DNA repair and could contribute to mitochondria ROS production and cerebellar degeneration.

Targeting *ATM* or smaller part of the gene to the Purkinje cells will be a difficult challenge but the development of strategies to generate hNSC that can differentiate into Purkinje cells or the identification of substrates specific to Purkinje cells will surely help direct these efforts. The emerging role of ATM in the cytoplasm as well as its functions in post-mitotic cells indicate that this multitask protein kinase is complex and versatile. The mechanisms that regulate its transitions from one function to another will probably include some post translational modifications affecting its structural characteristic and consequently its substrate preferences and interacting partners. Structural studies will allow a better understanding of the different ATM domains involved in these interactions and will likely result into the development of drugs that can specifically target a given pathway. Identification of the ATM gene was a major breakthrough and its studies will provide tools to better understand several fundamental cellular mechanisms that have implications far beyond the AT syndrome.

See also: Cell Responses to DNA Damage. Genetic Instability. Radiation Therapy-Induced Metastasis and Secondary Malignancy. TP53.

References

Amariglio, N., Hirshberg, A., Scheithauer, B.W., Cohen, Y., Loewenthal, R., Trakhtenbrot, L., Paz, N., Koren-Michowitz, M., Waldman, D., Leider-Trejo, L., Toren, A., Constantini, S., Rechavi, G., 2009. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Medicine 6, e1000029.

Ambrose, M., Gatti, R.A., 2013. Pathogenesis of ataxia-telangiectasia: the next generation of ATM functions. Blood 121, 4036–4045.

Carlessi, L., Fusar Poli, E., De Filippis, L., Delia, D., 2013. ATM-deficient human neural stem cells as an in vitro model system to study neurodegeneration. DNA Repair (Amst) 12, 605–611.
Carrier, F., Fornace Jr., A., 2002. Ataxia Telangiectasia Syndrome. In: Bertino, J. (Ed.), Encyclopedia of Cancer, 2nd edn. Academic Press, San Diego, CA.
Carrier, F., 2013. Chromatin modulation by Histone Deacetylase Inhibitors; impact on cellular sensitivity to ionizing radiation. Molecular and Cellular Pharmacology 5 (1), 51–59.
Carrillo, F., Schneider, S.A., Taylor, A.M., Srinivasan, V., Kapoor, R., Bhatia, K.P., 2009. Prominent oromandibular dystonia and pharyngeal telangiectasia in atypical ataxia telangiectasia. Cerebellum 8, 22–27.
Chan, S.F., Sances, S., Brill, L.M., Okamoto, S., Zaidi, R., Mckercher, S.R., Akhtar, M.W., Nakanishi, N., Lipton, S.A., 2014. ATM-dependent phosphorylation of MEF2D promotes neuronal survival after DNA damage. Journal of Neuroscience 34, 4640–4653.
Cosentino, C., Grieco, D., Costanzo, V., 2011. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. EMBO Journal 30, 546–555.
Davis, M.Y., Keene, C.D., Swanson, P.D., Sheehy, C., Bird, T.D., 2013. Novel mutations in ataxia telangiectasia and AOA2 associated with prolonged survival. Journal of the Neurological Sciences 335, 134–138.
Driessen, G.J., Ijspeert, H., Weemaes, C.M., Haraldsson, A., Trip, M., Warris, A., Van Der Flier, M., Wulffraat, N., Verhagen, M.M., Taylor, M.A., Van Zelm, M.C., Van Dongen, J.J., Van Deuren, M., Van Der Burg, M., 2013. Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity. Journal of Allergy and Clinical Immunology 131 (1367–75) e9.
Gatti, R.A., Berkel, I., Boder, E., Braedt, G., Charmley, P., Concannon, P., Ersoy, F., Foroud, T., Jaspers, N.G., Lange, K., et al., 1988. Localization of an ataxia-telangiectasia gene to chromosome.11q22-23. Nature 336, 577–580.
Guo, Z., Deshpande, R., Paull, T.T., 2010. ATM activation in the presence of oxidative stress. Cell Cycle 9, 4805–4811.
Halaby, M.J., Hibma, J.C., He, J., Yang, D.Q., 2008. ATM protein kinase mediates full activation of Akt and regulates glucose transporter 4 translocation by insulin in muscle cells. Cellular Signalling 20, 1555–1563.
Hoche, F., Seidel, K., Theis, M., Vlaho, S., Schubert, R., Zielen, S., Kieslich, M., 2012. Neurodegeneration in ataxia telangiectasia: what is new? What is evident? Neuropediatrics 43, 119–129.
Kriaucionis, S., Heintz, N., 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324, 929–930.
Lavin, M.F., 2008. Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nature Reviews. Molecular Cell Biology 9, 759–769.
Mckinnon, P.J., 2012. ATM and the molecular pathogenesis of ataxia telangiectasia. Annual Review of Pathology 7, 303–321.
Montella, S., Mollica, C., Finocchi, A., Pession, A., Pietrogrande, M.C., Trizzino, A., Ranucci, G., Maglione, M., Giardino, G., Salvatore, M., Santamaria, F., Pignata, C., 2013. Non invasive assessment of lung disease in ataxia telangiectasia by high-field magnetic resonance imaging. Journal of Clinical Immunology 33, 1185–1191.
Palau, F., Espinos, C., 2006. Autosomal recessive cerebellar ataxias. Orphanet Journal of Rare Diseases 1, 47.
Sharma, N.K., Lebedeva, M., Thomas, T., Kovalenko, O.A., Stumpf, J.D., Shadel, G.S., Santos, J.H., 2014. Intrinsic mitochondrial DNA repair defects in Ataxia Telangiectasia. DNA Repair (Amst) 13, 22–31.
Shiloh, Y., Ziv, Y., 2013. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nature Reviews. Molecular Cell Biology 14, 197–210.

Autophagy and Cancer

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Glossary

Macroautophagy The cellular degradation process by which cytoplasmic content is delivered to the lysosome via double membrane vesicles (autophagosomes) for degradation by lysosomal hydrolases.

Microautophagy Direct invagination of the lysosome to engulf and degrade cytoplasmic material.

CMA (chaperone mediated autophagy) The targeted delivery and degradation of proteins containing a KFERQ motif by the lysosome.

Mitophagy A form of selective macroautophagy that specifically degrades damaged mitochondria.

Lysosome A cellular organelle that maintains an acidic pH and contains degradative enzymes that can function at a low pH to degrade proteins and other cellular material into amino acid and other macromolecular building blocks that can be recycled.

ATG genes Genes that encode the regulators of autophagy. ATG proteins function in various complexes to cause the initiation, elongation, and fusion steps of autophagy.

Autophagic flux The formation and degradation (via fusion with the lysosome) of autophagosomes.

LAP (LC3-associated phagocytosis) A form of noncanonical autophagy used by phagocytic cells that utilizes parts of the autophagic machinery but is autophagosome-independent, and plays important roles in immunity.

Introduction

The award of the 2016 Nobel Prize for Medicine or Physiology to Yoshinori Ohsumi for his work uncovering the mechanisms of autophagy highlighted the recognition in recent years that autophagy is critically important in biology and many diseases including cancer. The term "autophagy," literally translated as "eating oneself," describes three mechanistically distinct processes by which cellular material is delivered to the lysosome for degradation by the lysosomal hydrolases. The resulting macromolecular precursors (amino acids, fatty acids etc.) are then recycled to make new macromolecules or used to fuel metabolic pathways. In addition, it has recently become apparent that the machinery that controls autophagy is also involved in nondegradative processes, for example in cellular secretion and in controlling signal transduction pathways.

The three types of autophagy that have been described are macroautophagy, chaperone mediated autophagy (CMA) and microautophagy. Macroautophagy involves the formation of double membrane vesicles called autophagosomes, which engulf proteins and other cellular material including organelles and parts of organelles. Autophagosomes go on to fuse with lysosomes in order to expose the engulfed material to the lysosomal hydrolases, which digest the contents of the autophagosome. CMA is the process by which specific proteins that contain the pentapeptide amino acid motif, KFERQ, are directly delivered to the lysosome. The KFERQ motif is recognized by the cytosolic chaperone protein hsc70, targeting the KFERQ-containing protein to the lysosomal surface, where the lysosome-associated membrane protein 2A (LAMP-2A) acts as a receptor to mediate translocation of the targeted protein into the lumen of the lysosome. One important difference between macroautophagy and CMA is that CMA only delivers proteins to the lysosome while macroautophagy delivers proteins as well as other cellular components. Additionally, CMA only delivers specific KFERQ motif-containing, proteins, while macroautophagy, although often selective for specific substrate proteins can, in principle, cause the turnover of any protein by the lysosome. Microautophagy traps cellular material in the lysosome by direct invagination of the lysosomal membrane. Hereafter, in this article when we say "autophagy" we will refer to macroautophagy, which is by far the best understood type with CMA being less well studied and microautophagy even less so.

Christian de Duve, who shared the 1974 Nobel Prize for his discoveries of the lysosome and peroxisome, first coined the term "autophagy" in the early 1960s. However, there was little work on this process until the late 1990s when genetic studies in yeast by Professor Ohsumi (and also other investigators especially Daniel Klionsky and Michael Thumm) led to the identification of genes (now called *ATG* genes) that control autophagy. This identification of autophagy regulators opened up the field for further study and it was rapidly discovered that other organisms had orthologs of the yeast genes and that in many cases the ATG proteins encoded by these genes had equivalent functions in mammals and other organisms. The discovery of the ATGs also allowed the development of better methods to study and measure autophagy and, since the early 2000's, there has been an explosion of interest in autophagy research in many areas of biomedicine and in normal biology. Thus, we now know that autophagy is important in regulating metabolism and homeostasis, it controls longevity, and is involved in infectious, metabolic, cardiovascular and neurodegenerative diseases as well as cancer.

Autophagy is affected by diverse physiological and pathological stimuli and is often affected positively or negatively by drugs that were developed for other purposes. For example, nutrient deprivation and starvation, hypoxia, hypothermia and rewarming, glucose deprivation, redox stress and many other stressful stimuli have all been shown to induce autophagy. A high

proportion of approved drugs including many anticancer drugs with different mechanisms of action including DNA damaging agents, antimetabolites kinase inhibitors, proteasome inhibitors, epigenetic modifiers and apoptosis targeted drugs like the BH3 mimetics also induce autophagy. Conversely some other anticancer drugs including microtubule-targeted agents can inhibit autophagy. Normal physiological processes such as exercise also activate autophagy. Many such stimuli will apply in cancer patients where, for example, nutrient deprivation and hypoxia of tumors is common and, perhaps more importantly, variable in many patients. Given the broad physiological stimuli that tumors are exposed to, combined with the fact that many anticancer drugs affect autophagy, it is impossible to avoid variations in autophagy impacting tumor behavior. This highlights the importance in obtaining a comprehensive understanding of the effects of autophagy on cancer cell behavior and also opens up an opportunity to deliberately manipulate autophagy to improve cancer treatment.

Regulation and Cellular Functions for Autophagy

Signaling Pathways and ATG Proteins That Control Autophagy

The process of autophagosome formation is highly regulated and complex involving multiple different protein complexes that contain ATG proteins and other regulators and only a brief and simplified summary is provided here. For more details please see the Further Reading section of this article.

Not surprisingly given that autophagy is affected by a large number of different stimuli, numerous signaling pathways regulate autophagy. However, the majority of these pathways converge on the mTOR/PI3 Kinase pathway where the mechanistic target of rapamycin (mTOR and specifically the mTORC1 complex) serves as a master negative regulator that controls autophagy (**Fig. 1**). This function might be expected since mTORC1 coordinates cellular growth and metabolism in response to diverse environmental signals including nutrients and growth factors. Thus by simultaneously promoting anabolic pathways while suppressing catabolic pathways like autophagy, coordinated cellular growth can occur. A key early step in the way that mTORC1 regulates autophagy is to control the activity of pair of protein kinases, ULK1 and ULK2, which form a complex with several other autophagy regulators including ATG13, FIP200 and ATG101. When nutrients are plentiful, mTORC1 phosphorylates ULK1. This prevents activation of ULK1 by AMPK, which itself is a key mediator of amino acid signaling. Thus relative levels of mTORC1 and AMPK activity control ULK1 activity to regulate autophagy and mTOR inhibitors like rapamycin are potent autophagy inducers.

Such posttranscriptional signaling largely explains how acute changes in autophagy—for example, an increase in the rate of formation of autophagosomes in response to acute amino acid starvation, are controlled. In addition transcriptional regulation of multiple components of the autophagy pathway (and also of lysosomal proteins) occurs. This is achieved through members of the TFEB/MITF and FOXO families of transcription factors, along with a number of epigenetic modifiers including BRD4 and repressors of gene expression. These transcriptional regulators directly regulate multiple *ATG* genes in a coordinated manner to allow a more sustained regulation of relative autophagy levels. This allows some cells to have a higher or lower level of sustained autophagy compared with other cells. Transcriptional regulation of autophagy is also coordinated with the mTOR pathway. For example, TFEB is a direct target of mTORC1 and its phosphorylation leads to sequestration of the transcription factor in the cytoplasm, thus preventing it from activating its target genes in the nucleus. By targeting both long term transcriptional control of the autophagy regulators and directly controlling the kinase complex that initiates formation of autophagosomes, coordinated control of the pathway is possible. In cancer these effects are thought to be especially important. For example in pancreas cancer, elevated activity of the MITF family leads to sustained increase in autophagy, that is critical for allowing tumor cell survival and pancreas tumor growth.

The ULK complex controls another complex that includes a protein called Beclin1 (BECN1), which is the mammalian ortholog of the yeast Atg6 protein. The BECN1 complex, which serves to initiate nucleation of the vesicle that will become the autophagosome, also contains a class III phosphatidyl inositol kinase, VPS34 as well as other regulators including AMBRA, RUBICON and UVRAG. Activation of the BECN1 complex increases VPS34 activity to phosphorylate phosphatidylinositol to form phosphatidylinositol (3)-phosphate (PtdINs(3)P) and this is critical for autophagy as well as other trafficking events including endocytosis.

The next steps in autophagosome generation involve protein conjugation systems that are conceptually similar to the systems that conjugate proteins with small peptides like ubiquitin. Two conjugation events take place. In the first, a small protein, ATG12, is conjugated to ATG5. This is catalyzed by an E1-like activating enzyme, ATG7, which works together with ATG10 (the E2 conjugating enzyme) to create the ATG12-ATG5 conjugate. The ATG12-ATG5 conjugate noncovalently interacts with another protein, ATG16, to create a complex that has an E3 like activity that together with the E2 enzyme ATG3 and ATG7 again serving as the E1, catalyzes another conjugation event that adds phosphatidylethanolamine (PE) to members of the MAP1LC3 and GABARAP families of proteins. These proteins are homologs of yeast Atg8. The most commonly studied of these proteins is MAP1LC3B (often referred to as LC3). PE conjugation occurs after proteolytic cleavage of LC3 by one of four members (A–D) of the ATG4 family of proteases. The LC3 conjugation event to create LC3-PE, which is usually referred to as LC3-II, is important for formation of autophagosomes (though perhaps not completely necessary since cells that have deletion of many of the above ATGs can still make autophagosome like vesicles). This event is also important in a practical sense because the conjugated LC3-II form runs faster on polyacrylamide gels than the unconjugated LC3-I protein and forms foci in cells. These easily detected changes in LC3 serve as the primary way that autophagosomes are quantified. Thus the conjugation events that drive autophagosome formation involve the same E1 enzyme (ATG7), different E2s (ATG10 and ATG3) and create a protein–protein conjugate (ATG12-ATG5) and a protein–lipid conjugate (ATG8/LC3/GABARAPs-PE). Researchers can target these molecules (most commonly ATG7 or ATG5) as a way to inhibit autophagy

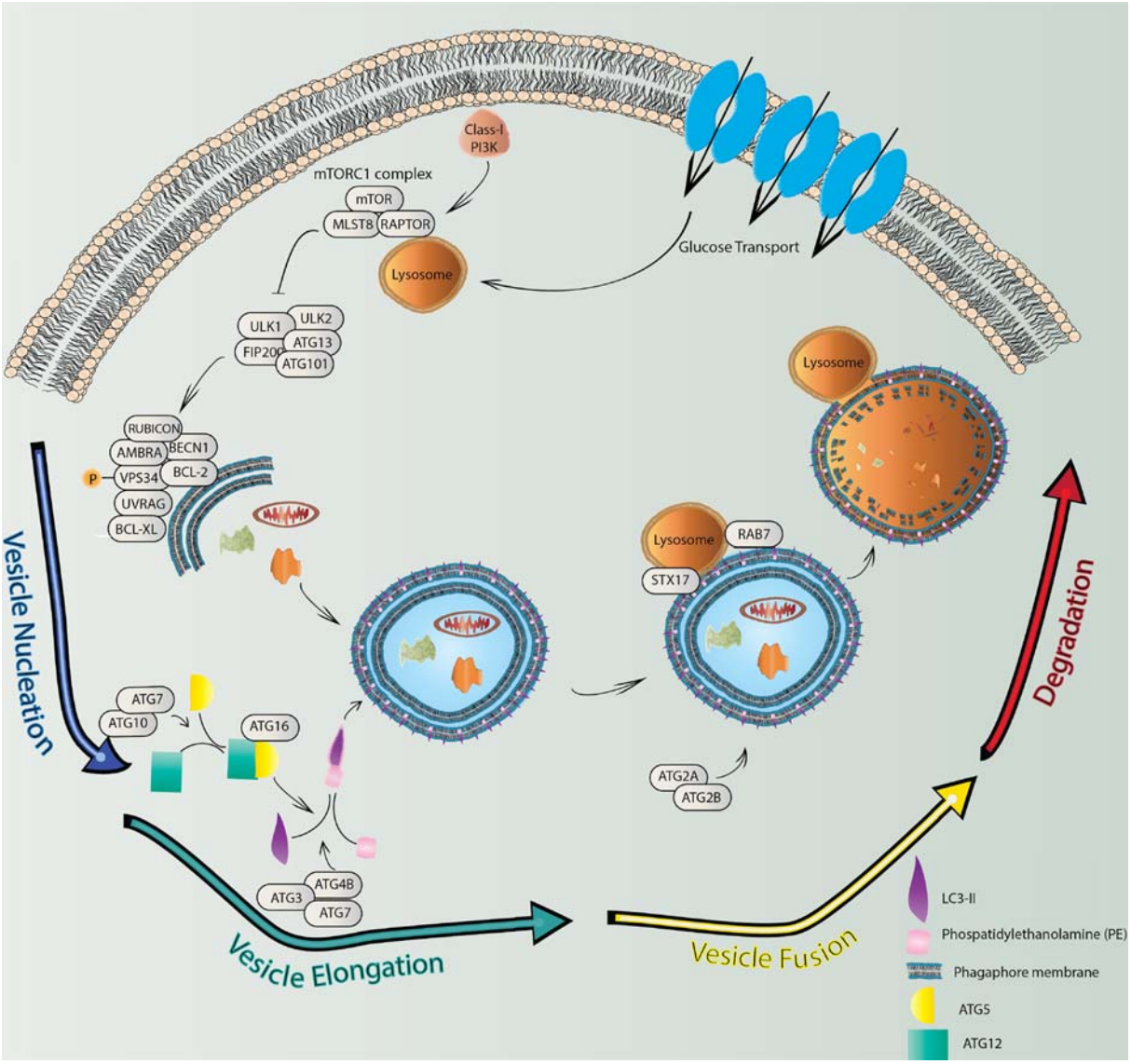

Fig. 1 A schematic of the autophagy pathway and the key proteins involved in the induction and nucleation of autophagosomes as well as the fusion of autophagosomes with lysosomes.

and there have been many preclinical animal studies to assess tumor development and progression following deletion of one or other of these genes in a particular tumor type.

Following the conjugation events, the autophagosome marked with LC3-II then closes. This step involves the RAB5 GTPase, and also requires two other proteins ATG2A and ATG2B, which if deleted, prevent closure. LC3-marked intact autophagosomes with their cargo that is destined for degradation then fuse with lysosomes. This requires another small GTPase, RAB7. Eventually the LC3-II protein that is on the inside of the autophagosomal membrane is degraded along with the rest of the cargo while LC3-II on the outside is cleaved by ATG4B releasing the LC3-I protein for re-use in another round of conjugation to PE to make new autophagosomes. Lysosome fusion also requires the snare protein, STX17, as well as proteins on the lysosome itself, especially LAMP2. Fusion also requires a fully functional lysosome that maintains an acidic pH. This means that drugs that affect the lysosome such as the antimalarials chloroquine, hydroxychloroquine or quinacrine as well as the V-ATPase inhibitor Bafilomycin A1, can function as autophagy inhibitors by preventing fusion of lysosomes to autophagosomes.

From the cancer biology perspective, there are a number of important issues that should be noted. First, the multistep nature of the autophagy pathway means that there are many potential opportunities for manipulation of this process. Such manipulations can be done both genetically by using shRNA, gene knockouts or dominant negative mutants to inactivate or block the activity of specific ATG proteins or many of the same proteins can be targeted pharmacologically. Thus selective inhibitors of the protein kinases (ULK1 and ULK2) as well as the lipid kinase VPS34 have been tested for antitumor effects, as have inhibitors of the protease ATG4B and the E1 conjugating ATG7 protein. At the current time however, the only drugs that have been used in people to inhibit

autophagy are the lysosomal inhibitors chloroquine and hydroxychloroquine. It is also possible to manipulate autophagy by interfering with the complexes themselves. For example, a cell permeable peptide that disrupts a negative regulator of the BECN1 complex has been used to activate autophagy. The BECN1 complex also contains antiapoptotic proteins including BCL2 and BCL-XL. These proteins interact with BECN1 via a BH3 domain. This is the same type of domain that controls BCL family activity at the mitochondria during mitochondrial outer membrane permeabilization (MOMP) to regulate the rate-limiting step in canonical apoptosis. The discovery of this interaction led to the suggestion that BH3 mimetics (e.g., Venetoclax), which are drugs that were designed to disrupt BH3 protein interactions to push tumor cells closer to their apoptotic threshold, could also disrupt the BECN1 complex and induce autophagy. Specific details of the mechanism are incomplete and while it is generally agreed that BH3 mimetics activate autophagy, the idea that they do so by disrupting the BECN1–BCL2 interaction has been challenged. Even if the details of the BH3 mimetics have been questioned however, the important point is that there are multiple potential strategies by which autophagy may be inhibited or induced and these include both interference with enzymatic activities and altering protein complexes; both strategies are conducive to selective pharmacological interventions.

Thus there are many potential ways by which autophagy could be manipulated and this brings up a second important point: the various potential interventions to block autophagy can do so at different steps in the process (Fig. 2). This is important because while blocking autophagy at any step should be similar in terms of its ability to prevent degradation of cargo, stopping the autophagy process before formation of autophagosomes may have different effects than blocking after formation of autophagosomes but before fusion with the lysosome when the autophagosome is serving as a signaling hub (see below for further discussion).

Selective and Nonselective Autophagy

Ultimately, the function of autophagy is to degrade cellular material. This is often described as nonselective, implying that the material that is engulfed by autophagosomes and degraded by the lysosomal hydrolases is completely random. This idea is incorrect. While there undoubtedly is random capture of cytosolic proteins in autophagosomes with their level in the autophagosome being determined simply by their cytosolic concentration, many proteins are specifically targeted for autophagic degradation or specifically excluded from autophagic degradation. Thus, although a large proportion of the proteome can be degraded by autophagy, autophagy is often quite specific. Global proteomic analysis suggests that short-lived proteins tend to be degraded by the proteasome while longer-lived proteins tend to be degraded by autophagy. Moreover some proteins and protein complexes seem to be especially resistant to autophagic turnover (e.g., the ribosome). Specific proteins can also be targeted for autophagic degradation and this can be regulated by external stimuli. A good example is ferritin, which binds iron. Ferritin must be degraded in the lysosome (a process that has been named ferritinophagy) for iron to be released. Thus under iron-deficient conditions, ferritin is specifically targeted to autophagosomes to be degraded. This is achieved by an adaptor protein called NCOA4, which binds to ferritin under iron-deficient conditions targeting it for recognition by the developing autophagosome. Oncogenic signals can also cause specific proteins to be degraded by autophagy. For example, the nuclear lamin B1 protein is selectively targeted to autophagosomes in RAS-transformed tumor cells. There are likely many other cases where specific proteins are targeted for autophagic degradation but identifying such proteins is technically difficult and to date most cases of selective autophagy of particular proteins have been identified by chance or by taking a very targeted candidate approach.

Autophagy also targets protein complexes (e.g., toxic aggregated proteins including those associated with glutamine expansion diseases like Huntington's disease and other neurodegenerative diseases), specific organelles (mitochondria, peroxisomes,

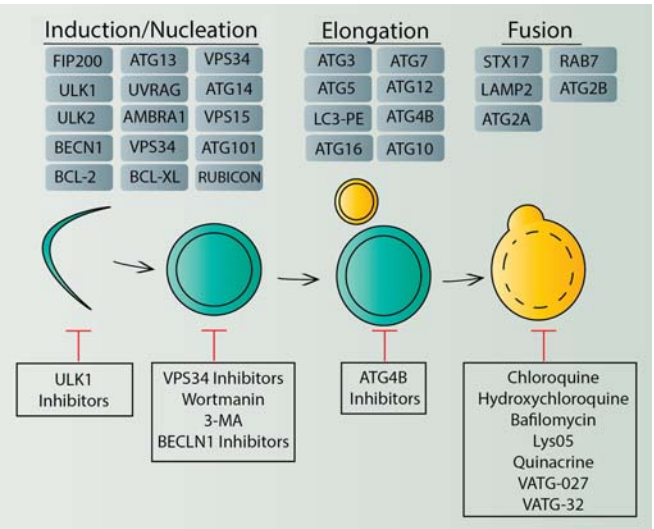

Fig. 2 The autophagy pathway can be targeted at multiple steps, either pharmacologically or genetically.

lysosomes and portions of the endoplasmic reticulum) and other organisms (bacteria and viruses). Many of these selective forms of autophagy have specific names (e.g., mitophagy refers to degradation of mitochondria while pexophagy is the degradation of peroxisomes). Specificity is achieved by particular cargo receptors that recognize the cargo and direct it to the forming autophagosome ensuring that the target in question is preferentially engulfed in autophagosomes. The best-characterized autophagy cargo receptor is SQSTM1 commonly known as p62, which binds ubiquitinated molecules to shuttle them to autophagosomes. It is likely that many other cargo receptors exist, and for example, the TRIM family of proteins can regulate different cargo targeting events. One focus of current research in cancer and other diseases is to try to understand and eventually find ways to enhance or inhibit specific forms of selective autophagy rather than affecting the process as a whole.

Measuring and Manipulating Autophagy

The most common way to measure autophagy is to assess levels of LC3-II. This is done either by western blotting, taking advantage of the faster mobility of the PE-conjugated protein in polyacrylamide gels, or by counting LC3 foci after immunostaining or, sometimes in laboratory studies, after exogenous expression of a fluorescent version of LC3. A common misunderstanding in the autophagy literature is that an increase in LC3-II levels by these measures must be associated with an increase in autophagy. The problem is that autophagy is a dynamic process whereby autophagosomes are formed (i.e., increasing LC3-II) but then fuse with lysosomes resulting in degradation and a decrease of LC3-II. This formation then degradation of autophagosomes is termed autophagic flux and a true increase in autophagy actually means an increase in the flux. The practical problem is that increased autophagic flux could lead to more LC3-II, less LC3-II or the same amount of LC3-II depending on the relative rates of formation, fusion, and degradation of the autophagosomes. In the literature there are many studies where misinterpretation of such experiments has occurred and in some cases incorrect conclusions have been made (e.g., on whether autophagy has been induced or inhibited).

As with any dynamic process involving turnover of biological molecules, in order to assess whether it is increased or not, one must block flux through the system. In the laboratory the most common way to definitively test if there is truly an effect on autophagy is to block the lysosome with chloroquine or Bafilomycin A1. When fusion is prevented, LC3-II should accumulate and, if there is also a stimulus-dependent increase in autophagosome formation (i.e., increased autophagy), this will be manifested by further accumulation of LC3-II and more LC3 foci. Autophagic flux is also measured using LC3 or other autophagosome-targeted proteins with fluorescent markers that change based on the acidity of their environment, thus allowing one to follow fusion with the acidic lysosomes by fluorescence microscopy or flow cytometry. An important unsolved problem in the field is that while such assays can be easily performed in cell culture experiments, it is much harder to do this kind of experiment in a living animal. It is even harder still to do such measurements in tissue samples such as might be available from clinical specimens. This means that there are many situations (e.g., when examining biopsy samples obtained as part of a clinical study) where these approaches to measure autophagy are not feasible. Alternative methods to measure autophagic flux have therefore been proposed. The levels of p62/SQSTM1 can be measured, which as noted above is an important cargo receptor for a number of selective autophagy substrates. An increase in p62 can be suggestive of autophagy inhibition while a decrease in p62 is interpreted as an increase in autophagy. A problem here is that p62 is also regulated in other ways. For example it is transcriptionally activated by many stimuli. At this time we have no truly specific marker whose levels alone are indicative only of the amount of autophagy that is taking place.

Functions for Autophagy: Controlling Metabolism and Cell Death

Two of the main related functions of autophagy that impinge on cancer are to control cellular metabolism and cell death. One way that autophagy controls metabolism is by providing breakdown products when macromolecular autophagy substrates are degraded by lysosomal hydrolases. These products are then used for biosynthesis or energy generation. For example, amino acids can feed into the TCA cycle to generate ATP through oxidative phosphorylation. This use of internally generated nutrients and metabolic precursors by the cell is especially important when external nutrients are not available, for example, during starvation. Not surprisingly, adult mice that are suddenly made completely defective in autophagy (by inducible knockout of the *Atg7* gene) display marked changes in metabolism and are very sensitive to starvation. Autophagy is also important in regulating the overall types of metabolism that cells rely on. For example, autophagy has been shown to promote glycolysis in RAS-transformed cancer cells. Autophagic degradation of specific substrates, for example, lipid droplets (a process that is called lipophagy) also affects cellular metabolism. Autophagic turnover of specific organelles (especially mitochondria) that themselves control metabolic pathways also impacts the overall metabolic state of the cell and organism. In cancer biology, another important aspect of autophagy's ability to control metabolism arises because autophagy in one cell can affect the behavior of another cell. One such non cell-autonomous effect occurs when autophagy in pancreatic stellate cells feeds amino acids (particularly alanine) to neighboring pancreatic cancer cells to promote metabolism in the tumor cells that is necessary for tumor cell growth.

Another frequently studied function for autophagy related to cancer is its ability to control cell death with both pro-death and antideath functions having been identified. Indeed autophagy-dependent cell death (sometime called autophagic cell death or Type 2 programmed cell death) is one of the currently defined versions of programmed cell death along with apoptosis (Type 1 programed cell death) and various types of programmed necrosis (Type 3 programmed cell death) such as necroptosis. Autophagy-dependent cell death is also important during normal development as has been best demonstrated in *Drosophila*. In the cancer literature there are many examples where autophagy has been proposed to promote tumor cell killing by anticancer

drugs. In most cases, this conclusion is based on two observations. First, the drug induces autophagy that is then followed by cell death. Second, inhibition of autophagy by knockdown of ATGs and/or the use of pharmacological inhibitors of autophagy reduces the number of tumor cells that are killed. One problem with such studies is that autophagy regulators may be able to regulate cell death/apoptosis by autophagy-independent mechanisms. For example both ATG5 and ATG12 can regulate mitochondrial apoptosis in ways that do not involve the process of autophagy itself. If the cell death mechanism that is being studied requires multiple different ATG proteins, then it is more likely that this is indeed a type of death that requires autophagy. In that event it is also important to determine if this is a case of autophagy itself killing the cell (i.e., true autophagic cell death) or a case of autophagy promoting another form of death such as apoptosis.

Such effects can be highly specific. For example, it has been shown that even in the same population of tumor cells, autophagy is able to specifically promote or inhibit two very similar apoptotic stimuli (the death receptor ligands Fas Ligand/CD95 Ligand and tumor necrosis factor-related apoptosis inducing ligand, TRAIL). In this case, the ability to promote or inhibit apoptosis was achieved through selective degradation of specific proteins. Thus autophagy sensitizes cancer cells to Fas Ligand because autophagy degrades a negative regulator of the Fas Receptor (the protein phosphatase PTPN13). Because the substrate protein that is being degraded only affects one apoptotic stimulus, this mechanism serves to make autophagy-mediated promotion of apoptosis highly stimulus-specific. Such effects can also be cell type-specific because only cells that express PTPN13 showed autophagy-dependent apoptosis by Fas Ligand. The important point is that by targeting specific proteins for autophagic degradation, it is possible for autophagy to display very specific effects on apoptosis. Other specific autophagy substrates that negatively control apoptosis may exist.

Conversely, the ability of autophagy to protect against apoptosis is more general and it is this activity that allows autophagy inhibitors to function as sensitizers to other anticancer drugs. Indeed this is the basis for numerous clinical trials based on preclinical studies where autophagy inhibitors have been shown to promote tumor cell apoptosis by other anticancer agents. Recently it was shown that one of the main mechanisms by which this occurs is through autophagy regulating a core component of the apoptosis machinery that controls release of mitochondrial proteins such as cytochrome *c* during apoptosis. Autophagy targets the protein PUMA/BBC3, but does so indirectly, as autophagy controls the basal transcription rate of the *PUMA* gene by degrading an upstream transcription factor. Thus when autophagy is low, basal PUMA levels are higher and this serves to prime a cell to undergo mitochondrial apoptosis more easily. Interestingly, in this case the ability of chloroquine to sensitize to conventional cytotoxic cancer drugs like etoposide or doxorubicin is achieved through this mechanism via just one transcription factor binding site in the human genome.

Nondegradative Functions for ATG Proteins and Autophagosomal Structures

Another important source of confusion in autophagy research comes from the fact that the ATG proteins that control autophagy have other biological effects that are autophagy-independent. Many of these other functions have important implications for cancer. For example ATG7 regulates the tumor suppressor p53 through autophagy-independent mechanisms. Similarly BECN1 controls cytokinesis, while as noted above, ATG12 and ATG5 control apoptosis and ATG5 also controls MAP kinase signaling. These kinds of effect usually involve a single autophagy regulator having an effect that other ATGs do not. It is therefore imperative to test multiple ATGs, particularly from different parts of the pathway when assessing the role of autophagy in a given system, as results from knock down/knock out of a single protein may not be due to autophagy related functions.

In other cases, we know that there are distinct autophagy-related processes that involve many of the ATGs but are not true autophagy. For example a type of phagocytosis called LC3-associated phagocytosis (LAP) involves most of the ATG proteins that are required for autophagy. LAP can be distinguished from autophagy by its dependence on a component of the Beclin complex called RUBICON, which is a negative regulator of autophagy but a positive regulator of LAP.

Our mechanistic understanding of autophagy-independent roles for ATG proteins and the autophagy machinery lags behind our understanding of how degradative functions of autophagy elicit biological effects. Studies with the FIP200 protein provide a model for how we can know whether the cancer-promoting effects of a protein that works in both autophagy and other cell pathways is autophagy-dependent. FIP200 is required for autophagy induction as part of the ULK1 complex where it binds to ATG13. It is also an important component of focal adhesions where it regulates focal adhesion kinase activity to alter integrin signaling. FIP200 knockout mice are embryonic lethal but this is due to FIP200's autophagy-independent effects as shown by the generation of a mouse with point mutations in FIP200 that ablate only its autophagy-dependent functions by disrupting ATG13 binding without affecting its other roles; these mice survive longer than the FIP200 knockouts and have a similar phenotype to *Atg5* or *Atg7* knockout mice that are deficient in autophagy but not in integrin signaling. In contrast, the same mutant protein was used to show that FIP200's ability to regulate breast tumor growth is in fact dependent on its autophagy function.

In other cases, evidence suggests that the roles of an autophagy regulator that are important for cancer cell behavior may be via functions that are independent of autophagic degradation. For example, the autophagy machinery regulates cellular secretion of cytokines and other molecules and this activity, independent of the degradative functions of autophagy can explain why interference with ATGs prevents breast tumor cell motility and invasion. The important issue as regards cancer is that although the ATGs that regulate autophagy can have important cancer specific functions, these may or may not be due to autophagy and it is necessary to understand the molecular mechanisms on a case-by-case basis to understand how the cancer phenotype that is of interest is controlled.

Autophagosomes and autophagosome-like structures can also serve as signaling hubs or scaffolds. At this time, the full range of signaling pathways that may be affected by autophagosomal scaffolding are incompletely understood. However we do know that two distinct types of cell death—apoptosis and necroptosis—can be regulated by association of signaling molecules on the

autophagosome. These physical associations activate either caspases to induce apoptosis or regulate the necroptosome, which is a protein kinase complex that controls necroptosis. Such mechanisms also demonstrate an important concept mentioned above—autophagy inhibition can have varying effects, depending on which stage of the pathway is inhibited. Thus, in a particular type of cancer (prostate tumor cells with loss of the tumor suppressor gene *MAP3K7*, which encodes the protein kinase TAK1) TRAIL kills cancer cells by necroptosis rather than apoptosis. In this case, necroptosis is caused by recruitment of components of the necroptosome to autophagosomes via the p62 adaptor protein mentioned above. However, because the underlying mechanism is autophagosomal scaffolding rather than autophagosomal degradation, interventions to inhibit autophagy that prevent formation of the scaffold can have opposing effects compared with those that prevent degradation of the scaffold. Thus inhibition of ATG5, ATG7 or the lipid kinase VPS34 that are all needed for formation of autophagosomes inhibit tumor cell necroptosis while inhibition of the lysosome to prevent autophagosome fusion increases tumor cell necroptosis. This establishes the concept that it may not just be important whether or not one inhibits autophagy, but exactly how autophagy is inhibited may determine usefulness in cancer treatment.

Roles of Autophagy in Cancer Development and Progression

Autophagy as an Inhibitor of Cancer Initiation and Development

The first note-worthy connection of autophagy to cancer came when Beth Levine's laboratory identified Beclin 1 as the human homolog of yeast Atg6 based on its ability to interact with the oncogene BCL2. This work also suggested that Beclin 1, which is often deleted in human tumors, could function as a tumor suppressor. The importance of the *BECN1* gene in human cancer has been challenged however because in humans it is located next to the well known tumor suppressor *BRCA1;* this has led to the suggestion that the genomic loss that is often seen in human tumors is actually just a consequence of this proximity rather than an indication that Beclin 1 itself is important for suppressing tumors. However, inactivation of one copy of the *Beclin 1* gene in mice is sufficient on its own to cause cancer showing that in mice at least *Beclin 1* can function as a bona fide tumor suppressor. Pro-tumorigenic effects of genetic deletion of other autophagy regulators in mice including *AMBRA-1*, *Bif-1*, and *Atg4c* and deletion of *Atg5* and *Atg7* have also been shown to induce tumor formation. Interesting puzzles have also arisen from these studies. For example in mosaic *Atg5* −/− animals, tumors (which tended to remain small and not progress) were seen only in the liver despite the fact that autophagy deficient cells occurred in other tissues in the animal as well.

Many cancer risk factors including aging, chronic inflammation and obesity are associated with reduced autophagy. Although such an association suggests that defective autophagy contributes to the increase in cancer risk, it is often difficult to rigorously demonstrate that such associations are causal. However, the current consensus is that although we have much to learn regarding the underlying mechanisms, autophagy can prevent tumor initiation (**Fig. 3**). This ability involves multiple mechanisms. Tumor suppressor activity is due at least in part to autophagy's ability to remove old and damaged organelles such as mitochondria and peroxisomes, which could generate genotoxic reactive oxidative species (ROS). Autophagy also removes damaged proteins that would otherwise cause increased endoplasmic reticulum (ER) stress. These effects in turn result in autophagy being able to reduce genomic mutations and other cell damage. Autophagy has also been implicated in causing tumor cell senescence, which can block tumor progression.

Autophagy's ability to remove specific intracellular material also plays a role in its ability to protect against cancer. A good example is that cancer-causing infectious agents such as *Helicobacter pylori* can be eliminated by a selective form of autophagy called xenophagy. Autophagy's ability to control p62 levels also affects cancer initiation because p62 can act as a pro-tumorigenic protein. In addition to targeting specific substrates for autophagy, p62 is an important signaling molecule that regulates NFκB and the transcription factor NF-E2-related factor-2 (NRF2), which is a transcriptional regulator of antioxidant genes. p62 is also critical in chronic inflammatory states that promote cancer development including pancreatitis and liver inflammation. One study directly implicates p62 as the critical mediator of autophagy's role in regulating cancer development as liver tumor development caused by deletion of the *Atg7* gene is reduced by simultaneous deletion of the *p62* gene.

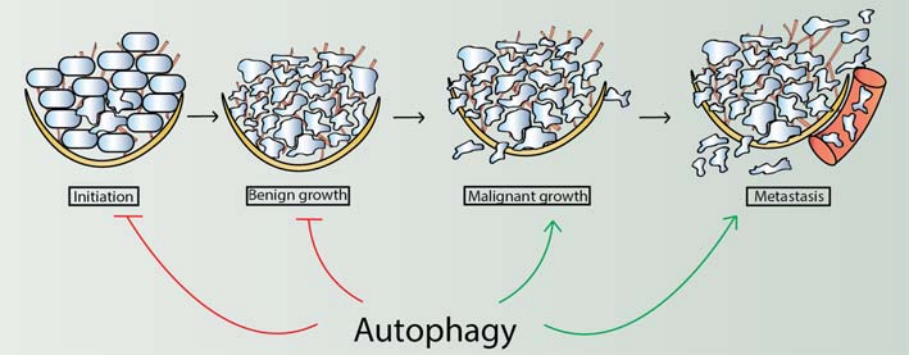

Fig. 3 Autophagy plays a complex role in tumor development where it can be tumor suppressive during early stages of disease progression but is tumor promotional at later stages.

Autophagy as a Promoter of Cancer Progression and Metastasis

Most current evidence suggests that after tumors have formed, the primary role of autophagy is to promote cancer progression. In several mouse models, it has been demonstrated that loss of autophagy regulators (often, but not exclusively through deletion of either *Atg5* or *Atg7* genes) in established tumors can reduce tumor growth and enhance cancer cell death. In lung tumors it has also been shown that deletion of essential autophagy genes can lead to a switch from more aggressive adenocarcinomas to less aggressive tumor types—specifically oncocytomas, which are notable for being associated with elevated levels of damaged mitochondria due to a lack of mitophagy.

In most of the animal studies that have examined the effect of inactivating autophagy regulators on tumor behavior, the autophagy gene knockout has taken place simultaneously with the genetic mutation that drives tumor development. This makes it hard to determine if there are different roles for autophagy at different steps in the tumor evolution. These studies also poorly model the effects of a clinical intervention as most patients are not treated until after they present with an established tumor.

To tackle this issue, more complicated animal models have been used where autophagy inhibition occurs subsequent to tumor development. In one study, this allowed the effects of complete whole body autophagy inhibition by *Atg7* knockout to be determined before or after KRAS-driven lung cancers developed. This approach models a scenario where a perfect autophagy inhibitor that affects all cells in the body and directly targets the autophagy machinery is administered. The effects were dramatic and clearly demonstrate that complete autophagy inhibition in an animal is uniformly lethal but with differing kinetics. Some mice died within a few days while the others died after a 2–3 month period. The first group died due to *Streptococcus* infection, consistent with a known role for xenophagy in eliminating pathogenic *Streptococcus* from cells. The remaining mice all died within a few months due to neurodegeneration. Again, this might have been expected because autophagy protects against aggregated protein-induced neurotoxicity. Additionally, and also as we would expect, the *Atg7*-deleted mice were very sensitive to nutritional stress as fasting for 24 h, a stress that wildtype mice can survive with no problem, was lethal for most of the animals. These toxicities could suggest that autophagy may be too toxic to be useful for cancer therapy. However, when the authors studied the effect of total body inhibition of autophagy after tumor formation specifically during the window of time when animals were healthy prior to neurodegeneration, they discovered that the lung tumors regressed. Interestingly, the antitumor effect was significantly greater when *Atg7* was deleted in all tissues compared with the effect seen when *Atg7* was deleted only in tumor cells. This suggests that autophagy has important effects in nontumor cells that affect the behavior of the tumor cells. Thus, although autophagy is indeed critical for survival of the organism, there may be a potential therapeutic window where autophagy inhibition can eradicate tumors. Gene knockouts, which are permanent and irreversible, are not going to have the same toxicities as drugs, which could be stopped for a period of time and are unlikely to ever completely inhibit a process. Another model used inducible expression of a dominant negative mutant ATG4 molecule that blocks autophagy in a pancreas cancer mouse model. This model is important because it allows reversible inhibition of autophagy by removing the inducer that activates expression of the dominant negative and also allowed intermittent treatment that better mimics the way pharmacological inhibitors of a process are used. Important conclusions from this study were that autophagy inhibition is effective at inhibiting tumor growth (i.e., autophagy is necessary for tumor progression and maintenance) and that these effects involve both tumor cell autonomous and nonautonomous mechanisms of autophagy, raising the issue of exactly what these mechanisms are.

As with its ability to suppress tumor initiation, multiple mechanisms have been identified by which autophagy may promote tumor progression and it is likely that many different mechanisms all play a role in the final behavior of the tumor. Cancer cells are generally sensitized to apoptosis, and one important mechanism by which autophagy protects tumor cell survival is by keeping tumor cells below their apoptotic threshold. Tumor cells are often exposed to nutrient stress and hypoxia; autophagy's well-established roles in protecting cells against these stresses can promote tumor cell survival under stress. Autophagy has also been shown to be important in tumor cell motility through multiple mechanisms including autophagy-dependent secretion of cytokines, turnover of cytoskeletal proteins that are important in regulating cell motility and regulation of small GTPases of the Rho family. Autophagy has been reported to be important for maintaining cancer stem cell-like activity and for allowing dormant tumor cells to survive. All of these mechanisms could contribute to increased metastasis and there are numerous reports that metastatic cancer cells have elevated autophagy. Most of these effects are tumor cell autonomous, however, there have been several studies that show important nontumor cell autonomous functions for autophagy. The best understood example shows that autophagy in the tumor microenvironment (e.g., pancreatic stellate cells) produces amino acids that are used to feed metabolic pathways in cancer cells.

Autophagy and the Immune Response to Cancer

Another important aspect of how autophagy affects tumor progression comes from the relationships between autophagy and the cancer immune response. However, as with cell death both tumor-promoting and tumor-suppressing functions have been identified. Autophagy can stimulate antitumor immune responses through several mechanisms. The adaptive antitumor immune response requires that tumor antigens are recognized by the immune system. Autophagy is important in antigen presentation by MHC class I and II molecules to T cells. Autophagy is also important in ensuring T cell maturation and survival. Activation of an effective T cell response to tumors requires that cancer cells die via an "immunogenic" type of death that involves exposure of calreticulin on the surface of the dying cell and it releasing damaged-associated molecular patterns (DAMPs) such as HMGB1 as well as ATP. Both DAMP and ATP release are affected by autophagy in the dying cell. All these effects suggest that autophagy is necessary for creating an effective antitumor immune response. Conversely autophagy can work against other forms of immune

control of cancer, by for example, reducing the ability of natural killer (NK) cells to kill cancer cells. Emerging data on the nondegradative role of autophagosome scaffolding on the apoptotic/necroptotic switch in cell death adds new issues to consider since necroptosis is especially potent at inducing antitumor immunity.

Targeting Autophagy in Cancer Treatment

Autophagy Inhibition as an Anticancer Treatment

It is clear that autophagy has context dependent roles in cancer that often compete with each other, thus it may seem unclear whether we should try to inhibit or enhance autophagy as a cancer treatment. Despite these controversies, there are dozens of clinical studies where autophagy inhibition is being tested as a cancer treatment. Indeed, although autophagy is involved in many diseases, cancer treatment with autophagy inhibitors make up the majority of all the clinical studies where attempts to deliberately manipulate autophagy to treat disease are being pursued. In addition, because autophagy is affected by many other treatments, autophagy is inadvertently being manipulated in other cases as well—for example, when a patient is treated with an mTOR inhibitor or a microtubule inhibitor. To date, attempts to deliberately inhibit autophagy as a cancer therapy have all used the lysosomal inhibitors chloroquine or hydroxychloroquine and most of the studies have involved combination therapy where the autophagy inhibitor is combined with another anticancer drug. These studies are based on a large number of in vitro and xenograft studies that followed an initial study by Amaravadi and colleagues showing that by inhibiting autophagy, another drug can be made more effective at killing cancer cells. As noted above this usually involves direct regulation of the apoptosis machinery by autophagy. This kind of chemosensitization implies that the other drug must have some activity on its own. In addition, it has been shown that autophagy inhibition can reverse drug resistance and thus make a drug that has no activity for a given tumor cell become active. For example, acquired resistance to a kinase inhibitor that targets mutated BRAF can be reversed by autophagy inhibition. Important issues that are under investigation include determination of which drugs work best in combination with autophagy inhibitors and which patients are best candidates for these therapies.

Although the current efforts to deliberately target autophagy in clinical trials focus on autophagy inhibitors, it has also been suggested that we should try to further enhance autophagy in cancer patients. The most persuasive argument for this strategy comes from studies showing that interventions that mimic caloric restriction can enhance the antitumor immune response through mechanisms that are dependent on induced autophagy.

Clinical Experience in Targeting Autophagy

Clinical response to autophagy inhibition using hydroxychloroquine and chloroquine has varied widely (see Levy et al. in the Further Reading section for more details) with some signs of clinical improvement but many cases where there was no apparent benefit. It is important to note that many of these studies have been performed in patients with advanced disease and this may have limited the potential for any benefit to be seen. Additionally, we know from several of the clinical studies that there is inconsistent autophagy inhibition in patients at clinically achievable doses of the lysosomal inhibitors that have been used. There is also marked variation in the bioavailability of hydroxychloroquine and different tumors accumulate very different levels of the drug. Encouragingly, most of the studies have not caused serious toxicity in patients. In particular, there have been no signs in patients of the toxicities that were observed in adult mice with inducible and complete inhibition of autophagy by *Atg* gene knockout (i.e., susceptibility to bacterial infection, greatly altered metabolism and neurodegeneration). This likely reflects the fact that unlike gene knockout, chloroquine and hydroxychloroquine are not 100% effective as autophagy inhibitors. Interestingly, the toxicities that have been seen, for example, in a canine clinical trial with doxorubicin plus hydroxychloroquine or in human patients treated with BRAF inhibitor plus chloroquine, were enhancement of the toxicities for the drug that was combined with the autophagy inhibitor. This suggests that systemic autophagy inhibition might also sensitize normal tissues to the anticancer drug that was used in combination. In most of the clinical studies to date there has been no attempt to define a particular subgroup of patients that may benefit most from autophagy inhibitors. This may explain the variability and low levels of clinical benefit that have been seen—perhaps only some defined subtypes of cancer will respond. One such subgroup may be patients whose tumors harbor a BRAF mutation. For example, all the BRAF mutant pediatric brain cancer patients whose tumors had become resistant to the RAF inhibitor, Vemurafenib, that were subsequently treated with RAF inhibitor plus chloroquine experienced clinical improvement. While only a few patients have been treated with this therapy, these initial studies suggest promising results for BRAF mutant tumors.

Current Efforts to Improve Autophagy-Based Therapies

The preclinical and clinical studies outlined above suggest that our current autophagy inhibitors are not effective enough. This has led to a significant effort to make more potent and selective inhibitors. These efforts have focused on targeting other molecules (e.g., the kinases ULK1/ULK2 or VPS34) as well as making more potent lysosome inhibitors. An exciting recent advance was the discovery that dimerization of antimalarials such as quinacrine can markedly enhance their potency and specificity as autophagy inhibitors. There are many options for autophagy enhancers (e.g., mTOR inhibitors and many other drugs, but also nutraceuticals such as trehalose and even potential behavioral interventions like exercise) but they all have other effects and there is, as of yet, no available agent that can be used in patients that would be considered a specific autophagy inducer.

Another major effort is to identify useful biomarkers where autophagy based interventions will be most useful. The BRAF mutation status may be useful and certain tumor types that are especially dependent on autophagy such as pancreas cancer may respond better than other tumor types. However at this time we have no definitive biomarker that would serve as a selection method for autophagy-based treatment. Perhaps the biggest barrier to improving autophagy-based therapy is that we lack an effective method to measure changes in autophagy in clinical samples. Measuring changes in autophagy in cultured cells is often problematic even when molecular biology techniques are readily available in these systems including the ability to introduce fluorescent proteins and acutely block the lysosome to measure autophagic flux. These problems are magnified significantly when it comes to studies in vivo in laboratory animals and magnified again in clinical studies where available tissue is often limited to biopsy samples before and after treatment. At this time the field lacks a reliable way to assess differences in autophagy in small tissue samples and instead must rely on correlative and usually nonquantitative studies. As progress is made on these problems and more effective manipulators of autophagy are developed it should become more feasible to rigorously test the value of autophagy-based treatments.

Prospective Vision

In the last 20 years there has been very rapid growth in our understanding of autophagy and its roles in cancer. And, although cancer is only one of very many diseases where autophagy plays a role, efforts to translate our basic understanding of autophagy to the clinic are further advanced in cancer than they are in other diseases. However, we have many areas for improvement. We have little understanding of exactly how autophagy elicits its biological effects and numerous important questions remain. What cargos are being degraded for autophagy to have a given biological effect? What is the role of nondegradative functions of the autophagy machinery in the biology? Is it possible to selectively manipulate autophagy so that only some cargos are affected? Would this make autophagy-based treatments better? How do we reconcile the various competing effects of autophagy in cancer to maximize the benefit of any intervention? Continued progress in answering these and other questions seems assured given the high level of activity in this field.

Further Reading

Amaravadi, R., Kimmelman, A.C., White, E., 2016. Recent insights into the function of autophagy in cancer. Genes and Development 30, 1913–1930.
Bento, C.F., Renna, M., Ghislat, G., et al., 2016. Mammalian autophagy: How does it work? Annual Review of Biochemistry 85, 685–713.
Fitzwalter, B.E., Thorburn, A., 2015. Recent insights into cell death and autophagy. The FEBS Journal 282, 4279–4288.
Galluzzi, L., Pietrocola, F., Bravo-San Pedro, J.M., et al., 2015. Autophagy in malignant transformation and cancer progression. The EMBO Journal 34, 856–880.
Kimmelman, A.C., White, E., 2017. Autophagy and tumor metabolism. Cell Metabolism 25, 1037–1043.
Klionsky, D.J., Abdelmohsen, K., Abe, A., et al., 2016. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12, 1–222.
Levy, J.M.M., Towers, C.G., Thorburn, A., 2017. Targeting autophagy in cancer. Nature Reviews Cancer 17, 528–542.
Mowers, E.E., Sharifi, M.N., Macleod, K.F., 2017. Autophagy in cancer metastasis. Oncogene 36, 1619–1630.
White, E., 2012. Deconvoluting the context-dependent role for autophagy in cancer. Nature Reviews Cancer 12, 401–410.
Zhong, Z., Sanchez-Lopez, E., Karin, M., 2016. Autophagy, inflammation, and immunity: A troika governing cancer and its treatment. Cell 166, 288–298.

Bladder Cancer: Pathology, Genetics, Diagnosis, and Treatment

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Abbreviations

BCG Bacillus Calmette-Guerin
BTA Bladder tumor-associated antigen
CIS Carcinoma in situ
CK Cytokeratin
CMV Cisplatin, methotrexate, and vinblastine
CT Computed tomography
ddMVAC Dose-dense MVAC
FDA US food and drug administration
FISH Fluorescence in situ hybridization
GC Gemcitabine plus cisplatin
hCFHrp Human complement factor H-related protein
MIBC Muscle-invasive bladder cancer
MRI Magnetic resonance imaging.
MVAC Methotrexate, Vinblastine, Doxorubicin (Adriamycin), Cisplatin
NCCN US National Comprehensive Cancer Network
NMIBC Non-muscle-invasive bladder cancer
PET Positron emission tomography
PUNLMP Papillary urothelial neoplasm of limited malignant potential
TURB Transurethral resection of the bladder
TURBT Transurethral resection of bladder tumor

Definition and Classification

There are five major histological types of urinary bladder cancer, including non-invasive and invasive urothelial carcinoma, as well as squamous cell carcinoma, adenocarcinoma, and small cell carcinoma.

By far the most common type is urothelial carcinoma which accounts for about 80%–90% of all bladder cancers worldwide (90% in industrialized countries). Urothelial carcinoma of the urinary bladder (ICD-O code: 8120/3; also called transitional carcinoma or transitional cell carcinoma) is a malignant neoplasm arising from the transitional epithelium lining the bladder. It may have a flat or papillary configuration. According to the TNM classification system, papillary tumors confined to the mucosa are classified as stage Ta and those invading the lamina propria as stage T1. Flat, high-grade tumors that are confined to the mucosa are classified as carcinoma in situ (CIS or Tis). For therapeutic purposes, some of the current treatment recommendations group these three tumor categories under one heading of non-muscle invasive bladder cancer (NMIBC) as opposed to muscle-invasive bladder cancer (MIBC; T2 and higher). However, the malignant potential of Cis and T1 lesions has been shown to differ from that of Ta lesions. Therefore, the term "NMIBC" may be considered to be suboptimal and should be used with caution.

Presentation and Diagnosis

Approximately 70%–75% of bladder cancer patients present with a disease confined to the mucosa (stage Ta, carcinoma in situ (CIS)) or—less often—to the lamina propria (stage T1). The most common symptom and presenting complaint is hematuria. However, the severity of symptoms depends on tumor stage and early-stage non-invasive urothelial carcinomas are often asymptomatic or associated with non-specific symptoms.

The classical clinical presentation of bladder carcinoma is painless gross hematuria. Depending on the size and the surface condition of the lesion (e.g. ulceration), increased urination frequency (nocturia) and urgency as well as dysuria may also occur. If the

cancer involves the site of implantation of the ureter(s), hydronephrosis may develop, which may be complicated by pyelonephritis. More advanced tumors may be associated with pelvic pain and urinary obstructive symptoms as well as—in case of large tumor masses—with a palpable suprapubic mass or lower extremity edema.

Urine cytology has a relatively low sensitivity for detecting bladder cancer. Therefore, a number of urine biomarker tests have been developed (see "**Biomarkers**" section). However, due to conflicting data on their accuracy and clinical utility, their use is still limited and none of them has been recommended for diagnosis or follow-up of bladder cancer patients by any official guidelines. Cystoscopy, bimanual examination, and biopsy or transurethral resection remain the highest sensitivity techniques for the diagnosis and staging of urinary bladder carcinoma. Newer magnetic resonance imaging (MRI) techniques may be useful in staging, particularly in patients with tumors in difficult locations, or to distinguish between confined tumors and extravesical fat invasion. Although lymph node size is not a reliable indicator of metastasis, lymph node enlargement is considered to be predictive of metastatic disease. Positron emission tomography (PET) imaging has been reported to be superior to computed tomography (CT) and MRI in the detection of lymph node metastases.

The most important element in pathologic evaluation of bladder cancer is recognition of the presence and extent of invasion. Invasive urothelial carcinoma shows a remarkable diversity of morphological manifestations. It can present with a wide range of architectural patterns, including variably sized nests with smooth borders, sheets, trabeculae, cords, and single cells. There is often a mixture of patterns. In most tumors, there is no specific feature that resembles normal urothelium. Some data have indicated that invasion patterns have prognostic significance, with more infiltrating cords and single cell patterns being associated with a worse prognosis.

Epidemiology and Risk Factors

Bladder Cancer Burden

Nearly 430,000 new bladder cancer cases were diagnosed worldwide in 2012 (330,380 in men and 99,413 in women), making it the 11th most common cancer in the world when considering both sexes together and the 7th most common in men. The highest age-standardized incidence rates were recorded in Turkey (15.24), followed by Hungary (14.08), Spain (13.92), and Norway (13.45). Overall, 72% of cases were diagnosed in countries with a high or very high human development index (**Fig. 1**).

Despite high recurrence rates, bladder cancer has a relatively good prognosis compared to many other cancers, if treated rapidly following diagnosis. With 165,000 deaths in 2012, it was the 13th cause of cancer-related mortality.

By the year 2030, the global bladder cancer incidence is supposed to rise by about 280,000 cases, with approximately 135,000 in less developed countries. The predicted number of deaths is nearly 280,000 (156,000 in less developed countries).

There has been a decline in bladder cancer incidence and mortality in developed countries but some rising trends have been observed in Eastern Europe and in certain countries in developmental transition. However, the trends are difficult to interpret due to much variability in diagnostics and treatment practices as well as the availability of treatments between different countries. Moreover, the reported variations may also reflect differences in the quality of data collection and cancer registration.

Etiology and Risk Factors

Men are more than four times more likely to get bladder cancer than women. Ethnicity does not seem to play a role. Recent studies have identified a number of putative genetic susceptibility loci. However, a number of conflicting results have been reported and the data remain to be clarified.

Smoking, and in particular cigarette smoking, is the leading cause of bladder cancer, with up to 40%–50% of male cases attributable to smoking. The risk of developing bladder cancer is two- to sixfold higher in smokers than never-smokers, and it is correlated with the duration and intensity of smoking, while smoking cessation reduces the risk over time. The underlying carcinogenic mechanisms have not been fully elucidated but they are clearly linked to mutagens contained in the tobacco smoke, such as aromatic amines which trigger carcinogenesis through the formation of DNA adducts. Along the same line, occupational exposure to aromatic amines has been shown to cause bladder cancer and it has been suggested that their carcinogenic effect may be synergistic with that of smoking. A number of other occupational exposures have also been linked to an increased risk of bladder cancer (**Table 1**) which has been widely recognized as an occupational disease.

Arsenic-contaminated drinking water, in particular in parts of South-East Asia and in Chile, has been proven to cause bladder cancer. There is also evidence that disinfection by-products, like chemicals generated by water chlorination, could increase the risk of the disease.

Chronic bladder inflammation is a major risk factor for developing squamous cell carcinoma of the bladder. Schistosomiasis, chronic infestation with the parasitic trematode (blood fluke) *Schistosoma haematobium*, has been associated with bladder cancer development in *S. haematobium* endemic countries, with affected individuals having a 2–15-fold increased risk of developing bladder cancer compared with non-affected subjects. Continuous mucosal irritation and chronic inflammation, associated with parasitic infestation but also with the use of indwelling catheters, can cause squamous metaplasia, dysplasia, and eventually

squamous cell carcinoma of the bladder. For this reason, patients with long-term paraplegia are also at a markedly elevated risk for this tumor type.

Pathology and Genetics

Most neoplasms of the bladder are epithelial. Epithelial neoplasms are most often of transitional epithelial (urothelial) type. However, as stated in the introduction, adenocarcinomas, squamous cell carcinomas and small cell carcinomas also occur, although much less frequently than urothelial neoplasms. Urothelial neoplasms are not limited to the bladder, they occur in the entire urinary tract (including renal pelvis and ureters).

The transitional epithelial (urothelial) tumors are rather heterogeneous in morphology and behavior. Non-invasive urothelial neoplasms are precursor lesions of invasive urothelial carcinoma. Two types of precursor lesions have been identified: papillary neoplasms and flat urothelial carcinoma in situ. Papillomas represent the benign end of the spectrum while—at the other end—high-grade papillary carcinomas are very aggressive lesions. These will be addressed extensively whereas squamous cell carcinoma will be only briefly discussed. Mixed carcinomas are composed of a mixture of cell types. Adenocarcinomas are rare; most show an intestinal phenotype. Some adenocarcinomas occur in the urachal vestige of the bladder and presumably

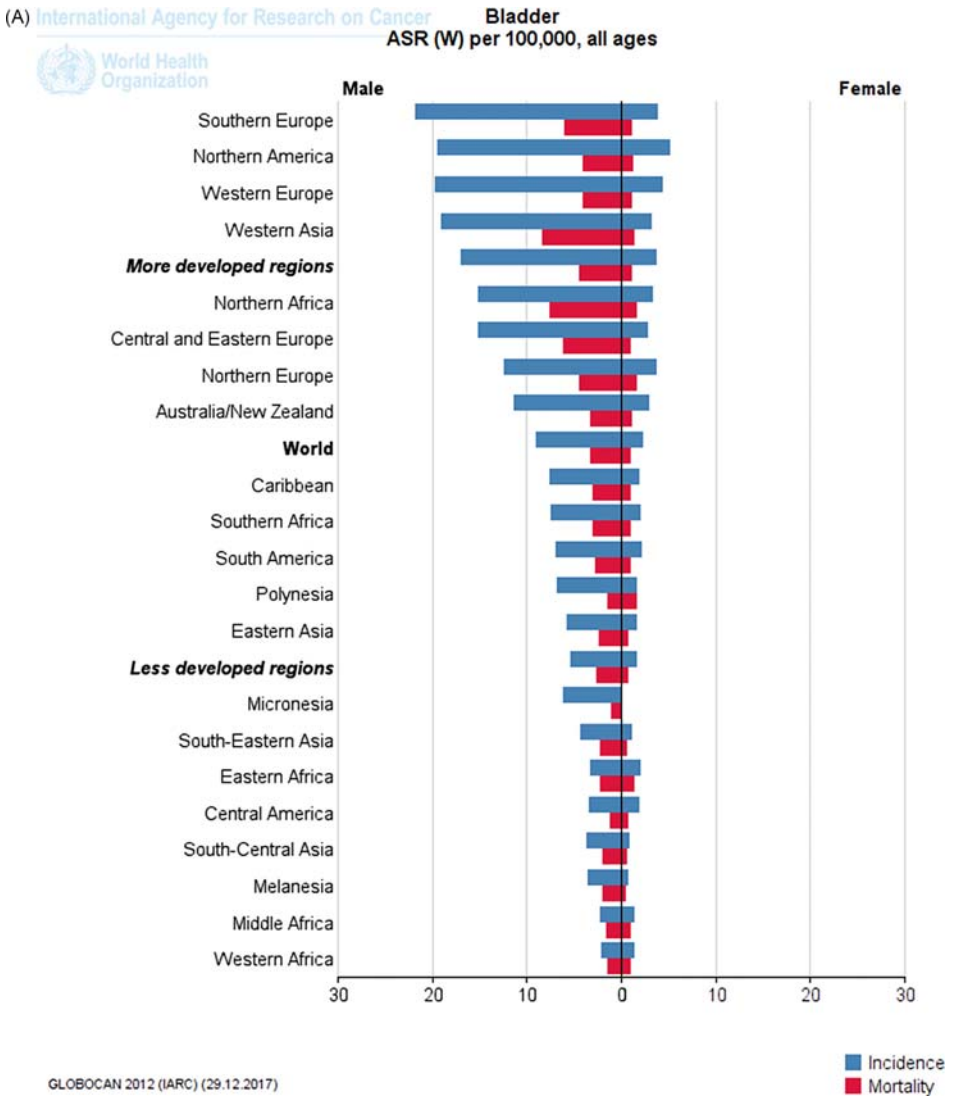

Fig. 1 Incidence and mortality of bladder cancer worldwide. (A) Age-standardized incidence and mortality rates (ASR) by gender and geographical area. (B) Incidence distribution worldwide. From Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. and Bray, F. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet].* Lyon, France: International Agency for Research on Cancer. Available from http://globocan.iarc.fr (accessed on February 20, 2018).

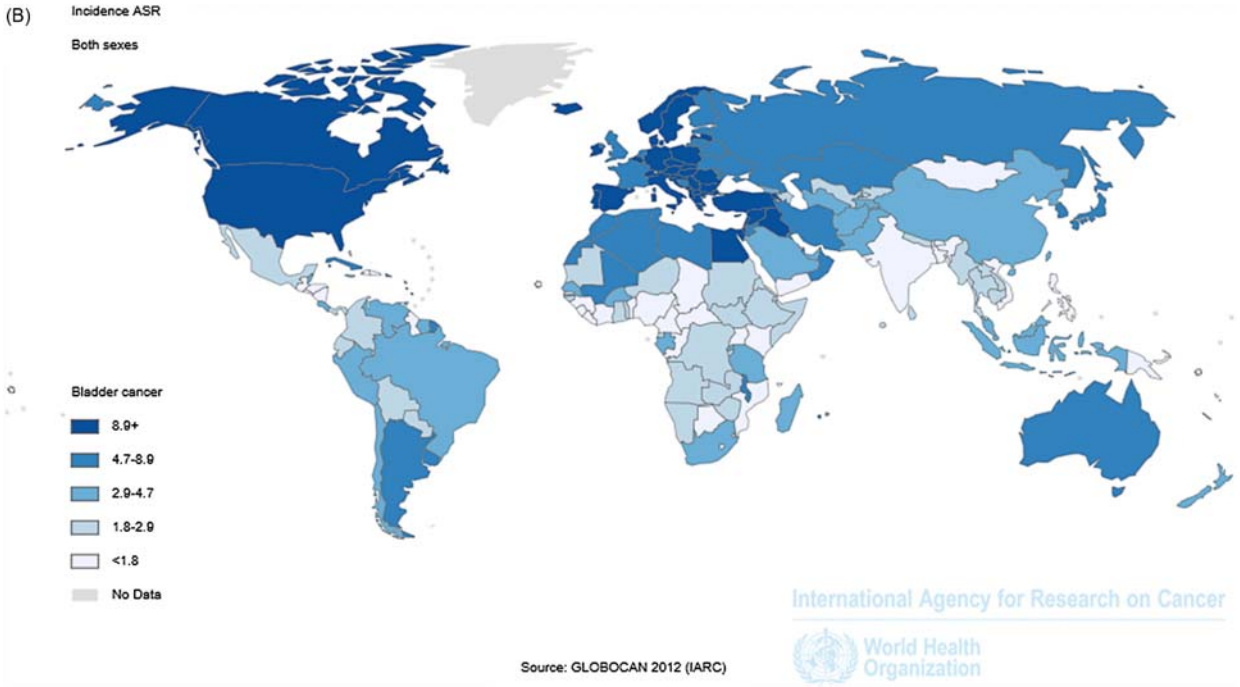

Fig. 1 (*continued*).

develop from areas of intestinal metaplasia. These will not be further discussed. Small cell carcinoma is rare and is similar to small cell carcinoma of the lung in morphology, biological behavior and molecular characteristics. Sarcomas are rare and will not be further discussed.

Table 1 Risk factors for urinary bladder cancer

Carcinogenic agents classified by *IARC Monographs on the evaluation of carcinogenic risks to humans* (volumes 1–120)
Agents of sufficient evidence for bladder carcinogenicity in humans
Aluminum production
4-Aminobiphenyl
Arsenic and inorganic arsenic compounds
Auramine production
Benzidine
Chlornaphazine
Cyclophosphamide
Magenta production
2-Naphthylamine
Painting
Rubber production industry
Schistosoma haematobium infection
Tobacco smoking
ortho-Toluidine
X-radiation and gamma-radiation
Agents of limited evidence for bladder carcinogenicity in humans
4-Chloro-ortho-toluidine
Coal-tar pitch
Dry cleaning
Diesel engine exhaust fumes
Hairdressers and barbers, occupational exposure
2-Mercaptobenzothiazole
Pioglitazone
Printing processes
Soot
Textile manufacturing
Tetrachloroethylene

Pathology of Non-Invasive Urothelial Neoplasms

Papilloma

Papillomas are relatively rare, representing about 1% of bladder tumors. They typically appear in young patients. Cystoscopically, papillomas are small (0.5–2.0 cm) lesions protruding with papillary fronds into the lumen of the bladder. Histologically, these are composed of a central fibrovascular stalk, covered with bland epithelial cells much like normal urothelium. Occasionally, such lesions do not extend exophytically but endophytically into the underlying stroma, but without passing through the epithelial basement membrane. These are called inverted papillomas. Papillomas are benign lesions which rarely recur or progress after endoscopic excision. Nonetheless, long-term follow-up is necessary as there are no morphological characteristics identifying those papillomas with a tendency to recur.

Papillary urothelial neoplasm of limited malignant potential (PUNLMP)

PUNLMPs mostly occur at advanced age (mean: 65 years). They are cystoscopically and histologically very similar to papilloma, but the epithelium covering the papillary fronds shows minimal atypia: it is thicker and the nuclei are slightly enlarged (Fig. 2A). PUNLMPs tend to be somewhat larger than papillomas. There is very limited proliferative activity; mitoses are rare and limited to the basal layer. PUNLMPs have a slight tendency to recur but—as a rule—the morphology does not change in recurrent lesions. Only rarely do they progress to high-grade lesions with invasive and metastatic capacity. This category has been developed to avoid a diagnosis of cancer for patients with a lesion that has a very low tendency for progression. Long-term follow-up, however, is advised.

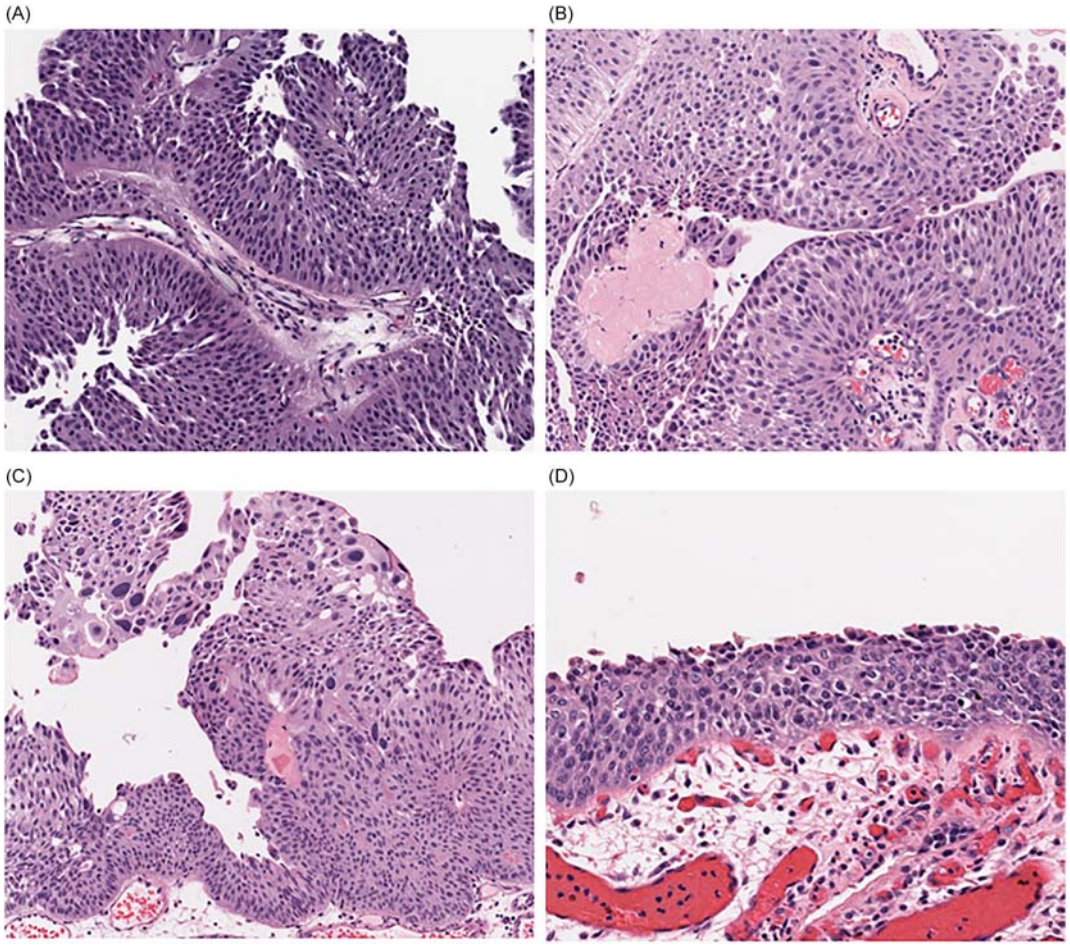

Fig. 2 Non-invasive urothelial neoplasms. (A) Papillary urothelial neoplasm of unknown malignant potential (PUNLMP). Papillary fronds are composed of a central fibrovascular core covered with normal looking urothelial cells but slightly thickened. (B) Papillary urothelial carcinoma, low-grade. Irregular papillary architecture and thickening of the epithelium. Epithelial cells show some loss of cohesion and focally of polarity. There is slight nuclear pleomorphism. (C) Papillary urothelial carcinoma, high-grade. The most important difference with low-grade papillary urothelial carcinoma is the striking nuclear pleomorphism and the presence of mitoses. There is no tendency towards invasive growth. (D) Urothelial carcinoma in situ. The mucosa is covered with an irregular urothelium with loss of epithelial stratification and cytonuclear atypia; no invasive growth. Note hyperemia and edema in the underlying lamina propria.

Low-grade papillary urothelial carcinoma

Low-grade papillary urothelial carcinomas characteristically show a rather regular architecture and limited cytonuclear atypia. The papillary fronds tend to branch. The epithelium is thickened and the epithelial cells may show some loss of polarity at higher magnification (Fig. 2B). Nuclei show some hyperchromasia and pleomorphism. Some mitotic activity is noted, not necessarily limited to the basal layer. Atypical mitoses are not found. Low-grade papillary urothelial carcinoma can recur and ultimately invade, although the latter is rare (10%).

The prognosis of papillomas, papillary urothelial neoplasms of low malignant potential, and low-grade papillary urothelial cancer is excellent, with 10-year survival rates of 98%. Even though there may be several recurrences, the tendency for progression is low.

High-grade papillary urothelial carcinoma

High-grade papillary urothelial cancers show more architectural disturbance. Papillary fronds tend to fuse, giving them a more solid appearance. The epithelium is often thickened but thickness can be quite variable. There is more striking cytonuclear atypia with loss of cell polarity, loss of cellular cohesion, nuclear hyperchromasia and pleomorphism (Fig. 2C). Mitotic activity is brisk and atypical mitoses are easily found. High-grade urothelial cancers have a much stronger tendency to progress and invade the muscular layer (80%). Once invasive, they have significant metastatic potential.

Carcinoma in situ

As stated above, invasive urothelial carcinoma has two precursor lesions, the second one being flat non-invasive urothelial carcinoma in situ (CIS). The histological term "CIS" is used for epithelial lesions that have cytonuclear characteristics of malignancy but do not invade through the epithelial basement membrane, being limited to the epithelium, hence the suffix in situ. CIS is invariably considered as high-grade. CIS tends to be multifocal and occasionally spreads diffusely over the bladder mucosa. Cystoscopically, it often appears as slightly elevated erythematous plaque but some CIS have more the aspect of an erosion.

CIS is composed of cytologically atypical cells that may occupy the full thickness of the epithelium or spread into normal urothelium in a pagetoid fashion. There is striking nuclear pleomorphism, with some nuclei 5–6 times as large as those of a lymphocyte. They tend to have one or more irregular nucleoli. Mitotic activity is significant and atypical mitoses can be found (Fig. 2D). Foci of micro-invasion are occasionally present. A lack of cohesiveness of CIS cells is responsible for shedding of the cells which may then colonize other areas of the bladder mucosa, one of the purported mechanisms of multifocality. CIS has a high tendency to progress into muscle-invasive cancer, with over 50% of cases which progress if untreated.

Molecular Carcinogenesis of Non-Invasive Urothelial Lesions

Molecular characteristics of the various non-invasive urothelial lesions are rather similar. The current working hypothesis regarding bladder carcinogenesis suggests that the initial event is a field effect, involving multiple sites in the urothelium. Supposedly, multiple oncogenic events transform stem cells in the affected areas of the urothelium into cancer stem cells. When these clonally expand, tumors are formed. Two different pathways have been singled out as affected by the initial event: the FGFR3- and p53-associated pathways which generate lesions differing in biological behavior and clinical outcome. Activating *FGFR3* mutations are associated with non-invasive lesions, while *TP53* mutations, or mutations in genes regulating p53, such as *CDKN2A*, are associated with high-grade lesions. Loss of heterozygosity of (part of) chromosome 9 is strongly associated with non-invasive lesions. Interestingly, this has been found also in normal mucosa adjacent to non-invasive lesions, supporting the field effect concept.

Pathology of Invasive Urothelial Carcinoma

Around 90% of invasive urothelial carcinomas are localized to the bladder, the remainder in the upper urinary tract. Invasive urothelial carcinomas can be unifocal or multifocal. Grossly, they may be sessile, polypoid, or ulcerative. Occasionally, invasive urothelial cancer is accompanied by papillary urothelial cancer which will be typically high-grade, or by CIS. The carcinoma usually extends into the bladder wall but, given the proximity of these structures, the adjacent prostate, seminal vesicles, ureters, or retroperitoneum may be involved. Aggressively invasive tumors with extensive necrosis may develop fistulae to rectum or vagina. Deeply invasive tumors are often metastatic to local lymph nodes (40%). Late-stage tumors show hematogenous dissemination to the liver, lungs and bone marrow.

Microscopically, urothelial carcinoma shows an astonishing array of growth and differentiation patterns. Growth pattern may be in sheets, nests, trabeculae, or single cells (Fig. 3A). As a rule, the tumor cell characteristics are those of a high-grade carcinoma, with striking nuclear pleomorphism, high mitotic activity and atypical mitoses. Often, the cytomorphology bears no resemblance to urothelium. Various divergent differentiation patterns have been recognized (squamous, glandular, trophoblastic, nested, microcystic, and micropapillary). These patterns have been associated with differences in prognosis but with the advent of molecular typing these may lose relevance.

A very important element is the presence and, if present, the extent of invasion of the carcinoma. Invasion starts with breaking through the epithelial basement membrane and migration of the invasive cells into the lamina propria (Fig. 3B). This worsens the prognosis, but invasion through the muscularis mucosae has been found to be a major prognostic determinant. In a significant proportion of biopsies, muscularis mucosa is not present and this may lead to understaging. In fact, on a small biopsy of a large

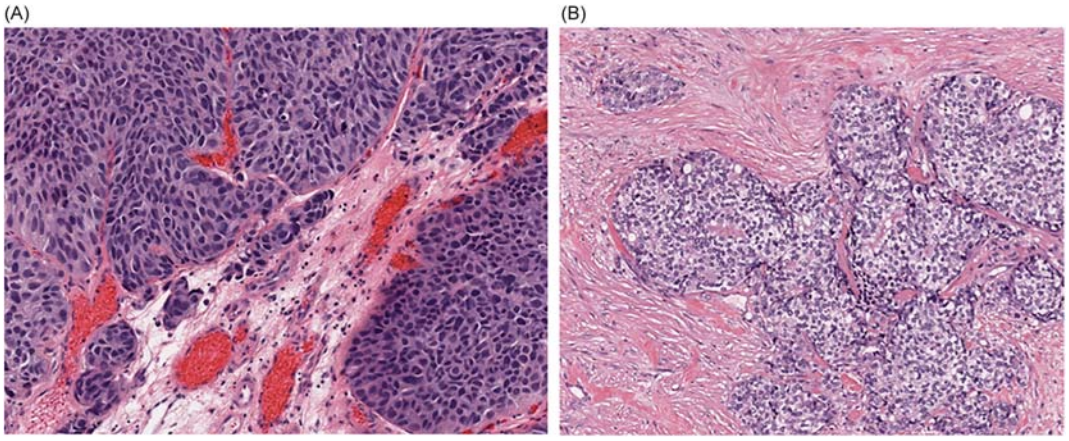

Fig. 3 Invasive urothelial carcinoma. (A) Large clusters of highly atypical urothelial cells are seen. Small clusters of carcinoma cells are found in the adjacent stroma, as evidence of invasion into the lamina propria. (B) Muscle-invasive urothelial carcinoma. Large clusters of atypical urothelial cells are surrounded by bundles of smooth muscle cells, evidence of invasion into the muscularis propria of the bladder.

lesion, the absence of invasion through the muscularis mucosae bears no significance. Only when this can be found already in the biopsy, this is significant information. Grading of invasive urothelial carcinomas is of minor importance as these are almost invariably high-grade. This is in contrast to the role the assessment of invasion plays in biopsies of non-invasive papillary urothelial carcinoma. For invasive lesions, the next step is invasion into the muscularis propria (detrusor) of the bladder wall (**Fig. 3**C). The 5-year mortality rate of patients with a detrusor muscle-invasive urothelial carcinoma is around 30%.

After resection of a urothelial carcinoma, there is a high recurrence rate, whatever the grade of the resected tumor. Tumor size, stage, grade, and multifocality or CIS in the surrounding mucosa are determinants of the risk of recurrence. Recurrence might not be the most appropriate term for this finding as studies have shown that many of the recurrent lesions are not clonally related to the original carcinoma and represent new lesions, in accordance with the field-effect concept. Some distant new lesions may share driver mutations with the original carcinoma, which can be taken as evidence of intravesical shedding and implantation of cancer cells at distant intravesical sites.

Urothelial carcinoma metastases may pose diagnostic difficulties in terms of identification of the primary tumor. The International Society of Urologic Pathology suggests that positivity for GATA3, CK20, p63, and either high molecular weight cytokeratin or CK5/6 as determined by immunohistochemistry may have some value to confirm urothelial differentiation and support differential diagnosis in the appropriate context. However, they clearly state that there is no single ideal biomarker or a panel to equivocally confirm urothelial differentiation (see also "**Biomarkers**" section).

Genetics of Urothelial Bladder Carcinoma

Unsupervised analysis of RNA-Seq data from a cohort of 1372 patients with non-muscle invasive bladder cancer (NMIBC) has led to the identification of three molecular subtypes (class 1–3) that differ by their histomolecular characteristics and by their risk of progression to MIBC. Class 1 tumors resembled the "luminal" subtype and had the best prognosis, class 2 was enriched with carcinoma in situ (CIS) and with high epithelial-to-mesenchyme transition markers, whereas class 3 tumors frequently expressed markers of bladder cancer stem cells and were more similar to "basal cell-like" subtype. T1 and high-grade tumors were more frequently seen in classes 2 and 3 than in class 1, and the majority of progression events were observed in patients with class 2 tumors. Since progression to MIBC is associated with a 50% 5-year survival, the early identification of tumors with a high risk of progression is a significant clinical challenge. Because MIBC likely arises from CIS, these findings suggest that class 2 tumors may have a genomic profile associated with an increased risk for progression. If this is validated prospectively, patients with class 2 NMIBC may benefit from definitive therapy with radical cystectomy.

A recent comprehensive analysis of 412 muscle-invasive bladder cancer (MIBC) by the Cancer Genome Atlas Research Network and collaborators has identified 58 often mutated genes as well as an overall high mutational load associated with *APOBEC*-signature mutagenesis (**Table 2**). Copy number gains occurred at loci involving *E2F3*, *PPARG*, and *MDM2*. The most commonly deleted genes were *CDKN2A* and *RB1*, whereas fusions commonly involved *FGFR3* and *PPARG*. The top most frequently mutated genes were *TP53*, *KMT2D*, *KDM6A*, *ARID1A*, *PIK3CA*, *KMT2C*, *RB1*, *EP300*, *FGFR3*, *STAG2*, and *ATM*. A classification of MIBC based on their mutagenic signatures identified four clusters (MSig1 to MSig4) characterized by *APOBEC a* and *b*, *ERCC2* and C>T at CpG sites, respectively, which were associated with overall survival. In particular, patients with MSig1 cancers (high *APOBEC* signature and high mutation burden) showed an exceptional 75% 5-year survival probability, whereas the *ERCC2* signature was more prevalent in smokers than in non-smokers.

Several groups have proposed integrative classifications of bladder cancers based on histomolecular features. An analysis of four independently published comprehensive gene expression datasets has identified several intrinsic molecular subtypes of high-grade

Table 2 Gene loci found to be most frequently altered in muscle-invasive bladder cancer in the recent Cancer Genome Atlas (TCGA) Research Network study

Pathway	*Gene (locus)*	*Alteration type*	*Estimated frequency (%)*
p53 signaling/regulation of cell division, DNA repair and apoptosis	*TP53* (17p13.1)	Point mutations	48
	E2F3 (6p22.3)	Amplifications	12
	MDM2 (12q15)	Amplifications	12
	RB1 (13q14.2)	Deletions and point mutations	17
	CDKN2A (9p21.3)	Deletions	22
	STAG2 (Xq25)	Point mutations	14
	ATM (11q22.3)	Point mutations	14
Chromatin remodeling/histone methylation	*KMT2D* (12q13.12)	Point mutations	28
	KDM6A (Xp11.3)	Point mutations	26
	KMT2C (7q36.1)	Point mutations	31
	ARID1A (1p36.11)	Point mutations	25
	EP300 (22q13.2)	Point mutations	14
PIK3CA/PTEN/Akt metabolism	*PIK3CA* (3q26.32)	Point mutations	22
	PPARG (3p25.2)	Fusions	3
		Amplifications	6
RAS-MAP-MEK signaling	*FGFR3* (4p16.3)	Fusions	2
		Point mutations	14

disease, agnostic to clinical stage or outcome. These subtypes fall into two broad classes of high-grade bladder cancer, termed "luminal" and "basal cell-like." These categories have characteristics of different stages of urothelial differentiation and have clinically meaningful differences in outcomes, with luminal subtypes having a more favorable prognosis than basal subtypes. Interestingly, these two subtypes recapitulate some of the biological characteristics of luminal and basal-like breast cancer, underscoring that common mechanistic events may underlie the development of solid tumors, beyond overlapping mutational spectra.

Pathology and Genetics of Squamous Cell Carcinoma

Squamous cell carcinoma is relatively infrequent in the Western world. It represents about 3%–7% of bladder cancers in the United States and in Europe, but in countries with endemic urinary schistosomiasis it is much more frequent. Pure squamous cell carcinoma is nearly always associated with chronic cystitis. Most squamous cell carcinomas are invasive, fungating, or ulcerative tumors. Cytonuclear characteristics vary widely, from highly differentiated keratinizing lesions to poorly differentiated tumors with only focal squamous differentiation.

Tumor staging is according to the American Joint Committee on Cancer (AJCC) TNM system. The tumor spreads by direct extension to adjacent organs or by lymphovascular invasion. Squamous cell carcinoma has a lower tendency to lymphogenous or hematogenous distant metastasis than urothelial carcinoma. It is graded as moderately or poorly differentiated, based on the criteria of extent of keratinization and nuclear morphology. Pathologic stage is the most important prognostic factor, with the overall 5-year survival of 55%–65% for pT1/pT2 tumors, dropping to 19% for pT3 and pT4 tumors. To date, there is no convincing evidence of genetic factors affecting the outcome.

Genetics of squamous cell carcinoma has been studied mostly in cases associated with Schistosoma infestation. Increased copy numbers have been found at 5p, 6p, 7p, 8q, 11q, 17q, and 20q and deletions at 3p, 4q, 5q, 8p, 13q, 17p, and 18q by comparative genomic hybridization. By immunohistochemistry, evidence has been obtained of p53 overexpression and this correlated with *TP53* mutations, base transitions at CpG dinucleotides being more frequent than in urothelial carcinomas. Furthermore, mutations of *HRAS*, and overexpression of EGFR and HER2 have been found. One study reported increased DNA methylation.

Biomarkers

Diagnostic and Monitoring Markers

For more than half a century, the detection of urothelial tumors has been dominated by cystoscopy. This rather unpleasant endoscopic examination is unavoidable for the initial workup of a patient presenting with symptoms which raise suspicion of a urothelial carcinoma. The relative frequency of precursor lesions, for which the tendency towards progression cannot be accurately assessed, has called for procedures to monitor potential future oncogenic event in the urothelium. Some claim that this causes urothelial cancer to generate the highest cost per patient of all cancers. For patients, follow-up approaches avoiding repeated cystoscopies would be a major step forward.

Urinary cytology-based diagnostics has been extensively used but its main problem is low sensitivity for detecting urothelial carcinoma, which requires repetition of the tests at relatively high frequency. In recent years, research attempts have been made

to identify and validate (cell- or protein-based) biomarkers in urine which might improve diagnostic sensitivity. Few of these biomarkers have made it into clinical use but recently developed molecular parameters hold great promise.

The most commonly used of these monitoring biomarkers are presented in Table 3.

The most established commercially available assays for detection and follow-up of bladder cancer include the Urovysion kit (Abbott) and ImmunoCyt/uCyt+ assay (Diagnocure). Urovysion is a fluorescence in situ hybridization (FISH) detection of chromosomal aberration specific to bladder cancer, designed to improve the specificity and sensitivity of cytology-based diagnostics. ImmunoCyt/uCyt+ is an immunofluorescent test using a cocktail of monoclonal antibodies against surface proteins expressed by bladder cancer cells. When used in conjunction with cytological testing, this monitoring tool has a sensitivity for the detection of bladder cancer recurrence that reaches 100%. NMP22 bladdercheck® and BTA stat/BTA TRAK have also been widely used. Moreover, the expression of different cytokeratins (CKs), intermediate filaments expressed by epithelial cells, has been used in the diagnostics of bladder cancer as markers of urothelial differentiation in a specific morphological and clinical context. A number of cytokeratin-based bladder cancer assays are commercially available, with UBC® tests and CYFRA 21-1 which are well-known and have been approved by the US Food and Drug Administration (FDA). However, their clinical utility is limited by a high rate of false positives due to other health conditions.

Overall, there are a number of either used or emerging putative diagnostic and monitoring biomarkers for bladder cancer. However, biomarker studies are frequently biased or insufficiently reported and so far, none of this plethora of putative biomarkers has been validated as an independent diagnostic or monitoring tool. Therefore, they are only recommended for use in conjunction with existing routine diagnostic methods and their results can be interpreted exclusively in the appropriate morphological and clinical context.

Prognostic and Predictive Biomarkers

Bladder cancer is a very heterogeneous disease with high recurrence rates. Therefore, reliable prognostication and risk assessment are crucial to guide treatment decisions. However, the same heterogeneity which renders the need of biomarkers so important, makes their identification extremely difficult.

Despite a number of studies on putative molecular prognostic markers, clinical features of the tumor remain the main criteria for predicting the likely course of the disease and prognostication, and thus inform treatment decisions. The simplest and most commonly used prognostic factors are the tumor grade as defined by the TNM staging system and the disease stage. Higher tumor grade and/or disease stage are associated with a higher risk of recurrence and higher progression rates, and so they prompt more aggressive treatments. The presence of associated CIS is an adverse prognostic factor. CIS progresses to invasive carcinoma in 80% of patients within 10 years of diagnosis. Also invasion of muscle, lymphatics, or perivesical fat is associated with a poor prognosis. Invasive cancer is associated with a 50% mortality rate in the first 18 months after diagnosis. Delaying cystectomy for over 12 weeks following the diagnosis of muscle invasive disease (stage T2) may hamper patient survival.

Numerous prediction tools using multiple clinical and morphological criteria have been developed, for example the nomograms by the International Bladder Cancer Nomogram Consortium. Many of them have been shown to be more accurate in predicting recurrence, disease progression, and/or survival than current staging systems. However, it is not always clear how to translate the results they provide into daily patient management decisions and therefore the adoption rate of these models for routine clinical practice is still quite low.

Among numerous putative molecular biomarkers, survivin, an anti-apoptotic protein expressed by embryonic and tumor cells but not by well-differentiated cells, holds great promise as a prognostic biomarker as its expression in bladder cancer has been associated with higher tumor grade. However, standardized detection tests with clearly defined cut-offs need to be established before any validation studies. Moreover, several new targets which are directly or indirectly linked to survivin expression, such as Nuclear factor kappa-B (NF-κB), miR-138-5p or Human HLA-F adjacent transcript 10 (FAT10), are yet to be explored. Many studies have pointed to receptor tyrosine kinases, such as FGFR3 or ERBB2 (HER2/neu) as potential prognostic and predictive biomarkers. Mutations in the *FGFR3* gene and FGFR3 overexpression have been associated with a favorable outcome in non-invasive disease. Furthermore, receptor tyrosine kinases are promising drug targets and a number of pan-FGFR inhibitors and HER2-targeted therapies are currently under clinical trials. "Molecular grade" combining *FGFR3* mutation status and Ki-67 expression has been proposed as an alternative to predict progression of non-muscle invasive bladder cancer. Ki-67 expression as detected by immunohistochemistry is a recognized measure of a tumor proliferation index. Elevated tumor proliferation index has been shown to be correlated with worse prognosis (shorter progression-free survival and disease-specific survival) in both non-invasive and invasive bladder cancer, whereas the molecular grade independently predicts disease-specific survival. Adding the molecular grade to the existing multivariable progression model increases its accuracy.

A number of putative biomarkers predicting tumor responsiveness to platinum-based therapies have been depicted by research studies. Cancers with high expression levels of ERCC1 and ERCC2, the proteins involved in the nucleotide-excision repair, have been shown to be more resistant to these therapies, whereas low ERCC1 and ERCC2 expression have correlated with responsiveness to cisplatin treatment. Multiple studies have suggested *TP53* status/p53 expression as a predictive marker for cisplatin chemosensitivity/chemoresistance, however, with conflicting results. Many other genetic alterations have also been suggested as putative prognostic and/or predictive biomarkers. However, with the emergence of large-scale genomic data which further confirm the heterogeneity of bladder cancers, it becomes clear that efficient prognostic and predictive biomarkers need to be multi-variable models which combine multiple clinical, histological and molecular parameters to reliably stratify

Table 3 Biomarkers for the detection and management of bladder cancer

Biomarker	*Characteristics and clinical utility*
Diagnostic and monitoring biomarkers[a]	
UroVysion Bladder Cancer Kit (UroVysion Kit; Abbott)	FISH-based detection of aneuploidy for chromosomes 3, 7, 17, and loss of the 9p21 locus in urine samples; designed as an aid for initial diagnosis of bladder carcinoma in patients with hematuria and for monitoring previously diagnosed patients for recurrence (FDA-approved)
ImmunoCyt/uCyt+ assay (UCyt+; Diagnocure)	Fluorescent immunohistochemistry test with three monoclonal antibodies against mucin glycoproteins found on the surface of malignant epithelial cells and a carcinoembryonic antigen expressed by bladder tumor cells; intended as a tool to monitor for bladder cancer recurrence in conjunction with standard cytology techniques to improve the sensitivity of the latter (FDA-approved)
Cytokeratins (CK)	A number of tests using different techniques exist (e.g., various UBC® tests (iDL Biotech; available both as quantitative and point-of-care assays) and CYFRA 21-1); increased CK expression is associated with urothelial differentiation and can be of added diagnostic value; not an independent biomarker due to a high rate of false positives resulting from other clinical conditions
Cytokeratin 7, 20	Expressed in bladder cancer; its immunohistochemical detection used for diagnostic purposes; not very specific
P63	Nuclear expression found in bladder cancer; its immunohistochemical detection used for diagnosis; not very specific
GATA3	Nuclear expression found in bladder cancer; its immunohistochemical detection used for diagnostic purposes; not very specific
Nuclear matrix Protein 22 (NMP22)	Overexpressed in bladder carcinoma, low sensitivity for lower-grade tumors; a quantitative sandwich ELISA test commercialized under the name NMP22 Test Kit (Maritech; FDA-approved for surveillance of non-invasive bladder cancer following surgery) a qualitative point-of-care test commercialized under the name NMP22 bladdercheck® (Alere; FDA-approved for both initial diagnosis of non-invasive bladder cancer in symptomatic and/or high-risk individuals and for monitoring for recurrence after surgery)
BTA *stat* and BTA TRAK assay	Quantitative immunoassays (FDA-approved for bladder cancer surveillance in combination with cytoscopy) which identify the bladder tumor-associated antigen (BTA; also called human complement factor H-related protein (hCFHrp)) in urine
Cxbladder tests (Cxbladder Triage, Cxbladder Detect, and Cxbladder Monitor)	Combined tests using both genetic data (five mRNA markers) and clinical data gathered during standard diagnostic procedures; Cxbladder Monitor seems to be the most promising as a follow-up test
Prognostic biomarkers	
Tumor grade and disease stage (as defined by WHO/TNM)	Higher grade and stage are associated with higher recurrence and progression rates
Presence of CIS	Associated with worse prognosis and high rates of progression to invasive disease
Ki-67 expression (as detected by immunohistochemistry)	A recognized measure of tumor cell proliferation index; high proliferation index correlates with worse prognosis (shorter progression-free survival and disease-specific survival) in both non-invasive and invasive bladder cancer
Alterations in the *FGFR3* gene	Mutations and overexpression associated with a favorable outcome in non-invasive disease
"Molecular grade": A combination of the *FGFR3* mutation status and Ki-67 expression	Proposed as an alternative to predict progression of non-muscle invasive bladder cancer; independently predicts disease-specific survival; adding the molecular grade to existing models improves their accuracy
Survivin (emerging biomarker)	Expression associated with higher grade tumors; still needs to be standardized (detection method and threshold) and validated
Predictive biomarkers	
Alterations in the *FGFR3* gene	*FGFR3*-mutant tumors may respond to pan-FGFR inhibitor treatments
ERBB2 (*HER2/neu*) expression	HER2-expessing tumors may respond to HER2-targeted therapies
ERCC1 and *2* expression (putative biomarker)	High expression levels associated with increased resistance to platinum-based therapies and low expression associated with good responsiveness

BTA, Bladder tumor-associated antigen; *CIS*, carcinoma in situ; *CK*, cytokeratin; *FDA*, US Food and Drug administration; *FISH*, fluorescence in situ hybridization.
[a]None of these biomarkers has been validated for clinical practice and none is recommended by clinical guidelines.

patients into risk groups and allow prescribing personalized therapeutic regimens. In particular, recent genomic and transcriptomic studies have identified molecularly distinct subtypes of bladder cancer that might predict therapeutic sensitivity. Clinical trials using molecularly-guided therapy selection will determine the clinical efficacy of using various predictive biomarkers to guide therapeutic decision-making.

Microenvironment and Host Response

Microenvironment of bladder cancer has been a focus of intense research for many years. Of late, this has focused on the host response to the tumor in terms of stromal reaction and immune contexture. However, urothelial carcinoma is an example of a cancer type for which therapeutic use of the host immune response has been effectively applied to treat the carcinoma or to reduce the risk of recurrence. For decades now, patients with a high risk of recurrence and/or progression of a urothelial carcinoma (CIS, papillary urothelial carcinoma high grade, multifocality, lamina propria invasive) are further treated by intravesical instillation of Bacillus Calmette-Guérin (BCG), an attenuated strain of Mycobacterium tuberculosis. These elicit an inflammatory response that also targets the carcinoma. With the emergence of immunotherapeutic modalities targeting the PD-L1/PD-1 immune checkpoint by interference with the binding of PD-L1 ligand to its two receptors, PD-1 and B7.1, in the last 5 years, interest in the immune contexture of bladder carcinoma has intensified. What was known from earlier studies is that the composition of the immune-cell infiltrate in bladder carcinoma (co)determines the response to BCG instillation: BCG failure is associated with low density of CD4+ and GATA3+ T-cells and high numbers of FOXP3+ and CD25+ regulatory T-cells (Tregs) as well as of CD68+ and CD163+ tumor-associated macrophages.

It has now been confirmed that also in bladder carcinoma clinical response to PD-L1/PD1 blocking immunotherapy is correlated with the presence of appropriate pre-existing tumor infiltrating immune cells. This has led to accelerated FDA approval of a PD-L1 blocking agent as second-line therapy for advanced bladder cancer. In initial studies, better response was associated with higher PD-L1 expression on the tumor-infiltrating leukocytes. Several studies have addressed the question of PD-L1 expression evaluated by immunohistochemistry as a biomarker for response to immunotherapy. The inhibitory molecule PD-1 is expressed after T cell activation. When this is persistent, it induces T cell dysfunction. Tumor cells as well as tumor-infiltrating immune cells can express the PD-1 ligand, PD-L1. In bladder carcinoma, PD-1 expression by tumor cells is infrequent ($<10\%$), while PD-L1 is expressed on tumor-infiltrating immune cells in about 30% of cases. This is associated with improved overall survival in metastatic disease. However, studies on the value of immunohistochemical expression of PD1/PD-L1 using different antibodies have not (yet) led to developing a convincing set of rules to guide check-point blocking immunotherapy for advanced bladder carcinoma, notably as up to 10% of patients with PD-L1-negative tumors have been shown to respond to anti-PD-L1 therapy. What is needed is a biomarker predictive of response to all drugs in the anti-PD-1/PD-L1 class. Achieving this goal will need further study.

Management and Therapy

Differentiating between non-muscle invasive tumors which have a good prognosis and aggressive muscle-invasive tumors is key to making proper management decisions (see "**Presentation and Diagnosis**" and "**Pathology and Genetics**" sections). The treatment of the tumors from the first group aims at reducing the recurrence rate and at preventing progression to a more advanced stage. The goal of therapy in patients with muscle-invasive disease is to determine whether the bladder should be removed or if it can be preserved without compromising survival, and to determine if the primary lesion can be managed independently or if the patient is at high risk for distant spread, and so requires systemic approaches to improve the likelihood of cure.

Non-muscle invasive bladder cancers (also referred to as superficial bladder tumors; the use of this term, however, is not recommended by the latest NCCN guidelines which judge it to be imprecise) which are not associated with CIS are commonly managed by transurethral resection of the bladder (TURB; also called TURBT: transurethral resection of the bladder tumor), usually segmental (partial cystectomy), and—when indicated—intravesical instillations, most often with mitomycin C. Fulguration may be effective for smaller lesions. Cystoscopy is used to follow up patients after surgery.

CIS, which has a higher malignant potential and by definition may evolve into invasive carcinoma is effectively treated by cystoscopic resection or fulguration. However, as these tumors are usually flat, they may not be visible by cystoscopy. Therefore, CIS patients receive additional treatment with intravesical Bacillus Calmette-Guerin (BCG). Induction BCG has been shown to prevent bladder cancer recurrences following TURB. Given the high risk of recurrence and progression, CIS patients need to be followed up closely with cystoscopy (every 3 months) and by routine cytology. Maintenance intravesical therapy may be considered following induction with chemotherapy or BCG. However, the efficacy of maintenance chemotherapy is controversial. Moreover, BCG toxicity inducing potentially severe local and systemic side effects is an important concern.

Invasive tumors that grow into the muscularis propria (T2 and higher) require more radical treatment. The gold standard is radical cystectomy, that is the excision of the bladder, perivesical fat, and attached peritoneum. In men, this is accompanied by removal of the entire prostate and seminal vesicles (radical cystoprostatectomy), whereas women undergo *en bloc* removal of the uterus, adnexa, and cuff of the vagina (anterior pelvic exenteration). Pelvic lymph node dissection (pelvic lymphadenectomy) is performed to stage the nodes, while extended lymphadenectomy remains controversial. Segmental resections of the bladder may be used in highly selected cases.

Radiotherapy may be an alternative to surgery for highly motivated patients who desire to retain their bladder and potency following a bladder preservation protocol. However, these multiple modality treatment protocols mandate aggressive treatments, frequent follow-up visits and close coordination of multiple subspecialties, and are therefore conducted only in selected dedicated institutions. Moreover, a salvage cystectomy is required following an attempt at bladder sparing in up to 20% of cases. Radiotherapy does not appear to improve expected survival beyond that achieved by radical surgery alone, although local recurrence is reduced.

One of the most noteworthy issues in the treatment of bladder cancer is the optimal use of perioperative chemotherapy for muscle-invasive disease in an attempt to provide the earliest possible treatment of micrometastatic disease and to facilitate definitive local therapy. Several studies have shown that neoadjuvant therapy is associated with a longer survival. The NCCN panel recommends neoadjuvant chemotherapy followed by radical cystectomy as the first-choice treatment for patients with T2, T3, and T4a bladder cancer without lymph node involvement. The neoadjuvant combination which is most common and also supported by best evidence so far is MVAC (Methotrexate, Vinblastine, Doxorubicin (Adriamycin), Cisplatin).

The data on the use of adjuvant chemotherapy are less conclusive. Adjuvant therapy with systemic cytotoxic agents for patients undergoing cystectomy has been associated with a delay in time to disease progression but there is no clear evidence for an improved survival. Therefore, neoadjuvant therapy remains the preferred option for treatment of early disease patients. Chemotherapy may be considered in case of late-stage disease and/or lymph node involvement. Different cisplatin-based combinations are used for perioperative chemotherapy, such as dose-dense MVAC (ddMVAC), gemcitabine plus cisplatin (GC), or cisplatin, methotrexate, and vinblastine (CMV).

Out of novel targeted therapies, immunotherapeutics are the future of bladder cancer treatment. In particular checkpoint inhibitors, like nivolumab (targeting PD-1) or avelumab (targeting PD-L1; both FDA-approved), have been shown to improve outcomes of patients with advanced and metastatic bladder cancer (see also "**Microenvironment and Host Response**" section).

Further Reading

D'Costa, J.J., et al., 2016. A systematic review of the diagnostic and prognostic value of urinary protein biomarkers in urothelial bladder cancer. Bladder Cancer 2 (3), 301–317.
Duquesne, I., et al., 2017. The contemporary role and impact of urine-based biomarkers in bladder cancer. Translational Andrology and Urology 6 (6), 1031–1042.
European Association of Urology. Guidelines on non-muscle-invasive bladder cancer, 2017: http://uroweb.org/guideline/non-muscle-invasive-bladder-cancer/(accessed on February 19, 2018).
European Association of Urology. Guidelines on muscle-invasive and metastatic bladder cancer, 2017: http://uroweb.org/guideline/non-muscle-invasive-bladder-cancer/ (accessed on February 19, 2018).
Moch, H., Humphrey, P.A., Ulbright, T.M., Reuter, V.E. (Eds.), 2016. WHO classification of tumors of the urinary system and male genital organs, 4th edn. International Agency for Research on Cancer, Lyon, France.
Jones, R.T., et al., 2016. Pharmacogenomics: Biomarker directed therapy for bladder cancer. The Urologic Clinics of North America 43 (1), 77–86.
Kluth, L.A., et al., 2015. Prognostic and prediction tools in bladder cancer: A comprehensive review of the literature. European Urology 68 (2), 238–253.
National Comprehensive Cancer Guidelines (NCCN). NCCN Clinical Practice Guidelines in Oncology. Bladder Cancer. Version 1.2018 – January 8, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site.
van Rhijn, B.W., 2012. Combining molecular and pathologic data to prognosticate non-muscle-invasive bladder cancer. Urologic Oncology 30 (4), 518–523.
Robertson, A.G., et al., 2017. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell 171 (3), 540–556.e25.

Relevant Websites

http://www.auanet.org/—American Urological Association.
www.euroweb.org—European Association of Urology.

Bone and Soft Tissue Sarcoma: From Molecular Features to Clinical Applications

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Glossary

Chromosomal translocation Genetic alteration resulting from the exchange of genetic material between nonhomologous chromosomes. Translocations can be balanced or unbalanced depending on the loss of chromosomal fragments during the rearrangement, and can lead to the constitution of fusion genes.

Ewing sarcoma The second most frequent bone sarcoma after osteosarcoma. Ewing sarcoma affect mostly children and teenagers, and is predominantly found in long bones and in the pelvis. Ewing sarcoma is the first sarcoma subtype for which a specific translocation was identified.

Gene amplification Copy number increase in a restricted region of a chromosome arm containing a limited number of genes. The number of gene copies available for transcription is increased and this leads to overexpression of the gene product.

GIST Gastro intestinal stromal tumors—the most frequent subtype of sarcoma affecting exclusively the gastrointestinal tract. GIST arise from the interstitial cells of Cajal, the latter representing the smooth muscle pacemaker of the intestinal lining.

LMS Leiomyosarcoma—one of the most frequent subtype of soft tissue sarcoma. LMS derive from smooth muscle cells and that are mostly found in the uterus, the stomach, the small intestine, and the retroperitoneum.

LPS Liposarcoma—one of the most frequent subtype of soft tissue sarcoma, originating from fat tissue. The most frequent tumor locations for LPS are the retroperitoneum and other deep soft tissues. Several subtypes of LPS are observed both from pathological and molecular points of view.

MRT Malignant rhabdoid tumors—a subset of very aggressive sarcoma affecting mostly young children. Most cases of MRT develop in the kidney or in the brain, where they are referred as atypical teratoid rhabdoid tumor (AT/RT).

MSC Mesenchymal stem cells—a population of pluripotent stromal cells located in the bone marrow or in adipose tissue, which can differentiate into various cell types including osteocytes, chondrocytes, adipocytes, tenocytes, or myocytes, and which are thought to be the cell of origin of most sarcomas.

NGS Next generation sequencing—a number of different modern sequencing technologies that allow to sequence DNA and RNA in a quicker and more efficient way than the traditional Sanger sequencing. Over the last decade, NGS development has revolutionized the study of genomics and molecular biology and is currently becoming a major tool for diagnostic and theranostic strategies in cancer patients

Oncogenic mutation Genetic alteration consisting in the modification of the normal sequence of a proto-oncogene, the latter being a gene with a potential to favor cancer development. Activating oncogenic mutations are gain-of-function mutations that lead to activation of proto-oncogenes and increase cell proliferation and survival.

Small round cell sarcomas A group of highly aggressive sarcomas affecting children and adults. Small round cell sarcomas are composed of undifferentiated small round blue tumor cells of mesenchymal origin. More than 90% of these tumors are associated with the presence of a recurrent chromosomal translocation leading to the expression of a specific fusion oncogene. Famous examples include Ewing sarcoma, desmoplastic small round cell tumors, alveolar rhabdomyosarcoma, and synovial sarcoma.

Introduction

Sarcomas constitute a group of rare tumors composed of malignant cells of mesenchymal origin. These tumors are characterized by their high heterogeneity both from a clinical and biological point of view. Sarcomas can arise in children, teenagers, and adults, and develop from all kinds of tissue. Histologically, sarcomas are composed of mesenchymal tumor cells with multiple features of differentiation, leading to the pathological description of more than 50 different tumor types, and making them the most heterogeneous tumors. Over the last decades, major breakthroughs have considerably increased our understanding of sarcoma biology. Advances in cytogenetic and molecular techniques have enabled to identify specific alterations related to particular sarcoma subtypes and to decipher their oncogenic mechanisms. The understanding of sarcoma biology has led to refining the diagnostic approaches of many subtypes of tumors and to developing successful targeted therapies that have become standard of care for patients. As a result, sarcomas have become a major model in oncology by combining biological and molecular data to immediate translational and clinical applications.

From an embryologic point of view, sarcoma develop from tissues of mesodermal origin. The mesoderm is the middle layer of the three primary germ layers in the early embryo and originates from the process of gastrulation happening at 3 weeks of development. Upon the course of embryogenesis, the mesoderm develops into the paraxial mesoderm, the intermediate mesoderm, and

the lateral mesoderm. The intermediate and lateral mesoderm will ultimately develop into the primitive urothelial tract and circulatory system, respectively, while the paraxial mesoderm thus develops into somites. The somites give rise to three major structures: the dermatome, the myotome, and the sclerotome, themselves leading to the formation of connective tissues and dermis, muscles, and cartilage and bone, respectively (**Fig. 1**A).

At the cellular level, most sarcomas are thought to originate from mesenchymal stem cells (MSC). MSC are multipotent stem cells located mostly in the bone marrow that have the ability to differentiate into various cell types, including osteocytes, chondrocytes, myocytes, or adipocytes. MSC differentiation toward a defined cell type involves sequential signaling regulation and several stages regulated by the expression of specific transcription factors (**Fig. 1**B). There is a now substantial evidence that a consequent number of sarcomas is related to the transformation of MSC, as reflected by the correspondence between the differentiation capacities of MSC and the histological spectrum of sarcomas. In vitro and in vivo studies have shown that the type of sarcoma arising from transformed MSC depends on many factors, including the originating tissue of MSC, the differentiation commitment of the cells, and the targeted molecular pathway. It is now widely thought that most sarcomas derive from the inability of MSC to undergo proper differentiation into specific cell lineages, due to the emergence of oncogenic molecular alterations.

This cellular origin of sarcomas explains the histological variety and heterogeneity of sarcomas. In the last World Health Organization (WHO) classification, hundreds of different pathological subtypes of bone and soft tissue tumors are described, primarily based on their malignancy potential and differentiation characteristics. Yet, during the last decades, major advances in cytogenetics and molecular biology have led to the discovery of a broad variety of specific alterations involved in sarcoma development, and thus to the emergence of new classifications of tumors.

Genetics of Sarcomas

In clinical practice, sarcomas are commonly divided into two main groups related to the primary tumor location, with the usual separation between "bone sarcomas" and "soft tissue sarcomas." Over the last decade, a new classification has emerged, dividing tumors in two broad categories, each of them including a range of diverse sarcoma subtypes (**Table 1**). The first category comprises sarcomas with diploid karyotypes and simple and recurrent genetic alterations, most of the time translocations and simple activating or inactivating mutations. The second category includes sarcomas with complex, unbalanced karyotypes, characterized by the presence of multiple genomic aberrations within a single tumor, and heterogeneity of genomic aberrations within a given tumor subtype.

Sarcomas With Simple Genomic Profiles

Sarcomas with simple karyotypes represent 40%–50% of all sarcomas. Those tumors are characterized by the presence of recurrent and specific molecular alterations, most of the time pathognomonic of a given tumor subtype, such as chromosomal translocations, oncogenic mutations, or amplifications.

Sarcomas with recurrent chromosomal translocations

Sarcomas with recurrent chromosomal translocations account for 10%–15% of all sarcomas. In 1983, chromosomal banding on Ewing Sarcoma tumors led to the discovery of a recurrent chromosomal translocation t(11;22)(q24;q12), that later proved to drive the expression of the fusion oncogene EWSR1–FLI1 in tumor cells. Since then, numerous translocations and resulting gene fusions have been identified in various sarcoma subtypes (**Table 2**). Each translocation results in the expression of a chimeric fusion gene directly involved in the pathogenesis of the sarcoma in which it is expressed, by three main mechanisms (**Fig. 2**).

First, the most frequent mechanism consists in the expression of an aberrant chimeric transcription factor, resulting from the fusion of a gene under the control of a strong promoter which confers the expression level, with a gene carrying a DNA-binding domain and transcription factor activity. The resulting chimeric transcription factor can bind DNA and regulate its own transcription program to promote tumorigenesis. Famous examples of translocation-positive sarcoma subtypes with aberrant transcription factor activity are small round cell sarcomas (Ewing sarcoma, synovial sarcoma, alveolar rhabdomyosarcoma, or desmoplastic small round cell tumors), myxoid liposarcoma, or clear cell sarcoma. Of note, the chimeric transcription factor drives the regulation of a highly specific transcription program involved in sarcomagenesis. For example, in Ewing sarcoma, several variants of the initial *EWSR1-FLI1* fusion gene have been described. Some of them involve genes belonging to the same families as *EWSR1* and *FLI1* (TET family of proteins and ETS transcription factors, respectively). The resulting fusion genes *EWSR1-ERG*, *EWSR1-ETV1*, *EWSR1-ETV4*, *EWSR1-FEV*, *FUS-ERG*, and *FUS-FEV* are expected to keep the same functions as *EWSR1-FLI1*, and to regulate the same transcriptional programs. On the other hand, other fusions involving genes not related to the TET and ETS families have been reported, such as *CIC-DUX4* and *BCOR-CCNB3*. Those last few years, several studies have shown that those alternative chimeric transcription factors are responsible for transcriptional regulation of target genes highly different from TET–ETS fusions. The resulting tumors, previously referred to as "Ewing-like sarcoma" based on their clinical and pathological similarities, are now being considered as independent tumor entities.

Second, translocations can result in the expression of a fusion protein carrying an aberrant kinase activity, as seen in congenital fibrosarcoma and inflammatory myofibroblastic tumors. In those cases, the translocation leads to the fusion of a strongly expressed protein to the catalytic domain of a tyrosine kinase receptor. The resulting chimeric tyrosine kinase protein is thus constitutively

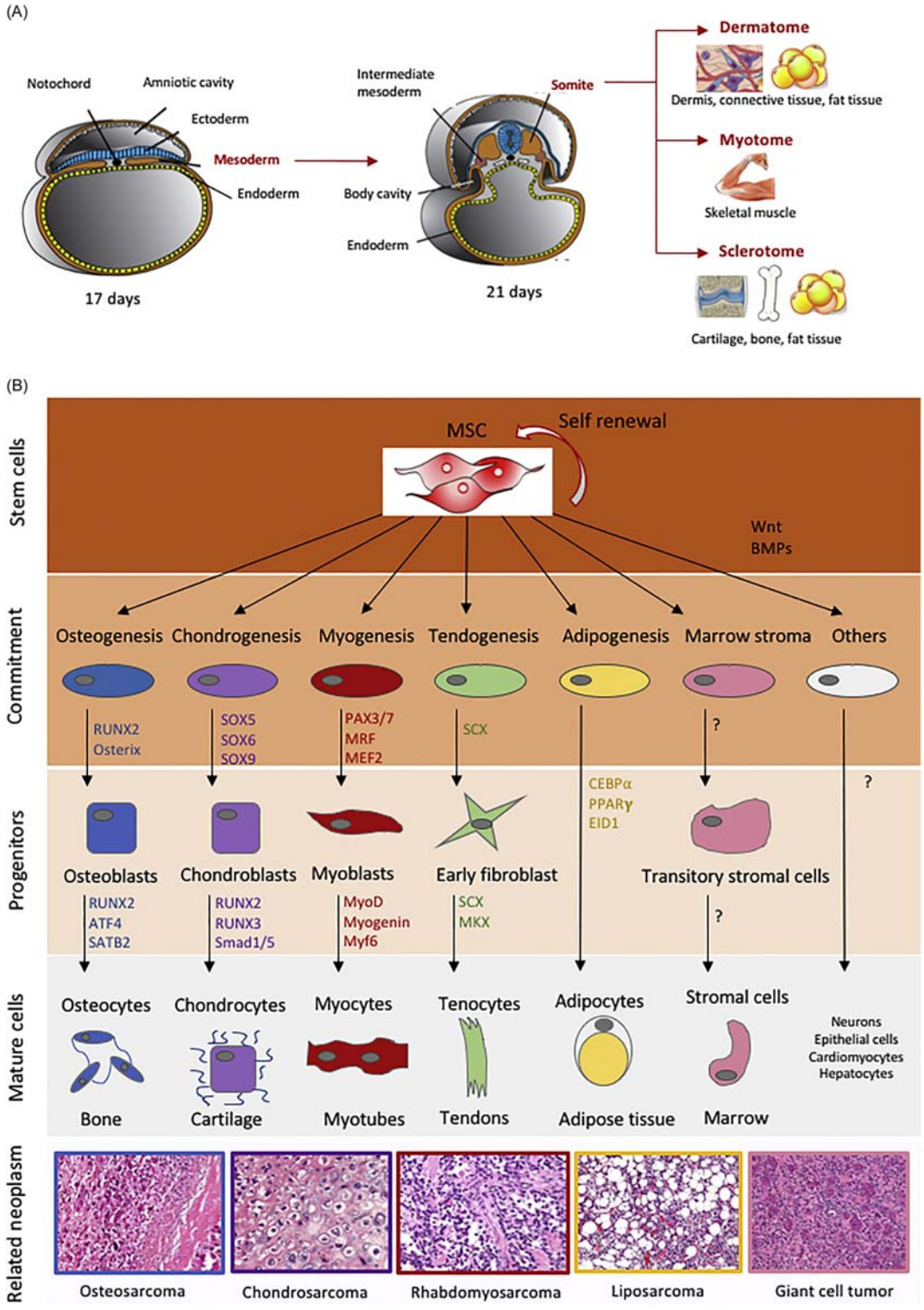

Fig. 1 Embryonal and cellular origin of sarcomas. (A) Schematic view of the embryonal development at 17 and 21 days postfertilization and the resulting derivative tissues. (B) Representation of the different steps of mesenchymal stem cells (MSC) differentiation and related sarcoma subtypes. The main transcription factors involved in each step of differentiation towards specific cell lineages are indicated.

Table 1 Molecular classification of sarcomas

	Molecular alteration	*Frequency (%)*	*Example*
Simple genomic profile	Recurrent translocation	10–15	Ewing sarcoma
	Activating mutation	15	GIST
	Inactivating mutation	5	MRT
	12q14-15 amplification	10–15	WDLPS/DDLPS
Complex genomic profile	Multiple/heterogeneous genomic aberrations	55	Leiomyosarcoma

Abbreviations: *GIST*, gastrointestinal stromal tumors; *MRT*, malignant rhabdoid tumors; *WDLPS*, well-differentiated liposarcoma; *DDLPS*, dedifferentiated liposarcoma.

Table 2 Most common translocations and gene fusions associated with specific subtypes of sarcomas

Sarcoma subtype	*Translocation*	*Main fusion genes*	*Oncogenic mechanism*	*Prevalence (%)*
Ewing sarcoma/PNET	t(11;22)(q24;q12)	EWSR1–FL1	TF	90
	t(21;22)(q22;q12)	EWSR1–ERG	TF	5
Synovial sarcoma	t(X;18)(p11;q11)	SS18–SSX1	TF	65
		SS18–SSX2	TF	35
ARMS	t(2;13)(q35;q14)	PAX3–FOXO1A	TF	80
	t(1;13)(p36;q14)	PAX7–FOXO1A	TF	15
DSRCT	t(11;22)(p13;q12)	EWSR1–WT1	TF	>90
Myxoid liposarcoma	t(12;16)(q13;p11)	TLS–DDIT3	TF	95
Clear cell sarcoma	t(12;22)(q13;q12)	EWSR1–ATF1	TF	95
ESMCS	t(9;22)(q22;q12)	EWSR1–NR4A3	TF	75
	t(9;17)(q22;q11)	TAF2N–NR4A3	TF	20
Angiomatoid fibrous histiocytoma	t(2;22)(q34;q12)	EWSR1–CREB1	TF	90
	t(12;22)(q13;q12)	EWSR1–ATF1	TF	10
Low grade FMS	t(7;16)(q32-34; p11)	TLS–CREB3L2	TF	90
	t(11;16)(p11;p11)	TLS–CREB3L1	TF	10
ASPS	t(X;17)(p11.2;q25)	ASPL–TFE3	TF	>90
Solitary fibrous tumor	inv12(q13;q13)	NAB2–STAT6	TF	>90
Infantile fibrosarcoma	t(12;15)(p13;q25)	ETV6–NTRK3	TK	85
IMT	2p23 rearrangements	Various partners-ALK	TK	60
DFSP	t(17;22)(q22;q13)	COL1A1–PDGFB	GF	>90

Abbreviations: *PNET*, primitive neuroectodermal tumor; *ARMS*, alveolar rhabdomyosarcoma; *DSCRT*, desmoplastic small round cell tumor; *ESMCS*, extraskeletal myxoid chondrosarcoma; *FMS*, fibromyxoid sarcoma; *ASPS*, alveolar soft part sarcoma; *IMT*, inflammatory myofibroblastic tumor; *DFSP*, dermatofibrosarcoma protuberans; *TF*, transcription factor; *TK*, tyrosine kinase; *GF*, growth factor.

activated in a ligand-independent mechanism and can stimulate downstream signaling pathways to promote cell proliferation and survival.

A third mechanism consists in the expression of a chimeric autocrine growth factor, as seen in dermatofibrosarcoma protuberans (DFSP) and giant cell fibroblastoma. In those tumors, the translocation results in putting platelet-derived growth factor beta (*PDGFB*) under the control of the strong *COL1A1* promoter, thus removing all elements repressing *PDGFB* transcription and leading to uncontrolled signal activation.

Translocation-positive sarcomas have become a model for oncogenesis, since their simple genomic profile has often been associated to simple oncogenic mechanisms and thus to promising therapeutic targets. Indeed, in sarcomas with chimeric tyrosine kinase receptor activity and chimeric autocrine growth factor, targeted therapies based on tyrosine kinase inhibitors have been successfully developed and have rapidly become standard of care for patients.

However, sarcomas with chimeric transcription factor activity are still a challenge for targeted therapies, since their mechanism of action is rather highly complex and not fully understood and since, at the difference of kinases, they do not present enzymatic sites that can be targeted by small molecules. For example, in synovial sarcoma, recurrent translocations between chromosomes X and 18 lead to the expression of SS18–SSX1/2 fusion protein. Both SS18 and SSX proteins lack known DNA binding motifs, yet they appear to be acting through transcriptional regulatory mechanisms. The fusion protein keeps the N-terminal domain of SS18 that is responsible for interaction with the SWI/SNF nucleosome remodeling complex, and the C-terminal domain of SSX1 or SSX2 which can interact with the polycomb repressing complex 1 (PRC1), an essential epigenetic regulator (Fig. 3A). The resulting SS18–SSX1/2 fusion proteins disrupt the assembly of the functional SWI/SNF complex by competing with normal SS18 and lead to the formation of an altered SWI/SNF complex lacking the tumor suppressor BAF47 subunit (Fig. 3B). The altered complex binds DNA on regions normally repressed by PRC1 epigenetic marks such as SOX2, activates transcription, and promotes cell proliferation.

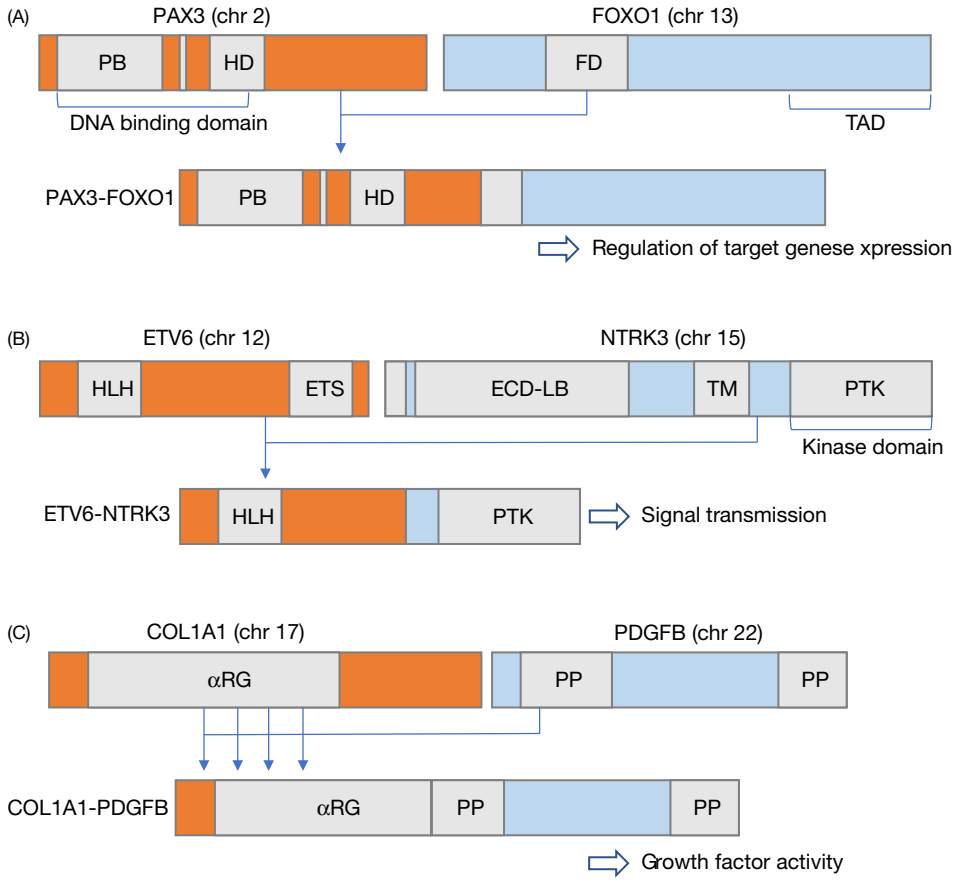

Fig. 2 Main oncogenic functions of chimeric proteins involved in sarcoma development. (A) Fusion gene encoding an aberrant transcription factor: the example of PAX3-FOXO1 in alveolar rhabdomyosarcoma. (B) Fusion gene encoding an aberrant kinase receptor: the example of ETV6–NTRK3 in congenital fibrosarcoma. (C) Fusion gene encoding an aberrant growth factor: the example of COL1A1–PDGFB in dermatofibrosarcoma protuberans. Abbreviations: *αRG*, alpha helical region; *ECD-LB*, extra cellular ligand biding domain; *FD*, fork head domain; *HD*, homeobox; *HLH*, helix loop helix; *PB*, paired box; *PP*, propeptide; *PTK*, protein kinase domain; *TAD*, transactivation domain; *TM*, transmembranous domain.

Such complex mechanisms of action for chimeric transcription factors are a hallmark of sarcomas. Thus, their simple genomic profiles do not properly reflect the level of complexity of molecular regulation at other levels. There is now growing evidence that most chimeric transcription factors in sarcoma exhibit epigenetic regulator activity. Their mechanism of action will have to be further characterized to develop successful targeted therapies.

Sarcomas with recurrent activating or inactivating mutations

Gastrointestinal stromal tumors

The presence of a specific oncogenic activating mutation is the central event in most cases of gastro intestinal stromal tumors (GIST). GIST are the most frequent mesenchymal tumors developing in the gastrointestinal tract and originate from the transformation of interstitial cells of Cajal (ICC). GIST were historically characterized by their poor prognosis, mainly due to their primary resistance to cytotoxic chemotherapy. In 1998, the discovery of recurrent activating mutations in the proto-oncogene *c-KIT* led to dramatic prognostic and therapeutic changes for GIST patients. *c-kit* encodes a type III tyrosine kinase (KIT), which is physiologically activated upon the binding of its ligand SCF. The SCF–KIT interaction is a crucial regulator of erythrocytes, germ cells, melanocytes, mast cells, and ICC growth and development, by activating major downstream pathways such as the RAS–RAF–MAPK pathway to promote cell proliferation and survival.

c-KIT mutations are found in 80%–85% of GIST and lead to constitutive activation of the receptor. *KIT* mutations in GIST are clustered in four regions, the most frequent being the one encoding the juxta-membrane domain (exon 11), and include deletions, point mutations, and amplifications. The discovery of *KIT* activating mutations led to the rapid and successful development of clinical trials evaluating the tyrosine kinase inhibitor imatinib mesylate in patients with KIT mutated GIST. Imatinib is a competitive inhibitor of BCR-ABL, KIT, ARG, PDGFRα, and PDGFRβ tyrosine kinases. With dramatic responses and prolonged survival compared to standard chemotherapy, imatinib became the most rapidly FDA-approved drug in patients with advanced GIST in 2002, only 4 years after the discovery of *c-KIT* alterations.

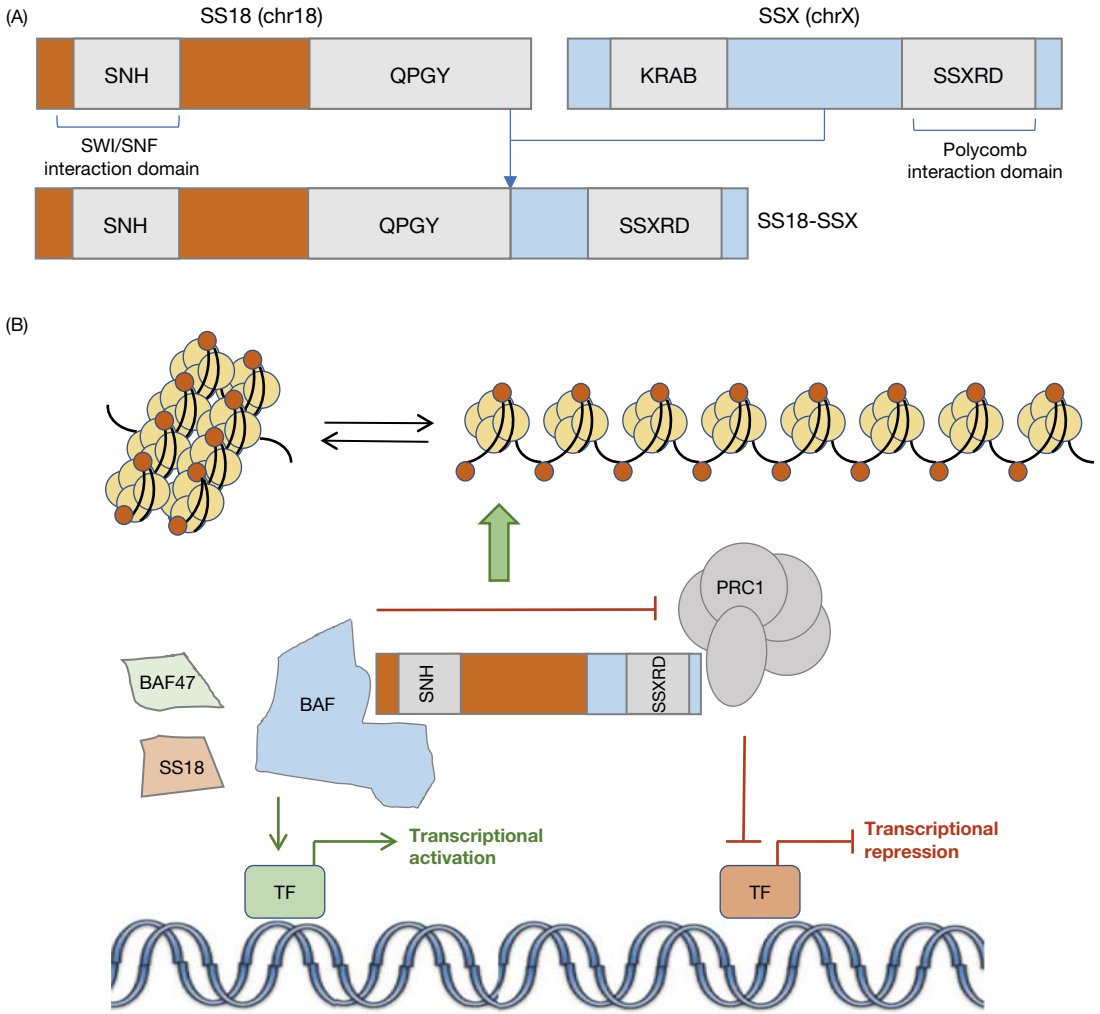

Fig. 3 Structure and oncogenic function of SS18–SSX fusion protein in synovial sarcoma. (A) Structure and main domains of SS18–SSX chimeric protein; the fusion proteins keeps the SNH SWI/SNF interaction domain of SS18 and the PRC1 interaction domain of SSX. (B) Putative molecular functions of SS18–SSX and selected protein–protein interactions; the fusion protein competes with normal SS18 for the assembly of the SWI/SNF complex, which results in an aberrant complex lacking BAF47 subunit. The fusion protein also interacts with the PRC1 complex via the SSXRD domain. The oncogenic functions of SS18–SSX protein are thus mediated by aberrant chromatin remodeling due to the disruption of the normal balance between SWI/SNF and PRC1 complexes to mediate transcription activation and repression. Abbreviations: *SNH*, SYT N-terminal homology domain; *QPGY*, glutamine, proline, glycine, and tyrosine repeat domain; *KRAB*, Krüppel associated box domain; *SSXRD*, SSX repression domain; *PRC1*, polycomb repressing complex 1; *SWI/SNF*, switch/sucrose nonfermentable complex.

In 2003, recurrent activating mutations in the gene encoding the related receptor platelet-derived growth factor receptor alpha (*PDGFRα*) were identified in 35%–40% of *KIT*-wild type GIST. Notably, *KIT* and *PDGFRα* mutations were shown to be mutually exclusive, and *KIT* and *PDGFRα*-mutated GIST to be indistinguishable with respect to activation of downstream signaling pathways and cytogenetic features. The most frequent *PDGFRα* mutations are located in the activation loop of the receptor (exon 18), and lead to the D842V amino-acid change (Fig. 4A). D842V mutation confers primary resistance to imatinib, whereas the remaining non-D842V *PDGFRα* tumors are known to be sensitive to the drug. As a result, therapeutic strategies in GIST have now become entirely guided by the detection of the primary molecular alterations, with algorithms of treatment based on the tumor genetics and the development of new drugs dedicated to specific KIT of PDGRα mutants that are resistant to conventional tyrosine kinase inhibitors.

However, approximately 10% of GIST show no detectable mutation in either *KIT* of *PDGFRα*. These correspond to three clinicopathological entities: pediatric GIST, GIST associated to neurofibromatosis type 1 (NF1), and sporadic wild-type GIST. Sporadic *BRAF* and *NRAS* mutations have been reported in a few cases of these "wild-type" GIST.

More recently, a small subset of GIST, mostly pediatric GIST and GIST associated to rare syndromes have been shown to be characterized by the intratumoral loss of function of the succinate dehydrogenase (SDH) complex. SDH-deficient GIST are exclusively located in the stomach, and they typically occur in children and young adults with a spectrum of behavior from indolent to aggressive. SDH complex is normally ubiquitously expressed. It consists of four subunits (SDHA, SDHB, SDHC, SDHD) localized in the

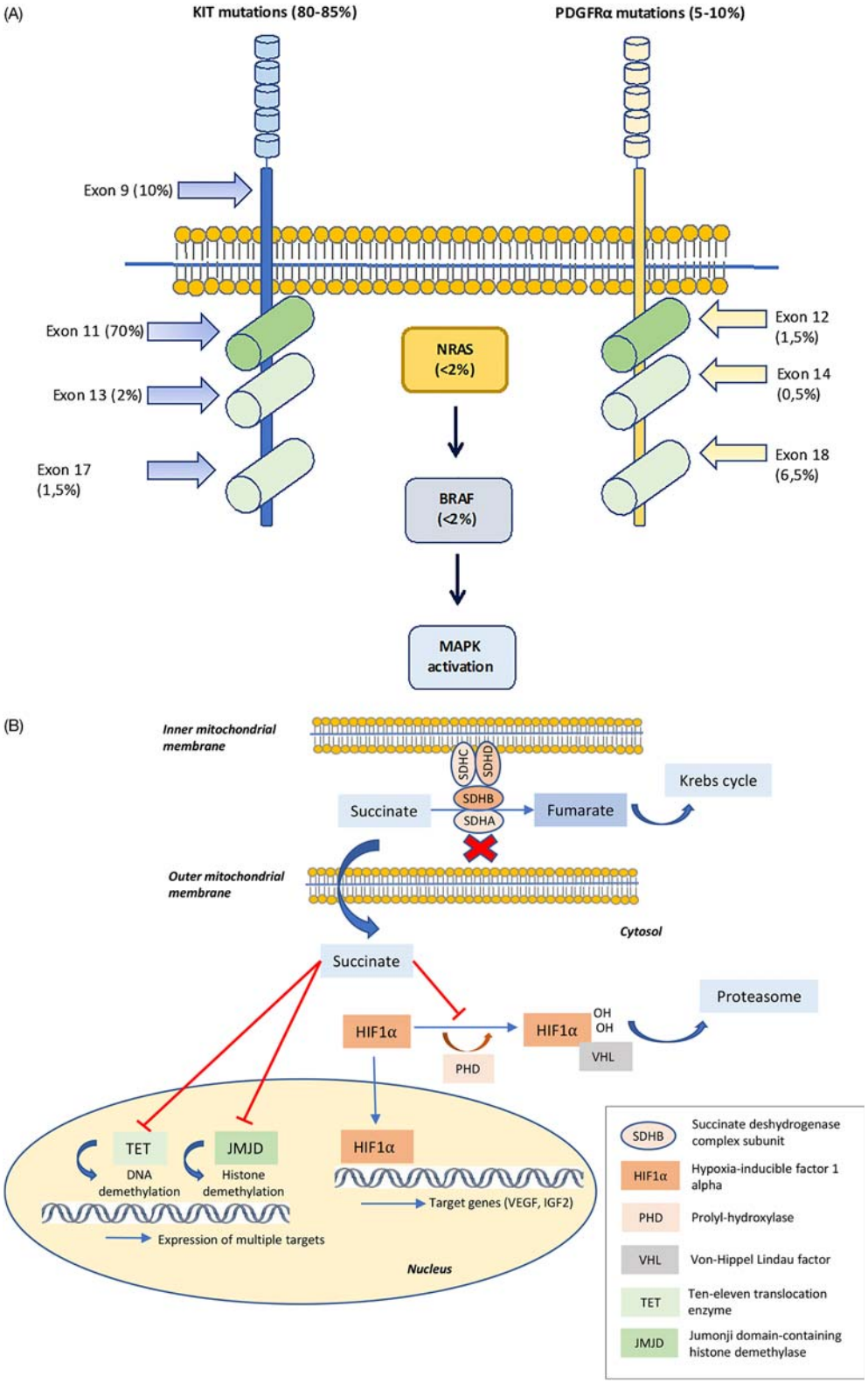

Fig. 4 Main oncogenic mutations found in gastrointestinal stromal tumors (GIST). (A) Schematic representation of c-KIT and PDGFRα receptors and downstream targets. The *arrows* show the domains most frequently mutated in GIST and the *percentages* indicate the frequency of mutations in GIST patients. (B) Representation of the SDH oncogenic pathway in patients with SDH-deficient GIST. SDH deficiency leads to the absence of transformation of succinate into fumarate in the inner mitochondrial membrane. The accumulation of succinate drives the overexpression of HIF1α through the inhibition of PHD which targets HIF1α to proteasomal degradation and results in the expression of HIFα target genes. Succinate accumulation also inhibits the DNA demethylase TET and the histone demethylase JMJD, resulting in aberrant hypermethylation profiles within tumor cells and oncogenic epigenetic changes.

inner mitochondrial membrane and acts at the interphase of the tricarboxylic acid cycle and electron transport chain, by catalyzing the transformation of succinate into fumarate (Fig. 4B). Pediatric GIST and GIST associated to hereditary paraganglioma syndromes, Carney–Stratakis syndrome, and Carney triad have been found to be SDHB deficient. SDHB deficiency is the consequence of any bi-allelic loss of function of SDH subunit, via the destabilization of the SDH complex. About half of the patients have SDH subunit gene mutations, often germline, with both alleles inactivated in the tumor cells typical of classic tumor suppressor genes. The remaining cases are not associated with *SDH*-mutations and epigenetic silencing of the *SDH* complex genes is the probable mechanism for SDH loss.

SDH loss results in the accumulation of succinate within tumor cells, which leads to the overexpression of hypoxia inducible factor 1 alpha (HIF1α) due to the inhibition of its proteasomal degradation and thus to activation of pseudohypoxia signaling and expression of target genes. Moreover, SDH-deficient tumors are characterized by a high frequency of gene methylation when compared to *KIT* or *PDGFRα* mutant GIST. This is probably the consequence of the inhibition of crucial DNA and histone demethylase enzymes caused by the accumulation of succinate within tumor cells. Both HIF1α signaling and hypermethylation are thought to be the central events in the oncogenesis of these SDH-deficient GIST.

From a practical point of view, the diagnosis of SDH-deficient GIST can be done by immunohistochemistry, with the loss of expression of SHDB subunit in tumor cells showing neither *KIT* nor *PDGFRα* mutation. Even though there is still only few data related to therapeutic strategies in SDH-deficient GIST, traditional tyrosine kinase inhibitors seem to be inactive in those tumors. New therapeutic options include alternative tyrosine kinase inhibitors and heat shock proteins (HSP) inhibitors, but still need to evaluated in clinical practice.

Malignant rhabdoid tumors

Apart from GIST, malignant rhabdoid tumor (MRT) is the most famous example of sarcoma showing a recurrent and specific mutation as main oncogenic mechanism. MRT is a rare childhood sarcoma which commonly develops in the kidney but can also arise from other soft tissues or from the brain, where it is referred as atypical teratoid rhabdoid tumor (AT/RT). MRT development was initially found to be associated to monosomy 22 and subsequently to deletions and translocations involving band 22q11.2. Early positional cloning studies managed to identify *SMARCB1* (*INI1*, *SNF5*, *BAF47*) loss as the initial oncogenic event for MRT development. *SMARCB1* functions as a classical tumor suppressor gene, with germline mutations or deletions predisposing to the disease, and somatic loss or mutation of the second allele constituting the second event. 15%–30% of *SMARCB1* germline mutations are found in MRT patients. Germline mutations or deletions can be de novo postzygotic. In such a case, related siblings are unaffected. However, these alterations can be prezygotic, mostly due to germinal mosaicism in one of the parents. In such a case, recurrence can be observed in siblings. Given the extreme aggressiveness of the disease, inherited *SMARCB1* mutation in multigeneration families associated to multiple affected siblings with MRT are extremely rare. Therefore, molecular genetic testing for a germline mutation and genetic counseling are highly recommended for MRT patients.

The majority of *SMARCB1* coding sequence mutations are point or frameshift mutations that introduce a premature stop codon and predict protein truncation. Several mutation hotspots have been described, some of them specific to AT/RT. Yet, most cases of MRT are characterized by *SMARCB1* homozygous deletions, regardless of tumor location. Copy number neutral loss of heterozygosity for most of the long arm of chromosome 22 is common, as well as more complex patterns of deletion, duplication, or translocation involving the *SMARCB1* locus.

Bi-allelic inactivation of *SMARCB1* leads to loss of expression of the protein which hence cannot be detected by immunohistochemistry in tumor cells and constitute a diagnostic tool in clinical practice.

SMARCB1 encodes a core subunit of the ATPase SWI/SNF chromatin remodeling complex, a major ubiquitous complex regulating gene expression by relieving repressive chromatin structure. The SWI/SNF complex uses ATP hydrolysis to displace nucleosomes in response to signaling and differentiation, thus regulating transcription of specific target genes. Notably, in the rare cases of MRT showing conserved expression of SMARCB1, loss of SMARCA4, another core subunit of the SWI/SNF complex, is common by bi-allelic mutations. Moreover, a new subtype of adult thoracic sarcomas characterized by SMARCA4 deficiency (SMARCA4-DTS) has recently been shown to present a transcriptional signature very close to MRT, suggesting that the loss of functional SWI/SNF complex in both cases leads to common oncogenic consequences, whatever the tissue of origin. The mechanisms by which the disruption of a functional SWI/SNF complex leads to tumor development are still being investigated, but several targets have been identified that may play a crucial role in tumor development. Among them, *CYCLIN-B1* and *c-MYC* are highly expressed in MRT, and known to be regulated by SWI/SNF and SMARCB1. Modulation of the Interferon pathway, blocking of differentiation, and increased migration have also been shown as consequences of SMARCB1 loss, and represent attractive therapeutic targets.

The most promising innovative strategy in MRT consists in the development of EZH2 inhibitors. EZH2 is the catalytic subunit of the multiprotein HMT complex known as polycomb repressing complex 2 (PRC2). PRC2 catalyzes the methylation of lysine 27 of Histone 3 (H3K27), H3K27 trimethylation marks leading to repression of gene expression. There is substantial evidence that SWI/SNF and PRC2 interact in an antagonist way to regulate gene expression at the epigenetic level. Indeed, EZH2 is highly expressed and PCR2 target genes are broadly repressed in SMARCB1-deficient tumors, which suggests an EZH2 oncogenic dependency. Moreover, in vitro and in vivo studies have shown that tumorigenesis in *SMARCB1*-deficient cells can be completely abrogated by specific codeletion of EZH2. These findings have led to the development of early clinical trials evaluating the benefit of EZH2 inhibitors such as tazemetostat in MRT patients. With promising and sustained responses observed in both SMARCB1 and SMARCA4 deficient tumors, EZH2 inhibitors are now being evaluated in larger clinical trials and could rapidly represent a new therapeutic standard for those chemo-resistant tumors.

Sarcomas with 12q13-15 amplicons

About 10%–15% of sarcomas are characterized by a simple genomic profile associated to 12q13-15 amplicons. These are mostly atypical lipomatous tumors/well-differentiated liposarcomas (ALT/WDLPS) and dedifferentiated liposarcomas (DDLPS).

ALT/WDLPS is considered a low-grade malignancy that rarely metastasizes, but needs careful follow-up because recurrence or dedifferentiation may occur. From a pathological point of view, ALT/WDLPS may be difficult to distinguish from benign lipomatous tumors, since both are composed of mature fat, variably-sized adipocytes and fibromyxoid stroma, with low cellularity and uncommon mitotic figures. On the other hand, DDLPS is a high-grade liposarcoma that arises in a background of preexisting ALT/WDLPS. Histologically, DDLPS is mostly composed of pleomorphic malignant fibrous histiocytoma-like figures, although other sarcomatous phenotypes have been described, arising by an abrupt transition from an ALT/WDLPS region.

90% of ALT/WDLPS and DDLPS have amplification of chromosome 12q13-15, which contains the oncogenes *MDM2*, *HMGA2* and *CDK4*, while additional genes located in this amplicon may also play a role in liposarcomagenesis. In most cases, the amplicon is located on neochromosomes or ring chromosomes. The 12q13-15 amplicon causes accumulation of MDM2 and CDK4 in tumor cells, so that immunohistochemical staining for these proteins can be used as a diagnostic marker for these diseases. The oncogenic role of MDM2, HMGA2, and CDK4 in liposarcoma has been widely documented both in vitro and in vivo and is as a result an active area for the development of targeted therapies. MDM2 drives the ubiquitination and degradation of the tumor suppressor P53, through a direct interaction via its RING domain. Thus, *MDM2* amplification results in reduced levels of P53 favoring transformation of progenitor cells. Competitive inhibitors of the MDM2–P53 interaction have been developed and are currently being evaluated in early phase clinical trials in patients with DDLPS. CDK4 is a protein kinase that promotes the G1/M cell cycle transition. CDK4 inhibitors have been developed and tested in patients with DDLPS. A recent Phase 2 study evaluating palbociclib, a CDK4 inhibitor, in 29 patients with refractory WDLPS and DDLPS reported a median progression-free survival of 4.7 months and only one case of partial response. This may be due to the fact that CDK4 overexpression is associated to a 12q amplicon distinct from the one containing *MDM2* and *HMGA2*, as a small subset of good prognosis WDLPS do not show increased levels of CDK4. Thus, CDK4 may be a key factor for progression, but not initiation, of liposarcoma, and targeting CDK4 alone may not be sufficient to induce tumor cell death.

Apart from the 12q13-15 amplicons, additional genomic alterations have been described in DDLPS, including 3p14-21 loss, 11q23-24 loss, and 19q13 loss. However, the mechanisms by which those alterations contribute to dedifferentiation and tumor progression are still being investigated and do not constitute current therapeutic targets.

Sarcomas With Complex Genomic Profiles

About 50%–60% of sarcomas are characterized by complex genomic profiles with multiple mutations, gene losses, and gains. This category is mainly composed of osteosarcoma, leiomyosarcoma, myxofibrosarcoma, pleomorphic liposarcoma, malignant peripheral nerve sheat tumor (MPNST), and spindle cell/pleomorphic unclassified sarcoma (previously referred as malignant fibrous histiocytoma). Some of these tumors can develop from a less aggressive sarcoma subtype, with increased genomic complexity paralleling stages of progression. Examples of such genetic evolution include the progression from neurofibroma to MPNST, of the progression from enchondroma to chondrosarcoma. However, in most cases, cytogenetically complex sarcomas arise de novo, without preexisting low-grade lesions.

Contrary to sarcoma with simple genomic profiles, these tumors present with multiple numerical and structural chromosomal aberrations that are reminiscent of epithelial tumors. Notably, some chromosomal aberrations are often unspecific and common to multiple subtypes of sarcomas. For example, broad amplifications of several chromosome arms (such as 5p) combined with deletions of the tumor suppressor genes *TP53*, *RB1*, *NF1*, *PTEN*, or *CDKN2A*/*CDKN2B* are observed in various tumor types. Inactivating mutations in the remaining allele of the deleted genes are frequent. These genetic alterations are thought to be an early event in sarcomagenesis, as the loss of function of these genes is believed to impair chromosome integrity and lead to genomic instability.

In the last few years, new molecular techniques have managed to identify prognostic signatures in cytogenetically complex sarcomas, and to point at oncogenic pathways that might be activated in specific subtypes, for example in leiomyosarcoma (LMS), osteosarcoma, and conventional chondrosarcoma.

Leiomyosarcoma

LMS is a subtype of sarcoma showing varying degree of smooth muscle differentiation, which can develop ubiquitously and represents 8%–10% of adult soft tissue sarcoma. From a cytogenetic point of view, LMS is characterized by complex karyotype alterations such as gains, losses and amplifications, often different from tumor to the other. However, some alterations are more frequent, such as loss of *RB1* and amplification of 5p13-15, while others have been associated to poor outcome and metastatic dissemination, such as loss of 1p, 2p, 13q14-q21, 10q, 16q, and gains of 17p, 8q, or 5p. At the molecular level, activation of the PI3K–AKT pathway seems to drive LMS development and maintenance, as a result of several distinct mechanisms including inactivation of PTEN. Mutations in *P53*, inactivation of *P16* and upregulation of *HIF1α* are associated with increased metastatic risk. So far, few studies have managed to identify specific alterations that may constitute attractive targets in LMS. An exception may be the amplification of myocardin (*MYOCD*), observed specifically in a subset of aggressive retroperitoneal LMS. *MYOCD* encodes a transcriptional cofactor of the serum response factor (SRF), the latter being a crucial regulator of smooth muscle differentiation, suggesting that MYOCD may be lineage-survival oncogene and could represent an attractive therapeutic target.

Osteosarcoma

Osteosarcoma is the most frequent primary bone tumor. It affects mostly children and teenagers and is thought to originate from osteoblastic cells. From a genetic point of view, the mutational landscape of osteosarcoma is highly complex, and great heterogeneity is observed between tumors. However, a hallmark of osteosarcoma is chromosome instability, with the presence of multiple aberrations in chromosome number and structure. Frequent losses of portions of chromosomes 3, 6, 9, 10, 13, 17, and 18 are observed, and lead to the loss of major tumor suppressor genes such as *RB* and *TP53*. Loss-of-function *RB* mutations are observed in up to 70% of osteosarcoma, the most common mechanisms for gene inactivation being loss-of-heterozygosity, structural rearrangements, and point mutations. *RB* inactivation leads to cell cycle progression through increased G1-to-S phase transition. Similarly, bi-allelic *TP53* mutations can be detected in almost 75% of cases of osteosarcoma. Allelic loss is the most frequent mechanism but *TP53* rearrangements and point mutations are also common. The loss of these tumor suppressor genes plays a critical role in osteosarcoma initiation and progression, as illustrated by the fact that germline *RB* and *TP53* mutations lead to osteosarcoma predisposition.

Conventional chondrosarcoma

Conventional chondrosarcoma is the most frequent subtype of chondrosarcoma and represents approximately 25% of primary bone tumors. These tumors can arise de novo or develop on preexisting benign cartilage neoplasms such as enchondromas. While enchondromas and low-grade chondrosarcomas show few cytogenetic abnormalities, high-grade chondrosarcomas are characterized by aneuploidy and complex karyotypes. A few recurrent but inconstant cytogenetic aberrations have been identified in those tumors, including 12q13-15 (containing *MDM2*) and 9p21 (containing *CDKN2A*) rearrangements, but their roles in chondrosarcoma initiation and progression still need to be clarified. The most significant advances in the understanding of conventional chondrosarcoma biology were done with the discovery of recurrent heterozygous mutations in isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) in approximately 50% of cases of sporadic central and periosteal chondrosarcomas. The same mutations were detected in tumor cells of patients with Ollier disease and Maffuci syndrome, two syndromes characterized by development of multiple enchondromas, suggesting that *IDH1*/*IDH2* mutations are early events in the oncogenesis of cartilage tumors. IDH1 and IDH2 are two homodimeric enzymes localized in the cytoplasm and peroxysomes, and in the mitochondrial matrix, respectively. They catalyze a redox reaction that converts isocitrate to α-ketoglutarate (α-KG), and are thought to play a major role in the regulation of glucose, fatty acids, and glutamine metabolism, and to maintain normal cellular redox status. Mutant IDH enzymes acquire a neomorphic activity in which α-KG is converted to 2-hydroxyglutarate (2-HG), with an exclusive production of the D- enantiomeric form of the metabolite (D2HG). As a result, elevated levels of D2HG can be detected in patients with *IDH1*/*IDH2* mutated tumors. Those findings support the concept that D2HG acts as an oncometabolite to promote tumor progression, as it has been previously shown in gliomas and acute myeloid leukemias showing the same *IDH1*/*IDH2* mutations.

Apart from those examples, much work remains to be done to identify and clarify the genes involved in the genesis of complex genomic sarcomas. This will require large scale genomic studies combined with gene expression profiling and functional genetics for target discovery. Of note, even if the identification of genetic alterations in sarcomas with complex karyotypes has not yet been associated with therapeutic applications, the analysis of genomic complexity can provide key elements for predicting patients' prognosis. For example, the CINSARC (complexity index in sarcomas) expression signature, composed of 67 genes involved in mitosis and chromosome management, has been shown to be associated with genome complexity and to predict metastatic outcome in patients with nontranslocation soft tissue sarcomas. This signature has thus been proposed for improving the selection of patients that could benefit from adjuvant chemotherapy after surgery of the primary tumor.

Perspectives in Sarcoma Biology

Diagnostic and Theranostic Approaches

The increasing knowledge concerning sarcoma biology has already led to tremendous advances in both diagnostic procedures and therapeutic strategies for specific subtypes. The high specificity of the molecular alterations found in a particular sarcoma subtype, especially for sarcomas with a simple genomic profile, has led to the development of dedicated molecular diagnostic techniques, that have proven to be an essential complement to standard pathological approaches. The current molecular dismantling of bone and soft tissue sarcomas is associated to major evolutions in the diagnostic techniques, that are currently shifting from research approaches to mandatory translational and theranostic tools for direct use in clinical practice.

Historical and traditional diagnostic methods

The detection of specific molecular abnormalities has progressively become key diagnostic markers to define sarcoma entities, due to their high specificity and prevalence within a given tumor subtype. Over the last decades, diagnostic methods have been developed and validated to detect these alterations, whether at the DNA, RNA, or protein level, and are commonly used in clinical practice in complement to standard pathological analyses.

Immunohistochemical (IHC) staining is widely used to assess for the presence or absence of a given protein within tumor cells. IHC is a good technique for detecting some fusion proteins in translocation-associated sarcomas, when a portion of a protein is overexpressed as a result of the gene fusion. For example, the diagnosis of alveolar soft part sarcoma and inflammatory myofibroblastic tumor can be done by IHC staining withTFE3 and ALK antibodies, respectively. However, all fusion proteins cannot be

detected by IHC, due to the lack of specificity of some antibodies, such as FLI1 antibodies for Ewing sarcoma, or when the fusion leads to protein truncation and loss of reactive epitopes. IHC staining is also a good technique for detecting inactivating mutations resulting in protein loss, such as the loss of SMARCB1 in MRT (Fig. 5A) or the loss of the SDHB unit in SDH-deficient GIST. Overexpression of MDM2 and CDK4 in sarcomas with 12q13-15 amplicon can also be highlighted by IHC, but IHC specificity and sensitivity in these cases is less efficient than alternative methods.

Fluorescence in situ hybridization (FISH) has long been the most frequently used technique to show gene rearrangements such as translocations and amplifications. FISH consists in the detection of a specific DNA target by a dedicated fluorescent probe in the nuclei of interphase cells. Break-apart probes specific for *EWSR1*, *SS18*, *DDIT3*, *FOXO1A*, *TLS*, *ETV6*, or *ALK* are commercially available are frequently used to detect rearrangements of corresponding genes in translocation-associated sarcoma (Fig. 5B). However, this technique does not enable to identify the second gene partner involved in the translocation. The definitive diagnosis of a specific sarcoma subtype may thus need additional investigations, for example in case of a rearrangement of *EWSR1* possibly related to Ewing sarcoma, clear cell sarcoma, desmoplastic small round cell tumor, and myxoid liposarcoma. FISH is currently the most frequently used molecular approach to detect MDM2 amplifications. The combination of MDM2 probes and probes specific for the centromeric region of chromosome 12 is highly sensitive and specific for detecting 12q13-15 amplicons in WDLPS and DDLPS (Fig. 5C).

Reverse transcription-polymerase chain reaction (RT-PCR) is the gold standard approach for detecting fusion transcripts in translocation-associated sarcomas. Contrary to IHC and FISH that can be routinely performed on formalin fixed paraffin-embedded tissues, RT-PCR is more suitable with RNA extracted from frozen tissue, and requires strictly controlled laboratory conditions for primer design, RNA extraction, and reduced risk of PCR contamination. It enables to detect known fusion transcript with high sensitivity and specificity which enables the detection of circulating tumor cells. Using quantitative or real-time RT-PCR (qRT-PCR) enables to improve both the sensitivity and specificity compared to standard RT-PCR, and is more appropriate for daily diagnosis (Fig. 5D and E).

Last, the detection of KIT and PDGFRα mutations in GIST is usually done by Sanger sequencing of PCR amplified-exons on tumor DNA extracted from paraffin-embedded or frozen tissue.

Thus, all those usual techniques enable to detect specific molecular abnormalities with high sensitivity and have been widely used during the last decades for sarcoma diagnosis. However, these techniques are targeted towards known and previously characterized alterations, and do not enable a broad screen of multiple gene fusions, or to detect new abnormalities and to established unbiased tumor molecular profiling. During the last few years, new unbiased genetic and molecular techniques have been developed and are becoming key tools both for diagnosis, prognosis and therapeutic strategies.

Next generation sequencing for genetic and molecular characterization of sarcomas

Next generation sequencing (NGS), also known as high-throughput sequencing, refers to a group of new sequencing technologies that have been developed over the last decades. These techniques enable to produce millions of sequences in one run and in a cheaper way than conventional sequencing. NGS allows to get away from cloning and genomic bank constitution, and enables to sequence isolated nucleic acid molecules.

Transcriptome analysis by RNA sequencing (RNAseq)

RNAseq is a recently developed approach that uses NGS for transcriptome profiling. It consists of converting a population of RNA (total or isolated) into a bank of cDNA fragments with adaptors attached at one or both ends. cDNA are then sequenced in a high throughput manner to get sequences from either one end (single-end sequencing) or both ends (pair-end sequencing). The reads can then be aligned to a reference genome or reference transcripts to produce a transcriptional map, both at the qualitative (structure of the transcriptome) and quantitative (level of expression of each transcript) levels.

RNAseq is a highly suitable technique for the identification of fusion events, especially to detect fusion genes coding for chimeric proteins. Indeed, using transcriptome enables to focus on the fusion genes that are expressed, and thus more likely to be functional and/or involved in the disease, and RNAseq enables to identify fusion genes resulting from nongenomic events, such as trans-splicing or read-through. Pair-end sequencing facilitates the detection of novel rearrangements and fusion genes compared to single-end sequencing. A chimeric gene can be identified when a mate pair spans the fusion junction, or if the mate pairs encompass the fusion junction, and additional evidence of a fusion transcript can be detected due to the quantitative aspect of RNAseq. Concerning data analysis, several algorithms of gene fusion detection have been developed in the last decade, which enable to predict the statistical probability of the detected fusion events. In sarcomas, RNAseq has proven to be a highly efficient technique to detect fusion transcripts in translocation-associated tumors (Fig. 6A,B). Moreover, since RNAseq is not limited to detecting transcripts corresponding to known genomic sequences, this technique has enabled to discover new fusion genes corresponding to new sarcoma subtypes, such as *BCOR-CCNB3* fusion in a group of tumors previously referred to as "Ewing-like sarcomas."

The quantitative aspect of RNAseq has also made it an attractive method to study global gene expression profiles. Several supervised and unsupervised bioinformatics techniques have been developed, some the most famous being hierarchical clustering (Fig. 6C) and principal component analysis, that enable to group or divide samples based on their expression profiles. Over the last decade, this has enabled to provide a wide description of multiple sarcoma subtypes, and to establish new biological convergences between previously unrelated subtypes. For example, RNAseq has enable to discover a new group of aggressive thoracic sarcomas characterized by deficiency in SMARCA4, the ATPase subunit of the SWI/SNF complex. Transcriptomic profiling has

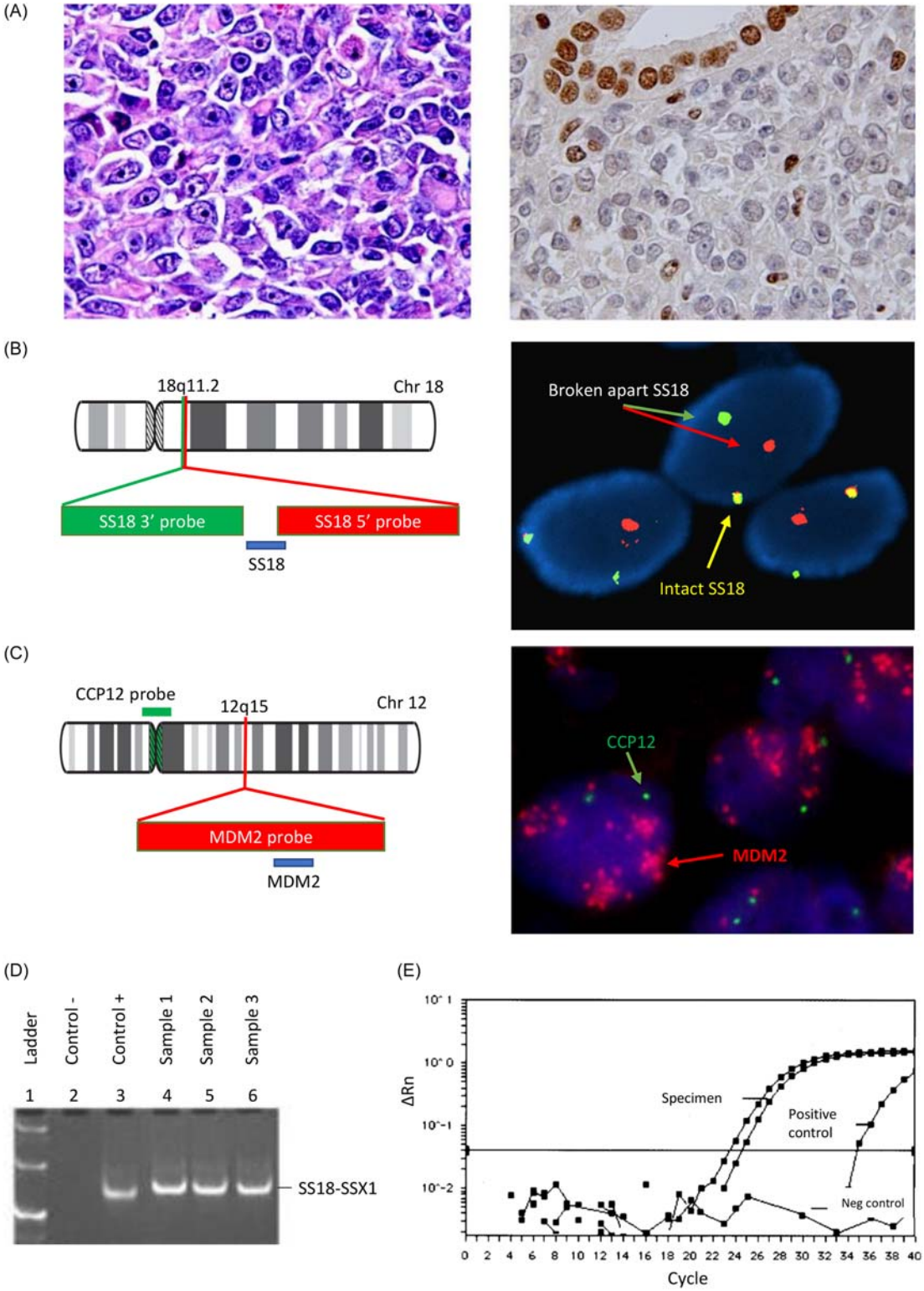

Fig. 5 Historic cytogenetic and molecular techniques for sarcoma diagnosis. (A) Immunohistochemistry (IHC) for SMARCB1-deficiency in malignant rhabdoid tumors. *Left panel*: Hematoxylin and Eosin staining (HES) of a classical malignant rhabdoid tumor; *right panel*: IHC with SMARCB1 antibody shows the absence of expression of SMARCB1 within tumor cells, whereas a normal nuclear expression is retained within endothelial cells. (B) Fluorescent in situ hybridization (FISH) for translocation-associated sarcomas. *Left panel*: representation of the *SS18* broken-apart probes used for the detection of *SS18* rearrangements. *Right panel*: example of FISH with the *SS18* broken-apart probe in a case of synovial sarcoma with *SS18-SSX1* fusion. The detection of two distinct *red* and *green* signals shows the rearrangement of *SS18* gene within tumor cells. (C) FISH for sarcomas with *MDM2* amplification. *Left panel*: representation of *MDM2* and chromosome 12 centromeric (CCP12) probes used for the detection of *MDM2* amplification. *Right panel*: example of *MDM2* amplification detected by FISH in a case of well-differentiated liposarcoma. The detection of multiple red signals shows the presence of the *MDM2* amplification within tumor cells. (D) Reverse-transcription polymerase chain reaction (RT-PCR) for the diagnosis of fusion-positive sarcomas. Line 1, ladder; line 2, negative control; line 3, positive control for SS18–SSX1 fusion transcript; lines 4–6, three samples of synovial sarcoma showing the presence of the SS18–SSX1 specific amplicon. (E) Quantitative RT-PCR for the diagnosis of fusion-positive sarcomas. The panel shows the amplification of a specific SS18–SSX1 amplicon in two synovial sarcomas specimens, whereas the amplicon is not detected in the negative control. The cycle at which the amplicon is detected gives a quantitative aspect of the expression level of the fusion transcript.

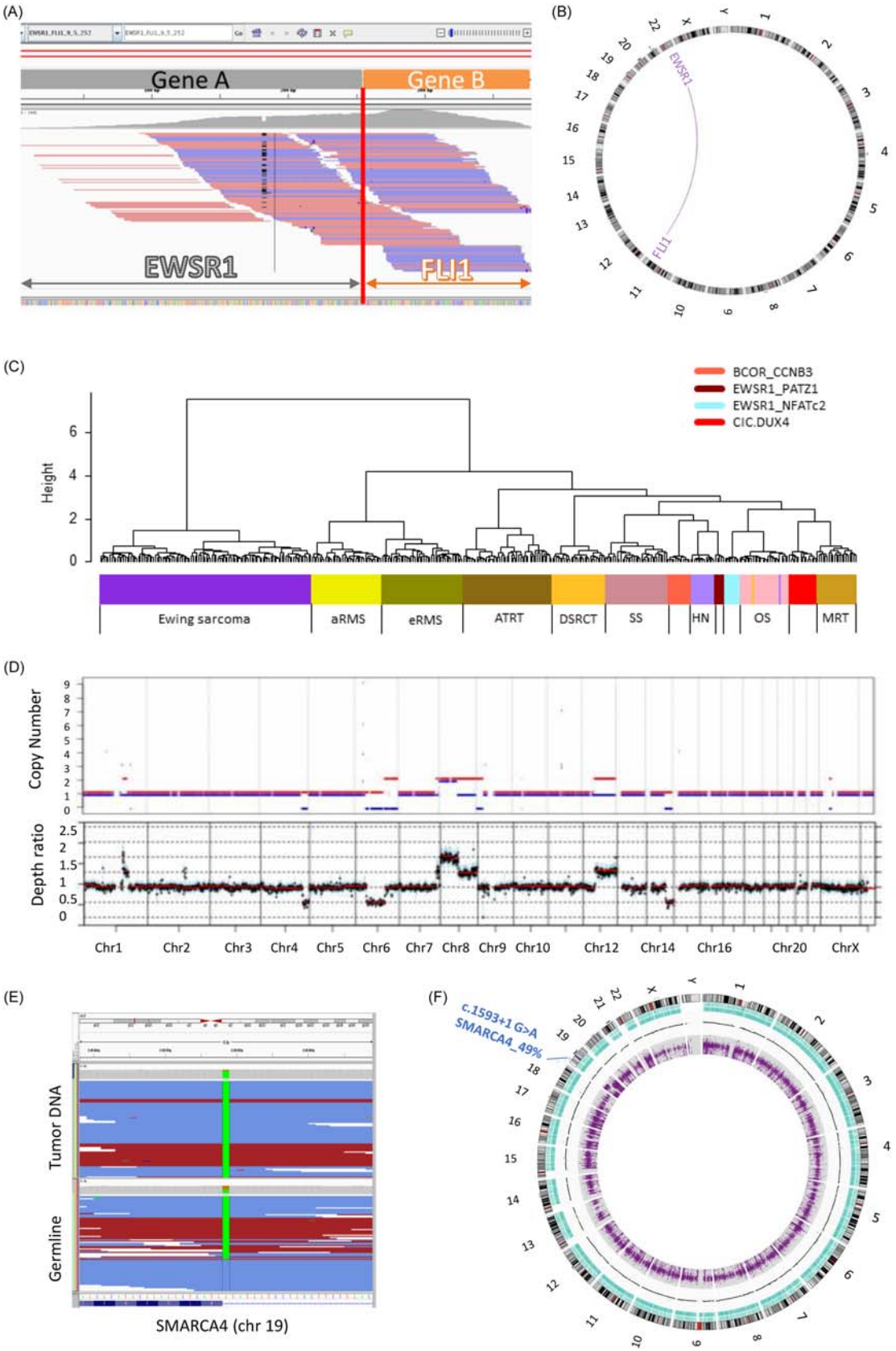

Fig. 6 Next generation sequencing for sarcoma diagnosis and theranostics. RNAseq is the gold standard NGS method for detection of previously known and/or new gene fusions. (A) Example of the detection of an EWSR1–FLI1 fusion transcript by RNAseq in a case of Ewing sarcoma. (B)

revealed that those tumors were biologically related to the other SWI/SNF-deficient MRT and small cell carcinoma of the ovary, hypercalcemic type (SCCOHT), but distinct from SMARCA4-mutated lung carcinoma.

Whole-exome and whole-genome sequencing

Apart from transcriptome sequencing, both whole-exome (WES) and whole-genome sequencing (WGS) are becoming key NGS approaches to study sarcoma genomics. WES captures and then deeply sequences all protein-coding exons of the human genome, while WGS is an unbiased technique that provide sequencing of all accessible genomic regions. Both techniques enable to detect point mutations, small insertions and deletions (indels), and copy number variations (**Fig. 6D–F**), while WGS can also simultaneously capture genome structure and intergenic variations.

Although those techniques are not currently used in clinical practice, they have already enabled to provide a genomic description of specific sarcoma subtypes and to detect previously unknown mutations that may play a major role in sarcomagenesis. For example, WES on series of matched tumor and germline samples of Ewing sarcoma patients enabled to detect recurrent mutations in the *STAG2* gene in approximately 20% of patients, the latter being associated with poor prognosis and suggesting a role of STAG2 loss-of-function in tumor progression.

In summary, NGS techniques are becoming major tools to study sarcomagenesis. Although not currently routinely used in clinics, NGS approaches have already enabled to detect previously unknown alterations in multiple sarcoma subtypes, including fusion genes and mutations, and thus to add new diversity and complexity to the usual tumor classification. Chromatin-immunoprecipitation coupled to sequencing (ChIP-seq) data are also being generated, and will facilitate target genes discovery in sarcomas with chimeric transcription factors. The current pathological classification of sarcomas is thus currently being entirely refined with the increasing number of biological data emerging from those approaches. Undoubtedly, NGS techniques combined to pathological analyses will soon become gold standard approaches for sarcoma diagnosis and classification.

Circulating-tumor DNA for sarcoma prognosis and follow-up

Circulating-tumor DNA (ctDNA) refers to fragments of DNA derived from tumor cells and circulating in the blood together with cell-free DNA from other sources. Although the mechanism leading to ctDNA release have not been fully characterized so far, it is believed that most ctDNA fragments originate from tumor cells necrosis or apoptosis. With an average size of 167 bp, ctDNA constitutes a major noninvasive genomic reservoir of tumor clones and gives a dynamic representation of tumor genomic diversity. As a result, several methods have been developed to capture and analyze ctDNA for specific molecular tumor alterations, including allele-specific PCR, droplet digital PCR (ddPCR) or targeted NGS, and are successfully used in several cancer types to monitor genomic alterations in a dynamic way.

Due to the low incidence of sarcomas, only few studies have evaluated the potential of ctDNA for genomic profiling and follow-up of patients. Some studies have suggested that ctDNA was not as frequent in sarcomas (especially soft tissue sarcomas) as in epithelial cancers. However, successful ctDNA monitoring has been reported in several subtypes of bone and soft tissue sarcomas.

Indeed, the presence of a specific gene fusion can be detected in ctDNA with a high level of sensitivity for translocation-associated sarcomas. For example, a study carried out on 20 patients with Ewing sarcoma undergoing multiple ctDNA assessments before and during multimodal treatment, revealed that *EWSR1* gene rearrangements could be detected in ctDNA in 90% of patients. Notably, ctDNA levels were correlated to tumor volume both in patients with localized or metastatic disease, and the kinetics of *EWSR1*fusion sequence copy number in the plasma correlated to response to initial chemotherapy or tumor recurrence. Similarly, successful detection and monitoring of *EWSR1-WT1* fusion gene in ctDNA of patients with desmoplastic small round cell tumors has been reported. Thus, ctDNA appears to be particularly appropriated for the follow-up of patients with fusion-positive sarcomas and by extension with karyotypically-simple sarcomas, due to the high specificity of the molecular alterations and to their expression in all tumor clones.

Apart from gene fusions, other genomic alterations have been successfully detected in ctDNA from sarcomas patients. These include *IDH1* mutations in conventional chondrosarcoma, *MYOD1* mutations in spindle cell rhabdomyosarcoma, *H3F3A* mutations in giant cell tumor of bone, or *KRAS* and *NF1* mutations in high-grade malignant spindle cell sarcoma. On the contrary,

Representation of the gene fusion detected by RNAseq in a CIRCOS representation. CIRCOS enables to give a circular representation of the transcriptomic finding, with the line indicating the fusion between genes located on separate chromosomes, the latter being distributed along the main circle. RNAseq also enables to establish transcriptional signatures and to compare the expression profiles of a pool of different samples thanks to unsupervised clustering methods. (C) Clustering of a cohort of pediatric sarcomas, showing the distinction of multiple tumor subtypes based on their expression profiles. In particular this figures shows that the tumors previously referred to as "Ewing-like sarcoma" such as CIC-DUX4 and BCOR-CCNB3 fusion-positive sarcomas, constitute distinct tumor entities clearly apart from classical Ewing sarcoma. Whole-exome-sequencing enables to establish copy number profiles on tumor DNA. (D) Copy number profiling by WES of a case of undifferentiated sarcoma showing gain of losses of multiple chromosomal fragments. WES also enables to look for mutations both in somatic and germline DNA. (E) Example of detection of a bi-allelic SMARCA4 inactivating mutation in a case of malignant rhabdoid tumor; the same mutation is found at the hemizygous level in matched germline DNA. (F) CIRCOS representation of the genomic data obtained by WES of the SMARCA4-mutated tumor presented in (E). Mutations are indicated along the outer circle, *while* copy number variations are plotted along the inner circles. Abbreviations: *aRMS*, alveolar rhabdomyosarcoma; *eRMS*, embryonal rhabdomyosarcoma; *ATRT*, atypical teratoid rhabdoid tumor; *DSRCT*, desmoplastic small round cell tumor; *SS*, synovial sarcoma; *HN*, HEY1-NCOA2 fusion-positive sarcoma; *OS*, osteosarcoma; *MRT*, malignant rhabdoid tumor. Courtesy from Gaëlle Pierron for CIRCOS plots.

for other tumors carrying well characterized mutations such as *CTNNB1* mutations in desmoid-like fibromatosis or *GNAS* mutations in fibrous dysplasia and intramuscular myxoma, no ctDNA could be detected. The absence of ctDNA detection in those cases could be due to the low aggressive potential of those tumors which do not metastasize. Indeed, there is growing evidence that ctDNA amount is directly correlated to tumor grade and metastatic potential in sarcoma patients. For example, in a recent study on patients with conventional chondrosarcoma harboring *IDH1* mutations, mutant *IDH1* ctDNA could be detected in all dedifferentiated and grade III tumors, but much more inconstantly in grade II and low-grade cases.

Concerning patients with cytogenetically complex sarcomas carrying multiple genomic aberrations in common genes such as *TP53*, RB1 pathway or Hedgehog pathway, numerous gene loci are commonly involved in these alterations, which will require the development of complex assays for ctDNA detection. In those patients, ctDNA will probably lead to a better understanding of tumor biology, with the possibility to follow multiple genomic alterations in the blood and thus to better explain their role in sarcoma development and progression.

To sum-up, ctDNA will probably become a major tool for genomic characterization, prognostic classification and follow-up of high-grade sarcoma patients undergoing multimodal treatment. Due to the low incidence of sarcomas, the inclusion of patients in systematic clinical trials will facilitate the evaluation of the impact of ctDNA assessment both for prognostic and therapeutic impacts.

Predisposition to sarcomas and genetic counseling

Although most cases of sarcoma arise sporadically with somatically-restricted genetic alterations, a proportion of tumors are integrated within well-defined heritable cancer predisposition syndromes. Since the 1880s and the first characterization by Heinrich Von Recklinghausen of the multiple neurofibromas affecting patients with neurofibromatosis type 1 (NF1) syndrome, multiple cancer predisposition syndromes have been described that can favor sarcoma development (**Table 3**). Although the first characterizations of those syndromes relied only on careful clinical observation and annotation, the tremendous advances in genetics and molecular biology have enabled to refine their description and to give insight into the biological mechanisms of sarcomagenesis for several tumor subtypes.

For example, Li–Fraumeni syndrome was first described in 1969 with the description of early onset cancers transmitted in an autosomal dominant way in a few families. More than 20 years later, germline inactivating mutations in the tumor suppressor gene *TP53* were identified as the responsible genetic aberrations. Since then, reports of large cohorts of LFS patients have enabled to widely refine the clinical and genetic characteristics of the syndrome and have led to the identification of several mutational subtypes with different clinical implications. Even if those inherited syndromes are rare, a key issue is that using common clinical criteria leads to cancer diagnosis before syndrome diagnosis, with no opportunity for preventive intervention. Thus, the complete familial medical history should be carefully examined in case of early onset sarcoma, and genetic counseling should be largely proposed in case of evocative history.

Apart from those rare inherited syndromes, several recent studies have reported a high frequency of germline mutations in cancer predisposition genes in patients with sporadic sarcomas. In a large case-control study involving 1162 sarcoma probands and 6545 Caucasian controls, an excess of potentially pathogenic germline variants was detected by targeted exome-sequencing in 55% of sarcomas patients. The most frequent mutated genes were *TP53*, *ATM*, *ATR*, *BRCA2*, and *ERCC2*, and 25% of probands carried variants with potential therapeutic significance. The excess risk was suggested to lie in both classic monogenic and previously unrecognized polygenic rare variations. Of note, the cumulative burden of multiple variants was associated with earlier age at sarcoma diagnosis. This still needs to be further confirmed in new studies but points out the attention that is required for possible genetic predisposition in sarcoma patients.

Thus, the frequency of putatively pathogenic germline variations in cancer genes may be particularly high in sarcoma patients, even in apparently sporadic cases. If recognized risk management strategies have been successfully validated for hereditary syndromes, there is now evidence that genetic counseling should be proposed more widely for sarcoma patients, and that sarcoma families carrying high-risk genetic variants might also benefit from dedicated surveillance and prevention strategies.

Therapeutic Perspectives in Sarcomas

Despite the tremendous advances in the understanding of sarcoma biology and the molecular dismantling of numerous sarcoma subtypes, those progress have not yet been associated to dramatic changes in the therapeutic strategies for most sarcoma patients. Indeed, apart from tumors for which an aberrant tyrosine kinase activity can be successfully targeted with specific tyrosine kinase inhibitors such as GIST or DFSP, most sarcomas are currently still being treated with unspecific multimodal therapeutic strategies including wide surgery, radiation therapy, and cytotoxic chemotherapy. However, new therapeutic strategies relying on biological data are emerging, both for sarcomas with simple and complex cytogenetic profiles.

Targeting gene expression reprogramming in sarcoma

There is now substantial evidence that some sarcoma subtypes are associated to a specific transcriptional signature due to the reprogramming of normal gene expression, particularly in the case of chimeric proteins with transcription factor activity. Targeting this aberrant transcriptional program represents a key therapeutic challenge, since contrary to chimeric tyrosine kinase receptors, transcription factors are not easily targetable. This is due notably to the fact that transcription factors usually lack ligand-binding domains or substrate-binding enzymatic pocket that constitute excellent opportunities for drug development. Moreover, the surface

Table 3 Main inherited syndromes associated with increased risk of sarcoma

Syndrome	*Gene*	*Transmission*	*Main clinical features*	*Associated sarcoma*
Li-Fraumeni	P53	AD	Early onset of multiple cancers including breast cancer, CNS tumors, adrenocortical carcinomas, leukemia	Osteosarcoma, RMS, LMS and others high-grade STS
Hereditary retinoblastoma	RB1	AD	Retinoblastoma before 5 years, often bilateral	Osteosarcoma and other high-grade STS
Neurofibromatosis type 1	NF1	AD	Café-au-lait spots, neurofibromas, optic gliomas, iris harmarthomas, skeletal defects	MPNST, RMS, GIST
Constitutional mismatch repair deficiency syndrome	MLH1, MSH2, MSH6, PMS2	AR	Predisposition to hematological and CNS malignancies, gastrointestinal tumors	Embryonal RMS
Familial adenomatous polyposis	APC	AD	Multiple colic adenomatous polyps, colic cancer before 40 years, osteoma, hepatoblastoma	Desmoid tumor
Carney-Stratakis	SDHB, SDHC	AD	GIST and paraganglioma	GIST
Familial GIST	KIT, PDGFRα	AD	ICC hyperplasia	Multifocal GIST
Familial rhabdoid predisposition syndrome	SMARCB1	AD	MRT and CNS tumors	MRT
Tuberous sclerosis	TSC1, TSC2	AD	Hamartomas, angiomyolipomas, renal cysts, RCC, angiofibromas	PEComa, chordoma
Hereditary leiomyomatosis and renal cancer	FH	AD	Type 2 papillary RCC, cutaneous and uterine leiomyoma	Uterine LMS
DICER1 syndrome	DICER	AD	Pleuropulmonary blastoma, cystic nephroma, ovarian sex cord-stromal tumors, thyroid neoplasia	Embryonal RMS
Gorlin syndrome	PTCH	AD	Basal cell carcinoma, odontogenic keratocyst, ovarian and heart fibroma, skeletal defects	Embryonal RMS
Bloom	RECQL3	AR	Short stature, sun sensitivity, skin changes	Embryonal RMS, osteosarcoma
Rothmund-Thomson syndrome II	RECQL4	AR	Poikiloderma, telangiestasias, congenital bone defects, cataract, hypogonadism	Osteosarcoma
Beckwith-Wiedeman	CDKN1C, IGF2, H19, KCNQ1OT1	AD, sporadic	Overgrowth syndrome, hepatoblastoma, Wilms tumor	Embryonal RMS
Costello	HRAS	AD	Rasopathy: Faciocutaneoskeletal syndrome, mental retardation	Embryonal RMS

Abbreviations: *AD*, autosomal dominant; *AR*, autosomal recessive; *RMS*, rhabdomyosarcoma; *LMS*, leiomyosarcoma; *STS*, soft-tissue sarcoma; *MPNST*, malignant peripheral nerve sheat tumor; *GIST*, gastrointestinal stromal tumors; *PEComa*, perivascular epitheliod cell tumor; *RCC*, renal cell carcinoma; *ICC*, interstitial cells of Cajal; *MRT*, malignant rhabdoid tumor; *CNS*, central nervous system.

of protein–protein and protein–DNA interactions is generally large for transcription factors and difficult to target, which creates issues for the development of small inhibitory molecules. However, several new therapeutic strategies are currently being developed for interfering with transcription factors' activity in sarcomas (**Fig. 7**).

Targeting transcription factors

Trabectedin is a marine-derived alkaloid initially isolated from the Caribean tunicate *Ecteinascidia turbinata*. Trabectedin binds covalently to DNA minor groove triplets, whereas another part of the molecule protrudes out of the DNA helix and interacts with the transcriptional machinery in a gene and promoter-dependent way. Although objective response rates to trabectedin in advanced anthracycline-resistant soft tissue sarcomas do not exceed 10%, a significantly higher proportion of responses have been reported in myxoid liposarcoma (MLPS) patients. MLPS accounts for 30%–35% of liposarcomas and is characterized by characteristic translocations resulting in the expression of a chimeric transcription factor, most of the time FUS-DDIT3 and less frequently EWSR1-DDIT3. Recent studies have shown that trabectedin blocks the trans-activating functions of FUS-DDIT3 by displacing the oncogenic fusion protein from its target promoters, which enables to induce adipogenic differentiation of MLPS cells both in experimental in vitro and in vivo models and in patient's biopsies. Trabectedin has also been shown to modulate the transcription program driven by EWSR1–FLI1 fusion and thus increase the susceptibility of Ewing sarcoma cells to topo-isomerase I poisons such as irinotecan. However, no significant response to trabectedin in Ewing sarcoma patients could be observed in a pivotal Phase II study, probably due to pharmacokinetic and pharmacodynamic issues. New drugs such as lurbinectedin, a second-generation trabectedin

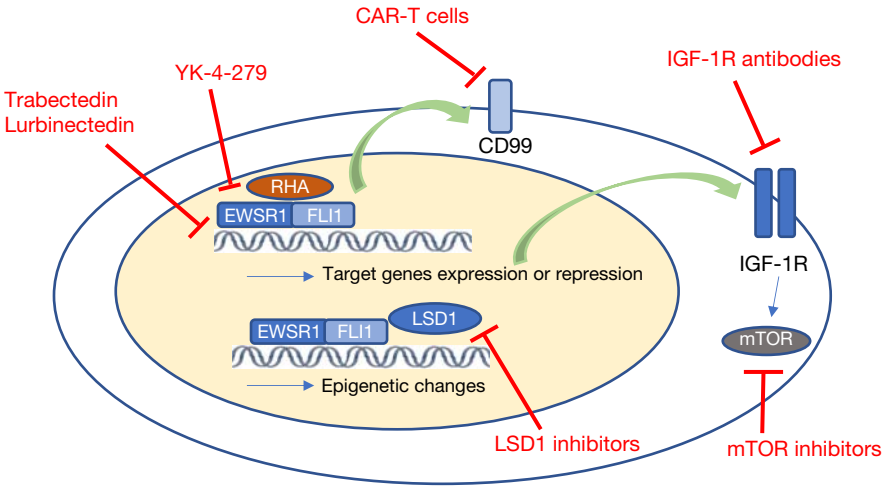

Fig. 7 Targeting transcriptional reprogramming in sarcomas: the example of Ewing sarcoma. Schematic representation of the therapeutic strategies aiming at modulating EWSR1–FLI1 transcription factor activity. EWSR1–FLI1 binding to the DNA helix can be directly targeted with the cytotoxic agents trabectedin and lurbinectedin, whereas its interaction with RNA helicase A (RHA), a major component of the transcription factor machinery, can be targeted with the small molecule YK-4-279. Epigenetic cofactors of EWSR1–FLI1 include the lysine-specific demethylase 1 (LSD1), which can be targeted with specific LSD1 inhibitors. Downstream targets of EWSR1–FLI1 such as the IGF1-R pathway also constitute therapeutic opportunities, as shown by the activity of IGF-1R inhibitors in Ewing sarcoma. Last, other EWSR1–FLI1 downstream targets such as the CD99 transmembranous protein can be targeted with immunomodulating agents, including chimeric antigen receptor gene-modified T cells (CAR-T cells) and specific antibodies.

analogue with a more favorable pharmacokinetic profile, are currently being developed and evaluated in Ewing sarcoma and other sarcoma subtypes with aberrant transcription factor activity.

Other approaches to directly target chimeric transcription factors include the development of small inhibitory molecules that interfere with the transcription machinery. For example, YK-4-279 is a small molecule which has been shown to block in vitro and in vivo the interaction between EWSR1–FLI1 and the RNA helicase A, a major component of EWSR1–FLI1 transcriptional complex, resulting in tumor growth inhibition.

Targeting epigenetic cofactors of transcription factors

Epigenetics refers to the heritable changes in gene expression that do not involve the DNA sequence, such as DNA methylation and histone modifications, and the understanding of its misregulation in cancer has significantly advanced over the last decade. There is now substantial evidenced that the oncogenic activity of chimeric transcription factors in sarcomas requires epigenetic cofactors.

For example, EWSR1–FLI1 activity depends upon histone deacetylases (HDAC) for both direct and indirect target gene repression, and treatment of Ewing sarcoma cells with the HDAC inhibitor vorinostat impairs the regulation of EWSR1–FLI1 target genes and reduces cell viability and transforming capacities in vitro. More recently, the lysine-specific demethylase 1 (LSD1) has become a major attractive target in several sarcoma subtypes including Ewing sarcoma, alveolar rhabdomyosarcoma and synovial sarcoma. More precisely, EWSR1–FLI1 mediated transcriptional regulation has been shown to require direct binding to an epigenetic complex containing LSD1. Several studies have reported that LSD1 inhibitors such as HCI-2509 upregulated EWSR1–FLI1-repressed targets and vice versa, and decreased cell viability in vitro. Moreover, LSD1 inhibition showed single-agent efficacy across multiple xenograft models for Ewing sarcoma. Several LSD1 inhibitors are thus currently being tested in early phase clinical studies including advanced Ewing sarcoma patients.

Apart from LSD1 inhibitors and HDAC inhibitors, other molecules targeting epigenetic cofactors such as BET bromodomain inhibitor and EZH2 inhibitors are currently being evaluated in sarcoma patients. Although the use of such therapeutic strategies shows promise, no reliable marker to predict clinical activity or either primary or acquired resistance has been identified so far.

Targeting downstream pathways of transcription factors

Another approach for modulating transcription reprogramming in sarcomas with aberrant transcription factor activity consists in the identification and targeting of downstream regulated pathways. This approach is highly complex due to the fact that transcription factors often regulate numerous different targets involved in various oncogenic processes. Thus, therapeutic strategies will necessarily require drug combinations for effective success in patients. For example, the insulin-like growth factor receptor 1 (IGF-1R) pathway has long been identified as an attractive downstream target of EWSR1–FLI1 chimera in Ewing sarcoma.

The IGF-1R pathway is part of a complex insulin network mostly involved in the regulation of growth, differentiation and development at the cellular, organ and organismal levels. Upon binding of the ligands IGF-1 and IGF-2, IGF-1R auto-phosphorylates which leads to subsequent activation of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) pathways. EWSR1–FLI1 regulates IGF1 and the IGF1-R pathway has long been known to be activated in Ewing sarcoma cell lines

and tumor tissues. Moreover, IGF-1 and IGF-2 bioavailability in the peripheral tissues is mostly regulated by IGF-binding proteins (IGFBP1-6), a group of proteins that bound the ligands in the circulation. More precisely, EWSR1–FLI1 fusion protein has been shown to directly bind the IGFBP3 promoter, thereby repressing its activity and leading to increased IGF-1R signaling.

These findings have led to evaluate several IGF-1R inhibitors in Ewing sarcoma patients, including monoclonal antibodies and tyrosine kinase inhibitors. The first early phase clinical trials evaluating anti-IGF-1R antibodies in advanced Ewing sarcoma patients have evidenced between 10% and 15% of objective tumor responses and approximately 25% of patients with stable disease, sometimes durable up to more than 2 years. Combining IGF-1R antibodies with the mTOR inhibitor temsirolimus resulted in higher antitumor activity with 35% of objective response. This example illustrates the relevance of targeting downstream molecular pathways in sarcomas with aberrant transcriptional programs. The successful development of such new therapeutic strategies will however require concomitant translational studies to prospectively identify potential responders.

Targeting metabolic regulators

The discovery of recurrent mutations in genes involved in metabolism regulation both in SDH-deficient GIST and IDH1/2-mutated chondrosarcoma has highlighted the role of metabolism balance in sarcomagenesis. Although SDH complex and IDH enzymes belong to different pathways, they are both involved in the regulation of glucose and fatty acid metabolism and in the maintenance of cellular redox status. Moreover, both loss of function of SDH and activating mutations of IDH1/2 result in the abnormal accumulation of a metabolic product within the cytoplasm, which is thought to act as an oncogene to promote tumor development.

Strikingly, aberrant hypermethylation patterns are observed in SDH-deficient GIST as well as in IDH1/2-mutated chondrosarcomas, suggesting that metabolic products can interfere with epigenetic cofactors to modulate gene expression. Since these mutations are thought to be early events in tumor biology, they thus represent attractive therapeutic targets.

Several selective inhibitors of the mutant forms of IDH1 and IDH2 have been developed over the last few years and have shown impressive clinical efficacy in IDH-mutated acute myeloid leukemia. For patients with solid tumors, no data are yet available in chondrosarcoma patients, but IDH inhibition has already proven to be efficient in a few IDH-mutated glioma patients. The results of ongoing clinical trials involving chondrosarcoma patients are thus eagerly expected.

Whether similar oncogenic mechanisms also occur in other sarcomas subtypes remains to be further investigated, but several studies have already highlighted abnormal metabolic regulation in both sarcomas with simple and complex genomic profiles. However, if those abnormalities are playing a key role in tumor initiation and progression or constitute only a stigmata of cancer cell activity needs to be elucidated.

Immunomodulating agents for sarcoma treatment

In the late 1800s, spontaneous sporadic remissions in sarcoma patients after severe infections were reported by William Coley, inspiring future generations of physicians and immunologist to use the immune system for cancer cure. Over a century later, new approaches of immunotherapy have become a cornerstone of cancer treatment, with more than a thousand clinical trials evaluating immunomodulating agents currently accruing on clinicaltrial.gov. In sarcoma patients, several types of immunomodulating strategies approaches are currently being developed.

The recent development of monoclonal antibodies targeting immune checkpoint blockers such as CTLA4 and the PD-1/PDL-1 axis has changed the landscape of immunotherapy, with major and sustained responses in nonsmall cell lung cancer, melanoma, urothelial cancer or head and neck carcinoma. Thus, these agents have rapidly been tested in several early phase clinical trials including sarcoma patients. SARC028 was a Phase II study evaluating the efficacy of the PD-1 antibody pembrolizumab in 80 patients with bone and soft tissue sarcoma (STS). The response rate was 19% in patients with STS, with an additional 40% of patients with stable disease. Whereas no activity could be observed in patients with LMS, most cases or tumor response were observed in patients with high-grade undifferentiated pleomorphic sarcomas and DDLPS. For bone sarcomas, only 5% of response were observed and concerned isolated patients with dedifferentiated chondrosarcoma and osteosarcoma. Since then, several studies have confirmed that monotherapy with immune checkpoint blockers should not be proposed to unselected sarcoma patients due to lack of efficacy and absence of predictive biomarker of response.

PDL-1 expression has been proposed as a predictive biomarker of efficacy of immune checkpoint blockers for several tumor types. In sarcoma, PDL-1 expression ranges from 12% to 65% depending on tumor subtypes and the type of antibody used for the analysis. In a large cohort of more than 320 patients with STS, only 20% of tumors expressed PDL-1, but 98% of PDL-1 expressing tumors showed CD8+ T cell infiltrates, supporting an immunosuppressive signature. Other immune infiltrates including Treg and tumor-associated macrophages (TAM) have been reported in sarcomas, but, so far, the role of those infiltrates to dictate prognosis and response to immune checkpoint blockers is still unknown.

Apart from immune checkpoint blockers, other immunomodulating agents are being developed and evaluated in distinct sarcoma subtypes. Vaccination strategies with tumor specific antigens and adoptive T cell therapy are currently being evaluated and constitute attractive approaches for sarcomas with simple genomic profile. Engineered TCR therapies such as specific chimeric antigen receptor gene-modified T cells (CAR T cells) directed against the ganglioside GD2 are also being evaluated in early phase trials in patients with Ewing sarcoma and osteosarcomas.

In conclusion, the use of immunomodulating agents in clinical practice in sarcoma patients is still at its infancy, and understanding the molecular features predicting the efficacy of modern immunotherapy approaches is mandatory to detect patients that could benefit from these therapeutic strategies. Since sarcomas are rare tumors, enrollment of patients with advanced disease in dedicated clinical trials will accelerate the optimal use of these new agents.

See also: Bones and Joints Cancer: Pathology and Genetics.

Further Reading

Ballinger, M.L., Goode, D.L., Ray-Coquard, I., et al., 2016. Monogenic and polygenic determinants of sarcoma risk: An international genetic study. Lancet Oncology 17, 1261–1271.

Chibon, F., Lagarde, P., Salas, S., et al., 2010. Validated prediction of clinical outcome in sarcomas and multiple types of cancer on the basis of a gene expression signature related to genome complexity. Nature Medicine 16, 781–787.

Delattre, O., Zucman, J., Melot, T., et al., 1994. The Ewing family of tumors–A subgroup of small-round-cell tumors defined by specific chimeric transcripts. New England Journal of Medicine 331, 294–299.

Erkizan, H.V., Kong, Y., Merchant, M., et al., 2009. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. Nature Medicine 15, 750–756.

Farid, M., Ngeow, J., 2016. Sarcomas associated with genetic cancer Predisposition syndromes: A review. The Oncologist 21, 1002–1013.

Fujii, T., Khawaja, M.R., DiNardo, C.D., et al., 2016. Targeting isocitrate dehydrogenase (IDH) in cancer. Discovery Medicine 21, 373–380.

Gaspar, N., Hawkins, D.S., Dirksen, U., et al., 2015. Ewing sarcoma: Current management and future approaches through collaboration. Journal of Clinical Oncology 33, 3036–3046.

Gianferante, D.M., Mirabello, L., Savage, S.A., 2017. Germline and somatic genetics of osteosarcoma—Connecting etiology, biology and therapy. Nature Reviews. Endocrinology 13, 480–491.

Hirota, S., Isozaki, K., Moriyama, Y., et al., 1998. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 279, 577–580.

Kadoch, C., Crabtree, G.R., 2013. Reversible disruption of mSWI/SNF (BAF) complexes by the SS18–SSX oncogenic fusion in synovial sarcoma. Cell 153, 71–85.

Le Loarer, F., Watson, S., Pierron, G., et al., 2015. SMARCA4 inactivation defines a group of undifferentiated thoracic malignancies transcriptionally related to BAF-deficient sarcomas. Nature Genetics 47, 1200–1205.

Taylor, B.S., Barretina, J., Maki, R.G., et al., 2011. Advances in sarcoma genomics and new therapeutic targets. Nature Reviews. Cancer 11, 541–557.

Wilky, B.A., Goldberg, J.M., 2017. From vision to reality: Deploying the immune system for treatment of sarcoma. Discovery Medicine 23, 61–74.

Bones and Joints Cancer: Pathology and Genetics

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Introduction

Secondary bone tumors (e.g., metastasis presenting as bone tumors without at that time a known primary tumor) and plasmacytoma outnumber by far primary bone tumors except at children's age. Here we focus on primary bone tumors.

Many different types of primary tumors and tumor-like lesions have been described in bones. These lesions vary widely in their clinical behaviour and pathological features. Benign and malignant lesions may present at virtually any age, though there is a certain predilection period per tumor type. Most bone tumors are rare and often asymptomatic and indolent. Therefore, their true incidence is unknown.

Bone sarcomas account for only 0.2% of all neoplasms. Their incidence rates are age-related with a first peak occurring during the second decade of life, in which age group osteosarcoma and Ewing sarcoma are most frequent, and a second peak occurring in patients >60 years of age, when chondrosarcoma is the most frequently encountered bone sarcoma.

Bone sarcomas have a very specific spatial distribution in the long bones. Most bone sarcomas present around the knee (up to 56% under the age of 20 years), followed by the pelvis. Most patients experience pain and/or swelling, and therefore symptoms are not very specific. All patients with suspected bone tumors should be discussed in multidisciplinary team meetings including the radiologist and pathologist involved in the diagnosis and the (orthopedic) surgeon and oncologist involved in treatment. Radiographic imaging is of utmost importance in diagnosing bone tumors. Good image quality of the entire lesion in at least two directions is essential to assess the nature of the process. Key-factors for making a relevant judgment and differential diagnosis include: location of the lesion in the bone as a whole, localisation in the affected part of the bone, destruction pattern, matrix mineralization, cortical damage, periosteal reaction and soft tissue extension.

Some lesions develop primarily in the metaphysis (e.g., osteosarcoma and giant cell tumor of bone), others have a mostly epi- (e.g., chondroblastoma) or diaphysial (e.g., Ewing sarcoma) origin (Fig. 1). Moreover, lesions can originate centrally (e.g.,

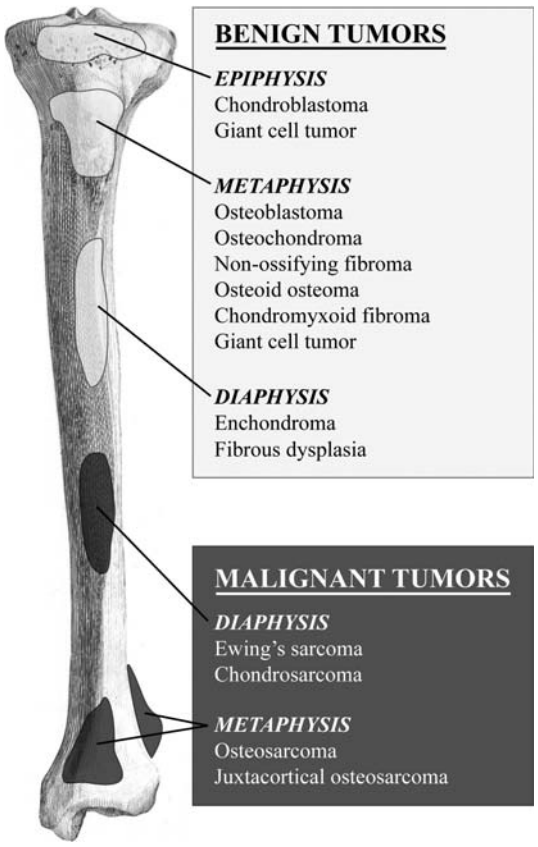

Fig. 1 Common location of bone tumors. Adapted from Fletcher, C. D. M., Bridge, J. A., Hogendoorn, P. C. W., et al. (2013). *WHO classification of tumors of soft tissue and bone*. Lyon, France: IARC Press.

enchondroma), eccentric (e.g., aneurysmal bone cyst), cortical (e.g. osteoid osteoma) or juxtacortical (e.g., parosteal/periosteal osteosarcoma). Multifocality is observed for enchondromas, osteochondromas, fibrous dysplasia, vascular tumors, Langerhans cell histiocytosis, multiple myeloma and metastases. The presence or absence of matrix (bone, cartilage) formation by the tumor assists in further characterization. Furthermore, the destruction pattern of a lesion (e.g., geographical, mottled-permeative) and the appearance of tumor margins (e.g., smooth, lobular, sharp, blunt, sclerotic) are important. The presence and amount of cortical damage (e.g., scalloping, irregular destruction or thickening) play a role in the interpretation. Presence or absence of a periosteal reaction (e.g., multi- or single layered, interrupted or not, Codman triangles and spiculation) further reduce the differential diagnosis.

As a rule, a reliable histological diagnosis cannot be made without adequate radiographical information and correlation, emphasizing the importance of multidisciplinary team meetings to establish a final diagnosis. One should always start with studying a conventional radiograph in two planes, which can be followed by MRI or CT to further define the extent of the lesion. Imaging should always be performed before the biopsy, because of the significant artifacts a biopsy results in on especially MR imaging.

While most bone sarcomas arise de novo, they can also develop within other conditions, such as Paget's disease of bone, or in preexisting benign lesions such as enchondromas and osteochondromas. Also, bone sarcomas can occur within hereditary syndromes such as Li-Fraumeni or retinoblastoma (osteosarcoma).

Classification of Bone Tumors

The current classification of bone tumors is based on the WHO consensus in which the differentiation of the tumor cells and the extracellular matrix that is being formed are important factors: (i) chondrogenic tumors (forming cartilaginous matrix), (ii) osteogenic tumors (forming osteoid), and (iii) osteoclastic giant cell rich tumors. In addition, fibrogenic, fibrohistiocytic, hematopoietic, notochordal, vascular, myogenic and lipogenic tumors are found, based on their line of differentiation. There are tumors of undefined neoplastic nature (e.g., fibrous dysplasia or aneurysmal bone cyst) and miscellaneous tumors (e.g., Ewing sarcoma, adamantinoma, and undifferentiated pleomorphic sarcoma of bone). At the current WHO classification attempts were made to harmonize the nomenclature with tumors of the soft tissues in those cases were appropriate. Immunohistochemistry plays a limited role in diagnosing primary bone tumors, while molecular diagnostics can be helpful in a subset of tumors.

The Fourth edition of the WHO classification of tumors of soft tissue and bone classifies bone tumors into four categories (similar to its soft tissue counterparts): benign, intermediate locally aggressive, intermediate rarely metastasizing and malignant. Benign tumors have a limited capacity for local recurrence, and if they do, recurrence will be nondestructive. Intermediate locally aggressive bone tumors, such as atypical cartilaginous tumor (previously called chondrosarcoma grade I), often recur locally, and display an infiltrative locally destructive growth pattern, although they do not have an evident potential to metastasize. They require wide excision, or application of a local adjuvant (e.g., phenol or cryotherapy) for local control. Intermediate—rarely metastasizing bone tumors, such as giant cell tumors of bone, bear in addition to their locally aggressive behaviour, a very small risk ($< 2\%$) of distant (lung) metastases. Such behaviour cannot be predicted on histological features. Malignant bone sarcomas carry a significant risk of distant metastases, ranging from 20% to 100% depending on the histotype and grade.

Osteogenic Tumors

Osteogenic tumors are characterized by the direct formation of osteoid or bone by the tumor cells. Bone formation by the tumor cells should be distinguished from metaplastic or reactive bone produced in nonosteogenic tumors or lesions (e.g., clear cell chondrosarcoma), or from growth plate like endochondral ossification (e.g., in osteochondroma, or fracture callus). Malignant transformation of benign bone forming lesions is extremely rare.

Osteoma

Osteoma is a benign tumor composed of compact mature bone arising on the surface of the bone. When developing in the medullary cavity, it is known as enostosis. Osteoma typically occurs in bones formed by membranous ossification, such as calvarial, facial, and jawbones, with a predilection for the paranasal sinuses. Osteoma is rare outside the skull, affecting predominantly adults between the ages of 30 and 50 years. Outside the craniofacial skeleton, parosteal osteosarcoma should be considered in the differential diagnosis. Multiple osteomas are found in the settings of the autosomal dominantly inherited Gardner syndrome (characterized by polyposis coli, osteomas/enostosis, impacted supernumerary teeth, odontomas, desmoid-type fibromatosis, epidermoid- and dermoid cysts). Gardner syndrome is a variant of familial adenomatous polyposis, caused by heterozygous mutation in the *APC* gene.

Osteoma is usually asymptomatic and does not require treatment because the lesions is slow growing and pursues an indolent clinical course. In the paranasal sinuses, it can cause obstruction or local swelling.

Radiologically, osteoma generally appears as a sharply marginated ossified mass projecting from a bony surface. Histologically, lamellar mature bone is seen, varying from compact to more trabecular. The trabeculae are lined by active or inactive osteoblasts within a well-vascularized moderately cellular fibrous stroma. If complicated by inflammation, usually due to an obstruction sinusitis, bone remodeling and a less mature bone can be found.

Osteoid Osteoma

Osteoid osteoma is a benign bone-forming tumor defined by small size (<2 cm), limited growth potential and disproportionate pain (Fig. 2A and B). Characteristically, patients with osteoid osteoma complain of localized and progressive pain that, generally, is worse at night. The lesion is thought to produce arachidonic acid and, as a result of this, non-steroidal anti-inflammatory drugs (NSAIDs) often relieve the pain. Although every bone can be affected, osteoid osteoma is more often found in the metaphysis or shaft of long bones and the cortex, particularly of the proximal femur.

Imaging studies are usually diagnostic. Osteoid osteoma is seen as dense cortical sclerosis surrounding a radiolucent nidus. The lesion is usually <1 cm, and per definition <2 cm (Fig. 2A). For lesions larger than 2 cm, osteoblastoma should be considered. Macroscopically, the nidus is seen as a small, red, granular, and softer area surrounded by sclerotic tissue. The interface between the nidus and the surrounding bone is very sharp. Histologically, the nidus is see as closely packed irregular trabeculae of woven bone, lined by active osteoblasts and occasional small, osteoclast-like giant cells (Fig. 2B). The stroma is loose, well-vascularized, and contains active fibroblasts, osteoblasts and osteoclast-like giant cells. The prognosis is excellent and recurrences are uncommon. In very rare circumstances lesions have been reported to disappear without surgical therapy.

Osteoblastoma

Osteoblastoma is a benign bone-forming lesion (>2 cm) characterized by woven bone spicules bordered by prominent osteoblasts. It is rare, accounting for about 1% of all bone tumors and is more common in males. While osteoid osteoma is most common at the long bones, osteoblastoma preferentially affects the posterior elements of the spine and sacrum. In the appendicular sites, the proximal femur, distal femur and proximal tibia are the most common site. In the spine, osteoblastomas and osteoid osteoma have similar symptoms, such as back pain, scoliosis and nerve root compression. However, in osteoblastoma the pain is usually not worse at night and is less likely to be relieved with NSAIDs.

Radiologically, osteoblastomas are lytic and well-circumscribed lesions, >2 cm (generally 3–10 cm). In those cases with secondary ABC, the lesions are generally much larger. Osteoblastoma is highly vascularized and appears red or red-brown at macroscopy with a pushing border. Histologically at high magnification, osteoblastoma is identical to osteoid osteoma, comprising interconnected trabeculae of woven bone, and distinction is made based on size (Fig. 3). The trabeculae are randomly arranged, lined by a prominent, single layer of osteoblasts. Osteoblasts may have mitosis, but they are usually not atypical. A highly vascularized stroma is present between the trabeculae. Diffusely scattered osteoclast-type, multinucleated giant cells are often observed. Epithelioid osteoblastoma (previously known as aggressive osteoblastoma) have large, plump osteoblasts with a larger nucleus containing a prominent nucleolus, accompanied by mitoses, causing confusion with osteosarcoma. Distinction can be made by careful examination of the interface between the lesion and pre-existing bone, which is not infiltrated in case of osteoblastoma. There is no evidence that epithelioid osteoblastoma has a worse prognosis than the conventional osteoblastoma.

Osteoblastoma has a higher rate of recurrence as compared to osteoid osteoma and requires surgical management. Genetically, recurrent chromosome 22 deletions affecting Wnt/Beta-Catenin signaling are found in osteoblastoma. Although these are not the hallmark of osteoblastoma, genetic analyses can be useful especially in case of a differential diagnosis between epithelioid osteoblastoma and osteosarcoma.

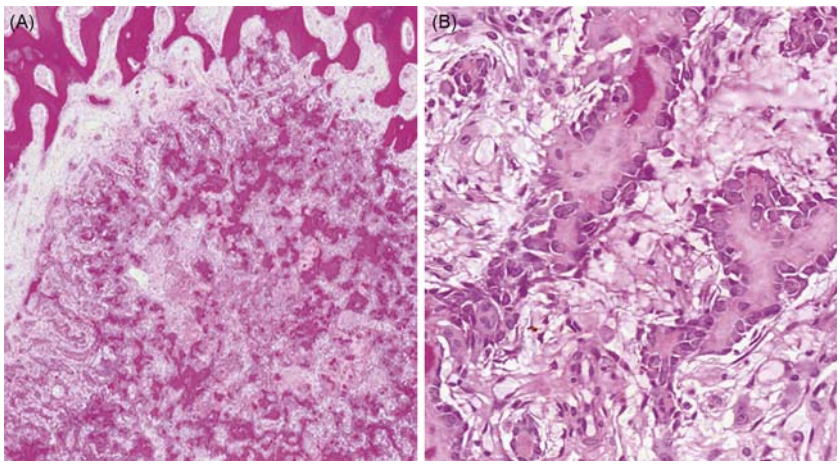

Fig. 2 Osteoid osteoma. (A) The lesion is characterized by a small nidus (<2 cm) (*right*) surrounded by reactive bone formation and fibrovascular tissue. (B) The nidus is composed of closely packed irregular trabeculae of woven bone, lined by active osteoblasts.

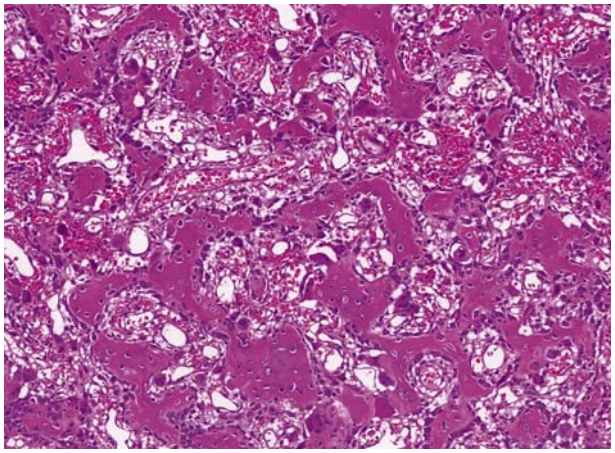

Fig. 3 Osteoblastoma. Interconnected trabeculae of woven bone lined by a prominent, single layer of osteoblasts.

Osteosarcoma

Osteosarcoma is a collective name for a group of sarcomas in which the hallmark is the production of osteoid directly by the malignant tumor cells. In addition to osteoid, the malignant cells may also produce cartilage and collagen. Osteosarcoma can be subdivided according to the site of origin (intra-osseous, surface or soft tissue), grade of malignancy, and differentiation (e.g., fibroblastic, chondroblastic etc.)

Low-Grade Central Osteosarcoma

Low-grade central osteosarcoma is defined as a low-grade malignant bone-forming neoplasm that arises within the medullary cavity of bone. It is rare and accounts for ~1%–2% of all osteosarcomas. In general, it is located in long bones, predominantly in the distal femur and proximal tibia. Radiologically, a large lytic lesion with focal aggressive features may be seen. Low-grade central osteosarcoma is histologically composed of an abundant hypocellular to moderately cellular fibrous tissue proliferation with variable amounts of osteoid production. The spindle-shaped tumor cells show little mitotic activity and slight cellular and nuclear atypia and are arranged in fascicles or interlacing bundles. Osteoclastic giant cells as well as foci of cartilage can be seen. In the majority of low-grade central osteosarcomas, some degree of cortical disruption and/or periosteal reaction and soft tissue extension is observed, which is helpful in the distinction from its mimics (e.g., fibrous dysplasia and desmoplastic fibroma). Moreover, low-grade central osteosarcoma may permeate surrounding cortical and/or cancellous bone, which is not seen in benign bone tumors.

Low-grade central osteosarcomas have a simple genetic profile with supernumerary ring chromosomes comprising amplification of chromosome 12q13–15, including the cyclin-dependent kinase 4 (CDK4) and murine double-minute type 2 (MDM2) gene region. Immunohistochemical expression of MDM2 and CDK4 is useful to differentiate low-grade osteosarcomas from benign fibrous and fibro-osseous lesions including fibrous dysplasia. Additionally, the overall lack of chromosomal aberrations and the low frequency of *TP53* mutations differentiate low-grade central osteosarcoma from the other osteosarcoma subtypes.

Low-grade central osteosarcoma has generally a good prognosis when resected with adequate margins, although progression to high grade osteosarcoma may occur (in 10%–36%).

Parosteal Osteosarcoma (Low-Grade)

Parosteal osteosarcoma is a rare, low-grade, bone-forming lesion that arises at the surface of a long bone. It accounts for ~4% of all osteosarcomas. Around 70% of parosteal osteosarcomas are found at the distal posterior femur. The tumor often presents with a long history of a localized, slow-growing, painless mass, in young adults, with a slight female predominance. The radiological presentation can be very typical with a heavily mineralized mass attached to the cortex of the femur, tibia or humerus. Parosteal osteosarcoma is histologically very similar to low-grade central osteosarcoma, being composed of a moderate cellular to cellular fibrous stroma with formation of long, thin and irregular bone trabeculae that are rather evenly spaced, parallel oriented and show ramifications (Fig. 4). The structure varies from fine lamellar to woven. The fibroblast-like cells are usually arranged in long, parallel oriented fascicles and have a rather uniform nucleus with coarse granular chromatin. Mitotic activity is low. Cartilaginous differentiation can be seen in ~50% of the tumors, either as nodules or as a cap-like arrangement (Fig. 4).

Similar to low-grade central osteosarcoma, >85% of parosteal osteosarcomas contain supernumerary ring chromosomes involving amplification of the 12q13–15 region, and immunohistochemistry or FISH detecting MDM2 or CDK4 amplification may be helpful in the diagnosis.

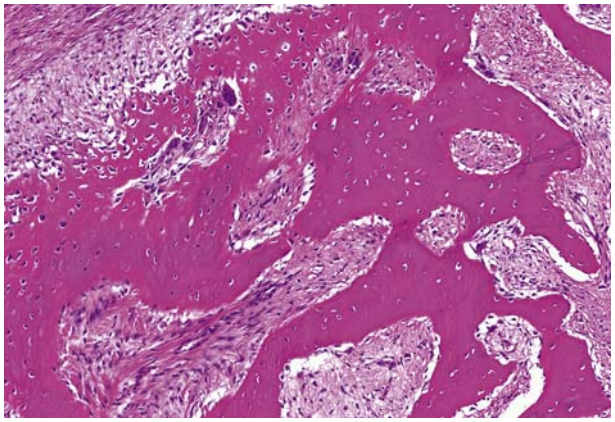

Fig. 4 Parosteal osteosarcoma. The lesion is characterized by a fibrous stroma with formation of long, thin and irregular bone trabeculae.

Progression to a high-grade sarcoma (dedifferentiation) occurs in ~15%–25% of the tumors. These areas resemble conventional osteosarcoma or high-grade spindle cell sarcoma/undifferentiated pleomorphic sarcoma. Dedifferentiation may occur at the time of diagnosis (synchronous) or at recurrence (metachronous), and worsens the prognosis.

Periosteal Osteosarcoma (Intermediate Grade)

Periosteal osteosarcoma is an intermediate grade, malignant, cartilage and bone forming neoplasm arising at the surface of bone. It accounts for < 2% of all osteosarcomas. Periosteal osteosarcoma is predominantly located in the diaphysis or diaphysis/metaphysis. It is usually seen as a sessile, anterior, medial lesion, which may almost wrap around the circumference of the bone, especially the distal femur and proximal tibia. Symptoms of pain and swelling are usually of short duration. Radiologically, the lesion is predominantly radiolucent but often contains focal areas of mineralization in the soft tissue component. There are spicules of reactive bone oriented perpendicular to the underlying bone cortex, which may be focally eroded or thickened. Invasion in the underlying medullary cavity is highly unusual. Histologically nodules of atypical cartilage predominate, occasionally with myxoid changes, separated by malignant spindle-shaped tumor cells (Fig. 5). Lace-like and woven bone is formed by the tumor cells and intermixed with cartilaginous elements and can sometimes be difficult to recognize. In addition, reactive periosteal bone formation, extending as spicules from the base of the tumor to its outer edge (periosteal surface), can be found in between lobules of tumor, but may be destroyed by tumor growth. It can be difficult to differentiate periosteal osteosarcoma from periosteal chondrosarcoma. Periosteal chondrosarcoma has a better prognosis. In periosteal chondrosarcoma, the cartilage is usually more lobular and well differentiated, while osteoid deposition by spindle cells is absent. Moreover, periosteal chondrosarcoma may contain *IDH* mutations, while for periosteal osteosarcoma no consistent genetic alterations have been reported. Reported overall 5- and 10-years survival is 89% and 83% respectively, and local recurrence is associated with an increased risk of metastases.

Conventional Osteosarcoma

Conventional osteosarcoma is a high-grade, intra-osseous, malignant neoplasm in which the neoplastic cells produce bone. It accounts for > 90% of all osteosarcomas. Conventional osteosarcomas usually arise de novo as primary bone tumors. Secondary

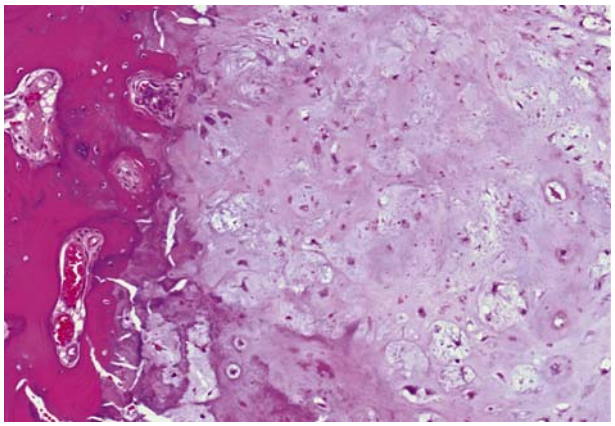

Fig. 5 Periosteal osteosarcoma. The lesion comprises of nodules of atypical cartilage, occasionally with myxoid changes, separated by malignant spindle-shaped tumor cells.

osteosarcomas are mainly associated with Paget's disease of bone or irradiation. Osteosarcoma has a bimodal age distribution, predominantly affecting adolescents and young adults (mean age is about 17 years), with a second peak in patients over 40 years of age. Its etiology is unknown and no benign precursor lesion has been identified. An increased incidence of primary osteosarcoma associated with several genetic syndromes (e.g., Li-Fraumeni, hereditary retinoblastoma, and Rothmund-Thomson). Osteosarcoma is characterized by deep pain that developed over a period of weeks to a few months. Osteosarcoma may affect any bone, but the vast majority is found in the metaphysis of long bones (**Fig. 6**A).

Radiologically, osteosarcoma presents as a lytic and/or sclerotic mass. Tumor expansion causes cortical infiltration and invasion of surrounding tissues. The neoplasm is usually located on the metaphysis and extends into the diaphysis; the epiphysis is generally spared or minimally involved. There is elevation of the periosteum with a prominent periosteal reaction. A characteristic radiological feature is the Codman's triangle that is produced when the tumor breaks through the periosteum.

Histologically, osteosarcoma is highly heterogeneous and has many different histological subtypes (see below), all of which share the diagnostic hallmark of deposition of osteoid by malignant tumor cells with a permeative growth pattern and erosion of preexisting bone trabeculae (**Fig. 6**B and C). Osteoid is un-mineralized bone matrix that is eosinophilic, dense, homogeneous and becomes bone as result of mineralization. No minimal amount of osteoid is required to establish the diagnosis of osteosarcoma. Conventional osteosarcomas are high-grade tumors containing pleomorphic neoplastic cells with large, abnormal, hyperchromatic nuclei. Mitotic activity is high and there can be numerous atypical mitotic figures. The histological heterogeneity of conventional osteosarcoma might be associated with its multipotent cell of origin, which is most likely the mesenchymal stem cell along its path of differentiation to the osteoblastic lineage. The immunohistochemical profile of osteosarcoma is however nonspecific. The tumor cells can express osteocalcin, osteonectin, S100, actin, CD99, keratin, EMA, and desmin, which could cause diagnostic confusion. SATB2 and DMP1 have recently been described as markers for bone-forming tumors as they demonstrate osteoblastic differentiation, which may be of help in cases with limited osteoid deposition and to distinguish hyalinised stroma closely mimicking osteoid.

Osteosarcoma is a highly genomically unstable tumor with as a consequence a complex, aneuploid karyotype. Chromothripsis, involving shattering of the chromosomes caused by a single event followed by their random re-assembly is a common phenomenon. Recurrent amplifications and DNA copy number gains and losses have been reported, such as deletion and LOH of *3q13* (including the *LSAMP* gene), amplification of *6p12–21* (containing the *RUNX2* and *VEGFA* genes), amplification and gain of 8q, and deregulation of the *RB* and p*53* pathway. However, thus far no single specific molecular marker is available to diagnose osteosarcoma.

Conventional osteosarcomas are usually treated with multimodal treatment including neoadjuvant chemotherapy. The histological response to chemotherapy is one of the most important prognosticators of overall and disease-free survival. For evaluating the response it is important to examine a full cross section through the largest tumor diameter, which can be divided in parts and as such fully submitted to microscopy. A good response is defined as $>90\%$ tumor necrosis and is strongly associated with improved survival in primary conventional osteosarcoma.

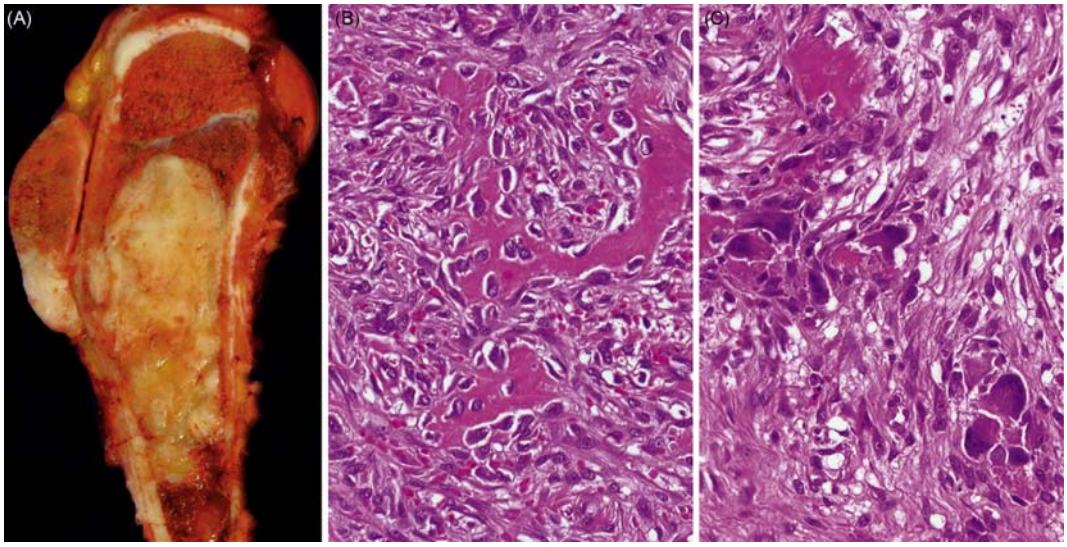

Fig. 6 High-grade conventional osteosarcoma. (A) Gross specimen of conventional osteosarcoma. Note that the epiphyseal plate acts as a relative barrier. (B) Deposition of osteoid by neoplastic tumor cells. (C) Hyperchromatic, pleomorphic tumor cells that produce osteoid.

Conventional Osteosarcoma, Histological Subtypes and Variants

O*steoblastic osteosarcoma* is the most common subtype in which neoplastic bone is the principal matrix component. The matrix varies from fine, lace-like osteoid to compact bone. The amount of matrix mineralization is variable. When the amount of compact bone is extensive, the neoplasm is subclassified as the sclerosing type. *Sclerosing osteosarcoma* is a high-grade sarcoma in which increase of tumor matrix is associated with a decrease of atypia ("normalization" of cells), giving the wrong impression of low grade malignant or even benign.

In *chondroblastic osteosarcoma* a significant amount of neoplastic cartilage is identified, which is usually hyaline and high-grade. Myxoid changes may be seen, particularly in tumors arising in jaw bones. In contrast to chondrosarcoma, the cartilage in chondroblastic osteosarcoma usually displays severe nuclear atypia. Moreover, in addition to the different clinical and radiological presentation, IDH mutation analysis can be helpful in distinguishing chondroblastic osteosarcoma from chondrosarcoma.

In *fibroblastic osteosarcoma* the neoplastic cells are purely spindle shaped, with only small foci of matrix.

Telangiectatic osteosarcoma is characterized by large spaces filled with blood, often with septations. Radiographically, the tumor is purely lytic with extensive bone destruction and soft tissue extension. Histologically, large vascular spaces separated by septa of fibrous tissue containing areas of hemorrhage and necrosis are seen. The neoplastic cells, some of which produce osteoid matrix or bone, are pleomorphic and show significant nuclear hyperchromasia and can be seen within the septa or in the lining of the cystic spaces. Osteoid can be hard to identify. Atypical mitoses are often seen. Numerous osteoclast-like giant cells are present within the tumor. Some of the blood-filled or empty cystic spaces closely resemble aneurysmal bone cyst.

Small cell osteosarcoma is composed of small cells with a variable degree of osteoid production. The small cells have round to oval nuclei and scant cytoplasm. Mitotic figures are often identified. A hemangiopericytoma-like vascular pattern may be found. Osteoid production is the key to the diagnosis and is found among sheet or nests of tumor cells. It has a lace-like pattern and should be distinguished from fibrin deposits that can be seen in Ewing sarcoma, which is the main differential diagnosis. CD99 can also be positive in small cell osteosarcoma, while FLI1 is negative which may help in the distinction. EWSR1 rearrangements are absent.

Chondrogenic Tumors

Osteochondroma

Osteochondroma is a benign cartilage capped bony projection arising on the external surface of bone containing a marrow cavity that is continuous with that of the underlying bone. The base of osteochondroma can be broad (sessile) or small (pedunculated). The lesion is entirely surrounded by perichondrium (Fig. 7A). Osteochondromas are the most common cartilage tumor, and found on the femur (21%), humerus (17%), and tibia (11%). Osteochondromas develop and increase in size during the first decade of life, and stop growing when growth plates close during puberty. The osteochondromas are often asymptomatic. They can be sporadic (solitary) or found in the setting of hereditary syndrome multiple osteochondromas (previously called hereditary multiple exostoses). Multiple osteochondromas is an autosomal dominant condition caused by mutations in *EXT1* or *EXT2*. Radiologically, the osteochondroma is typically found at the transition of the metaphysis to the diaphysis, projected away from the joint, the osteochondroma stalk is in continuity with the cortex of the underlying bone.

Histologically, three layers are seen: perichondrium, cartilage and bone. Chondrocytes are often arranged in columns, as is normally observed in the growth plate (Fig. 7B). Endochondral ossification is seen at the cartilage-bone interface. Cellularity is variable depending on the age of the patient. Binucleated cells, calcification, necrosis, nodularity and cystic changes can be seen. Malignant

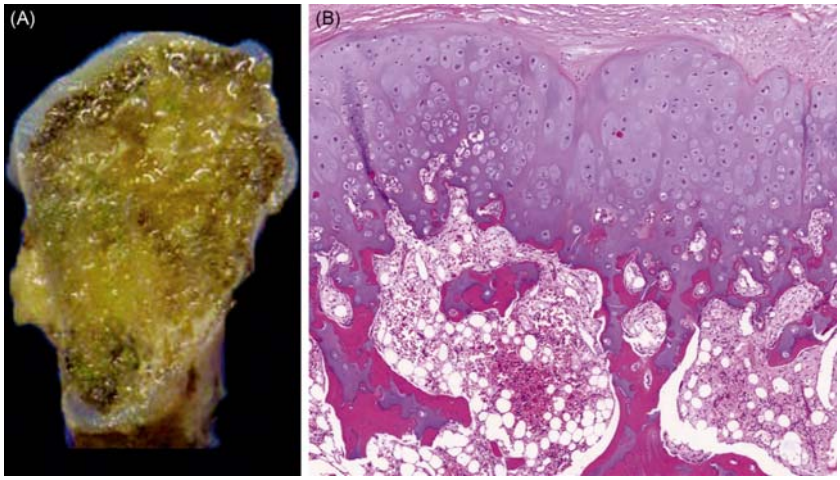

Fig. 7 Osteochondroma. (A) Gross specimen of osteochondroma. The tumor is formed by a stalk of medullary bone covered by a smooth, pale, blue-gray cartilage cap that has a thickness of <1 cm. (B) Three layers are seen: perichondrium, cartilage, and bone.

transformation to secondary peripheral chondrosarcoma occurs in 1%–5%. There are no accepted histological criteria to distinguish osteochondroma from low-grade secondary peripheral chondrosarcoma; the radiological and gross macroscopic documentation of the thickness of the cartilaginous cap is extremely important as a cartilage cap exceeding 1.5–2 cm in adults is suggestive of malignancy.

Enchondroma

Enchondroma is a benign hyaline cartilage neoplasm of medullary bone. Most tumors are solitary, however, they occasionally involve more than one bone or site in a single bone. Enchondromas are relatively common and have a wide age range. They are found in the long bones, and 50% of all cases are located in the bones of the hands and feet (Fig. 8A). Chondrosarcomas are only rarely seen at this location. The size of the enchondroma is often <3 cm. Radiologically, matrix mineralization in the form of "popcorn" calcifications is often seen. The edge of the lesion is most often lobular and sharp.

Histologically, enchondroma are lobular, relatively cell-poor hyaline cartilage, often surrounded by a zone of reactive bone formation (encasement). The chondrocytes have nuclei with condensed chromatin (Fig. 8B). Binucleated cells are infrequent and mitoses are absent. Occasionally degenerative features, such as necrosis or calcifications are found. The distinction between an enchondroma and atypical cartilaginous tumor/chondrosarcoma grade 1 can be difficult at radiology. Moreover, also on histology they are difficult to distinguish causing a large degree of interobserver variation. Mucomyxoid matrix degeneration and entrapment of preexisting host bone are most predictive of atypical cartilaginous tumor/chondrosarcoma grade I. Immunohistochemistry or molecular diagnostics cannot help in the differential diagnosis. Malignant transformation of enchondromas is rare ($<1\%$).

Multiple enchondromas are seen in the setting of non-hereditary Ollier disease (multiple enchondromas with a unilateral predominance) and Maffucci syndrome (multiple enchondromas combined with (spindle cell) hemangiomas), caused by somatic mosaic mutations in the *IDH1* or *IDH2* genes. In these conditions the risk on malignant transformation is increased to 18%–46%, depending on the extent and localisation of the lesions.

Periosteal Chondroma

Periosteal chondroma is a rare benign, small (<5 cm) hyaline cartilage neoplasm of bone surface that arises from the periosteum. Periosteal chondroma is often located at the fingers or proximal humerus.

Histologically, they display a mixture of low- and highly cellular areas. Myxoid changes of the matrix and nuclear pleomorphism can be seen. Some of these features would lead to a diagnosis of chondrosarcoma if it were located outside the periosteal area. The distinction between a periosteal chondroma and a periosteal chondrosarcoma is mainly based on the presence of cortical destruction in case of a periosteal chondrosarcoma. Furthermore, periosteal chondrosarcomas are usually >5 cm in size[4]. $\sim$70% of periosteal chondromas harbor mutations in *IDH1*.

Synovial Chondromatosis

Synovial chondromatosis is a rare benign neoplasm presenting as multiple hyaline cartilage nodules in the subsynovial tissue. The lesion is monoarticular, with the knee most often affected. There is a male predominance, with an average age of 41 years. At radiography, the presence of opaque bodies and/or stippled calcification is found. At macroscopy multiple gray-white nodules are seen. Histologically, nodules are seen surrounded by a thin layer of synovial tissue. Characteristically, the nodules are formed by chondrocytes that cluster together in nests. The cartilage is hypercellular with plump hyperchromatic nuclei, with numerous binucleate cells. Inflammation and ossification can be found.

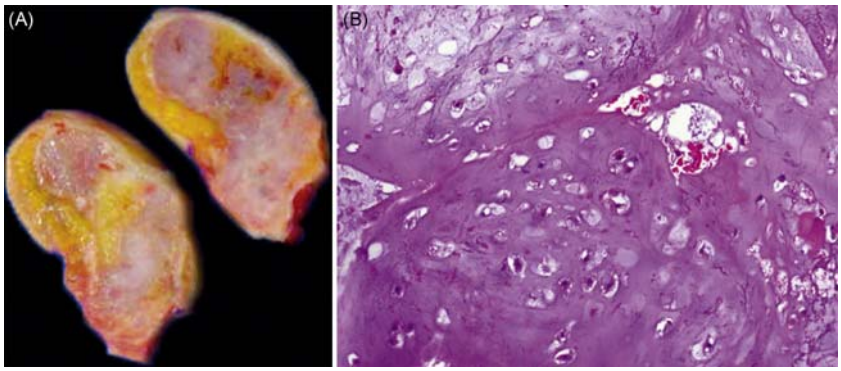

Fig. 8 Enchondroma. (A) Gross specimen of enchondroma. (B) The lesion consists of lobular, relatively cell-poor hyaline cartilage.

Chondromyxoid Fibroma

Chondromyxoid fibroma is a very rare benign cartilaginous neoplasm composed of lobules of spindle shaped or stellate cells with abundant myxoid or chondroid intercellular material. The lesions can affect any bone. Patients are adolescents or young adults.

Radiologically, the lesion is eccentric, oval, and radiolucent in the metaphysis. Histologically, the periphery of the lobules is more cellular, with spindle shaped or round cells, mixed with multinucleated giant cells. The centre of the lobules has more stellate cells found in a mucomyxoid matrix. Nuclear enlargement and hyperchromasia can be found. Mitoses are unusual but can be seen. The cells in chondromyxoid fibroma express S100, and the cells at the periphery of the lobules are myofibroblastic expressing MSA and SMA.

Genetically, chondromyxoid fibroma is characterized by upregulation of GRM1 through gene fusion and promoter swapping. This upregulation is due to genomic rearrangements that place GRM1 under the influence of a strong promoter.

Chondroblastoma

Chondroblastoma is a rare benign, chondroid-producing neoplasm composed of chondroblast-like cells usually arising in the epiphyses of skeletally immature patients.

At radiography chondroblastoma is an eccentric radiolucent lesion, with sharp sclerotic margin and areas of calcification, characteristically located in the epiphysis of the long bones. Histologically the tumor is highly cellular with relatively uniform, immature, rounded or polygonal chondroblast-like cells with a sharp cell demarcation, intermingled with multi-nuclear osteoclast-like giant cells (Fig. 9A). There is variable deposition of extracellular matrix, both cartilaginous and osteoid, and small areas of cartilage matrix with focal net-shaped ("chicken-wire") calcification. Occasionally, (non-atypical) mitoses are found. In about 15% of all cases the formation of a secondary aneurysmal bone cyst is observed. The cells express S100, and may express keratins.

Genetically, chondroblastoma is characterized by heterozygous mutation replacing lysine-36 with methionine-36 (K36M) in the histone H3 variant H3.3. A H3F3 K36M mutant monoclonal antibody (clone RM193) can be used to identify tumors that harbor this mutation (Fig. 9B).

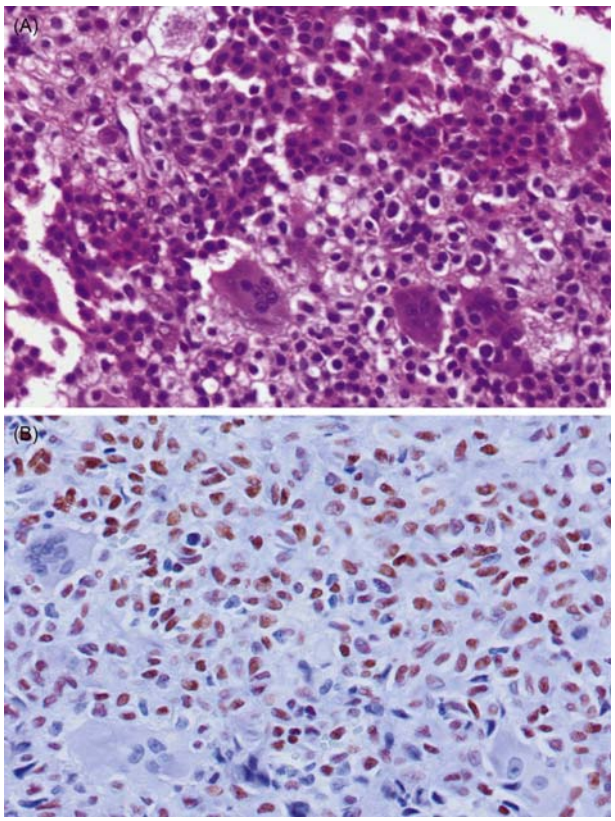

Fig. 9 Chondroblastoma. (A) Immature, chondroblast-like cells with sharp cell borders embedded in a chondroid-like matrix and admixed with multinucleated osteoclastlike giant cells. (B) A H3F3 K36M mutant monoclonal antibody (clone RM193) can be used to identify tumors that harbor this mutation.

Conventional Chondrosarcoma

Chondrosarcoma is a locally aggressive to malignant cartilaginous matrix producing neoplasm. Chondrosarcoma is mainly seen in adults in the third till sixth decade of life, with equal distribution among genders. They usually arise in the thoracic bones, pelvic and long bones. Surgery is the mainstay of treatment as chondrosarcomas are highly resistant to chemo- and radiotherapy.

Primary central chondrosarcoma arises centrally in previously normal bone as opposed to secondary chondrosarcoma arising in a pre-existing benign cartilaginous tumor. Primary central chondrosarcomas comprise about 75% of all chondrosarcomas and the majority is well differentiated.

Mutations in the isocitrate dehydrogenase genes *IDH1* and *IDH2* are found in 38%–70% of the primary central chondrosarcomas. Hotspot mutations are exclusively found at the *IDH1* R132 and the *IDH2* R172 position, of which the R132C mutation is most common in chondrosarcoma. Mutation analysis can be useful in the distinction between high-grade chondrosarcoma and chondroblastic osteosarcoma.

Secondary central chondrosarcoma is a chondrosarcoma arising in a preexisting enchondroma. The risk of malignant transformation in solitary enchondroma is estimated to be very low ($<1\%$). In patients with Ollier disease and Maffucci syndrome, the risk of developing secondary chondrosarcomas increases (18%–46%). IDH mutations are found in 86% of secondary central chondrosarcomas.

Secondary peripheral chondrosarcoma arises in a preexisting osteochondroma. A minority (up to 15% in referral centres) of chondrosarcomas develop through malignant transformation within the cartilage cap of a preexisting osteochondroma. Secondary peripheral chondrosarcoma is mainly found in the ilium (19%), followed by scapula (15%), tibia (12%), femur (11%), pubic bone (10%), and ribs (10%). The radiological and gross macroscopic documentation of the thickness of the cartilaginous cap is extremely important in the distinction of osteochondroma and secondary peripheral chondrosarcoma, as a cartilage cap exceeding 1.5–2 cm in adults is suggestive of malignancy.

While for osteochondroma formation inactivation of the *EXT1* or *EXT2* genes is required, most secondary peripheral chondrosarcomas arising in osteochondroma are *EXT* wildtype indicating that *EXT* is not important for malignant transformation and that the non-*EXT* mutated cells in osteochondroma are more vulnerable for as yet unknown (epi-)genetic changes promoting them to become malignant.

Histological Grading of Conventional Chondrosarcoma

Both conventional central and peripheral chondrosarcomas are histologically classified in three grades. Histological grade is so far the best predictor of clinical behaviour. What was previously called central chondrosarcoma grade 1 has now been renamed as atypical cartilaginous tumor, and the histology shows an abundance of predominantly hyaline cartilaginous matrix in which chondrocytes with small, condensed nuclei are seen (**Fig. 10**A). Atypical cartilaginous tumor/chondrosarcoma grade I (ACT/CS1) is distinguished from enchondroma by its higher cellularity, irregular distribution of the cells, and the occurrence of binucleated cells (**Fig. 10**B). An important criterion is the growth pattern, in which the presence of entrapment (of pre-existing lamellar bone) and the absence of encasement favor ACT/CS1 over enchondroma. Also the presence of $>20\%$ mucomyxoid changes of the matrix is an important argument for the diagnosis of ACT/CS1. For the distinction between osteochondroma and secondary peripheral chondrosarcoma no reliable histological criteria could be identified, indicating the diagnosis can only be made in a multidisciplinary team taking the thickness of the cartilaginous cap into account. In grade II chondrosarcomas the cellularity and nuclear atypia are increased, and mitoses, though scarce, can be seen (**Fig. 11**). The matrix becomes more mucomyxoid. In grade III chondrosarcomas the cellularity is high, with nuclear atypia, and mitoses are even more easily identified. At the periphery of the lobules spindle

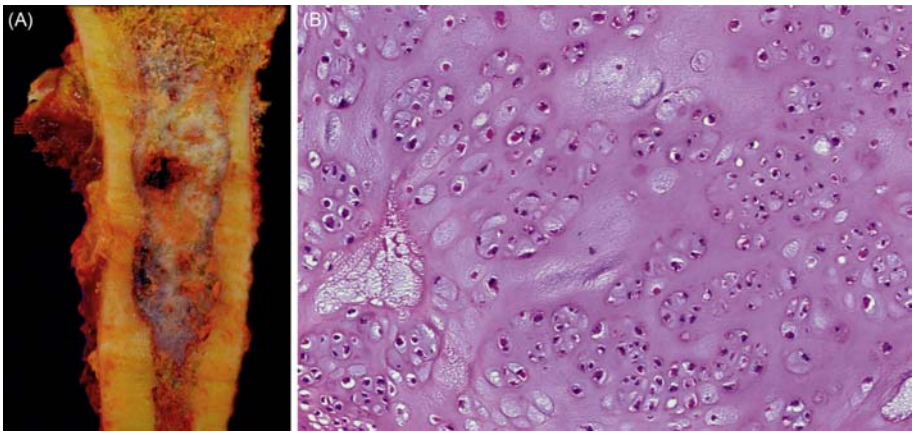

Fig. 10 Atypical cartilaginous tumor (ACT)/chondrosarcoma grade I (CS1). (A) Gross specimen, erosion of the cortex can be seen. (B) ACT/CS1 can be distinguished from enchondroma by its higher cellularity, irregular distribution of the cells, and the occurrence of binucleated cells.

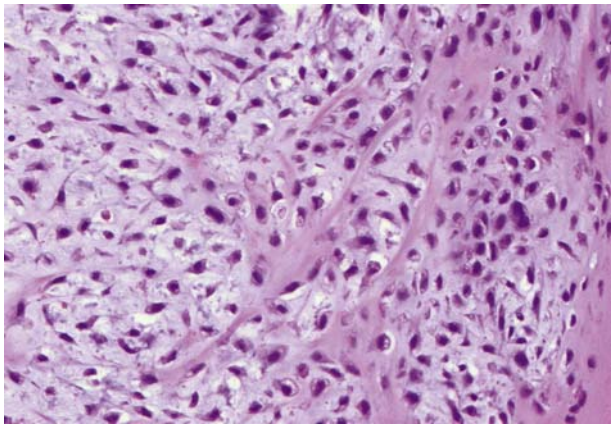

Fig. 11 Grade II chondrosarcoma. The cellularity is increased and myxoid change of the matrix can be seen.

cell changes may occur. The 10-year survival of grade I, II and III tumors is 83%, 64%, and 23% respectively. ACT/CS1 has a relatively high chance of recurrence in case of incomplete resection, but only rarely metastasize, whereas 10% of the grade II tumors and 71% of the grade III tumors metastasize. However, in approximately 13% of the recurring chondrosarcomas progression to a higher grade of malignancy as compared to the original neoplasm is described. Since considerable heterogeneity may occur in chondrosarcoma it is of importance to select several areas to be submitted for histology. The tumor needs to be graded on those areas demonstrating the highest grade.

Dedifferentiated Chondrosarcoma

Dedifferentiated chondrosarcoma is a highly malignant variant of chondrosarcoma, characterized by the occurrence of two clearly defined components; a well differentiated cartilage tumor, either an enchondroma, ACT/CS1 or grade II chondrosarcoma, juxtaposed to a high grade non-cartilaginous sarcoma. There is a histologically abrupt transition between the two components. Patients are often older than 50 years. The lesion is most often located in the pelvic bones, proximal femur or humerus, distal femur or the ribs. The prognosis is unfavorable, irrespective of the treatment. The metastases often only show the high-grade anaplastic component. The sharp transition between the two components is an important criterion in the distinction between grade III chondrosarcoma (in which the cells at the edge of the lobules show a gradual transition toward more cellular spindle shaped cells) and dedifferentiated chondrosarcoma (Fig. 12). The non-cartilaginous, anaplastic component can show characteristics of undifferentiated pleomorphic sarcoma, osteosarcoma, fibrosarcoma, leiomyosarcoma, rhabdomyosarcoma, or angiosarcoma. The demonstration of the presence of a low grade cartilaginous component is crucial for the distinction; however, on biopsy specimens one of the components can be absent. Radiological correlation is therefore crucial in the diagnosis. The rare reports on gene aberrations in dedifferentiated chondrosarcoma demonstrate that both components share identical gene aberrations with additional gene changes in the anaplastic component indicating a common precursor cell with early diversion of the two components. ~50% of dedifferentiated chondrosarcomas carry mutations in *IDH1* or *IDH2* in both components. The demonstration of an *IDH* mutation may

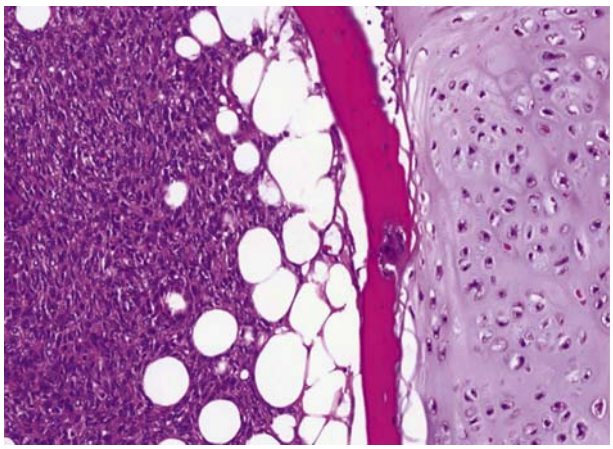

Fig. 12 Dedifferentiated chondrosarcoma. Abrupt transition between the conventional low-grade cartilaginous component (*right*) and the high-grade spindle cell sarcoma (*left*).

confirm the diagnosis of dedifferentiated chondrosarcoma in case the biopsy specimen shows the anaplastic component only, while the radiology suggests the presence of a low grade cartilaginous component as well.

Mesenchymal Chondrosarcoma

Mesenchymal chondrosarcoma is a rare malignant neoplasm characterized by a bimorphic pattern that is composed of poorly differentiated small round cells and islands of well differentiated hyaline cartilage. Mesenchymal chondrosarcoma comprises about 2% of all chondrosarcomas. The tumor can be found at all ages, with a peak in the second and third decade of life, without male or female preference. The majority (65%–86%) of mesenchymal chondrosarcomas is located in the skeleton, especially in the lower extremities such as the femur, facial bones and the pelvis. A minority (14%–34%) is primarily of extra-osseal origin, in which the meninges and the leg/thigh are mostly affected. Mesenchymal chondrosarcomas are in general of high grade malignancy, with a 10-year survival of $<30\%$. In contrast to conventional chondrosarcoma, metastases to lymph nodes and other bones are common. Radiologically, a lytical and destructive lesion is seen, with spot-like calcifications, resembling a conventional chondrosarcoma. Histologically, mesenchymal chondrosarcoma is characterized by the presence of areas with more or less differentiated cartilage mixed with vascular rich cellular areas containing undifferentiated small spindle-shaped or round cells with scant or no cytoplasm, with a haemangiopericytoma-like vascular pattern. Mitoses can be observed. The cartilaginous areas are often relatively small, sharply demarcated and cytologically benign or of low-grade. The small cell component is positive for SOX9 and variably for CD99, and can show expression of desmin. The undifferentiated small cell areas may morphologically resemble Ewing sarcoma. However, per definition, cartilage is absent in Ewing sarcoma. Furthermore, small cell osteosarcoma and dedifferentiated chondrosarcoma should also be considered in the differential diagnosis. Irregular fine trabecular deposition of osteoid, characteristic for small cell osteosarcoma, and a sharp demarcation between the two components, characteristic for dedifferentiated chondrosarcoma, are absent in mesenchymal chondrosarcoma. A recurrent HEY1-NCOA2 fusion has been identified in mesenchymal chondrosarcoma. When histologically the cellular undifferentiated component predominates, the lesions can be sensitive to chemotherapy and radiation.

Clear Cell Chondrosarcoma

Clear cell chondrosarcoma is a rare, low grade variant of chondrosarcoma, which predilects the epiphyseal ends of long bones. It is characterized histologically by bland clear cells, resembling the hypertrophic cells in the growth plate, in addition to hyaline cartilage. The tumor comprises about 2% of all chondrosarcomas and is often found in adults, with a slight preference for males, and a peak in the third and fourth decade of life. Similar to chondroblastoma, the proximal parts of the femur, humerus or tibia are most often affected. Radiologically the lesion is located in the epiphysis of the femur and humerus, can be lytic, demonstrates slight expansile growth, often with a sharp border. Macroscopically the tumor distinguishes itself by the absence of the typical glassy white-gray aspect of a conventional chondrosarcoma but is more red and granular, occasionally with cavities. Histologically round cells are seen with large round nuclei with centrally located nucleoli, and clear empty cytoplasm with sharp cell borders, which are positive for S100. Throughout the lesion metaplastic woven bone is regularly deposited, and osteoclast-like giant cells are present. Mitoses are rare. Focally, a hyaline cartilaginous matrix or even areas of more conventional chondrosarcoma can be observed. Areas of cystic degeneration resembling aneurysmal bone cyst are often seen. Based on the epiphyseal location chondroblastoma is often raised in the differential diagnosis, although clear cells are rare or absent in chondroblastoma. Metastasis of (renal) clear cell adenocarcinoma should be considered in the differential diagnosis, in which case immunohistochemistry for keratin and PAX8 may be helpful. *IDH* mutations are absent. Clear cell chondrosarcomas often recur after curettage or marginal excision, while metastases are rare.

Giant Cell Tumor of Bone

Giant cell tumor of bone is a locally aggressive bone tumor. The tumor is localized epi-metaphyseal ("end of the bone"), mainly in the long bones, especially around the knee (in $>50\%$), followed by the axial skeleton, especially the sacrum. It typically presents as an eccentric lytic lesion with a well-defined margin extending near the articular surface in patients >25 years. The gross appearance of a giant cell tumor is usually quite characteristic; the lesion is soft and dark brown, sometimes intermingled with areas that are yellow (corresponding to xanthomatous areas) or white (corresponding to fibrous areas). Hemorrhage and blood-filled cystic spaces can be seen. Histologically, giant cell tumor of bone contains an admixture of reactive round mononuclear histiocytic cells, pre-osteoclasts, numerous osteoclast-like giant cells and neoplastic spindle-shaped mononuclear stromal cells (**Fig. 13**A). Osteoclast-like giant cells have eosinophilic cytoplasm and vesicular nuclei (up to 20–50) with prominent nucleoli, and are often larger than normal osteoclasts. Mitotic activity is often seen (up to 20 per 10 high power fields). The stroma is often well vascularized with broad bands of cellular or collagenous fibrous tissue. Areas with regressive changes (e.g., hemorrhage, haemosiderin deposition, necrosis, and foamy macrophages) can be found. Areas of reactive bone formation are common especially after pathologic fracture or open biopsy. Secondary aneurysmal bone cysts (ABC) are present in 10%–14% of giant cell tumor of bone.

Metastasis of giant cell tumor to the lungs is rare. Primary malignant giant cell tumor of bone is seen at initial diagnosis as an area of high grade sarcoma containing highly pleomorphic mononuclear cells within an otherwise conventional giant cell tumor of bone. In secondary malignancy in giant cell tumor, a high-grade sarcoma arises subsequent to previous radiation or surgical therapy,

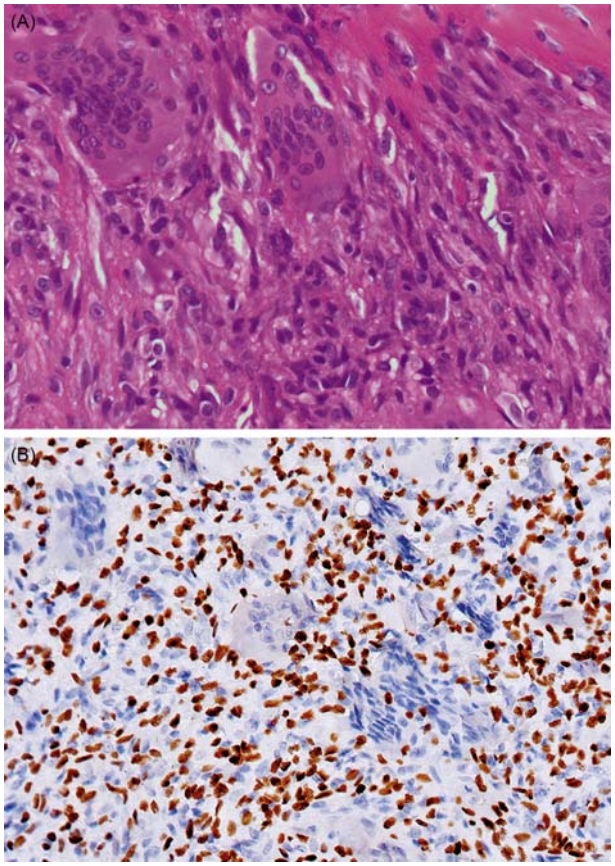

Fig. 13 Giant cell tumor. (A) The lesion is composed of a mixture of mononuclear round or spindle-shaped cells and giant cells with a large number of nuclei. (B) An anti-histone H3.3 G34 W monoclonal antibody (clone RM263) can be used to identify tumors that harbor this mutation.

and the preexisting giant cell tumor of bone is not always seen anymore. Atypical mitotic figures are suggestive of malignancy. To date, grading of histopathological features of giant cell tumor is not predictive for clinical behaviour including risk for recurrent or metastatic disease. Intra-lesioned curettage, with or without local adjuvant is the treatment of choice.

The giant cells originate from the blood and are reactive. The mononuclear cells are the proliferating and neoplastic cells, which strongly express receptor activator of nuclear factor kappa-B ligand (RANKL) thereby mediating osteoclast formation, differentiation and survival. This interplay can be blocked using denosumab (monoclonal antibody against RANK ligand) to which the tumors respond well. Denosumab treated tumors can be devoid of giant cells and mononuclear cells and composed of abundant woven bone and fibrous tissue.

Genetically, giant cell tumor of bone is characterized by a histone *H3F3A* (H3.3) gene mutation involving a substitution in glycine 34 in 96% of the cases, the vast majority of which are represented by p.Gly34Trp (p.G34W), although small numbers of G34L, V, and M variants have also been detected. An anti-histone H3.3 G34W monoclonal antibody (clone RM263) can be used to identify tumors that harbor this mutation (Fig. 13B).

Osteofibrous Dysplasia and Adamantinoma

Osteofibrous dysplasia is considered a benign fibro-osseous intra-cortical lesion of long bone, while adamantinoma is defined as a malignant biphasic tumor characterized by epithelial cells, embedded in a relatively bland osteofibrous proliferation.

Osteofibrous dysplasia is reported several times to be able to progress into adamantinoma of bone, suggesting that these lesions are part of a single spectrum. The lesions characteristically affect the cortex of the anterior midshaft of the tibia. Osteofibrous dysplasia occurs during infancy and childhood. Osteofibrous dysplasia-like adamantinoma affects children and adolescents with a relatively benign behaviour. Classic adamantinomas predominate in adults and have a more aggressive clinical course. Lesions can occur multifocal, bilateral and the fibula may also be involved.

Osteofibrous dysplasia is a well-defined lesion lying within an expanding cortex, sometimes with perilesional sclerosis. Histologically, woven bone is being formed by well-defined osteoblasts in a fibrous background. Osteoclasts and mature lamellar bone can be seen. The fibrous cells are bland and produce a myxoid or collagenous matrix. Single keratin positive cells can be seen. The

differential diagnosis includes fibrous dysplasia. In fibrous dysplasia, keratin positive cells are absent. In osteofibrous dysplasia, *GNAS* mutations are absent.

Osteofibrous dysplasia-like adamantinoma is very difficult to distinguish from osteofibrous dysplasia. In osteofibrous dysplasia keratin positive cells are sparse, and in osteofibrous dysplasia-like adamantinoma keratin positive cells are slightly more abundant and sometimes cluster together.

Classic adamantinoma demonstrates an easily distinguishable epithelial component, which can present in various patterns (basaloid, tubular, spindle cell, and squamous). The epithelial cells express keratin 5, 14, and 19, while keratins 1, 13, and 17 are variable. In addition, vimentin, EMA, p63, and podoplanin are also expressed in the epithelial component. The osteofibrous component is similar to osteofibrous dysplasia, with a storiform architecture and woven bone trabeculae prominently rimmed by osteoblasts. The epithelial component can undergo sarcomatoid transformation.

Undifferentiated High-Grade Pleomorphic Sarcoma of Bone

Previously designated as "malignant fibrous histiocytoma of bone", undifferentiated high grade pleomorphic sarcoma (UPS) of bone represents a rare, high grade malignant neoplasm characterized by pleomorphic tumor cells without obvious differentiation. The tumor predominantly affects adults, and males are more often affected than females. Histologically, either spindle cells or epithelioid cells can be seen. Pleomorphism is usually obvious and mitoses, including atypical mitoses, are often numerous. (Atypical) multinucleated giant cells and an inflammatory infiltrate can be found. UPS is a diagnosis of exclusion. Extensive sampling is required to exclude osteosarcoma. Immunohistochemistry should be used to exclude leiomyosarcoma of bone, metastatic carcinoma or melanoma. The tumors can focally express actin and keratin. Moreover, in case of a needle biopsy correlation with imaging is required to rule out dedifferentiated chondrosarcoma, in which case mutation analysis for *IDH1/2* might be helpful.

Chordoma

Chordoma is a rare malignant locally destructive tumor with characteristic morphology and immunohistochemical profile (cytokeratin 19 and T [brachyury] expression) (Fig. 14A and B). The expression of brachyury is exclusive to chordoma. Chordoma is typically located at the clivus, vertebral or sacral bones. It affects individuals of all ages and rarely occurs in the black African population. The brachyury gene (*T* gene, located on chromosome 6q27) is implicated in its pathogenesis.

Histologically, chordoma is composed of an abundant extracellular myxoid matrix among which are found columns, cords, and clusters of large round or polyhedral tumor cells with prominent vesicular nuclei and multivacuolated cytoplasm (physaliphorous cells) (Fig. 14A). Mitotic activity is rare or absent. The tumor cells may contain a single large vacuole, which gives a signet ring appearance, or are arranged as densely packed epithelioid cells, which may be mistaken for renal cell carcinoma. Tumor cells are often separated by fibrous septa. Foci of cartilage, fat, bone, and calcification may be seen within the tumor. Necrosis is frequently seen and may be extensive.

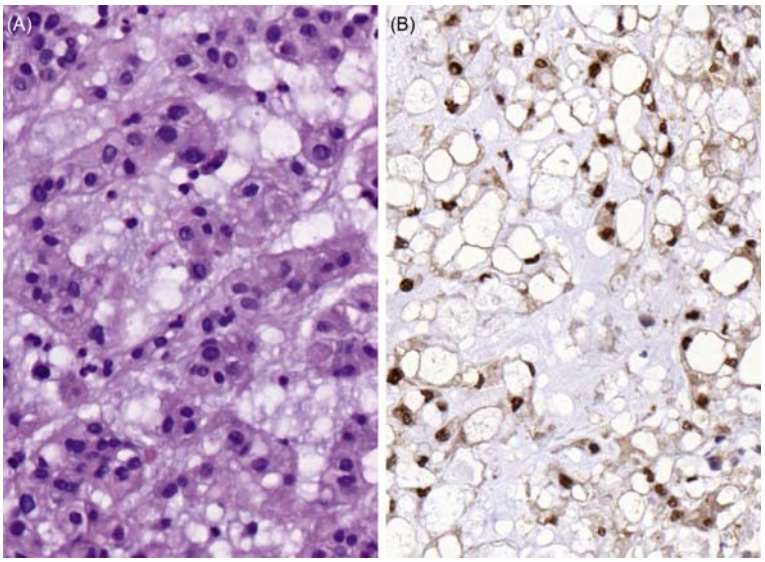

Fig. 14 Chordoma. (A) Vacuolated cells arranged in cords and nests in a mucomyxoid background. (B) Diffuse strong nuclear staining for brachyury.

Ewing Sarcoma

Ewing sarcoma is a small round blue cell tumor characterized by the pathognomonic *EWSR1* gene fusion to a member of the ETS family of transcription factors, creating a novel fusion oncogene crucial to its pathogenesis. Ewing sarcoma is the second most frequent bone sarcoma in children and young adults, about 80% of patients are younger than 20 years of age. It predominantly affects the diaphysis or the metaphyseal–diaphyseal portion of long bones but can also affect other bones or occur in soft tissue (10%–20%). Radiographically, Ewing sarcoma is an ill-defined, most often osteolytic lesion. Permeative bone destruction, periosteal reaction, and soft tissue extension are often seen. Histologically, undifferentiated uniform small round cells with round nuclei and fine chromatin are seen (**Fig. 15**A). Although not specific, immunohistochemistry typically shows diffuse membranous CD99 staining in Ewing sarcoma (**Fig. 15**B). Approximately 85% of Ewing sarcomas harbor the t(11;22)(q24;q12) translocation, fusing *EWSR1* to *FLI1*.

Recently a number of new rare translocations involving ETS family members have been described. Also, small blue round cell tumors harboring non-ETS translocations have been identified. The condensing opinion in literature is that the non-ETS translocation round cell tumors might best be considered a separate group of tumors as the clinical behaviour including reaction to chemotherapy may differ from that seen in classic Ewing sarcoma.

Tumors of Undefined Neoplastic Nature

Aneurysmal Bone Cyst

Aneurysmal bone cyst (ABC) is a cystic lesion of bone composed of blood filled spaces separated by connective tissue septations containing fibroblasts, osteoclast-type giant cells and reactive woven bone. It is considered to be of the intermediate category, locally aggressive based on its destructive and expansile growth pattern. ABC can occur at any age, although primary ABC usually affects the meta-epiphyseal region in long bones of patients in the first two decades. ABC-like areas can also be found in other benign and malignant bone tumors undergoing haemorrhagic cystic changes such as osteoblastoma or chondroblastoma (secondary ABC). Macroscopically, ABC is well circumscribed with blood filled cystic spaces separated by white septa. Solid areas can be seen. Histologically, the fibrous septa contain fibroblasts, with scattered osteoclastic giant cells and reactive woven bone, lined by osteoblasts (**Fig. 16**). The bone can be basophilic ("blue bone"). Mitoses can be found, although atypical mitoses are absent.

A recurrent translocation t(16;17) is found in primary ABC, relocating the promotor of *CDH11*, a gene that is strongly expressed in bone, in front of the *USP6*-gene (*TRE2*, *TRE17*). Later on, different translocations have been described, all resulting in oncogenic activation of the USP6 gene on chromosome 17p13. Thus, the pathogenesis of most primary ABCs involves upregulation of USP6 transcription driven by other, highly active promoters. The USP6 gene product inhibits the differentiation of pre-osteoblasts.

USP6 rearrangements were shown to be restricted to the spindle cells, and were absent in the multinucleated giant cells, inflammatory cells, endothelial cells and osteoblasts.

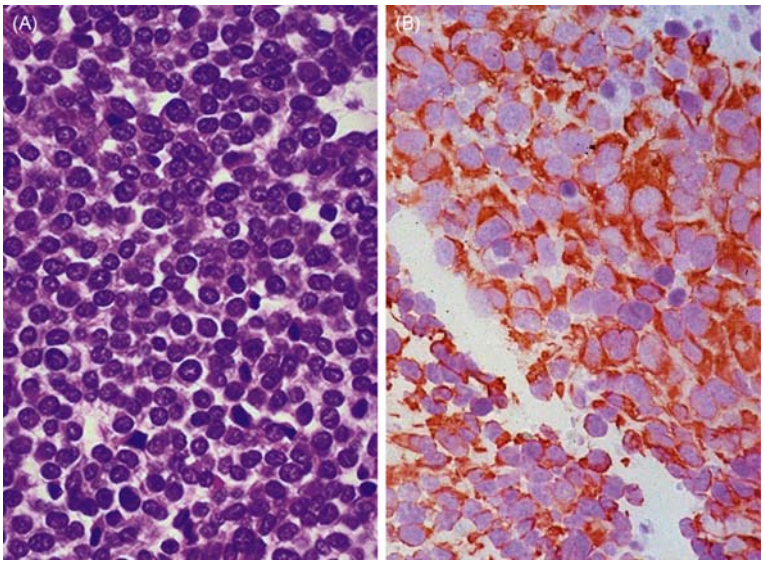

Fig. 15 Ewing sarcoma. (A) Undifferentiated uniform small round blue cells with round nuclei with fine chromatin. (B) Diffuse membrane staining for CD99.

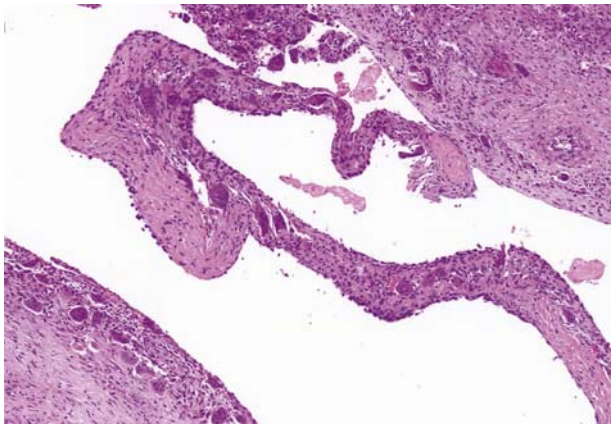

Fig. 16 Aneurysmal bone cyst. Blood-filled spaces separated by connective tissue septa with fibroblasts, osteoid, and osteoclast-type giant cells. A discontinuous layer of flattened cells lines the spaces.

Simple Bone Cyst

Simple bone cyst (unicameral bone cyst) is a benign, intramedullary, usually unicameral, cystic bone cavity lined by a fibrous membrane and filled with serous or serosanguinous fluid. The lesion mainly affects males in the first two decades of life. It can be found in any bone, specifically at the metaphyseal area of the proximal humerus and proximal femur. Lesions are typically asymptomatic. Radiology shows a lucent lesion adjacent to the growth plate. Histologically, the inner lining of the cyst consists of connective tissue, reactive new bone, hemosiderin, and scattered giant cells. Fibrinous deposits can be seen. Distinction from aneurysmal bone cyst can sometimes be difficult, especially in case of fracture. Recurrence is reported in 10%–20% of the cases.

Fibrous Dysplasia

Fibrous dysplasia is a benign fibro-osseous lesion in the medulla of bone, involving one or more bones. Fibrous dysplasia has no age and sex predilection. Craniofacial bones and the femur are most often involved, although any bone can be affected. The monostotic form is six times more common than poly-ostotic fibrous dysplasia. The latter can be associated with endocrine abnormalities and café-au-lait pigmentation in non-hereditary McCune Albright Syndrome. Fibrous dysplasia can also co-occur with intramuscular myxoma in Mazabraud syndrome. The lesion is most often asymptomatic. Radiologically, fibrous dysplasia is seen as a typical nonaggressive lesion with ground glass appearance. Histologically, fibrous dysplasia shows the typical proliferation of bland fibroblasts, intermingled with irregular, curvilinear trabeculae of woven bone, without lining by osteoblasts. The amount of ossification is variable; in the craniofacial bones ossification is usually more prominent and rounded psammomatous or cementum-like bodies can be seen. Secondary changes can be seen, including ABC-like changes, foam cells, multinucleated giant cells, or myxoid changes. Cartilaginous metaplasia can also be found.

Fibrous dysplasia is characterized by activating mutations in the Gs alpha (*GNAS1*) gene localized on chromosome 20q12–q13.3, which can be detected in 45%–93% of the cases. Mutation hotspots include R201H (57%), R201C (38%), and Q227L (5%).

Conclusion

Tumors of the bone and joints are a diverse and rare group of neoplasms. For diagnosis a experienced eye at the microscope is needed as well as communicative skills with radiological and clinical colleagues. Molecular diagnostic tests based upon the rapidly growing number of tumor specific abnormalities are becoming increasingly important and need to be interpreted in the context of morphology, clinical presentation and radiological appearance. Immunohistochemistry—while in the past of very limited use—is gaining its place in the context of the diagnosis of this challenging group of tumors.

See also: Bone and Soft Tissue Sarcoma: From Molecular Features to Clinical Applications.

Further Reading

Amary, M.F., Bacsi, K., Maggiani, F., et al., 2011. IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumors. The Journal of Pathology 224, 334–343.

Amary, F., Berisha, F., Ye, H., et al., 2017. H3F3A (histone 3.3) G34 W immunohistochemistry: A reliable marker defining benign and malignant Giant cell tumor of bone. The American Journal of Surgical Pathology 41, 1059–1068.

Atesok, K.I., Alman, B.A., Schemitsch, E.H., Peyser, A., Mankin, H., 2011. Osteoid osteoma and osteoblastoma. The Journal of the American Academy of Orthopedic Surgeons 19, 678–689.
de Andrea, C.E., San-Julian, M., Bovee, J., 2017. Integrating morphology and genetics in the diagnosis of cartilage tumors. Surgical Pathology Clinics 10, 537–552.
Fletcher, C.D.M., Bridge, J.A., Hogendoorn, P.C.W., et al., 2013. WHO classification of tumors of soft tissue and bone. IARC Press, Lyon, France.
Garcia, R.A., Inwards, C.Y., Unni, K.K., 2011. Benign bone tumors—Recent developments. Seminars in Diagnostic Pathology 28, 73–85.
Gelderblom, H., Hogendoorn, P.C., Dijkstra, S.D., et al., 2008. The clinical approach toward chondrosarcoma. The Oncologist 13, 320–329.
Gorlick, R., Khanna, C., 2010. Osteosarcoma. Journal of Bone and Mineral Research 25, 683–691.
Green, J.T., Mills, A.M., 2014. Osteogenic tumors of bone. Seminars in Diagnostic Pathology 31, 21–29.
Hazelbag, H.M., Hogendoorn, P.C., 2001. Adamantinoma of the long bones: An anatomo-clinical review and its relationship with osteofibrous dysplasia. Annales de Pathologie 21, 499–511.
Kresse, S.H., Rydbeck, H., Skarn, M., et al., 2012. Integrative analysis reveals relationships of genetic and epigenetic alterations in osteosarcoma. PLoS One e48262, 7.
Kuijjer, M.L., Hogendoorn, P.C., Cleton-Jansen, A.M., 2013. Genome-wide analyses on high-grade osteosarcoma: Making sense of a genomically most unstable tumor. International Journal of Cancer 133, 2512–2521.
Meijer, D., de Jong, D., Pansuriya, T.C., et al., 2012. Genetic characterization of mesenchymal, clear cell, and dedifferentiated chondrosarcoma. Genes, Chromosomes & Cancer 51, 899–909.
Mohseny, A.B., Hogendoorn, P.C., 2011. Concise review: Mesenchymal tumors: When stem cells go mad. Stem Cells 29, 397–403.
Szuhai, K., Cleton-Jansen, A.M., Hogendoorn, P.C., Bovee, J.V., 2012. Molecular pathology and its diagnostic use in bone tumors. Cancer Genetics 205, 193–204.
Szuhai, K., Ijszenga, M., de Jong, D., Karseladze, A., Tanke, H.J., Hogendoorn, P.C., 2009. The NFATc2 gene is involved in a novel cloned translocation in a Ewing sarcoma variant that couples its function in immunology to oncology. Clinical Cancer Research 15, 2259–2268.
Tabareau-Delalande, F., Collin, C., Gomez-Brouchet, A., et al., 2013. Diagnostic value of investigating GNAS mutations in fibro-osseous lesions: A retrospective study of 91 cases of fibrous dysplasia and 40 other fibro-osseous lesions. Modern Pathology 26, 911–921.
Vujovic, S., Henderson, S., Presneau, N., et al., 2006. Brachyury, a crucial regulator of notochordal development, is a novel biomarker for chordomas. The Journal of Pathology 209, 157–165.

Breast Cancer: Pathology and Genetics

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Glossary

Copy number alteration A type of genomic structural variation resulting in gain or loss of parts of, or whole arms of, chromosomes. The degrees of change are categorized as a gain—a moderate increase in copy number at the locus, or amplification—a high-level gain, increasing the copy number at the locus substantially. Loss of copies at a locus is described as either a single copy loss or loss of both copies, also known as homozygous deletion.

Endocrine therapy, or hormone therapy A major therapeutic modality employed in the management of certain breast and other cancers. Endocrine therapy modulates systemic hormone levels through the administration of hormones or hormone antagonists. In breast cancer, this approach is indicated tumors with detectable expression of the estrogen receptor.

Gene expression profiling A microarray-based molecular technique used to measure the transcriptome (all the detectable RNAs transcribed from the DNA). This array-based technology has been superseded by RNA sequencing technologies, but was critical to the definition of the breast cancer intrinsic subtypes.

Mutation signature A peculiar pattern of DNA changes indicative of the activity of a particular mutagen or of the inactivity of DNA repair and protection mechanisms. With the advent of whole genome sequencing, it is possible to define a pattern of alterations, the mutation signature, and extrapolate the cause of this phenomenon. The first known example is the causality between ultraviolet light and a specific pattern of pyrimidine dimers.

Prognostic signature A prognostic signature is a signature used to predict clinical outcome, or prognosis. In this instance, the signature maybe a mutation signature, or a gene expression signature, where the collective expression levels of a group of genes is characteristic of a phenotype.

Nomenclature

ACC Adenoid cystic carcinoma
ACCA Acinic cell carcinoma
DCIS Ductal carcinoma in situ
ER Estrogen receptor
FLCIS Florid lobular carcinoma in situ
GRC Glycogen rich carcinoma
GWAS Genome wide association study
HER2 Human epidermal growth factor receptor 2
IC-NST Invasive carcinoma—no special type
IDC Invasive ductal carcinoma
ILC Invasive lobular carcinoma
LCIS Lobular carcinoma in situ
LN Lymph node
LRC Lipid rich carcinoma
NEC Neuroendocrine carcinoma
PILC Pleomorphic invasive lobular carcinoma
PLCIS Pleomorphic lobular carcinoma in situ
PR Progesterone receptor
SNP Single nucleotide polymorphism
TNM Tumor staging (tumor size, number of positive lymph nodes, metastatic spread)
TC Tubular carcinoma

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Invasive Carcinoma of No Special Type (IC-NST)

Synonyms—invasive ductal carcinoma (IDC); ductal; IDC not otherwise specified (NOS).

IC-NST is diagnosed on the basis of exclusion of the special types of breast cancer; features indicative of a special type may only comprise up to 50% of the lesion. There are no defining inclusion characteristics of the group.

Burden: A diagnosis of IC-NST accounts for the largest proportion of breast cancers, as it includes all cases that do not fit into the so-called "special type" categories; reports put this proportion from 40% to 75%. The 5-year survival rate following a diagnosis of IC-NST is between 70% and 90%.

Risk factors: There are many contributing factors to the risk of developing breast cancer, including genetic risk, environment, and lifestyle. First and foremost, increasing age and female gender confer risk, as does a previous history of invasive or benign disease. Genetic risk is particularly important for breast cancer, with neoplasms arising from inherited mutations in high penetrance genes like *BRCA1* and *BRCA2*. A large number of genome wide association studies (GWAS) have been performed to identify genetic loci associated with breast cancer risk, which to date numbers more than 100 different loci, including for specific subtypes of breast cancer. The different risks afforded by genetic status at various loci have recently been combined into a polygenic risk score for *BRCA1/2* mutation carriers, which could improve current risk prediction algorithms and better inform current cancer risk management. Radiation and chemical exposure are thought to contribute a small increase to breast cancer risk, while increasingly, lifestyle factors such as alcohol consumption, high adiposity and reduced physical activity levels are negatively impacting risk levels. Reproductive history shows a strong association with breast cancer risk; women with early onset menarche, who are nulliparous or have a late age of first delivery, or late onset of menopause are more frequently diagnosed with breast cancer. Protective effects are specific to hormone receptor status; early age of first full-term delivery is only protective against ER (estrogen receptor) positive breast cancer. Lactation was once considered to have a strongly protective effect on risk, however this effect is not as straightforward as initially considered, and long term, cumulative breastfeeding may be most important. Parity and lactation alter endogenous hormone profiles, and the cessation of menstruation (including to some degree during lactation) minimizes exposure of the tissues to higher estrogen levels. Indeed, classes of steroids such as androgens, estrogens and progestogens play an important role in the development of breast cancer. In addition to the effects of endogenous hormones, exogenous hormones also alter a women's risk of breast cancer. Oral contraceptives and hormone-releasing intra-uterine devices have been variably demonstrated to alter a women's risk of breast cancer. Changing formulations of oral contraceptives have likely improved the risk status, as for post-menopausal hormone replacement therapies, however additional variables such as duration of therapy also influence risk. Recent changes to formulations have yet to be assessed in a prospective fashion.

Pathology: Diagnosis of an IC-NST is on the basis of exclusion of all the "special types"; IC-NST represents a collection of all non-"special" breast cancers. To this end, there are no additional inclusion criteria, and the term IC-NST can include tumors of all types of margins (infiltrative, pushing) and architectures (cords, trabeculae, clusters, solid growth). At the cellular level, variability continues, with nuclei either uniform or pleomorphic; with or without prominent nucleoli; possibly abundant cytoplasm. Mitoses may be extensive, or infrequent.

Molecular pathology and genetics: Due to the diagnosis through exclusion, there are no defining molecular pathology features of note; indeed, IC-NST encompasses the gamut of molecular features. Gene expression profiling studies have shown that IC-NST tumor profiles can be stratified into different intrinsic subtypes, such as basal, HER2 and luminal (A and B), an approach that has been developed into a prognostic test using the nanoString panel (ProSigna®). A landmark study from the International Cancer Genome Consortium, sequenced the whole genomes of 560 breast cancers and identified 93 somatic breast cancer driver genes. Together with the pioneering work from The Cancer Genome Atlas consortium, it is clear that the most commonly altered driver genes in breast cancer include; *TP53, PIK3CA, MYC, CCND1, PTEN, and ERBB2*. These mutations serve to activate the PI3 kinase/AKT signaling pathway, and to repress the JUN/MAPK pathways; critical pathways in breast cancer development. The landscape of mutational signatures was also defined, identifying 12 base substitution signatures and 6 rearrangement signatures, highlighting the importance of DNA damage repair processes and *BRCA1* and *BRCA2* mutational signatures, among others, in breast cancer.

Microenvironment including immune response: The breast tumor microenvironment comprises extracellular matrix, soluble factors (cytokines, hormones) and a variety of cell types including fibroblasts, endothelial cells, and immune cells. IC-NST may present with the spectrum of immune infiltrates (from scanty to brisk).

Staging and grading: For staging breast tumors, the American Joint Committee on Cancer (AJCC) TNM staging system is used. As of January 2018, the AJCC 8th edition will be implemented. It is updated to include biological markers (estrogen receptor, progesterone receptor and HER2 (human epidermal growth factor receptor 2)) as well as genomic tests (see below), which together with TMN could re-stage a tumor for treatment purposes. T is a measure of the tumor size (<2 cm, between 2 and 5 cm, and >5 cm) and whether the tumor has invaded the chest wall; *N* refers to the number of lymph nodes with evidence of tumor cells present (0, 1–3, 4–9, >10 positive nodes) and the position of the node in the nodal system; and *M* is a measure of distant metastasis. The status of the lymph nodes at presentation is a critical prognostic factor, as is the presence of distant metastasis; the greater the stage, the more advanced the disease. IC-NST tumors represent the spectrum of stages at diagnosis. Routine diagnostic assessment of breast tumors requires grading, an assessment of tubule or gland formation, nuclear pleomorphism and mitotic count. The Elston and Ellis modification of the Bloom and Richardson method is standardly employed, and grade is a highly prognostic component of a typical diagnostic pathology report. A final grading score (1, 2, or 3) is calculated from the scores of the individual components, broadly corresponding to well-differentiated, moderately differentiated and poorly differentiated, respectively. As for stage, IC-NST tumors represent the spectrum of grades at diagnosis (**Table 1**).

Table 1 The grading of breast tumors

Component	*Score*
Tubule/acinar/gland formation	
Majority of tumor (>75%)	1
Moderate (10%–75%)	2
Little to none (<10%)	3
Nuclear atypia	
Small, regular, uniform nuclei	1
Moderately larger nuclei with increasing variability	2
Marked variation; large nuclei, vesicular chromatin, often prominent nucleoli	3
Mitoses (per 10 high power fields; 0.50 mm diameter)	
7 or fewer	1
8–14 mitoses	2
15 or more	3

Prognostic and predictive biomarkers: The most well-known prognostic biomarkers in breast cancer are ER (estrogen receptor), PR (progesterone receptor), and HER2 (human epidermal growth factor receptor 2). The expression of these markers is assessed by immunohistochemistry and in situ hybridization, and forms part of the gold standard of breast diagnostic pathology, and indeed the clinical parameters for management. Positive expression of these biomarkers correlates with a good prognosis for the patient. Patients classed as triple negative for all three markers, tend to have a poor prognosis. ER and HER2 also classed as predictive biomarkers, with positive expression of ER indicating the use of anti-estrogen therapies such as selective estrogen receptor modulators (e.g., tamoxifen), selective estrogen receptor degraders (e.g., fulvestrant), and aromatase inhibitors (e.g., anastrazole). Providing the tumor has a significant amplification of gene dose at the *ERBB2* (HER2) locus, Herceptin, or another HER2-targetting drug can be prescribed. Trials are underway to investigate the efficacy of either tyrosine kinase small molecular inhibitors or monoclonal antibodies targeting mutated EGFR, especially in triple negative breast cancer.

The Nottingham Prognostic Index is also widely implemented, and is an algorithm based on the assessment of tumor size, number of positive nodes and the tumor grade. The generated score correlates with 5-year survival rates. Significant gene expression profiling studies published more than 15 years ago defined breast cancer intrinsic subtypes of luminal A, luminal B, HER2-enriched and basal-like, largely on the basis of proliferation and hormone related pathways. Array-based profiling of mRNA expression determined a 50-gene panel (PAM50) to refine this stratification, but immunohistochemical surrogates are also relatively widely used, and have been adopted by the St Gallen expert consensus. The four intrinsic subtypes are prognostic, and together with tumor size and lymph node status are the main factors predicting outcome for breast cancer patients. A number of molecular diagnostic signatures are available in the clinic and are variably able to predict likely benefit of chemotherapy, or more critically, whether withholding adjuvant chemotherapy would impact patient outcome. Tests include MammaPrint®, OncotypeDx®, ProSigna®, EndoPredict® and MapQuant Dx™, and have a range of inclusion criteria dependent on pathology features such as ER status, lymph node positivity and early tumor stage.

Invasive Lobular Carcinoma

Invasive lobular carcinoma (ILC) is characterized by cells lacking cohesion, which arrange in a linear fashion (single-file) within fibrous stroma (**Fig. 1**). Over 90% of the tumor must be morphologically lobular to be classified as such. There are also a number of histological variants of ILC, including classic, alveolar, solid, pleomorphic, tubulolobular and those of mixed phenotypes.

Burden: ILC is the second most common type of breast cancer (behind IC-NST), and accounts for up to 15% of all breast cancers. Together, classic and mixed ILC tumors account for ~75% of all ILCs. The 5-year survival rate for ILC is significantly better than for a ductal carcinoma however, over the longer term (15–20 years), the prognosis for ILC patients significantly worsens, compared to IC-NST.

Risk factors: Risk factors include those as described in IC-NST above. In addition, patients diagnosed with ILC have a slightly higher mean age than those with IC-NST (57–65 years). There is a small increase in ILC risk for women who have received a previous diagnosis of lobular carcinoma in situ (LCIS). Due to the highly hormone dependent nature of ILC (most are ER positive), there is an increased risk of ILC in patients with early menarche, late menopause and late age at first birth. ILC poses an increased risk of developing contralateral breast cancer compared to IC-NST. In female carriers of *CDH1* mutations (see below), the risk of developing ILC is approximately 50%. A genome-wide association study (GLACIER) to identify risk SNPs associated with lobular breast cancers identified a number of candidate polymorphisms, including a strong association between ILC and rs11977670 (7q34), and we await functional studies to better understand the impact of such associations.

Pathology: ILC are comprised of cells classed as either type A (small, with a classic appearance), or type B (larger cells with vesicular nuclei, demonstrating mild pleomorphism). The cells lack cohesion, and cause minimal disruption to the tissue architecture. In

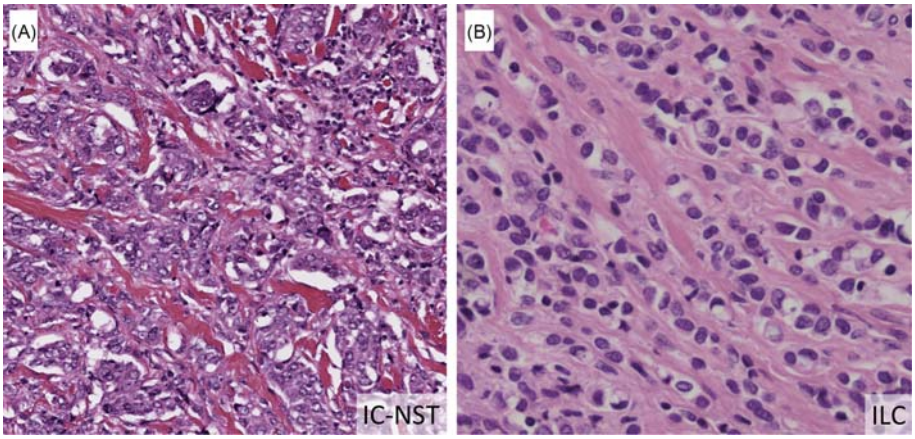

Fig. 1 The most common invasive breast cancer histologies. (A) Invasive carcinoma of no special type; grade 3. (B) Invasive lobular carcinoma; note the small, uniform cells in single files. Figure reproduced from Lal, S., McCart Reed, A.E., de Luca, X.M. and Simpson, P.T. (2017). Molecular signatures in breast cancer. Methods. **131**, 135–146. Doi: 10.1016/j.ymeth.2017.06.032, with permission from Elsevier.

classic ILC, the cells often form a targetoid or concentric pattern around ducts. Alveolar variants demonstrate cells in globular, circumscribed aggregates of more than 20 cells, while solid variants contain either irregular nests or sheets. Pleomorphic ILC (PILC) retain the typical growth pattern of classics, however there is marked cellular atypia and an increase in mitoses (and therefore a concomitant increase in grade). Tubulolobular variants have small, round tubules (*c.f.* angulated tubules of tubular carcinoma), admixed with lobular cells.

Molecular pathology and genetics: The archetypal feature of ILC is its lack of cellular cohesion, and this stems from the loss or mutation of E-cadherin, disrupting the adhesion complex. The *CDH1* gene encodes *CDH1* result in families with predisposition to both diffuse gastric cancer and ILC, and both tumor types share the typical discohesive phenotype. Other frequent genomic alterations in ILC include 1q gain, and 8p and 11q13 amplification, however ILC are considered to be diploid with relatively few genomic changes. *CDH1* is mutated in up to ~60% of ILCs, and promoter methylation is an alternative mechanism of loss. ILC are enriched for mutations in *FOXA1*, *PTEN* and *TBX3*, and activation of the AKT pathway. Mutations in the *ERBB2* gene are statistically associated with solid variants in HER2-negative ILC tumors. Indeed, mutation and amplification of *ERBB2* is frequent in ER-negative PILC tumors. Most ILC are classified as luminal tumors, and can be further stratified into "immune-related," "proliferative" and "reactive-like" on the basis of their gene expression profile.

Microenvironment including immune response: ILC tumors are often regarded as having a high stromal content, due their infiltrative pattern of growth. Lymphocytic infiltrate and composition has not been linked to prognosis in ILC, and ILC are generally associated with lower levels of lymphocytic infiltrate than other tumor types.

Staging and grading: ILC tumors are on average, larger than IC-NSTs, at 24 mm. At diagnosis, <10% of ILC have distant metastases, while ~40% have axillary lymph nodes with tumor cells present. The pattern of distant metastases differs in ILC compared to IC-NST, with cells also colonizing the gynecological and gastrointestinal tracts, in addition to the liver and bone, and less frequent presentation of lung and brain metastases. Around 75% of all ILC are histological grade 2, with grade 1 accounting for ~15%, and grade 3, 10%. Indeed, grade 3 ILC are largely non-classic, and often of the pleomorphic histotype.

Prognostic and predictive biomarkers: ER is expressed in as many as 90% of all ILC, with some of the variants showing either uniform positivity (e.g., alveolar, 100%) or much reduced (e.g., pleomorphic, ~10%). PR expression is less predictable in ILC than ER, with expression in up to 70% of tumors, a similar proportion to IC-NST. HER2 amplification is far less frequent in ILC than in IC-NST, however there is emerging evidence that the gene encoding HER2 (*ERBB2*) maybe mutated in a subset of ILC tumors. As for IC-NST, the NPI is also employed in ILC clinical management. Molecular signatures such as MammaPrint® have been shown to be of value in node-negative ILC, while the utility of other tests remains to be seen.

Tubular Carcinoma

Tubular carcinomas (TC) are defined by the presence of well-differentiated tubular structures, with a single layer of cells lining open lumen (**Fig. 2**).

Burden: TC account for approximately 2% of all invasive breast cancers.

Risk factors: The risks for developing tubular carcinoma are as detailed in IC-NST. TC are frequently detected in mammographic screening programs.

Pathology: The tumor is composed of characteristic tubules, either angulated or ovoid/round, arranged irregularly. The clear lumens are lined by a single layer of epithelium, often presenting with apical snouts, a non-pathognomonic feature. Tumor cells are typically small to moderately sized, with regular nuclei and infrequent mitoses.

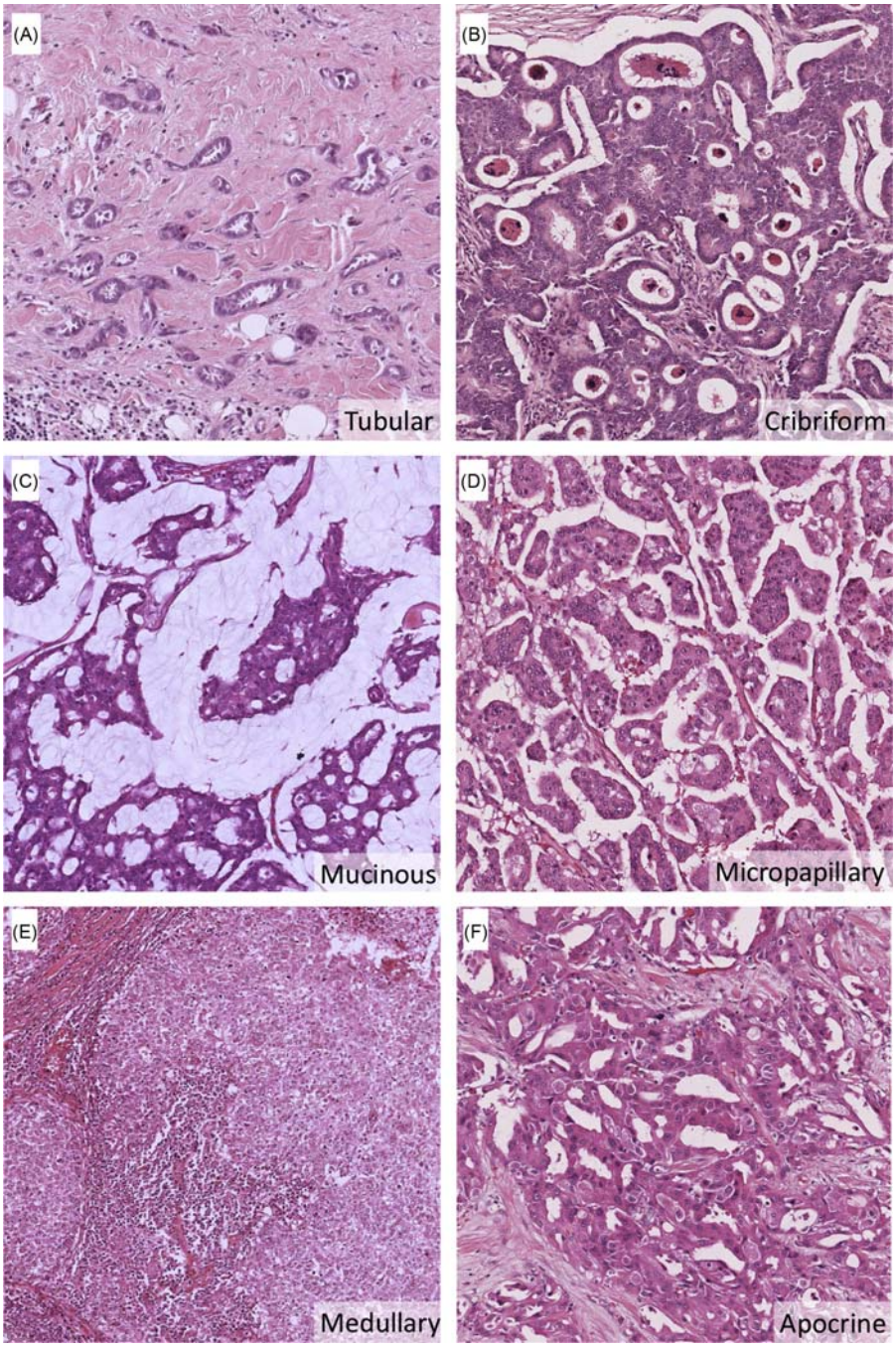

Fig. 2 Examples of special breast cancer morphologies. (A) Tubular; (B) cribriform; (C) mucinous, note the lakes of mucin; (D) micropapillary; (E) medullary; (F) apocrine carcinoma.

Molecular pathology and genetics: TC are diploid, with relatively few large scale genomic alterations. Most frequent is the concomitant loss of 16q and gain of 1p, however these are changes typical of many low-grade breast carcinomas. Only subtle differences at the transcriptomic level set TC apart from other low-grade tumors, and they are categorized as luminal A.

Microenvironment including immune response: The tubules in TC are surrounded by a characteristic desmoplastic stroma.

Staging and grading: Regarding tumor grade, it is implicit in their diagnosis that TC must score 1 for tubules, 1–2 for nuclear atypia, and 1 for mitoses, resulting in a grade of 1. Spread to the lymph nodes is detected in ~15% of TC, and correlates with tumor size, although typically TC are small (average 14 mm).

Prognostic and predictive biomarkers: TC as a histological subtype is in itself an independent prognostic indicator, and the favorable outcome associated with TC provides a survival rate comparable to that of the general population. Considering immunohistochemical biomarkers, TC are usually ER and PR positive, and HER2 negative.

Cribriform Carcinoma

Invasive cribriform carcinoma (ICC) is defined on the basis of its cribriform or "lattice-like" growth pattern (Fig. 2B).

Burden: Frequency of invasive cribriform carcinoma is reported at between ~1% and 4%.

Risk factors: The risks for developing cribriform carcinoma are as detailed in IC-NST.

Pathology: A fenestrated appearance is conferred by the presence of round or ovoid glandular spaces. The mild to moderately pleomorphic cells are arranged in angular islands; mitoses are rare. DCIS of the cribriform morphology is often present. Luminal secretions with microcalcifications may be present.

Molecular pathology and genetics: ICC share similar alterations as TC, and are also luminal A.

Microenvironment including immune response: Fibroplastic stroma features prominently in the ICC phenotype, and occasionally there may be osteoclast-like giant cells.

Staging and grading: This well differentiated tumor is normally of a low grade, with an average size of ~30 mm. Spread to the axillary lymph nodes is detected in approximately 14% of ICC, with the specialized growth architecture retained in the node.

Prognostic and predictive biomarkers: As for TC, ICC have an excellent prognosis, by virtue of their inherent low grade and stage, with a 5-year survival of 100%. They are routinely ER positive, and PR positive in approximately 70% of cases. HER2 positivity is very uncommon.

Mucinous Carcinoma

Mucinous carcinoma, also known as a colloid tumor, is identifiable by its characteristic clusters of small, uniform cells within lakes of extracellular mucin (Fig. 2C).

Burden: A diagnosis of pure mucinous carcinoma is made in up to 2% of all invasive breast cancer diagnoses.

Risk factors: Risk factors include those as described in IC-NST above. Mucinous carcinoma often occurs in older patients, with an average age of diagnosis of 65 years.

Pathology: Mucinous tumors present with a distinctive gelatinous cut surface, and are generally well-circumscribed. Neoplastic cells are arranged in nests or trabeculae within the mucinous lakes. Nuclear pleomorphism is mild to moderate. There are two recognized types of mucinous tumors; the classic type A, and a more aggressive, hypercellular form, type B, which also shows frequent neuroendocrine differentiation.

Molecular pathology and genetics: Mucinous carcinomas are frequently diploid, have minimal genetic instability and show few recurrent chromosomal alterations. Gene expression profiling stratifies mucinous tumors into the luminal A category.

Microenvironment including immune response: The microenvironment is remarkable due to the large amounts of extracellular mucin.

Staging and grading: Up to 14% of mucinous carcinoma show lymph node positivity, and there is a strong positive correlation with tumor size. Type A tumors tend to be of lower grade, and have a favorable 10-year survival prediction, while type B tumors tend towards a survival profile similar to IC-NST. Late, distant metastases have been recorded for pure mucinous tumors. Mixed mucinous tumors (where the mucinous compartment accounts for $<90\%$ of the tumor), have a high rate of lymph node metastases and a worse outcome when compared to their pure counterparts.

Prognostic and predictive biomarkers: Mucinous carcinomas are typically positive for ER and PR expression, and do not harbor HER2 amplifications.

Neuroendocrine Carcinoma

Primary neuroendocrine carcinoma (NEC) of the breast, is a tumor with neuroendocrine morphological features and positive immunoreactivity to neuroendocrine markers, such as neurone-specific enolase, chromogranin and/or synaptophysin, in at least 50% of tumor cells.

Burden: Breast NEC account for $<0.1\%$ of all breast cancers, and $<1\%$ of all neuroendocrine tumors. However, regions of neuroendocrine differentiation as defined by IHC are identified in up to 30% of IC-NST and special types (e.g., mucinous).

Risk factors: NEC tend to be diagnosed in women in their sixth or seventh decade of life.

Pathology: Classification of NEC tumors of the breast is controversial but currently follows that of NECs of other organ sites, in that there are three categories: (1) well differentiated neuroendocrine tumor; (2) poorly differentiated/small cell carcinoma; (3) invasive breast carcinoma with neuroendocrine differentiation. The well differentiated tumors (group 1) are densely cellular, with the cells arranged in nests or trabeculae. Cells vary from spindle to plasmacytoid and large clear cells, divided by fine fibrovascular stroma. The poorly differentiated/small cell carcinoma (group 2) are comprised of densely packed hyperchromatic cells with scant cytoplasm. Frequent mitoses are present, as are focal necrotic areas. Group 3 comprises invasive breast carcinoma with neuroendocrine differentiation; IHC identifies regions of neuroendocrine differentiation in approximately 30% of IC-NST and other special types like mucinous. Indeed, the hypercellular variant of mucinous carcinomas account for a quarter of breast cancers with neuroendocrine differentiation and these tumors are almost always low grade.

Molecular pathology and genetics: Recent molecular analyses have shown that NEC stratify equally into either luminal A or B intrinsic subtypes; approximately half had a high risk of score using Prosigna, but this did not completely correlate with luminal B status. NEC tumors showed a low rate of *PIK3CA* mutation. No correlation between histologic category and molecular features were observed.

Microenvironment including immune response: Lymphovascular invasion has been reported, however there is a paucity of data regarding lymphocytic invasion.

Staging and grading: The majority of NEC group 1 type tumors are grade 1 or 2; two thirds of NEC are grade 2.

Prognostic and predictive biomarkers: NEC are most often ER and PR positive, and HER2 negative.

Invasive Micropapillary Carcinoma

Invasive micropapillary carcinoma (IMPC) is composed of hollow or solid nests of epithelial cells, surrounded by a clear space (**Fig. 2D**). Characteristically, the cells display an "inside-out," or reverse polarity phenotype, where the apical pole is facing outwards.

Burden: Pure IMPC account for between 1% and 2% of all invasive breast cancers, however ~7% of all invasive mammary tumors display some micropapillary morphology.

Risk factors: The risks for developing IMPC are as detailed in IC-NST.

Pathology: The neoplastic cells of IMPC are cuboid-to-columnar, with an eosinophilic cytoplasm. The cells are arranged in tufts with a serrated-like edge, which can be either solid or hollow, but which are surrounded by clear spaces. These empty stromal spaces are not lined by endothelial cells (i.e., they are not dilated lymphatic channels, despite their appearance) and may actually be retraction artifact. Nuclear pleomorphism is variable, but unlikely to be overt; equally, the cells are not highly mitotic. A common feature of micropapillary tumors is that the cells have a reverse polarity or an "inside-out" growth pattern. To this end, the cells' apical pole is arranged to face the empty stromal space, rather than the central region of the neoplastic aggregate. MUC1 staining is useful to confirm this feature.

Molecular pathology and genetics: IMPC are typically classed as luminal A or B intrinsic subtype. Genomically, recurrent gains of chromosomes 8q, 17q, and 20q, and deletion of 6q and 13q are noted, and genes encoding polarity-regulating proteins reside in these loci.

Microenvironment including immune response: Immune infiltrate is variable but rarely brisk, and lymphoid follicles can occur in the breast stroma.

Staging and grading: IMPC are typically grade 3 (~75%) or 2 (~25%), and have an average size of 24 mm. Most importantly, IMPC display an angioinvasive phenotype, with a high rate of lymphovascular invasion. Equally, rates of lymph node positivity are high, recorded at between 69% and 95% of cases, and the total number of involved nodes is also high in IMPC. Extranodal extension in the axilla is also more frequent in IMPC than in IC-NST.

Prognostic and predictive biomarkers: ER positivity is noted in 70% of IMPC, with PR in 60%, and HER2 in 40%. In spite of these relatively good prognostic features, IMPC have a poor prognosis overall, due to their angioinvasive nature and frequent lymph node positivity. Indeed, the lymph node metastasis is the single most important prognostic feature in IMPC. Survival is comparative to other tumor types when matched for grade and stage, however skin involvement is strongly correlated with poor outcome.

Metaplastic Carcinoma; Metaplastic Breast Cancer

Metaplastic breast cancer (MBC) is a unique and lethal neoplasm of the breast. It encompasses a heterogeneous group of lesions exhibiting squamous differentiation and/or mesenchymal—looking components, such as spindle, chondroid, rhabdoid or osseous forms. A descriptive classification from the WHO working group categorized the tumor into low-grade adenosquamous carcinoma, fibromatosis-like metaplastic carcinoma, squamous cell carcinoma, spindle cell carcinoma, metaplastic carcinoma with mesenchymal differentiation, and mixed metaplastic carcinoma.

Burden: MBC constitute approximately 0.2%–5% of invasive breast cancers. The 5-year survival rate for this subtype is relatively 65%, less than conventional IDC. A uniform therapeutic approach is yet to be established owing to the tumor's heterogeneity and inherent special characteristics. In the spectrum of morphology reported in a series of 45 patients, chondroid (24%), spindled (20%), sarcomatoid (16%), squamous (11%), and mixed/others (29%) subtypes were identified.

Risk factors: The risk factors are covered in the IC-NST section. The tumor is found in women >50 years of age.

Pathology: Low-grade adenosquamous carcinoma is characterized by a stellate arrangement of highly infiltrative glandular and tubular cells, surrounded by foci of squamous differentiation in a spindle-cell background. Fibromatosis-like tumors are composed of banal fibroblasts arranged in sweeping fascicles, or long fascicles with finger-like extensions infiltrating neighboring tissues. Squamous cell carcinoma histologically reveals infiltrating nests and tongues of malignant squamous epithelium, with or without keratin pearls. The cystic areas are lined by neoplastic cells that are mildly to markedly pleomorphic with an extensive range of nuclear atypia. Spindle cell morphologies display varying degrees of differentiation, atypical-looking spindle cells with short fascicles forming a storiform architecture, or long fascicles in herringbone pattern. The nuclei are usually moderately to highly pleomorphic. The background stroma may be collagenous or focally myxoid in appearance. MBC with heterologous mesenchymal elements

include tumors composed of invasive adenocarcinomas with mesenchymal elements, the most common of which are chondromyxoid, chondroid, or osseous. Islands of chondrocytes with round-to-spindle-shaped nuclei within a homogeneous and basophilic matrix are seen singly or in groups. Bony structures that display minimal atypia to frankly malignant features are seen intermingling with carcinomatous areas, that are in the form of glandular structures and/or foci of neoplastic squamous cells. Mixed metaplastic carcinomas have been previously observed developing with other growths such as complex sclerosing lesions or papillomas.

Molecular pathology and genetics: MBCs segregate into either a basal-like or claudin-low molecular subtype. The claudin-low intrinsic subtype is associated with epithelial to mesenchymal transition, including positivity with cancer stem cells markers CD44 and Yes-associated proteins. *TP53* is frequently mutated (69%), as is the phosphatase and tensin homolog (*PTEN*; 11%); both are critical players in the mammalian target of rapamycin (mTOR) signaling pathway. Compared with triple-negative IC-NST, MBC have genetically discriminative mutation profiles in *PIK3CA*, *PIK3R1*, *PTEN*, and Wnt pathway genes. Almost 60% of MBCs harbor at least one nonsynonymous somatic mutation affecting PI3K/AKT/mTOR pathway-related genes, compared with 22% in triple-negative IC-NST. *PIK3CA* mutations are absent from chondroid variants. Molecular analysis of a small cohort identified *ERBB4*, *FLT3*, and *CSF1R* mutations, all of which are susceptible to targeted therapeutics in other cancers. Other mutations reported are *EGFR*, *TOP2A*, and *BRCA1*; however, a conclusive molecular signature has yet to be formulated. Copy number alterations are akin to that of triple-negative IC-NST; frequent copy-number gains of 1q, 3q, and 8q, copy-number losses of 1p, 3p, 5q, 8p, and 17p and high-level copy number amplifications of 8q. Evidence suggests that the different morphological regions of an MBC are genetically similar, and that epi-genetic and non-coding alterations may be important in the genesis of this breast cancer type.

Microenvironment including immune response: The tumor microenvironment is as diverse as the neoplastic cells themselves. MBC may contain a plethora of immune infiltrates, with lymphocytes and dendritic cells permeating or abutting the tumor area. The lymphocytes at the periphery may form a "cannonball" pattern, and may appear scant to brisk.

Staging and grading: A small study reported that 62% of patients (28 out of 45) had T2 disease (tumor size 2–5 cm), and metastasis to the lymph nodes were documented in 24% of patients. In many cases, distant metastasis occurs without positivity in the LN, and there is a predilection for lung and brain as sites of metastasis. The prognostic value of histologic grading in MBC is uncertain. For the low-grade adenosquamous and fibromatosis-like carcinoma, the grade is implicitly low, and prognostic outcome is better than for the majority of MBC.

Prognostic and predictive biomarkers: MBCs are of triple negative phenotype; therefore, they are not responsive to either hormone- or HER2 targeted-therapies. They are relatively resistant to conventional chemotherapeutic agents, with common recurrences and poor prognoses. The neoplastic cells in MBCs express stem cell markers ALDH-1 and $CD44^{+}/CD24^{-}$ and novel therapies for stem cell markers is presently an area of interest in clinical trials. There is currently no widely accepted prognostic significance to the morphologic subtype of MBC.

Medullary Carcinoma; Carcinoma With Medullary Features

The histologic criteria required for a medullary carcinoma diagnosis are: a predominant syncytial growth pattern; cells with high-grade nuclei; lymphoplasmacytic infiltration; and/or, a circumscribed or pushing border (Fig. 2E).

Burden: Medullary carcinoma accounts for $<5\%$ of breast cancers, however this is dependent on the stringency of diagnostic criteria employed.

Risk factors: Risk factors for medullary carcinoma are as described in IC-NST above. Additionally, tumors with medullary features occur more frequently in younger patients, and in women with a mutated *BRCA1* gene (up to 10% of this population).

Pathology: Four histologic features are used to define medullary tumors: (i) a predominant syncytial growth pattern ($>75\%$), (ii) cells with high-grade nuclei (iii) diffuse lymphoplasmacytic infiltration, and (iv) a circumscribed or pushing border. The tumor cells are generally round with pleomorphic, vesicular nuclei containing one or more nucleoli, and an abundance of cytoplasm. The inter-observer reproducibility of the application of said criteria has been poor, and the tumors are now more broadly considered to be "carcinomas with medullary features," to account for those tumors with fewer than four of the criteria present (e.g., atypical medullary carcinoma, and also, IC-NST with medullary features). These features are also seen in BRCA1-associated cancers.

Molecular pathology and genetics: Medullary tumors demonstrate high levels of genetic instability; broad copy number profile is consistent with age and grade-matched IC-NSTs. Considering the transcriptome, medullary carcinomas fall into the expression category of basal-like breast cancers, and are also recognized to over-express genes within the 12p13 and 6p21 loci. Approximately half of all carcinomas with medullary features show loss of *PTEN*, and overexpression of TP53 due to mutation is common.

Microenvironment including immune response: This tumor type is typified by a prominent lymphoid response. Characterization of the infiltrates identified a predominance of $CD3^{+}$ T-lymphocytes, with higher levels of $CD8^{+}$ cytotoxic T-lymphocytes.

Staging and grading: Medullary tumors are more likely to present at a higher grade than IC-NST tumors; however, they have lower rates of lymph node metastases.

Prognostic and predictive biomarkers: Carcinomas with medullary features infrequently express ER, PR and HER2 making them "triple negative," and thus precluding them from hormone- and HER2 targeted- therapies. They variably express basal markers such as keratin 5/6 and EGFR, as well as smooth muscle actin, p63 and vimentin (among other myoepithelial markers). In spite of these characteristics, medullary cancers tend to have a significantly better distant relapse free survival than IC-NST. Indeed, the presence of lymphoplasmacytic infiltrate correlates strongly with a good prognosis.

Adenoid Cystic Carcinoma

Adenoid cystic carcinoma (ACC) is the most common "salivary gland-like" tumor of the breast, and is considered to be of low malignant potential. They are neither adenomas, nor cysts.

Burden: These tumors are rare, and account for $<0.1\%$ of all breast tumors.

Risk factors: The risk factors for ACC are as described in IC-NST above. Additionally, 96% of patients are post-menopausal, and the incidence is $\sim$40% lower for black women than white women.

Pathology: ACC lesions are well circumscribed, and average 30 mm in size. Microscopically, epithelial and myoepithelial cells comprise typical architectures such as cribriform or tubular, and/or solid nests. The presentation is biphasic, with two forms of component structures. The first, true glandular spaces, are lined with epithelial cells with round nuclei and eosinophilic cytoplasm; they contain periodic acid-Schiff (PAS)-positive mucin. The second, the pseudolumina or pseudocysts, account for most of the tumor and result from stromal invagination. They are comprised of small, hyperchromatic basal/myoepithelial cells with sparse cytoplasm. The pseudolumina are generally round and Alcian blue positively stains the myxoid stromal substance.

Molecular pathology and genetics: ACCs harbor a recurrent chromosomal translocation, t(6;9) (q22–23;p23–24), which results in the generation of *MYB* and *NFIB* fusion transcripts in more than 90% of ACC cases. ACC have a basal-like expression profile. ACCs display a relatively stable genome, with few high-level alterations and recurrent 17q21–q25.1 gains and 12q12–q14.1 losses. In spite of a low mutation rate overall, these tumors show recurrent mutations in *TLN2*, *MYB*, and *BRAF*, and an absence of mutations in *TP53* and *PIK3CA*.

Microenvironment including immune response: ACCs are noted for their prominent myxoid secretions.

Staging and grading: ACC have an excellent prognosis, and rarely present with axillary lymph node metastases, or distant metastases. ACC are generally low grade, and in fact standard grading is of limited use. The salivary gland tumor grading system maybe more appropriate, whereby tumors with an increasing proportion of solid tumor architecture have an increasing grade. Grade 1 tumors are broadly cystic and glandular.

Prognostic and predictive biomarkers: ACC are typically triple negative for ER, PR, and HER2, while overexpression of EGFR has been described in 65% of cases.

Secretory Carcinoma

Also known as juvenile carcinoma, this rare subtype of breast cancer is composed of cells that produce intracellular and extracellular secretory material.

Burden: These tumors are rare, and account for $<0.15\%$ of all breast tumors.

Risk factors: Secretory carcinoma was originally described in children, but is now recognized to occur in both sexes, and a broad age range (median 25; range 3–87 years). Just under 40% of patients are younger than 40 years. No associations with hormonal deficiency have been described, neither has a link with pregnancy been proven. Some coexisting cases of juvenile papillomatosis have been described.

Pathology: The lesions present as a well-circumscribed, palpable mass of approximately 3 cm on average, frequently located near the areola in pre-pubertal or male patients. Three growth patterns are seen in a variety of combinations, with most tumors containing areas of all three: microcystic, solid, and tubular. Cells are polygonal, with regular nuclei and minimal mitoses. Prominent intracellular and extracellular amphophilic/granular eosinophilic cytoplasm.

Molecular pathology and genetics: Secretory carcinomas are diploid, with characteristic recurrent alterations including gain of 8q and 1q, and loss of 22q. The chromosomal translocation t(12;15) results in the expression of a fusion transcript *ETV6-NTRK3*, a chimeric tyrosine kinase. This alteration is unique to secretory carcinomas, but is not present in every case.

Microenvironment including immune response: Secretory carcinoma display prominent extra-cellular amphophilic/granular eosinophilic deposits.

Staging and grading: Secretory carcinoma are regarded as having a favorable prognosis, especially in children and young adults; they are generally low grade. Axillary lymph spread rarely involves more than three nodes, and distant metastasis is also infrequent.

Prognostic and predictive biomarkers: ER, PR and HER2 positivity is uncommon in secretory carcinomas. The tumors have a basal-like immunoprofile.

Apocrine Carcinoma

Tumors with more than 90% of neoplastic cells demonstrating apocrine differentiation are considered to be an apocrine carcinoma, as opposed to those with focal regions of apocrine differentiation (**Fig. 2F**).

Burden: Tumors with extensive apocrine differentiation account for up to 4% of all breast cancers; focal apocrine differentiation is a common feature in breast cancers.

Risk factors: The risk factors are as described in IC-NST above.

Pathology: Neoplastic cells with an apocrine phenotype demonstrate either a PAS-positive, eosinophilic, granular (type A cells) cytoplasm, an abundant foamy cytoplasm (type B cells) or a combination of both cell types. Nuclei are enlarged and feature prominent nucleoli. Cells have sharply defined borders.

Molecular pathology and genetics: Chromosomal alterations detected in Apocrine carcinomas include losses of 1p, 12q, 16q, 17q, 22q and gains of 1p, 1q, 2q; these changes are not specific to apocrine tumors. Microarray-based gene expression profiling has described a subtype of breast cancer named "molecular apocrine." This grouping is defined by increased androgen receptor (AR) signaling, and contains those HER2 positive tumors that are not classified as basal. Importantly, not all tumors with an apocrine phenotype stratify into this group.

Staging and grading: Apocrine breast cancers tend to have a similar or slightly better prognosis than grade-matched IC-NST.

Prognostic and predictive biomarkers: Apocrine carcinomas are typically ER and PR negative, and AR positive. HER2 may be over-expressed. These tumors may be candidates for AR-targeted therapies.

Inflammatory Carcinoma

Inflammatory carcinoma represents a clinical manifestation of breast cancer, rather than a histopathological subtype. The presence of abundant dermal lymphocytic emboli alone indicates the presence of an occult breast cancer.

Burden: Inflammatory carcinoma accounts for up to 2% of all breast cancers, and there is evidence to suggest that the incidence is rising.

Risk factors: Inflammatory breast cancer occurs most frequently in African-American women, and is far less frequent in Asian and Pacific Islander populations. A high body-mass index and younger age of disease have been correlated with this tumor type in African-Americans.

Pathology: The defining histology of inflammatory carcinoma is the presence of dermal lymphatic emboli, composed of tumor cells, in the skin encompassing the breast; however, an absence of emboli in a small diagnostic specimen does not preclude its diagnosis. The emboli cause significant obstruction of the lymph system in the skin, and consequent edema, however there is not an increase in inflammatory cell infiltrate. The tumor cell histology is often IC-NST, and high grade but can be any subtype.

Molecular pathology and genetics: The tumor emboli stain strongly for E-cadherin; a hallmark of the disease. Approximately 40% of inflammatory breast cancers overexpress HER2, with much greater numbers highly expressing p53 and sialomucin MUC1. Genetically, *HER2* amplification and *TP53* mutation are common, and the tumors' expression profiles are consistent with the basal-like or HER2 subtypes. There is increasing evidence for copy number gain of *ALK* in inflammatory breast cancer.

Microenvironment including immune response: As inflammatory breast cancer is a clinical presentation, rather than histological subtype, there is no specific microenvironment description; rather, it is determined by the tumor's histotype.

Staging and grading: Inflammatory carcinoma is considered to be stage T4d.

Prognostic and predictive biomarkers: Fifty percent of inflammatory breast cancers are triple negative for ER/PR/HER2, affording these tumors a poorer prognostic outcome. Approximately 40% overexpress HER2, indicating the use of HER2 targeted therapies. The role of ALK is under investigation, but there is potential for ALK over-expressing patients to be prescribed targeted therapies such as crizotinib.

Rare Carcinomas

Invasive Papillary Carcinoma

Invasive papillary carcinoma is recognized as an adenocarcinoma of the breast, demonstrating an exclusive papillary morphology. This tumor type excludes solid papillary and encapsulated tumors.

- *Burden*: This tumor type is rare, and accounts for less than 1% of breast cancers.
- *Risk factors*: Invasive papillary tumors are most frequently diagnosed in older, post-menopausal, non-white women.
- *Pathology*: Invasive papillary carcinoma has a well-defined border. Invasive cells are arranged in papules, or finger-like projections, and the tumor has a permeative front. Notably, invasive papillary lesions contain true fibrovascular cores, unlike micropapillary tumors. Due to the tumor's rarity, the primary diagnostic pathology goal is to exclude the possibility that the breast lesion is a metastatic deposit.
- *Staging and grading*: Clinical data on stage and grade, and indeed outcome, are lacking for this rare tumor type.
- *Prognostic and predictive biomarkers*: There is a paucity of data available for this rare tumor type.

Glycogen-Rich Carcinoma

Glycogen-rich carcinoma (GRC), a type of clear cell carcinoma, is diagnosed when more than 90% of a tumor contains cells an abundant, glycogen-rich cytoplasm.

- *Burden*: This tumor accounts for 1%–3% of breast cancers.
- *Risk factors*: The risk factors for GRC are as described in IC-NST above.
- *Pathology*: Neoplastic cells are arranged in sheets or nest, and contain clear cytoplasm. GRC usually present with DCIS of a solid, clear cell type.

- *Staging and grading*: GRC present with numerous mitoses, and are typically grade 3. Up to 20% of axillary dissections have shown detectable metastases.
- *Prognostic and predictive biomarkers*: ER is positive in approximately 50% of cases; PR expression is negative. HER2 positive cases have also been reported. Some evidence suggests that GRC have a worse prognosis than IC-NST, however after matching for grade, size and LN status, this association with poor survival is lost.

Lipid-Rich Carcinoma

Lipid-rich carcinoma (LRC), is a type of clear cell carcinoma, reported when at least 90% cells demonstrate abundant cytoplasmic lipids.

- *Burden*: This tumor type is rare, and accounts for less than 1% of breast cancers.
- *Risk Factors*: A case report demonstrated an association with neuroleptic drugs, which interfere with inhibition of prolactin secretion.
- *Pathology*: LRC present with infiltrating margins and cells contain numerous vacuoles of a range of sizes; the vacuoles are negative for PAS staining. LRCs stain positively for oil red O or Sudan black. LRC may present with associated DCIS or LCIS.
- *Staging and grading*: LRC tend to be grade 2 or 3 tumors, with over 50% presenting with lymph node metastases. Over 50% of LRC patients go on to develop distant metastasis, and an extremely poor first year mortality rate of 38.5% has been reported.
- *Prognostic and predictive biomarkers*: Although most cases are triple negative for ER/PR/HER2, there are cases of ER positive tumors, which indicate the prescription of endocrine therapy.

Acinic Cell Carcinoma

Acinic cell carcinoma (ACCA) of the breast shows strong similarity to the ACC of the parotid gland in terms of morphology and immunoprofile, with evident serous differentiation.

- *Burden*: This tumor type is rare, and accounts for less than 1% of breast cancers.
- *Risk factors*: There is a paucity of data available for this rare tumor type.
- *Pathology*: ACCA show small glandular structures composed of cells with irregular nuclei, varying from round to ovoid, and single nucleoli. The cells have abundant cytoplasm which is granular, consistent with zymogen granules, and ranges from amphophilic to eosinophilic in response to stains. Cells with a clear cytoplasm, reminiscent of "hypernephroid" or adrenal cortex histology, can predominate. This tumor types shows an infiltrating growth pattern.
- *Molecular pathology and genetics*: ACCA does not appear to harbor the t(6;9) (q22–23; p23–24) of the other salivary-like carcinoma of the breast (adenoid cystic carcinoma, see above)
- *Staging and grading*: tumors may range in size from 1 to 5 cm, and average 15 mitoses per 10 high powered fields however, no specific data is available for tumor grade frequencies.
- *Prognostic and predictive biomarkers*: ACCA are triple negative for ER/PR/HER2 and also negative for androgen receptor (AR).

Sebaceous Carcinoma

Sebaceous carcinoma is a rare tumor type presenting with prominent sebaceous differentiation in at least 50% of cells.

- *Burden*: This tumor type is rare, and fewer than 20 cases have been reported.
- *Risk factors*: The risk factors for sebaceous carcinoma are as described in IC-NST above.
- *Pathology*: Sebaceous carcinoma present with a nested growth pattern. There is an admixture of cell types; those with abundant, finely vacuolated cytoplasm (oil red O-positive), and smaller round to ovoid cell with eosinophilic, not vacuolated cytoplasm. There are numerous mitoses evident.
- *Staging and grading*: There is a paucity of data available for this rare tumor type.
- *Prognostic and predictive biomarkers*: ER, PR, and HER2 may be expressed.

Oncocytic Carcinoma

Oncocytic Carcinoma is defined when at least 70% of cells show an oncocytic phenotype, that is, they accumulate high numbers of mitochondria and have a highly eosinophilic cytoplasm.

- *Burden*: This tumor type is rare, and accounts for less than 1% of breast cancers.
- *Risk factors*: The risk factors for Oncocytic carcinoma are as described in IC-NST above.
- *Pathology*: Oncocytic carcinomas are circumscribed, with a solid growth pattern. Nuclei morphology varies from pleomorphic to monotonous, and may show prominent nucleoli. There is no polar condensation of the abundant mitochondria, which are dispersed throughout the cytoplasm.
- *Molecular pathology and genetics*: Chromosomal gains of 11q13.1–11q13.2 and 19p3 are reported for oncocytic carcinoma, as for the similar oncocytic tumors in the thyroid and kidney.

- *Staging and grading*: Oncocytic carcinoma afford the same prognosis as stage- and grade-matched IC-NST. LN metastases are reported at diagnosis in up to 44% of patients.
- *Prognostic and predictive biomarkers*: In a single series of 32 cases, ER positivity was calculated to be approximately 80%, PR positivity at ~60% and HER2 positivity at 25%.

In Situ Carcinoma

Ductal Carcinoma In Situ

Ductal carcinoma in situ (DCIS), also known as intraductal carcinoma or ductal intraepithelial neoplasia, is a heterogeneous group of proliferative, neoplastic lesions confined to the ductalobular network (Fig. 3).

- *Burden*: DCIS detection has increased significantly since the onset of mammographic screening programs, from previous palpably detected rates of 2%–3% of all breast cancers to up to a quarter of all newly diagnosed breast cancers.
- *Risk factors*: The risk factors for DCIS are as described above for IC-NST, and increases if a benign breast lesion (e.g., atypical ductal hyperplasia) has previously been diagnosed. DCIS is considered to be a non-obligate precursor to invasive breast cancer.
- *Pathology*: The definition of DCIS requires that the lesion is confined by the basement membrane and does not penetrate into the stromal areas. Depending on whether the lesion is low- or high-grade, the neoplastic cells may be small and regular, with infrequent mitoses, or large and pleomorphic with clumped chromatin and prominent nucleoli, respectively. Typically, DCIS can exhibit architectural morphologies including; comedo, cribriform, micropapillary and solid, but often present as a mixed phenotype. Micropapillary DCIS tends to a more extensive presentation of disease.
- *Molecular pathology and genetics*: Typically, low grade DCIS lesions are near diploid, with up to half of intermediate grade DCIS being aneuploid, and high-grade DCIS frequently aneuploid. Consistent with increased nuclear anomaly, high-grade DCIS harbor a much higher rate of chromosomal copy number abnormality than those with intermediate or low nuclear grade; the most frequent changes being losses of 1p, 8p, 17p, and gains of 1q and 8q. High grade lesions are most likely to have

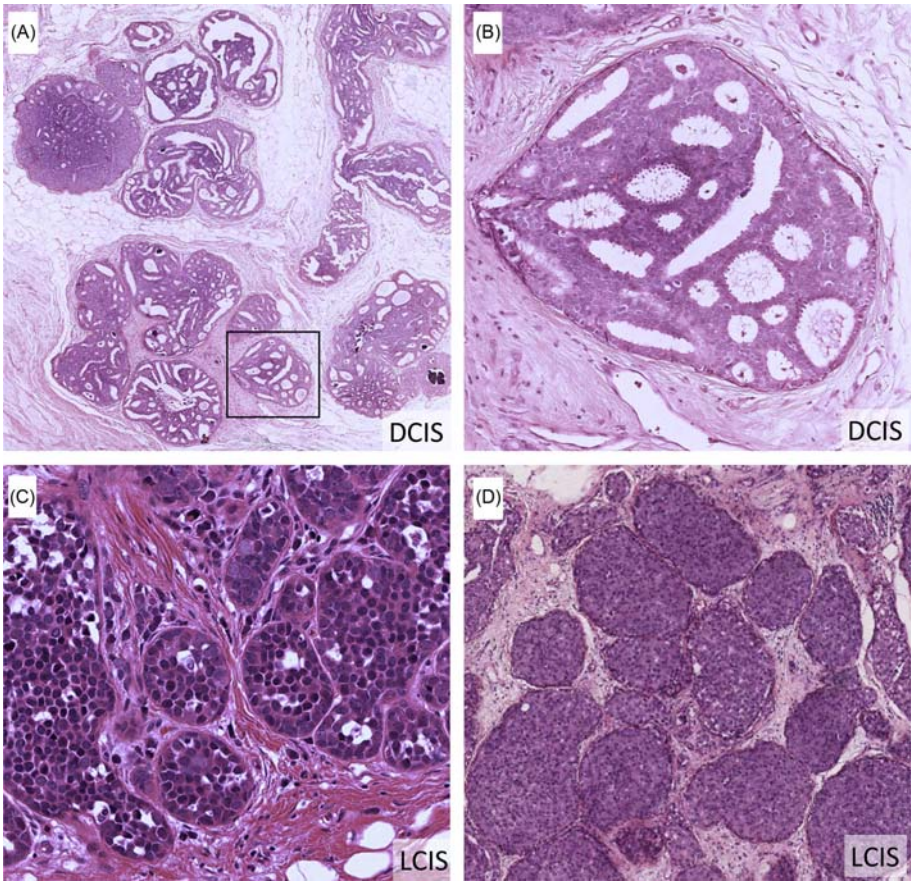

Fig. 3 In situ carcinoma of the breast. (A) Ductal carcinoma in situ, with a higher power view exemplifying the cribriform morphology in (B) (boxed region). (C) Classic lobular carcinoma in situ, with (D) showing bulky or florid disease, which would require E-cadherin and p120-catenin biomarker staining to distinguish from DCIS.

chromosomal amplifications; frequently at 1q, 8q, 17q, and 20q. In spite of significant advances in applying novel sequencing technologies to invasive breast cancer, still relatively few DCIS lesions have been adequately characterized. Most recently, targeted next generation sequencing elucidated that the cohort has a higher frequency of *PIK3CA* mutations than previously reported, and confirmed that a correlation between *GATA3* mutation and ER positivity holds true in pre-invasive lesions. While mutations in *PIK3CA* and *GATA3* are mostly mutually exclusive in invasive breast cancer, four cases harbored mutations in both genes. Intrinsic subtyping of DCIS lesions has shown that low-grade lesions are most often luminal A; intermediate lesions are luminal A or B; and, high-grade lesions are more likely to be luminal B, HER2 or basal.

- *Microenvironment including immune response*: Recent research has demonstrated that lymphocytic infiltrate is present in all DCIS to varying degrees, indicating an immune response even at this pre-invasive stage. While neoplastic DCIS cells do not express PD-L1 (the immune checkpoint marker), PD-L1 positive infiltrating lymphocytes were found in ~80% of DCIS lesions.
- *Staging and grading*: DCIS is defined as Stage 0, however the grading of DCIS is more complex. Historically, DCIS was classified based on architecture alone, however more recently a move to applying either nuclear grade alone, or in addition to necrosis and polarization has been made. Expectedly, high grade DCIS are more proliferative than the low and intermediate grades. As for invasive breast cancer, there is unlikely to be progression through the grades in a lesion's natural history, although this can occur.
- *Prognostic and predictive biomarkers*: DCIS recapitulates the variability of biomarker expression as seen in invasive carcinomas.

Lobular Carcinoma In Situ

Lobular carcinoma in situ (LCIS), like its ductal counterpart (DCIS), is a neoplastic lesion confined to the ductalobular network, that shows a characteristic lobular phenotype, with a lack of cohesion between neoplastic cells (**Fig. 3**).

- *Burden*: LCIS is not visible mammographically and is almost always diagnosed as an incidental finding through a biopsy for another indication, making estimation of true incidence difficult. However, less than 4% of excised invasive carcinoma specimens, and up to 4% of otherwise benign biopsies contain associated LCIS.
- *Risk factors*: The risk factors for LCIS are as described above for IC-NST, and increases if a benign breast lesion (e.g., atypical lobular hyperplasia) has previously been diagnosed. LCIS is considered to be a non-obligate precursor to invasive breast cancer, and a risk factor for developing ILC in the ipsilateral breast.
- *Pathology*: LCIS presents as a multicentric lesion in up to 85% of patients, and bilaterally in up to two thirds of patients. Pagetoid spread along the duct is common. Neoplastic cells can be either type A (uniform nuclei with inconspicuous nucleoli and scant cytoplasm) or type B (bigger cells with clearer cytoplasm, obvious nucleoli, and clumped chromatin). Lesions presenting with mild to moderate nuclear atypia are considered to be classic. Those lesions with larger cells with marked nuclear atypia and granular cytoplasm, are pleomorphic LCIS, and a third classification of LCIS is florid, or FLCIS.
- *Molecular pathology and genetics*: LCIS is highly similar to ILC at the genetic level. Common chromosomal changes include loss of 16p, 16q, 17p, 22q and gain of 1q and 11q13. As for ILC, LCIS are generally diploid, however the pleomorphic variant shows much greater genomic instability, as does FLCIS. The gene encoding E-cadherin is altered in a similar fashion, indeed, in co-occurring ILC and LCIS, the *CDH1* mutation status is identical, supporting that LCIS is a non-obligate precursor, not solely a risk indicator. In a large genome wide association study, a risk SNP was identified for LCIS in the *LGR6* gene (rs6678914), we await functional studies to further understand this finding.
- *Microenvironment including immune response*: There is limited information on this topic for this lesion.
- *Staging and grading*: LCIS is defined as Stage 0.
- *Prognostic and predictive biomarkers*: LCIS recapitulates the variability of biomarker expression as seen in ILC.

Prospective Vision

The advent of cost effective next-generation sequencing technologies, and the increasingly collaborative nature of research consortia, has resulted in the whole genomes of hundreds of breast cancers being characterized. With increasing optimization of these technologies for archival material, we anticipate that the specific genomic details of the various morphological subtypes of breast cancer will soon be delineated, affording clinicians detailed insights into the biology of different breast tumors, and hopefully informing the use of targeted therapies.

Further Reading

Hagemann, I.S., 2016. Molecular testing in breast cancer: A guide to current practices. Archives of Pathology & Laboratory Medicine 140 (8), 815–824. https://doi.org/10.5858/arpa.2016-0051-RA.

Horlings, H.M., Weigelt, B., Anderson, E.M., Lambros, M.B., Mackay, A., Natrajan, R., et al., 2013. Genomic profiling of histological special types of breast cancer. Breast Cancer Research and Treatment 142 (2), 257–269. https://doi.org/10.1007/s10549-013-2740-6.

Lakhani, S.R., Ellis, I.O., Schnitt, S.J., Tan, P.H., van de Vijver, M.J., 2012. WHO classification of tumors of the breast, 4th edn. IARC, Lyon.

Lavigne, M., Menet, E., Tille, J.C., Lae, M., Fuhrmann, L., Bonneau, C., Vincent-Salomon, A., 2018. Comprehensive clinical and molecular analyses of neuroendocrine carcinomas of the breast. Modern Pathology 31 (1), 68–82. https://doi.org/10.1038/modpathol.2017.107.

Marinova, L., Malinova, D., Vicheva, S., 2016. Primary neuroendocrine carcinoma of the breast: Histopathological criteria, prognostic factors, and review of the literature. Case Reports in Pathology 2016. https://doi.org/10.1155/2016/6762085. Article ID 6762085, 4 pp.

McCart Reed, A.E., Kutasovic, J.R., Lakhani, S.R., Simpson, P.T., 2015. Invasive lobular carcinoma of the breast: Morphology, biomarkers and 'omics. Breast Cancer Research 17, 12. https://doi.org/10.1186/s13058-015-0519-x.

Nik-Zainal, S., Davies, H., Staaf, J., Ramakrishna, M., Glodzik, D., Zou, X., et al., 2016. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature 534 (7605), 47–54. https://doi.org/10.1038/nature17676.

Pal, S.K., Lau, S.K., Kruper, L., Nwoye, U., Garberoglio, C., Gupta, R.K., et al., 2010. Papillary carcinoma of the breast: An overview. Breast Cancer Research and Treatment 122 (3), 637–645. https://doi.org/10.1007/s10549-010-0961-5.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., et al., 2000. Molecular portraits of human breast tumors. Nature 406 (6797), 747–752. https://doi.org/10.1038/35021093.

Rakha, E.A., El-Sayed, M.E., Lee, A.H., Elston, C.W., Grainge, M.J., Hodi, Z., et al., 2008. Prognostic significance of Nottingham histologic grade in invasive breast carcinoma. Journal of Clinical Oncology 26 (19), 3153–3158. https://doi.org/10.1200/JCO.2007.15.5986.

Reis-Filho, J.S., Lakhani, S.R., 2008. Breast cancer special types: Why bother? Journal of Pathology 216 (4), 394–398. https://doi.org/10.1002/path.2419.

Rojas, K., Stuckey, A., 2016. Breast cancer epidemiology and risk factors. Clinical Obstetrics and Gynecology 59 (4), 651–672. https://doi.org/10.1097/GRF.0000000000000239.

Yang, Y.L., Liu, B.B., Zhang, X., Fu, L., 2016. Invasive micropapillary carcinoma of the breast: An update. Archives of Pathology & Laboratory Medicine 140 (8), 799–805. https://doi.org/10.5858/arpa.2016-0040-RA.

Yates, L.R., Desmedt, C., 2017. Translational genomics: Practical applications of the genomic revolution in breast cancer. Clinical Cancer Research 23 (11), 2630–2639. https://doi.org/10.1158/1078-0432.CCR-16-2548.

Cancer Disparities

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Introduction

Cancer disparities may be described as systematic differences in cancer outcomes, such as incidence, survival, mortality, between certain population groups, disproportionally affecting the most disadvantaged individuals and occurring across many axes, socioeconomic (household income, education or occupation), racial/ethnic, gender or geographical. Social conditions and social factors account for a substantial part of the total burden of cancer and may be measured by different indicators, each of which may suggest different aspects and mechanisms for the role of social determinants. The consensus is that the observed disparities in cancer are largely driven by a complex set of causal processes. Multiple pathways are involved representing the consequence of a combination of social, economic, environmental, historical and political factors, including childhood experiences, that result in a differential exposure to risk factors, such as tobacco smoking, alcohol drinking, unhealthy diet, occupational exposures as well as differential access to the health care system. Although health care system should assure and regulate the access to prevention, early detection and treatment for the entire population, individuals with lower socioeconomic status (SES) benefit less from the health system compared to people with higher SES.

It is important to acknowledge that the systematic differences in cancer occurrence and on the patterns of the specific cancer types that are observed both within and between countries are thought to be in principle largely preventable. Here we provide a summary of cancer disparities between and within countries and a description of the reasons underlying these disparities.

Disparities in Cancer Within and Between Countries

Cancer disparities largely reflect the environment in which individuals are born, reside and work, and the uneven distribution of resources and services within and between countries. Cancer incidence and mortality patterns show distinct variations between and within more or less developed countries and regions of the world. From a global perspective, it has been observed that the overall cancer incidence rates increase with increasing level of human development, as measured by Human Development Index (HDI) (a summary measure of life expectancy, years of schooling and standard of living), Fig. 1. In 2012, the total number of new cases of cancer was 14.1 million, of which 41% observed in very high HDI countries, and 28%, 16%, and 6% in high, medium, and low HDI countries, respectively. Despite the larger burden of cancer incidence in highly developed countries, similar cancer mortality rates are observed between countries with different HDI levels, with the possible exception of low HDI countries that are at a slightly lower level compared to other categories, Fig. 2. The discrepancy between incidence and mortality is much higher in high-income countries (HICs) than in low- and middle-income countries (LMICs) and it roughly suggests that, despite the high cancer incidence rates, high-quality health systems in HICs were able to have a major impact in limiting mortality.

Not only the cancer burden but also the cancer profiles differ by HDI level. The relationship of cancer incidence and mortality with HDI may change substantially for specific cancer types, Fig. 3. For instance, cancer incidence is directly associated with HDI for cancers of the colorectum, breast, prostate, thyroid, melanoma, and inversely for cancers of cervix, lung, and stomach. The most

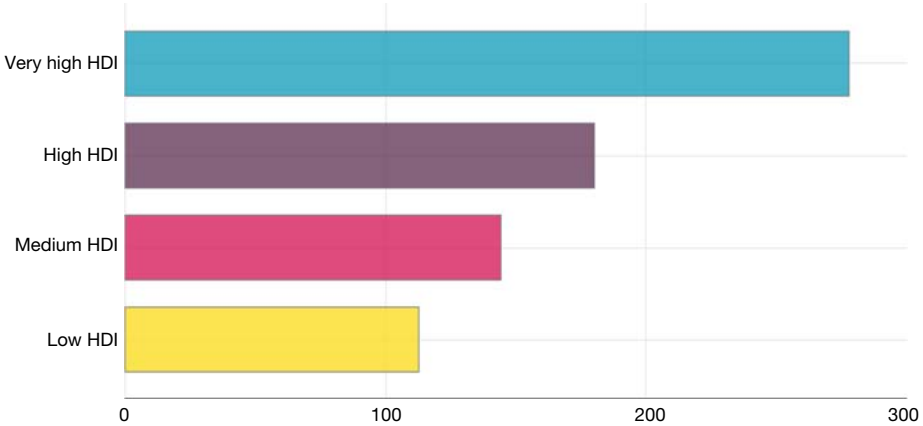

Fig. 1 Age-standardized (World population) incidence rates of all cancers worldwide in 2012. From Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. and Bray, F. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]*. Lyon, France: International Agency for Research on Cancer.

 https://doi.org/10.1016/B978-0-12-801238-3.65117-6

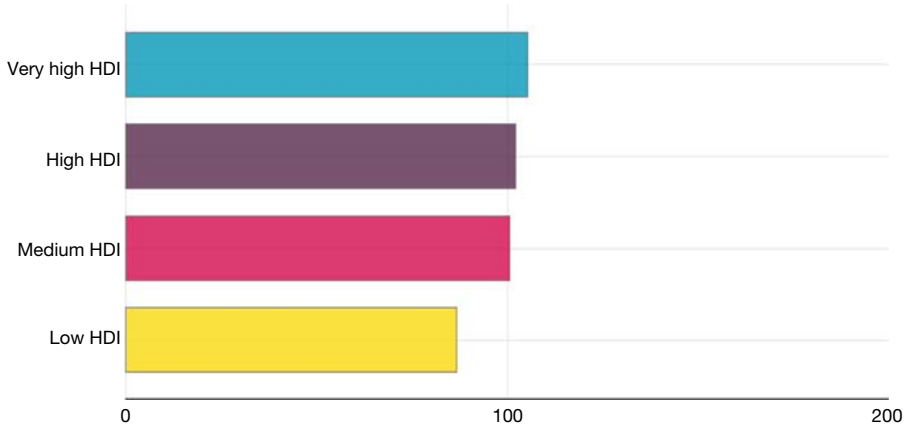

Fig. 2 Age-standardized (World population) mortality rates of all cancers worldwide in 2012. Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. and Bray, F. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]*. Lyon, France: International Agency for Research on Cancer.

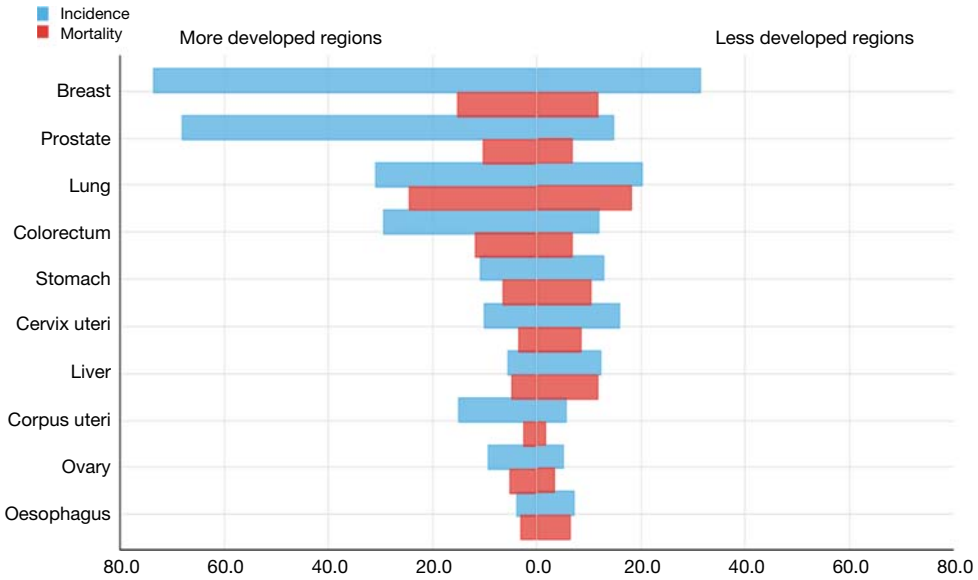

Fig. 3 Age-standardized (World population) incidence and mortality rates of the most frequent cancers types in 2012, by development level. Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. and Bray, F. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]*. Lyon, France: International Agency for Research on Cancer.

frequent cancers in high and very high HDI countries (prostate, breast, colorectal, and lung cancers) are related to increased exposure to certain risk factors such as smoking, obesity and unhealthy diet that are associated with "Westernization". Infection-related cancers (e.g., cervical, liver and stomach cancers) dominate the low and middle HDI countries. The patterns described above however are only general tendencies and there are several exceptions to these trends, depending on the country or area and cancer type.

Cancer and cancer disparities are not static. Changes in population structures, declining fertility and increasing longevity, with population growth and ageing, as well as declines in cardiovascular diseases, have already led cancer to be an increasingly more important non-communicable disease. The result of societal, economic and lifestyle changes associated with globalization have also led and will lead to major changes between and within countries in cancer occurrence. Although once considered a disease of affluent individuals and countries, cancer is a global problem affecting all countries, with approximately two-thirds of cancer deaths already occurring in transitioning LMICs. LMICs are increasingly suffering from cancers typical of more affluent countries, e.g., colorectum, breast and prostate. The future incidence of cancer in LMICs is expected to increase proportionally more than in HICs and the dose-response relationship between cancer incidence and HDI may anticipate the future scale of the problem that LMICs will face in the decades to come. It is not clear whether LMICs, already suffering from a disproportionate burden of infection-related cancers, will be able to contain the expected epidemic of cancer. Disparities between countries can only be expected to widen if efficient and cost-effective interventions will not be implemented.

Disparities in cancer incidence and mortality are not only observed between countries but also within countries, according to socioeconomic status and race/ethnicity regardless of the country economic development, Fig. 4. Although the social gradient for cancer incidence may show different directions, depending on the cancer site, mortality is usually higher among individuals with lower SES. There is a clear gradient of worsening overall cancer mortality and survival from high to low SES, and therefore almost everyone's health is affected to a certain degree. The existence of a gradient implies that possible interventions to reduce cancer disparities may be beneficial for whole populations, even though disadvantaged groups may clearly benefit the most from these interventions. Data in low-income countries are insufficient, but when available reveal poor cancer outcomes, including very high mortality and low cancer survival even for preventable or curable cancers (e.g., cervix and childhood cancers), which are due to limited or complete absence of resources and infrastructures at all steps of cancer control. It is however important to point out that even in high-income countries, cancer outcomes among individuals living in poverty, indigenous and racial/ethnic minorities, are much worse compared to other groups. Cancer mortality trends in the past decades have been generally more favorable for the

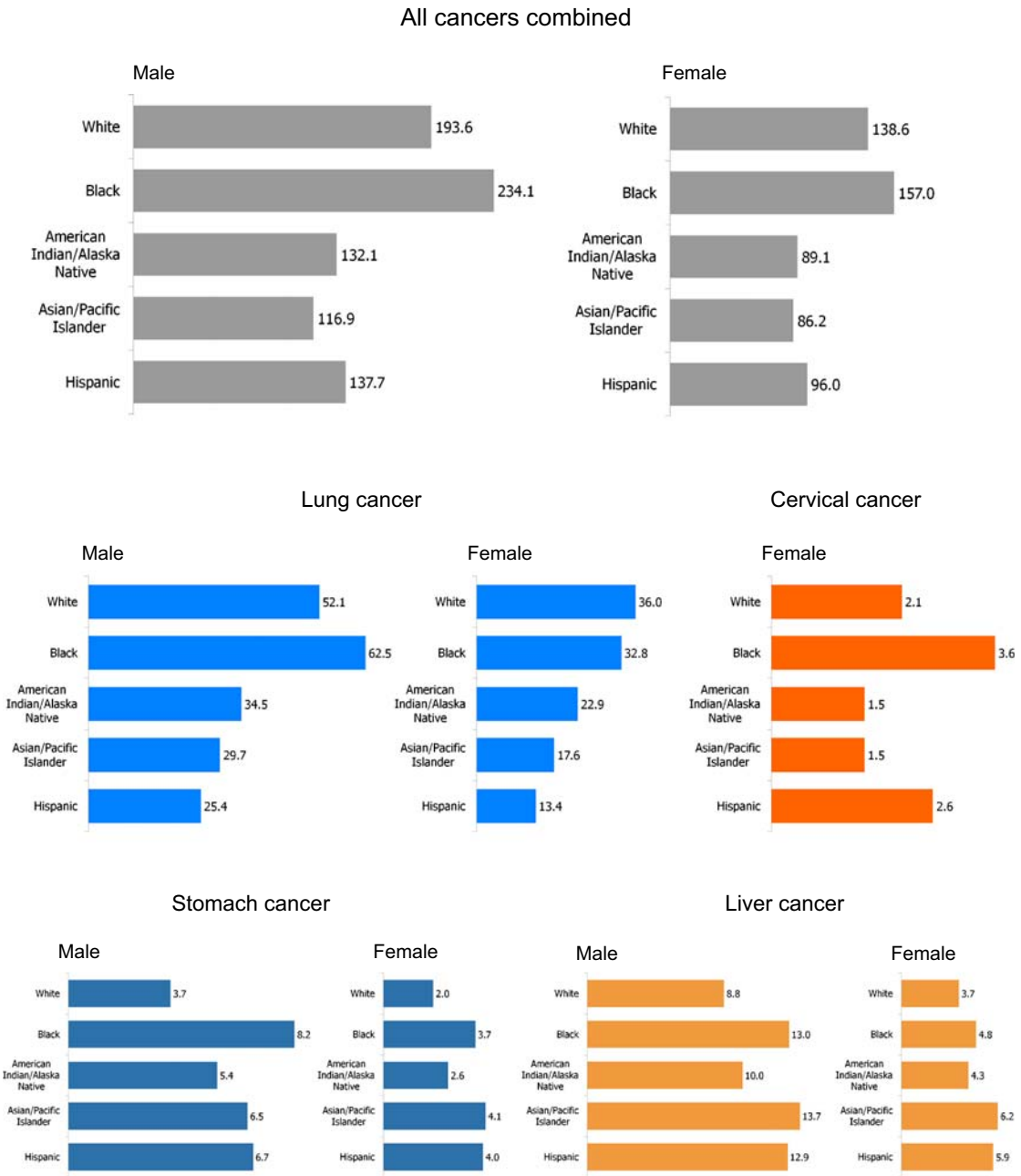

Fig. 4 Age-standardized (US population) mortality rates of all cancer combined and selected cancers types in the US in 2014, by demographic groups. Centers for Disease Control and Prevention (CDC).

higher socioeconomic groups, who apparently have benefited more from advances in prevention and treatment of cancer. Higher socioeconomic groups are in better position to benefit because they have access to a greater array of material and non-material resources, such as less financial barriers to health care or greater health literacy. As example can be mention that whereas cancer mortality among high educated men and women has largely declined, cancer mortality among low educated individuals declined with a lower speed, remained stable or even increased.

Regarding the cancer types, large socioeconomic inequalities with higher cancer incidence and mortality among low SES groups were reported for smoking-related cancers (such as lung, oral cavity and pharynx, larynx and esophagus), and infection-related cancers (such as stomach, liver, and cervical cancer).

Substantial cancer disparities have also been reported by race/ethnicity. In the US, blacks were observed to have an increased risk of cancer, especially for cervix uteri and prostate. Regarding indigenous population, a study from New Zealand showed an increased risk of lung, breast, stomach, endometrial, and liver cancer for Maori population.

Disparities between and within countries are also observed in cancer survival. The highest survival rates are usually observed in affluent countries, and within countries in affluent and high-educated patients. Social determinants influence cancer survival patterns in countries in transition, although survival data are not always available, particularly in LMICs. Over the most recent periods, improvements in 5-year survival are observed for most cancers in many LMICs, although with major differences between cancer types and between countries having different possibilities to benefit from high qualities health care services. The stage at diagnosis, the quality of treatment and the quality of health care services importantly contribute to disparities in cancer survival. Specifically the stage at diagnosis had the largest effect on racial/ethnic survival disparities in breast, prostate and colorectal cancer, showing that black patients had the lowest survival for all cancer sites. The characteristics of the patient, in particular comorbidities in people with low SES, may also contribute to reduce survival.

Disparities in Risk Factors and Access to Health Care

Disparities in cancer mortality are often linked to known risk factors which may have an impact at each stage of the cancer continuum, and include behavioral/lifestyle individual factors as well as factors related to the environment and to the context of culture, politics and policy, including price policies and tobacco, alcohol and soft drink taxations, of each country, depending on a specific cancer type. The prevalence of underlying cancer risk factors including cigarette smoking, cancer-related infections, occupational exposures, obesity, diet, or physical inactivity is not distributed equally by socioeconomic status or race/ethnicity. Exposure to these risk factors is higher in low SES and underprivileged populations, such as ethnic/racial minorities and indigenous populations. Several known occupational carcinogens, such as for example, asbestos, are concentrated among manual and industrial workers.

While for some cancers, like stomach, childhood circumstances play a key role, for others, like lung, breast or colorectal cancer, risk factors in adulthood are essential. In cancers such as breast or cervical cancer, access to screening is an important factor contributing to the cancer disparities.

Lung cancer is the most common cause of incidence and mortality in the past decades. Cigarette smoking is the most important cause of lung cancer causing about 90% of all lung cancers. Both lung cancer incidence and mortality rates were estimated to be higher among blacks than whites and in the individuals with lower SES. From this point of view, the social disparities in lung cancer incidence are closely related with social disparities in smoking prevalence. The diffusion of smoking in the population follows a process described as the smoking epidemic. According to the smoking epidemic, smoking had an initial higher uptake among affluent people but progressively became more concentrated among socially deprived individuals. The contribution of smoking to the cancer disparities increases as the smoking epidemic progresses. Countries at the most advanced stage of the epidemic such as the United States, Canada, Scandinavian countries, the United Kingdom and the Netherlands demonstrate the highest smoking-related cancer disparities. Based on the process of the smoking epidemic, an increase in smoking-related cancer disparities may be expected in countries at an earlier phase of the smoking epidemic, i.e., LMICs.

The incidence of breast cancer is usually higher among individuals with higher SES. The positive association between SES and breast cancer incidence may be explained by certain factors, such as delayed age at first birth, nulliparity, lower body mass index, higher use of oral contraceptives and hormone replacement therapy that are more common among women with a higher social status. Differences in diagnostic factors may also play an important role as high SES women are more likely to participate in breast cancer screening programs and therefore to be diagnosed with the disease. Breast cancer survival is however lower, and mortality higher, among women from poor socioeconomic background and from LMICs mainly because these women are more likely to be diagnosed at a later stage compared to high SES women and to experience delays in treatment.

Prostate cancer is a significant public health burden and a major cause of morbidity and mortality among men worldwide. Similarly to breast cancer, incidence of prostate cancer is generally higher in high SES individuals and affluent countries but low SES is associated with poorer survival and higher mortality, most likely as a consequence of differences in treatment across racial and socioeconomic strata. Particularly high prostate cancer incidence and mortality rates are observed among Africans and black Americans although the reasons for racial/ethnic differences remain unclear, as the risk factors of prostate cancer have not been clearly determined yet.

The incidence of colorectal cancer has markedly increased in the past decades in HICs, most likely due also to changes in lifestyle characteristics, including dietary habits linked to overweight/obesity, reduced physical activity, tobacco and alcohol consumption

("Westernized lifestyle"). There is clear positive association between the incidence of colorectal cancer and HDI values at the country-level, with rates at least six times higher in very high HDI countries relative to low HDI countries in both sexes. However, characteristics and risk factors for colorectal cancer typically cluster in low SES and disadvantaged populations, which are likely to have the highest mortality rates for this disease. In the very recent past, colorectal cancer incidence rates have stabilized or declined in a several affluent countries, this possibly due to several factors including colorectal cancer screening, which has the potential to protect against the disease. Even in this case, strong socioeconomic inequalities exist in the access and uptake of colorectal screening programs.

A large majority (86% or 453,000 cases) of the global burden of cervical cancer is recorded in LMICs, particularly in Africa, Asia and in Latin America, where one in nine new cancer cases are of the cervix. Cervical cancer has now become relatively rare in HICs, like North America, Northern and Central Europe, and Japan, where historical investments have been done in effective screening programs. Incidence rates of cervical cancer have been declining in the recent past in some populations in Asia and Latin America, whereas increasing rates have been recorded in other areas of the world where limited or no screening programs are available, for example, Baltic and Eastern European countries as well as some African countries, like Zimbabwe and Uganda, where high quality cancer registry data are available. Persistent infection of the ano-genital tract with high-risk types of human papillomavirus (HPV) is a known, necessary cause of pre-invasive and invasive cancer of the cervix. Although the prevalence of HPV may vary substantially between countries and it is higher in the African continent, no clear gradient is observed with average levels of socioeconomic development of the country. In addition, although cervical cancer risk is consistently associated with low SES in screened and unscreened population, this excess risk does not seem to be explained by a different prevalence of HPV infection, as HPV prevalence does not vary substantially across SES levels within countries. Rather, differences in cervical cancer are likely due to differential access to health care, in particular to (organized or even opportunistic) screening. Vaccination against HPV infection is now available with follow-up data showing safety and efficacy in protecting against high-risk HPV types 16 and 18 (as well as low risk types 6 and 11) and related cervical lesions for over 10 years. A nonavalent vaccine has also being developed that can additionally protect against HPV types 31, 33, 45, 52, and 58. HPV vaccination may reduce cervical cancer disparities by reducing the prevalence of HPV in the population. However, the roll out of HPV vaccination programs has been particularly challenging in most LMICs because of the high cost of the vaccine, and major inequalities in uptake of HPV vaccination still exist even in HICs. Although HPV vaccination is an important preventive measure to reduce cervical cancer, its impact on cancer in the population will be visible only after few decades after its implementation. It is therefore still necessary to implement high-quality screening programs, such as screening based on HPV-testing. Cervical cancer screening and HPV-vaccination programs certainly have the potential to reduce dramatically the future rates of cervical cancer. However, disparities in the uptake of screening and of vaccine exist, with lower compliance and coverage for low SES groups and racial/ethnic communities.

It is thus of great importance for reducing cancer disparities the availability and access to health care, from early detection to treatment, which, particularly in LMICs, may suffer from major weaknesses and fail to provide sufficient health care services. There are marked differences in access to health care and health care outcomes between LMICs and HICs as well as people of low versus high SES. Poorer countries typically provide the worst quality of care and spend the least amount of national resources on health care. Even within HICs, access to high-quality cancer care and treatment is far from equal across different population groups. It has been shown that racial and ethnic minorities and individuals from lower socioeconomic groups are less likely to receive appropriate cancer treatment. Disparities in cancer screening participation by SES and ethnicity were observed in all types (organized and opportunistic) of breast and colorectal cancer screening programs. The lower participation in cancer screening in more disadvantaged populations may result in further disparities in the stage at diagnosis, being at more advanced stage among individuals from lower SES or from racial/ethnic minorities.

The largely reported socioeconomic or racial/ethnic disparities in early detection and treatment may be the consequence of structural barriers such as geographical distance to the health care facilities and access to transportation, education, lack of health insurance or other financial support. Women from minority racial/ethnic groups have been shown to have longer waiting times from the abnormal cytology screening result to an appointment for a final diagnostic procedure. Furthermore, among women with abnormal cervical cytology screening results, Hispanic, Asian, and black women have significantly lower odds of being scheduled for treatment and clinical recommendations given by physicians often differ for patients of different race/ethnicity or lower SES.

Lack of private health insurance was associated with lower participation in mammography and with worse quality of diagnostic services. Women with private health insurance have access to facilities with better quality of breast cancer screening such as for example, academic facilities, breast imaging specialist, and digital mammography. Digital mammography may be better at detecting cancerous lesions in younger women and women with extremely dense breasts. As black and Hispanic women tend to be diagnosed with breast cancer at an earlier age and with more aggressive breast cancer than their white counterparts, the differences in access to digital mammography may play an essential role for the disparities in the stage at diagnosis.

Although financial aspects and lack of adequate health insurance may play a significant role in disparities in cancer screening attendance, disparities in cancer screening participation exist not only in countries with private health insurance like the United States, where health insurance status can be a barrier for lower income groups, but also in countries with free health care like in the United Kingdom or the Netherlands suggesting that other risk factors are an important contributor to these disparities.

Individuals from disadvantage groups such as, for example, lower SES, racial/ethnic minorities or immigrants perceive cancer screening tests as threatening and difficult to accomplish, or may feel embarrassed, afraid of pain and do not recognize the benefits

of early detection. Furthermore, lack of confidence in dealing with the health system may also contribute to the disparities in cancer screening participation. Not attending cancer screening may also be related to inadequate knowledge of cancer, illiteracy, and linguistic or cultural obstacles. Low health literacy rates are commonly observed in the ethnic minorities, elderly, immigrants and nonnative speakers. Immigration status functions as a predictor of screening uptake independent of ethnicity or race. For instance, immigrants in the Netherlands were found to have lower participation in cancer screening probably due to the lack of the Dutch language proficiency. Cultural differences including lack of acceptance of care from male providers contributes to low rates of cervical cytology screening in immigrant women.

Economic Considerations

Disparities in cancer have also large economic consequences. These disparities raise the total costs of health care and social security and reduce labor productivity and the gross domestic product (GDP). Elimination of disparities in cancer incidence would also result in cost savings attributable to improved labor market outcomes, that is, fewer disability days, more hours worked and higher wages for working age adults. In the United States, the costs of cancer disparities across racial or ethnic groups and across socioeconomic groups were estimated and quantified. The annual costs of racial and/or ethnic disparities were $193 billion for premature death, $2.3 billion for direct medical costs and $471.5 million for lost productivity. The reduction in the number of new cancer cases would result in $2.3 billion savings on annual medical expenditure for persons with cancer if racial disparities in cancer incidence were eliminated. The elimination of poverty disparities in cancer incidence would lower health expenditure by $236 million nationally.

Reducing Cancer Disparities

Many cancers in LMICs and excess cancers among disadvantaged groups within countries are amenable to prevention through policies and strategies at addressing risk factors of cancer such as comprehensive tobacco control policies, strategies to address obesity, including supporting a healthy diet and increased physical activity, and policies for equitable access to high quality vaccination and screening programs. Access to affordable, appropriate, effective health care with a focus on cancer prevention, diagnosis and treatment is critical. There are vast disparities between HICs and LMICs, with many of the latter having weak health systems and very poor access to cancer treatment services. Ongoing global urbanization and disorganized growth of urban areas is leading to substantial challenges for health generally. Higher levels of overcrowding, reductions in physical activity, increased consumption of unhealthy food leading to higher rates of obesity, and higher rates of tobacco and alcohol consumption, are risk factors for many cancers, and these trends tend to affect the most disadvantaged groups. In addition, conflicts and other catastrophic events have led to the massive dislocation of entire populations, and the dissolution of structures and process to protect the health of those people. Lack of universal health coverage in many LMICs is a barrier both to development, and to addressing cancer outcome disparities. Progress toward meeting the health-related SDG of UHC will likely have a major impact in reducing cancer disparities both between and within countries. The evidence to support interventions to reduce inequalities within HICs is largely focused on single aspects of complex systems. However, comprehensive systems-level approaches are most likely to succeed. There is some evidence that this broad, organized approach may be effective in reducing cancer disparities within HICs. For example, socioeconomic disparities in breast and cervical cancer screening participation are less likely to be found in countries with organized screening programs, socioeconomic disparities are more marked in the US than in Canada where universal healthcare is available, and in New Zealand, ethnic disparities in cancer care and survival have reduced over time in the context of organized screening programs that include an explicit focus on reducing disparities.

Conclusion

There are major disparities in cancer incidence and mortality between and within countries that change over time and that are potentially modifiable. Social determinants influence cancer risk factors such as for example tobacco and alcohol consumption, exposure to infections, occupational exposures, unhealthy diet, physical inactivity and obesity, and have an impact on the access to appropriate early detection, treatment, and palliative care, and contributing to the large cancer disparities between certain population groups. Cancer disparities between and within countries may be reduced or even eliminated by effective health policies targeting the disadvantage populations but without appropriate action, however, cancer disparities are likely to increase. It is of great importance to collect high-quality scientific evidence on the magnitude of these disparities and increase the understanding and the knowledge on the many dimensions of the problem. Also, although social determinants affect all stages of the cancer continuum, from prevention to end-of-life care, it is prevention that has the largest potential for reducing cancer disparities in all settings. Therefore, there is the need to expand both research focus and investments in prevention. Furthermore, all interventions and cancer control programs, from prevention to treatment measures, should be explicitly designed to account not only for the overall effect, but also to avoid increasing cancer disparities, and ideally to decrease or eliminate them. This represents an attainable and ethical goal.

See also: Cancer in Sub-Saharan Africa. Cancer in the Middle East. Cancer in Populations in Transition.

Further Reading

Bray, F., Jemal, A., Grey, N., Ferlay, J., Forman, D., 2012. Global cancer transitions according to the human development index (2008-2030): A population-based study. The Lancet Oncology 13, 790–801.

Center to Reduce Cancer Health Disparities (CRCHD), 2004. Economic Costs of Cancer Health Disparities: Summary of meeting proceedings. U.S. Department of Health and Human Services, National Institute of Health, Bethesda, MD.

Dachs, G.U., Currie, M.J., McKenzie, F., Jeffreys, M., Cox, B., Foliaki, S., Le Marchand, L., Robinson, B.A., 2008. Cancer disparities in indigenous Polynesian populations: Maori, native Hawaiians, and Pacific people. The Lancet Oncology 9, 473–484.

Elk, R., Landrine, H., American Cancer Society, 2012. Cancer Disparities: Causes and Evidence-Based Solutions. Springer Publishing Company, New York.

Farmer, P., Frenk, J., Knaul, F.M., Shulman, L.N., Alleyne, G., Armstrong, L., Atun, R., Blayney, D., Chen, L., et al., 2010. Expansion of cancer care and control in countries of low and middle income: A call to action. Lancet 376, 1186–1193.

Gadeyne, S., Menvielle, G., Kulhanova, I., Bopp, M., Deboosere, P., Eikemo, T.A., Hoffmann, R., Kovács, K., Leinsalu, M., Martikainen, P., Regidor, E., Rychtarikova, J., Spadea, T., Strand, B.H., Trewin, C., Wojtyniak, B., Mackenbach, J.P., 2017. The turn of the gradient? Educational differences in breast cancer mortality in 18 European populations during the 2000s. International Journal of Cancer 141, 33–44.

Koh, H., 2009. Toward the elimination of Cancer disparities. Springer, New York, USA.

Lopez, A.D., Collishaw, N.E., Piha, T., 1994. A descriptive model of the cigarette epidemic in developed countries. Tobacco Control 3, 242–247.

Mackenbach, J.P., Meerding, W.J., Kunst, A.E., 2011. Economic costs of health inequalities in the European Union. Journal of Epidemiology and Community Health 65, 412–419.

Stringhini, S., Carmeli, C., Jokela, M., Avendaño, M., Muennig, P., Guida, F., Ricceri, F., d'Errico, A., Barros, H., Bochud, M., Chadeau-Hyam, M., Clavel-Chapelon, F., Costa, G., Delpierre, C., Fraga, S., Goldberg, M., Giles, G.G., Krogh, V., Kelly-Irving, M., Layte, R., Lasserre, A.M., Marmot, M.G., Preisig, M., Shipley, M.J., Vollenweider, P., Zins, M., Kawachi, I., Steptoe, A., Mackenbach, J.P., Vineis, P., Kivimäki, M., LIFEPATH Consortium, 2017. Socioeconomic status and the 25×25 risk factors as determinants of premature mortality: A multicohort study and meta-analysis of 1·7 million men and women. Lancet 389, 1229–1237.

Thun, M., Peto, R., Boreham, J., Lopez, A.D., 2012. Stages of the cigarette epidemic on entering its second century. Tobacco Control 21, 96–101.

Ward, E., Jemal, A., Cokkinides, V., Singh, G.K., Cardinez, C., Ghafoor, A., Thun, M., 2004. Cancer disparities by race/ethnicity and socioeconomic status. CA: a Cancer Journal for Clinicians 54, 78–93.

World Health Organization, 2008. Closing the gap in a generation: health equity through action on the social determinants of health. Final Report of the Commission on Social Determinants of Health. World Health Organization, Geneva.

Relevant Websites

https://www.cancer.gov/about-cancer/understanding/disparities—Website of the U.S. National Cancer Institute.
http://gco.iarc.fr—Website of the Global Cancer Observatory IARC.

Cancer in Populations in Transition

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Abbreviations

AML Acute myeloid leukaemia
APC Annual percent change
ARC Alcohol-related cancers
ASpR Age-specific incidence rate
ASR Age-standardized (world population) incidence rate
CI5 Cancer incidence in five continents
CR Crude cancer incidence rate
EBV Epstein-Barr virus
FOBT Fecal occult blood tests
FVRC Low intake of fruits and vegetables-related cancers
HICs High-income countries
HIV Human immune deficiency virus
HPV Human Papilloma virus
IARC International agency for research on cancer
LE Life-expectancy
LMICs Low- and middle-income countries
MIR Cancer mortality-to-incidence ratio
OORC Overweight and obesity-related cancers
PAF Population attributable fraction
PIR Physical inactivity-related cancers
PSA Prostate-specific antigen
TRC Tobacco-related cancers

Introduction

Cancer is the leading cause of death in many developed countries and is set to become a major cause of morbidity and mortality in the next few decades in every region of the world. Transition of cancer in population has led the world a steep increase in the burden of the disease. It is evident that the transition of cancer burden in populations is a continuous transformation process, more complex, dynamic and not unidirectional as some cancers are declining and others increasing or re-emerging.

From the GLOBOCAN series, cancer estimates for the year 1990 were a total of 8.1 million new cases, which is an increase of 37% from the year 1975 (5.9 million new cases) (annual growth rate 2.1%, faster than annual world population growth rate of 1.7%). In 2012, these were 14.1 million new cases, which is an increase of 139% from the year 1975. The common cancers such as lung (13.0%), breast (12.0%), colo-rectum (9.7%), prostate (7.8%), stomach (6.8%), liver (5.6%), cervix-uteri (3.8%), urinary bladder (3.1%), non-Hodgkin's lymphoma (NHL) (2.7%), leukaemia (2.5%), kidney (2.4%) and oral cavity (2.1%), constitute 72% of the total burden (14.1 million) of cancers world-wide in 2012. The increasing burden is a changing spectrum of common cancers, which differs from high-income countries (HICs) to low- and middle-income countries (LMICs).

Several mechanisms are involved in the transition of cancer in populations. Demographic transition such as reduction in mortality and fertility, thereby increased life-expectancy, changes the structure of the global population and, in turn, in the scale of the increased burden of cancer. Moreover, it is widely held that 80%–90% of cancer in populations are attributed to environmental and life-style factors. Thus, epidemiologic transition in the prevalence of risk factors such as tobacco and alcohol consumption, certain dietary habits, obesity, physical inactivity and infection has mainly attributed to the transition of cancer in populations. Further, transitions in the detection practices, cancer screening and access to care have all resulted increased burden of many cancers now.

This article assesses the transition of cancer in populations according to the changes in the demographic, epidemiologic and detection practices occurred during the past 30 years. This article has four sessions such as (i) transition of leading cancers, (ii) cancer incidence due to demographic transition, (iii) cancer incidence due to transition of epidemiologic risk factors, and (iv) cancer incidence due to transition in access to care. All the comparisons were made separately by HICs and LMICs and by gender.

The data sources were cancer incidence in five continents (CI5), published by the international agency for research on cancer (IARC) (http://ci5.iarc.fr) volumes VI to XI. A total of 8 HICs [Canada (British Colombia and Ontario), United States (SEER black and White), United Kingdom (England, New South Wales), Germany (Saarland), Denmark, Finland, Norway, and Australia (New South Wales)] and 9 LMICs [Brazil (Goiania), Colombia (Cali), Costa Rica, Ecuador (Quito), China (Hong Kong and Shanghai), India (Chennai), Philippines (Manila), Thailand (Chiang Mai), and Uganda (Kyadondo)] were chosen for assessing the transition of cancer in populations during the past 30 years. The various registries mainly from LMICs were chosen according to the availability and quality of data in the CI5, volumes VI to XI (Parkin et al., 1992, 1997, 2002; Curado et al., 2007; Forman et al., 2014; Bray et al., 2017).

Further data sources were, GLOBOCAN series (http://globocan.iarc.fr) for the year 1990 and 2012 (Parkin et al., 1999; Ferlay et al., 2013), incidence and mortality data of various HICs and LMICs from http://ci5.iarc.fr/CI5plus/pages/online.aspx, http://www-dep.iarc.fr/WHOdb/WHOdb.htm, https://data.worldbank.org for life-expectancy at birth of the various countries, https://data.worldbank.org, Giovino (2002) and Gilmore (2000) for smoking prevalence, http://apps.who.int/gho/data/node.main (A1026, A900A and A893) respectively for per-capita consumption of alcohol, prevalence in overweight and obesity and prevalence of insufficient physical activity. Comprehensive estimates of the population attributable fractions (PAF) of cancer cases or deaths due to various risk factors such as tobacco, alcohol, low intake of fruits and vegetable, overweight and obesity, physical inactivity and infection were used (Ezzati et al., 2002; Danaei et al., 2005, de Martel et al., 2012, 2017; Plummer et al., 2015, 2016). Further references used were IARC (1987) and US Surgeon General Report (2004) for tobacco; Hamajima et al. (2002), Baan et al. (2007), Connor (2017) for alcohol; IARC (2016) for overweight and obesity; Schmid and Leitzmann (2014) for physical inactivity; Oyebode et al. (2014) for low-intake of fruits and vegetables and Parkin (2011), Plummer et al. (2015), de Martel et al. (2015) for infection-related cancers.

The various measures used in this article are (i) crude cancer incidence rate (CR) to assess the burden; (ii) age-specific incidence rate (ASpR), to assess the peak age at incidence; (iii) mean age at diagnosis of various cancers; (iv) age-standardized (world population) incidence rate, to compare the rates with various countries (ASR); (v) annual percent change, to assess the transition in rates (APC); and (vi) cancer mortality-to-incidence ratio (MIR), to assess whether a region/country has higher or lower cancer mortality, normalized to its incidence.

We fitted linear regression to the natural logarithm of the estimated rates APC. JointPoint linear regression was used to determine trends in cancer incidence (APC), a statistical algorithm detects joint points, or points in time where the slope of the regression line significantly changes. Thus, the model described trends during different time segments. At each segment, trends in rates were measured using the estimated APC, which assumes that rates changes by a constant percentage each year. The JoinPoint regression software was used for the trend analysis.

The following session presents the changing spectrum in the burden of all and common cancers between HICs and LMICs by gender using GLOBOCAN series for the year 1990 and 2012.

Transition of Leading Cancers

Among males, a total of 4.3 million new cases (49% in HICs and 51% in LMICs) in 1990, these were 7.4 million new cases (44% in HICs and 56% in LMICs) in 2012. Among females, 3.8 million new cancer cases (equally distributed in HICs and LMICs) in 1990 and these were 6.7 million new cases (42% in HICs and 58% in LMICs) in 2012. The top 10 cancers among males in HICs (mainly prostate, lung, and colo-rectum: 51%) and in LMICs (mainly lung, liver, and stomach: 40%) accounted for nearly 80% of the burden and the same among females in HICs (mainly breast, colo-rectum, and lung: 50%) and in LMICs (mainly breast, cervix uteri, and lung: 43%) accounted for nearly 77% in 2012 (**Figs. 1**A, B and **2**A, B).

Among males in HICs, relative proportion and rank ordering increased for prostate and kidney cancers, no change in the proportion and rank ordering for colo-rectum and bladder cancers, and melanoma, emerged as the 8th leading cancer in 2012. Relative proportion decreased for lung and stomach cancers, no change in the proportion, rank ordering declined for NHL, pancreas and liver. Oral cavity was not a leading site in 2012. Among males in LMICs, relative proportion increased for prostate, lung and colo-rectum, with increase in the rank ordering for prostate. Relative proportion decreased for stomach, esophagus, oral cavity and leukaemia (**Fig. 1**A and B).

Among females in HICs, relative proportion increased for breast, lung, corpus uteri, pancreas and thyroid cancers. Like in males, melanoma emerged as the 9th leading cancer (3.2%) in 2012. Relative proportion decreased for colo-rectal and stomach cancers. Cervix-uteri cancer was not a leading site in 2012. Among females in LMICs, relative proportion increased for breast, colo-rectum, lung and corpus-uteri cancers. Like in HICs, thyroid cancer emerged as the 9th leading site (3.6%) in 2012. Relative proportion decreased for cervix-uteri and stomach cancers (**Fig. 2**A and B).

Among males in the selected HICs, 30 years ago, lung was the leading site, which is replaced by prostate cancer. Lung is still the 2nd common cancer in HICs. Currently, colo-rectum/bladder shared the 3rd, bladder/colo-rectum/NHL/kidney, shared the 4th and NHL/bladder/esophagus/liver/kidney shared the 5th position in the selected HICs. Among the five leading cancers, kidney is an emerging cancer in the selected HICs. Among males in the selected LMICs, 30 years ago, stomach/lung was the leading cancer sites, which is replaced by prostate/lung cancer. Stomach/liver shared the 2nd, liver/prostate/colo-rectum/lung/NHL shared the 3rd; colo-rectum/lung/stomach/NHL shared the 4th position and NHL shared the 5th position in the various regions among the selected LMICs (**Table 1**).

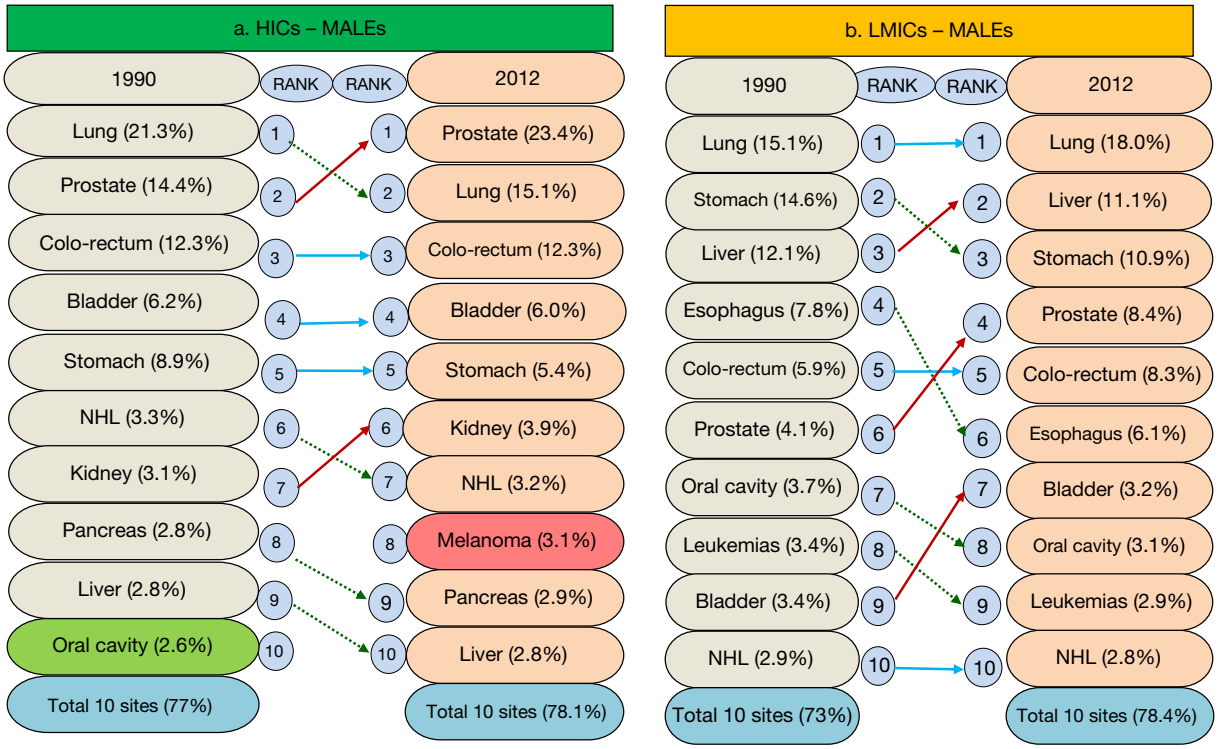

Fig. 1 Transition in rank ordering and relative proportion of leading cancer sites (1990 vs. 2014) in HICs and LMICs: males.

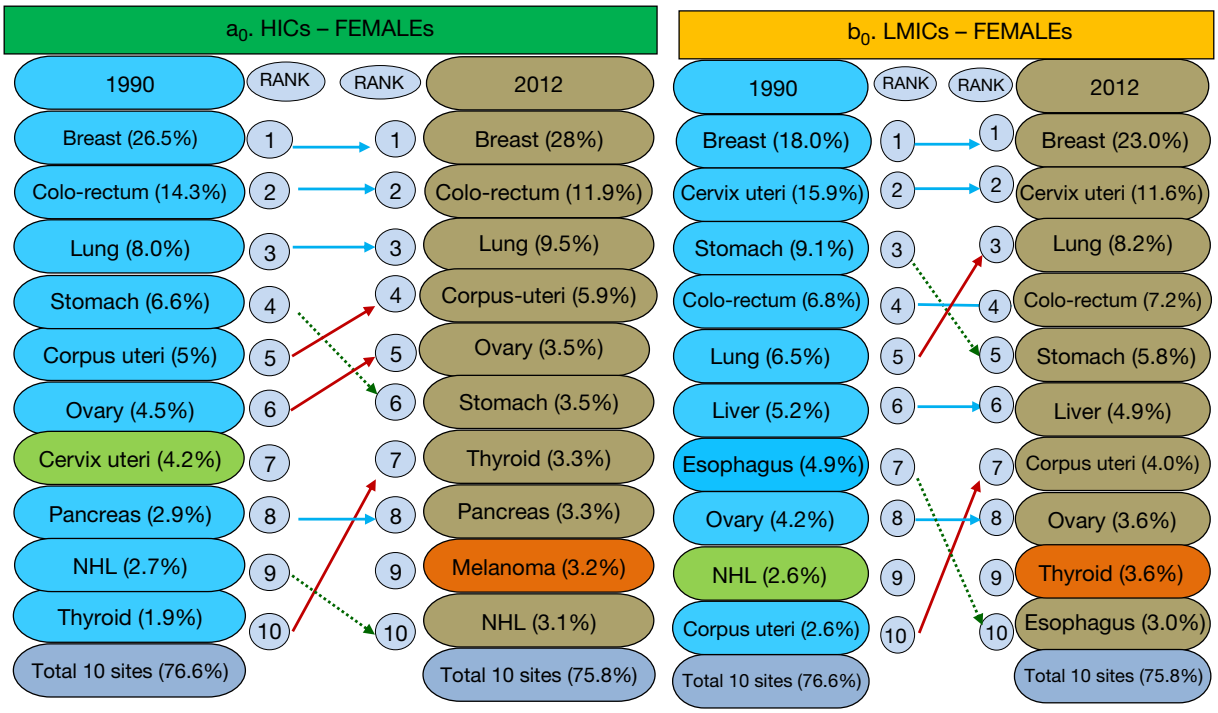

Fig. 2 Transition in rank ordering and proportion of leading cancer sites (1990 vs. 2014) in HICs and LMICs: females.

Among females in the selected HICs, breast cancer remains the leading cancer site in the past 30 years. Currently, lung/colo-rectum shared the 2nd, colo-rectum/corpus-uteri shared the 3rd, corpus-uteri/thyroid, shared the 4th and NHL/ovary shared the 5th position in the selected HICs. The emerging cancer site is thyroid in HICs. Among females in the selected LMICs, cervix uteri/lung cancers were the leading sites in 30 years ago, which is replaced by breast cancer now. Cervix-uteri/thyroid shared the 2nd and 3rd, stomach/liver shared the 4th and colo-rectum shared the 5th position in majority of LMICs (Table 1).

Table 1 Transition of leading cancer sites (2008–12 vs. 1983–87) in selected HICs and LMICs by gender

HICs	*Males*					*%*	*LMICs*	*Females*					*%*	*Period*
Canada, British Colombia	Prostate	Lung	Colo-rectum	Bladder	NHL	62.8	Brazil, Goiania	Prostate	Lung	Colo-rectum	Stomach	Bladder	40.1	2008–12
	Prostate	Lung	Colo-rectum	NHL	Bladder	44.8		Prostate	Stomach	Lung	Esophagus	Bladder	31.4	1983–87
Canada, Ontario	Prostate	Lung	Colo-rectum	NHL	Bladder	61.4	Colombia, Cali	Prostate	Stomach	Lung	Colo-rectum	NHL	57.4	2008–12
	Lung	Prostate	Colo-rectum	Bladder	NHL	62.3		Stomach	Prostate	Lung	Bladder	NHL	41.3	1983–87
United States, SEER: Black	Prostate	Lung	Colo-rectum	Kidney	Liver	68.5	Costa Rica	Prostate	Stomach	Colo-rectum	Lung	NHL	45.5	2008–12
	Lung	Prostate	Colo-rectum	Esophagus	Stomach	66.8		Stomach	Prostate	Lung	Bladder	NHL	42.5	1983–87
United States, SEER: White	Prostate	Lung	Bladder	Colo-rectum	NHL	59.3	Ecuador, Quito	Prostate	Stomach	NHL	Lung	Colo-rectum	49.1	2008–12
	Lung	Prostate	Colo-rectum	Bladder	NHL	65.1		Stomach	Prostate	Lung	NHL	Colo-rectum	42.1	1983–87
Denmark	Prostate	Lung	Colo-rectum	Bladder	NHL	45.4	China, Hong Kong	Lung	Liver	Prostate	Colo-rectum	Nasopharynx	63.1	2008–12
	Lung	Prostate	Bladder	Colo-rectum	Stomach	53.9		Lung	Liver	Nasopharynx	Stomach	Colo-rectum	61.6	1983–87
Finland	Prostate	Lung	Colo-rectum	Bladder	NHL	66.5	China, Shanghai	Lung	Stomach	Liver	Colo-rectum	Prostate	63.5	2008–12
	Lung	Prostate	Stomach	Bladder	Colo-rectum	53.4		Lung	Stomach	Liver	Esophagus	Colo-rectum	74.2	1983–87
Germany, Saarland	Prostate	Lung	Bladder	Colo-rectum	NHL	48.3	India, Chennai	Lung	Stomach	Mouth	Tongue	Esophagus	37.9	2008–12
	Lung	Prostate	Colo-rectum	Bladder	Stomach	56.1		Stomach	Lung	Esophagus	Mouth	Hypopharynx	43.6	1983–87
Norway	Prostate	Lung	Colo-rectum	Bladder	NHL	62.4	Philippines, Manila	Lung	Prostate	Liver	Colo-rectum	NHL	57.4	2008–12
	Prostate	Lung	Colo-rectum	Bladder	Stomach	61.1		Lung	Liver	Prostate	Stomach	Colo-rectum	56.9	1983–87
United Kingdom, England, NW region	Prostate	Lung	Colo-rectum	Bladder	Esophagus	61.9	Thailand, Chiang Mai	Lung	Liver	Prostate	Colo-rectum	NHL	61.5	2008–12
	Lung	Prostate	Bladder	Stomach	Colo-rectum	60.5		Lung	Liver	Stomach	Larynx	Bladder	39.8	1983–87
Australia, NSW	Prostate	Lung	Colo-rectum	NHL	Kidney	59.3	Uganda	Prostate	Esophagus	Liver	NHL	Stomach	45.6	2008–12
	Lung	Prostate	Colo-rectum	Bladder	Stomach	56.8		Prostate	Esophagus	Liver	Stomach	Colo-rectum	23.9	1983–87
Females							Females							
Canada, British Colombia	Breast	Lung	Colo-rectum	Corpus uteri	NHL	62.7	Brazil, Goiania	Breast	Cervix uteri	Thyroid	Lung	Colo-rectum	31.8	2008–12
	Breast	Lung	Colo-rectum	Corpus uteri	Ovary	46.6		Cervix uteri	Breast	Stomach	Lung	Colo-rectum	40.5	1983–87
Canada, Ontario	Breast	Lung	Colo-rectum	Corpus uteri	NHL	62.6	Colombia, Cali	Breast	Cervix uteri	Thyroid	Stomach	Colo-rectum	52.9	2008–12
	Breast	Colo-rectum	Lung	Corpus uteri	Ovary	60.9		Cervix uteri	Breast	Stomach	Lung	Gallbladder	44.2	1983–87
United States, SEER: Black	Breast	Lung	Colo-rectum	Corpus uteri	Thyroid	62.8	Costa Rica	Breast	Thyroid	Cervix uteri	Stomach	Colo-rectum	47.3	2008–12
	Breast	Lung	Colo-rectum	Cervix uteri	Corpus uteri	64.1		Breast	Cervix uteri	Stomach	Colo-rectum	Ovary	46.4	1983–87
United States, SEER: White	Breast	Lung	Corpus uteri	Thyroid	Colo-rect.	60.9	Ecuador, Quito	Breast	Thyroid	Cervix uteri	Stomach	NHL	48.6	2008–12
	Breast	Lung	Colo-rectum	Corpus uteri	Ovary	66.1		Cervix uteri	Breast	Stomach	Gallbladder	NHL	45.8	1983–87
Denmark	Breast	Lung	Colo-rectum	Corpus uteri	Ovary	44.6	China, Hong Kong	Breast	Lung	Colo-rectum	Corpus uteri	Thyroid	61.7	2008–12
	Breast	Lung	Colo-rectum	Cervix uteri	Corpus uteri	50.8		Lung	Breast	Cervix uteri	Colo-rectum	Nasopharynx	53.6	1983–87
Finland	Breast	Colo-rectum	Corpus uteri	Lung	NHL	60.9	China, Shanghai	Breast	Thyroid	Lung	Colo-rectum	Stomach	60.6	2008–12
	Breast	Corpus uteri	Stomach	Colo-rectum	Ovary	43.3		Stomach	Breast	Lung	Liver	Colo-rectum	60.1	1983–87
Germany, Saarland	Breast	Lung	Colo-rectum	Corpus uteri	Cervix uteri	45.8	India, Chennai	Breast	Cervix uteri	Ovary	Stomach	Corpus uteri	56.1	2008–12
	Breast	Colo-rectum	Corpus uteri	Stomach	Cervix uteri	52.1		Cervix uteri	Breast	Mouth	Stomach	Esophagus	67.9	1983–87
Norway	Breast	Lung	Colo-rectum	Corpus uteri	Ovary	56.1	Philippines, Manila	Breast	Cervix uteri	Lung	Ovary	Colo-rectum	61.6	2008–12
	Breast	Colo-rectum	Ovary	Cervix uteri	Corpus uteri	53.1		Breast	Cervix uteri	Lung	Ovary	Colo-rectum	56.8	1983–87
United Kingdom, England, NW region	Breast	Lung	Colo-rectum	Corpus uteri	Ovary	62.1	Thailand, Chiang Mai	Breast	Lung	Cervix uteri	Liver	Colo-rectum	63.2	2008–12
	Breast	Lung	Colo-rectum	Cervix uteri	Ovary	52.3		Lung	Cervix uteri	Breast	Liver	Stomach	51.8	1983–87
Australia, NSW	Breast	Colo-rectum	Lung	Thyroid	Corpus uteri	56.8	Uganda	Cervix uteri	Breast	Esophagus	Liver	Ovary	49.7	2008–12
	Breast	Colo-rectum	Lung	Cervix uteri	Stomach	53.3		Cervix uteri	Breast	Esophagus	Ovary	Corpus uteri	44.1	1983–87

Source: https://data.worldbank.org/indicator, Parkin et al. (1992, 1997, 2002), Curado et al. (2007), Forman et al. (2014), Bray et al. (2017).

In summary, burden of cancer has been increasing worldwide with higher relative proportion in LMICs for the last three decades and its transition is not uniform between HICs and LMICs. The emerging cancers among males in HICs are prostate, colo-rectum, kidney and melanoma and in LMICs are lung, liver, colo-rectum and prostate. Among females in HICs, the same are breast, lung, corpus-uteri, pancreas and thyroid and in LMICs are breast, colo-rectum, lung, corpus-uteri and thyroid.

The following session presents in demographic transition and cancer incidence rates (CR, ASpR and mean age) by gender in HICs and LMICs.

Demographic Transition and Cancer Incidence

Demographic transition over the past two or more decades has left the world struggling with the issues of an aging population. Life-expectancy, an estimate of how long a person can expect to live, is a statistic that applies to populations. Changes in life-expectancy can occur through changes in overall mortality rate in a population over time as well as shifts in relative mortality rates in particular age-groups within the population. Increased life-expectancy is leading to a major change in the structure of the global population. As it increases, so does the certainty that people will become more and more prone to develop cancer that are more common among older age groups.

Life-Expectancy and Cancer Incidence

Life-expectancy has been increased in both HICs and LMICs (Fig. 3). However, the current life-expectancy is <70 years in most of the LMICs and is as similar they were in HICs 30 years ago. Wide range in CRs (per 100,000 person-years) of all sites was observed in LMICs, the CRs and the common cancers such prostate, breast, colo-rectum in LMICs were only one-fourth or less than the rates, what we observed in HICs. CRs for lung cancer among males in HICs showed declining trend, contrary to this, CRs among females

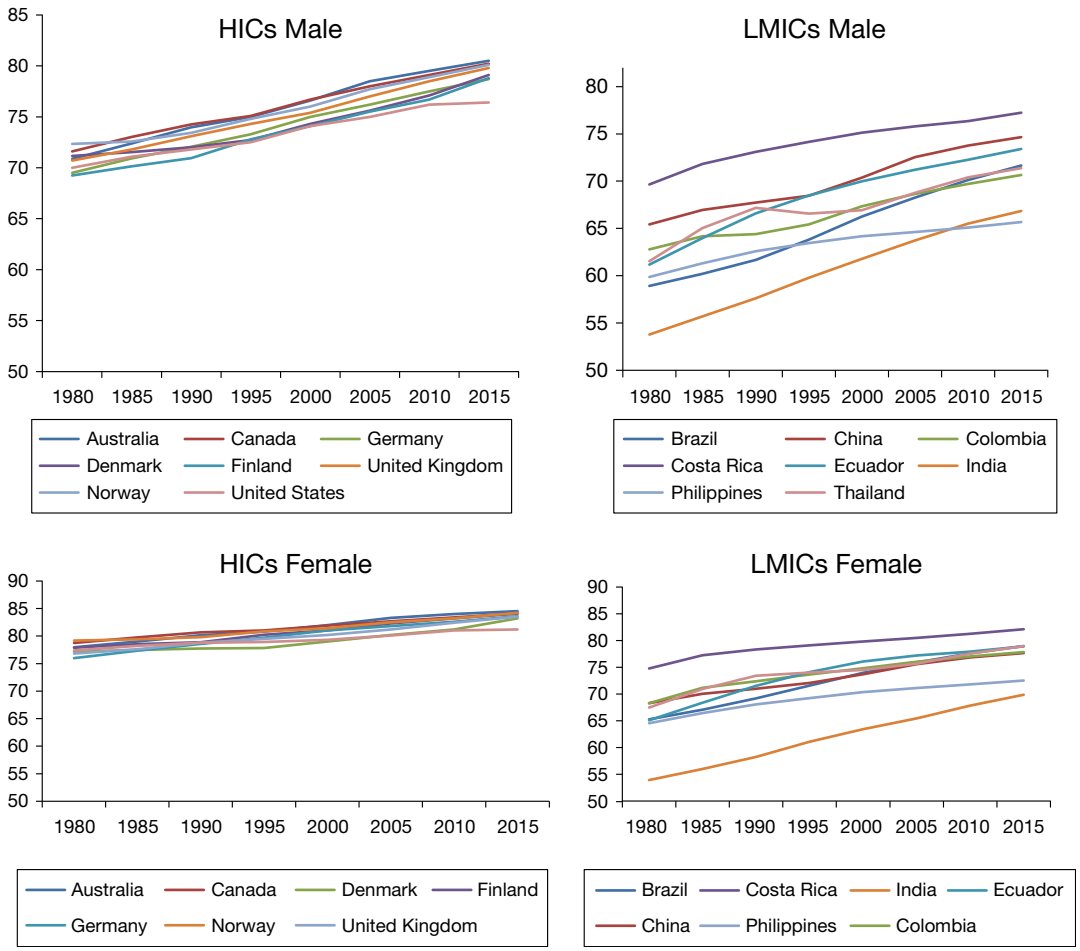

Fig. 3 Demographic transition (life-expectancy) (1980 vs. 2015) in selected HICs and LMICs.

showed increasing trend. CRs for liver cancer increased in most of the LMICs in males. Increased life-expectancy did not affect for stomach and cervix-uteri cancers as it showed a declining trend in HICs and most of the LMICs (Tables 2 and 3).

Even though the CRs of all sites and the common cancers were comparatively low in LMICs, the percent change in the rates was much higher in these countries compared to the HICs. Since the cancer registry data collection system is active, in most of the LMICs, it is possible that the case-ascertainment might not be complete and thereby low incidence rates in the early periods and later on improvements in the data collections system in LMICs, the rates in the initial period might be under-estimated and thus observed higher rate of increase in LMICs.

Age is an established risk factor in the occurrence of many cancers. The CRs increased with age for most cancers worldwide. The ASpRs of prostate cancer increased with age and reached to a peak age at incidence in 70s and then the rate decreased in both HICs and LMICs. The peak ASpR for breast cancer is in 65–70 years. For colo-rectal cancers, it increased with age with peak in the 75+ age group in both genders in HICs and LMICs. Contrary to this, the peak ASpR of cervix-uteri cancer is in late 30s in HICs where as it is in late 60s in LMICs. The peak ASpR of oral-cavity cancer is in late 60s in both HICs and LMICs (Fig. 4).

Transition in Age at Diagnosis of Cancer

Mean age at diagnosis of all cancer sites together has increased in HICs and LMICs with higher increase in LMICs in both genders in 2008–12. However, the mean age at diagnosis is much lower in LMICs than HICs in both genders. In the selected HICs, it is >66 years in males and >61 years in females (except United States, SEER black) where as in most of the LMICs, it is <63 years in males and <59 years in females (except China) in 2008–12. A downward shift in the mean age at diagnosis for prostate cancer in most of the HICs, and a few LMICs for colo-rectal cancers in the United States in both genders and for stomach and cervix uteri cancers in a few LMICs and an upward shift in the mean age at diagnosis for corpus-uteri cancers in HICs (except United States and Canada), for lung cancer in most of the HICs and LMICs in both genders (Tables 4 and 5).

Upward shift in the mean age at diagnosis of cancers is closely associated with the increase in the life-expectancy and thereby growth in the older age population and downward shift in the mean age, could be due to the availability of early detection programs. Another possible reason for this is that the cancer registry data collection system is active in majority of cancer registries in LMICs, the age of the patient is collected only the reporting age and not linked with their date of birth. Thus there is a possibility of incorrect reporting in the age at incidence.

Epidemiologic transition reflects shifts in cancer incidence that has taken place in the vast majority of countries over the past three to four decades. Transitions in many cancers are accompanied by changes in exposure due to certain factors. Adoption of a life-style characterized by a more "industrialized" diet, rise in obesity, chronic physical inactivity will attenuate the expected gains in life-expectancy. The following session deals with the comparison of the transition of various established risk factors and related cancers in the HICs and LMICs.

Epidemiologic Transition and Related Cancers

A link between a particular risk factor and cancers has been established. Comprehensive estimates of the fractions of cancer cases or deaths attributable to various risk factors (population attributable fraction) such as tobacco, alcohol, low intake of fruits and vegetable, overweight and obesity, physical inactivity, and infection have been made separately by HICs and LMICs. In the past three to four decades, a lot of changes in the prevalence of these risk factors and there by a lot of transition in the related cancers have been taking place world-wide.

Tobacco

PAF due to tobacco and related cancers

Of the several causes investigated for cancer, the use of cigarette smoking and smokeless tobacco has shown strong and consistent associations with cancer at several sites in the body. During the past 30 years, the number and strength of the conclusions on active smoking and exposure to second-hand smoke as a cause of cancer have increased markedly, moving from the specific causal conclusions on lung cancer in males. The top tobacco-related cancers (TRC) (exposure due to active and passive) include lung, oral cavity, other pharynx (oropharynx and hypopharynx), larynx, esophagus, and urinary bladder. Other than these cancers, the International Agency for Research on Cancer (IARC) monograph (1987) stated that tobacco smoking is associated with stomach, liver, pancreas, kidney, cervix-uteri, and acute myeloid leukaemia (AML). The US Surgeon-General's report for 2004 stated that the evidence is sufficient to infer a causal relationship between smoking and cancer of the lung, oral-cavity, larynx, esophagus, urinary bladder, kidney, stomach, pancreas, cervix-uteri, and AML. The population attributable fraction (PAF) of the various TRCs in HICs and LMICs varied markedly. The PAF for all TRCs (mouth, pharynx, esophagus, stomach, liver, pancreas, lung, cervix uteri, bladder, and leukaemia) together in HICs is 29% and in LMICs is 18%, the same for lung in HICs is 86% whereas the same in LMICs is 60%. Similarly, the PAF for esophagus/larynx/other pharynx/oral cavity in HICs is 71% and the same in LMICs is 37% (Fig. 5A).

Table 2 Life-expectancy and crude incidence rates of all three leading sites (1983–87 to 2008–12) of the selected HICs by gender

HIC	*Male*	*1983–87*	*1988–92*	*1993–97*	*1998–2002*	*2003–2007*	*2008–12*	*Female*	*1983–87*	*1988–92*	*1993–97*	*1998–2002*	*2003–2007*	*2008–12*
Canada, BC	LE	73.0	74.3	75.1	76.7	78.0	79.1	LE	79.7	80.7	81.0	81.9	82.7	83.4
	All	407.8	428.7	450.1	469.6	494.0	537.5	All	366.3	379.4	386.5	404.6	428.8	480.6
	Pr.	90.9	127.5	133.4	139.7	137.6	145.0	Br.	103.9	119.5	115.1	117.6	120.5	138.4
	Lu.	80.2	76.3	68.2	65.3	68.1	67.5	Co.R	52.5	47.4	44.7	47.7	50.8	55.7
	Co.R	56.8	52.7	52.8	57.7	63.1	69.3	Lu.	39.4	47.3	50.5	53.3	60.7	63.2
United States, SEER: White	LE	71.1	71.8	72.5	74.1	75.0	76.2	LE	78.2	78.8	78.9	79.3	80.1	81.0
	All	412.1	490.1	489.6	506.1	525.9	540.4	All	405.3	426.5	442.2	467.7	485.2	502.8
	Pr.	84.7	142.2	146.1	155.6	153.4	148.7	Br.	122.5	129.6	134.1	144.9	142.4	146.4
	Lu.	80.3	79.8	74.3	70.9	68.1	65.8	Co.R	59.2	54.8	51.1	52.0	47.8	42.7
	Co.R	60.2	57.4	53.1	53.4	49.7	45.3	Lu.	42.9	51.4	55.9	59.0	62.6	61.9
Denmark	LE	71.5	72.0	72.7	74.3	75.6	77.1	LE	77.5	77.7	77.8	79.0	80.2	81.2
	All	434.2	433.4	439.3	466.9	560.4	621.3	All	436.2	450.9	471.2	492.9	533.8	590.0
	Pr.	55.2	58.3	55.8	75.1	129.1	162.2	Br.	105.0	115.2	128.8	138.4	146.1	176.0
	Lu.	91.7	82.4	78.4	77.6	80.7	83.7	Co.R	63.2	64.8	62.5	63.1	68.2	71.9
	Co.R	61.9	63.0	63.0	67.2	76.0	81.7	Lu.	38.3	42.4	50.3	57.8	67.6	76.9
Finland	LE	70.1	70.9	72.7	74.1	75.5	76.7	LE	78.5	78.9	80.2	81.0	82.3	83.2
	All	316.6	333.2	379.5	430.9	505.5	503.3*	All	307.2	348.1	377.8	413.1	437.2	444.8*
	Pr.	47.6	59.0	96.4	136.9	184.3	178.7*	Br.	78.1	98.2	114.1	134.7	142.6	148.8*
	Lu.	82.6	72.1	65.8	61.7	63.3	58.8*	Co.R	33.3	37.0	39.9	42.7	44.6	44.9*
	Co.R	28.1	31.3	37.0	41.0	48.1	49.2*	Lu.	13.2	15.2	17.4	20.7	24.3	25.7*
Germany, Saarland	LE	70.9	72.1	73.3	75.0	76.2	77.5	LE	77.3	78.6	79.7	81.0	81.8	82.6
	All	433.1	479.8	539.5	596.4	702.7	659.7	All	399.2	439.7	487.2	510.8	555.5	554.1
	Pr.	45.2	58.0	87.2	127.7	163.6	134.7	Br.	95.5	109.7	128.3	143.8	158.9	167.7
	Lu.	101.9	108.8	106.5	110.7	119.2	109.4	Co.R	65.3	75.1	81.4	85.9	83.9	69.3
	Co.R	60.4	67.2	80.6	94.2	107.5	90.0	Lu.	14.5	20.9	26.1	35.9	44.9	49.8
Norway	LE	72.5	73.4	74.8	76.0	77.7	78.9	LE	79.4	79.8	80.8	81.4	82.5	83.2
	All	407.3	420.6	457.1	480.0	545.2	589.5	All	372.2	379.7	414.0	434.8	473.4	492.1
	Pr.	84.2	95.4	111.9	131.6	167.7	188.1	Br.	87.0	87.8	97.6	110.2	118.2	120.0
	Lu.	53.3	55.1	57.5	58.5	62.5	63.5	Co.R	58.5	62.9	67.9	69.5	73.9	76.3
	Co.R	59.1	64.1	65.8	69.4	74.4	79.7	Lu.	16.2	21.8	27.4	33.1	41.9	49.4
United Kingdom, England, NW	LE	71.8	73.1	74.3	75.4	77.0	78.5	LE	77.6	78.8	79.5	80.2	81.2	82.4
	All	413.6	421.9	471.4	510.6	541.1	568.1	All	390.0	412.9	451.9	470.3	494.6	523.9
	Pr.	40.6	47.3	76.3	98.3	124.7	133.4	Br.	91.9	106.3	118.3	129.9	141.6	147.3
	Lu.	127.4	112.5	101.2	90.3	88.7	91.8	Co.R	55.0	51.7	53.2	52.7	52.2	56.2
	Co.R	52.1	55.5	60.7	65.0	69.7	77.0	Lu.	48.5	53.4	55.3	58.3	67.4	77.1
Australia, NSW	LE	72.4	73.9	75.0	76.6	78.5	79.5	LE	79.0	80.2	80.8	82.0	83.3	84.0
	All	354.9	410.4	491.3	502.7	582.5	636.9	All	307.0	339.1	381.4	414.3	443.4	477.2
	Pr.	50.0	74.8	131.1	119.7	173.6	201.7	Br.	73.3	87.9	108.8	117.6	120.9	131.0
	Lu.	64.6	60.7	58.9	57.6	57.6	59.7	Co.R	47.6	48.1	51.8	56.2	59.6	61.2
	Co.R	52.2	60.0	64.5	68.7	73.0	76.4	Lu.	18.5	22.3	26.6	28.7	34.1	41.0

LE: life expectancy; Pr.: prostate; Lu.: lung; Co.R.: colo-rectum; Br.: breast, *: estimated.

Table 3 Life-expectancy and crude incidence rates of all three leading sites (1983–87 to 2008–12) of the selected LMICs by gender

LMIC	*Male*	*1983–87*	*1988–92*	*1993–97*	*1998–2002*	*2003–2007*	*2008–12*	*Female*	*1983–87*	*1988–92*	*1993–97*	*1998–2002*	*2003–2007*	*2008–2012*
Brazil, Goiania	LE	60.2	61.7	63.8	66.3	68.3	70.1	LE	67.1	69.2	71.6	73.9	75.9	77.6
	All	82.6	108.2	163.6	137.2	280.5	255.1	All	113.4	127.3	156.5	143.0	237.7	206.9
	Lu.	10.5	11.2	12.9	10.2	20.1	17.6	Br.	21.2	28.3	38.1	36.4	62.7	55.0
	Li.	0.8	2.4	2.2	1.9	4.9	4.5	Cx.	26.9	26.2	30.9	24.8	26.7	16.3
	St.	11.6	10.3	12.7	9.9	16.6	12.7	Lu.	5.4	5.9	5.6	6.0	11.5	12.5
Colombia, Cali	LE	64.2	64.4	65.4	67.3	68.7	69.7	LE	71.2	72.4	73.6	74.8	76.0	77.0
	All	109.2	124.8	136.6	179.1	199.9	193.7	All	144.2	153.1	159.3	197.6	211.8	214.5
	Lu.	14.0	15.3	14.8	16.4	16.8	13.0	Br.	24.3	29.4	29.6	44.0	50.8	50.8
	Li.	1.4	1.7	2.0	2.4	3.7	4.6	Cx.	30.9	27.1	25.0	27.0	21.9	17.7
	St.	21.0	21.4	21.3	21.6	22.8	19.4	Lu.	6.1	6.7	7.3	8.5	9.5	9.7
Costa Rica	LE	71.8	73.1	74.2	75.1	75.8	76.3	LE	77.2	78.3	79.1	79.8	80.5	81.2
	All	101.1	119.4	122.7	136.9	152.4	160.4	All	104.1	119.7	126.9	139.6	153.7	174.1
	Lu.	7.0	9.0	8.3	8.1	8.6	8.0	Br.	17.6	20.3	23.7	30.8	36.6	43.0
	Li.	2.6	4.2	3.8	4.5	4.7	4.8	Cx.	18.5	18.5	16.4	17.2	14.0	14.4
	St.	25.9	30.5	27.7	24.5	22.0	19.7	Lu.	3.0	3.7	4.0	3.8	4.3	4.4
Ecuador, Quito	LE	63.9	66.6	68.5	69.9	71.2	72.3	LE	68.4	71.5	74.1	76.1	77.2	77.9
	All	91.1	93.0	93.1	122.3	148.7	170.3	All	126.5	122.2	120.4	139.8	171.7	200.4
	Lu.	4.3	6.0	5.1	6.2	6.6	7.1	Br.	16.6	18.7	18.2	26.3	33.3	37.3
	Li.	1.8	1.7	2.3	2.7	3.8	4.8	Cx.	22.5	22.5	18.5	17.3	18.5	17.8
	St.	15.9	19.0	15.5	17.5	19.4	18.6	Lu.	2.6	2.5	3.1	3.7	5.5	6.2
China, Shanghai	LE	66.9	57.6	68.4	70.4	72.5	73.8	LE	70.0	71.0	72.1	73.7	75.6	76.8
	All	265.9	286.7	306.4	336.4	375.9	443.0	All	196.2	220.1	244.7	293.4	348.3	401.9
	Lu.	63.1	71.5	76.3	80.2	79.8	92.1	Br.	26.4	35.0	41.6	54.9	69.8	78.6
	Li.	13.8	35.5	35.9	37.7	39.4	39.6	Cx.	6.1	4.9	3.8	4.7	6.8	9.6
	St.	60.8	59.2	54.6	52.6	51.2	53.3	Lu.	25.3	28.2	31.1	36.9	40.7	49.3
India, Chennai	LE	55.7	57.6	59.8	61.8	63.7	65.5	LE	56.0	58.3	61.1	63.4	65.4	67.8
	All	64.1	77.2	81.3	88.5	100.4	112.2	All	91.9	91.6	93.9	100.1	112.2	123.5
	Lu.	5.6	8.0	8.3	8.7	10.7	11.8	Br.	14.3	16.6	19.5	23.6	30.8	34.8
	Li.	1.6	1.6	2.1	2.4	2.6	4.3	Cx.	34.2	27.7	24.3	24.2	19.4	17.4
	St.	9.9	10.0	10.1	10.2	10.4	10.9	Lu.	0.9	1.5	1.8	2.2	3.1	4.1
Philippines, Manila	LE	61.3	62.6	63.5	64.2	64.6	65.1	LE	66.4	68.0	69.2	70.3	71.1	71.8
	All	95.5	101.6	103.3	108.7	112.3	98.0	All	109.9	114.2	126.1	138.6	150.4	140.9
	Lu.	23.3	26.0	24.3	24.4	22.0	19.4	Br.	27.3	29.1	34.2	38.3	43.5	43.9
	Li.	11.7	12.3	11.5	12.1	11.7	10.0	Cx.	14.7	13.6	14.7	14.6	13.4	12.9
	St.	5.7	4.6	4.5	3.8	3.7	2.7	Lu.	7.5	8.5	8.0	8.5	9.3	8.8
Thailand, Chiang Mai	LE	65.0	67.2	66.6	66.9	68.8	70.4	LE	70.9	73.4	74.0	74.5	75.7	77.6
	All	110.2	134.2	141.4	151.5	175.5	211.1	All	122.8	145.9	153.3	172.8	193.8	215.5
	Lu.	24.5	32.8	34.5	33.3	42.9	47.9	Br.	10.2	14.2	17.5	25.3	34.0	42.9
	Li.	13.1	19.0	19.1	19.7	33.9	45.4	Cx.	22.8	25.7	27.5	34.2	32.0	30.4
	St.	6.9	7.1	6.7	6.1	7.6	7.8	Lu.	20.5	28.0	25.6	24.6	31.1	33.4

LE: life expectancy; Lu.: lung; Li.: liver; St.: stomach; Br.: breast; Cx.: cervix uteri

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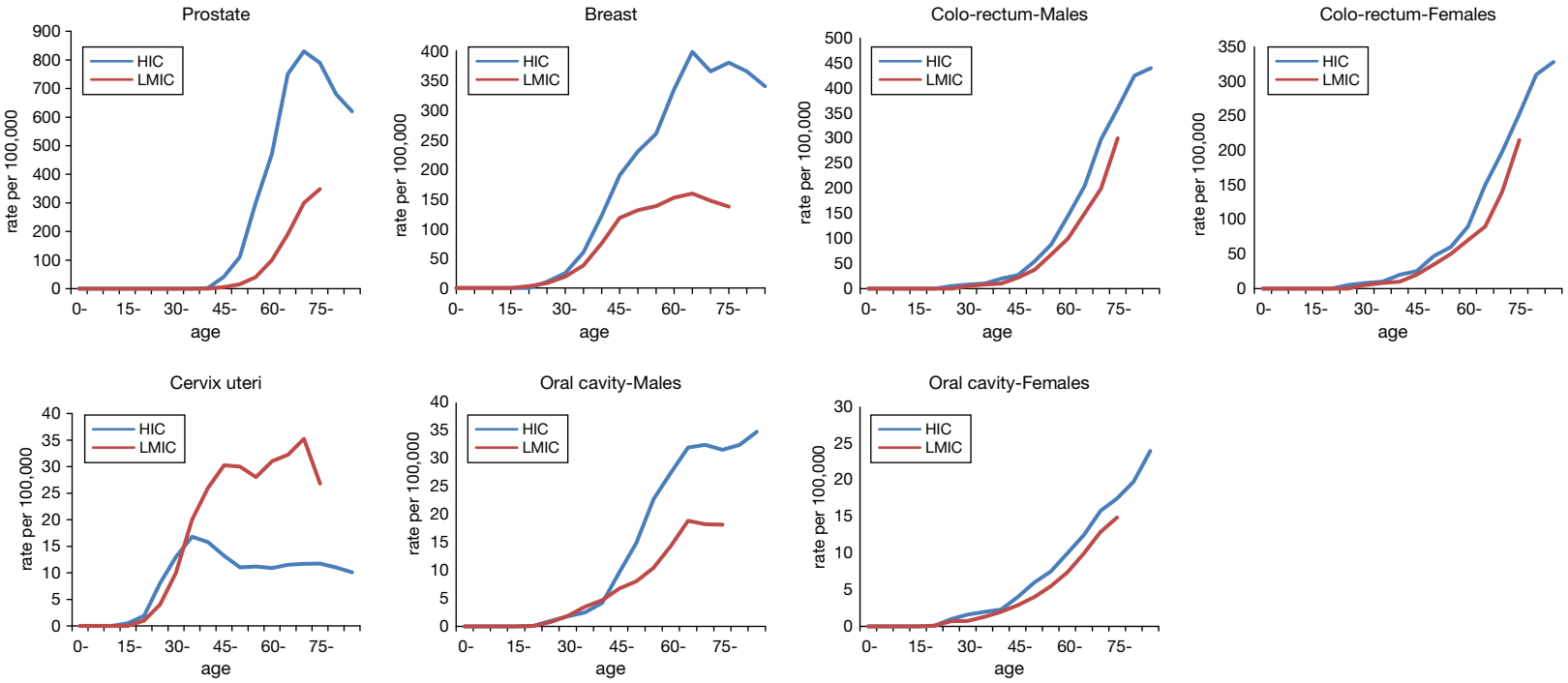

Fig. 4 Age-specific incidence rates of leading cancers (prostate, breast, colo-rectum, oral cavity, and cervix-uteri) in 2012 (HICs vs. LMICS).

Table 4 Transition in life expectancy (years), crude rate (% change) and average age at diagnosis (years) (1983–87 vs. 2008–12): HIC vs. LMIC, males

		Crude rate (% change)						*Average age shift (years)*					
HIC	*LE shift (years)*	*All sites*	*Prostate*	*Lung*	*Colo-rectum*	*Bladder*	*Stomach*	*All sites*	*Prostate*	*Lung*	*Colo-rectum*	*Bladder*	*Stomach*
Canada, BC	6.1	31.8	59.5	−15.8	22.0	110.6	−28.4	1.7 (68.0)	−3.1(69.5)	3.3(71.1)	1.1(69.5)	3.7(72.3)	0.6(69.3)
Canada, Ontario	6.1	28.2	114.8	−20.0	10.1	−18.2	−20.7	0.8 (66.0)	2.5(67.3)	3.3(69.8)	0.4(67.7)	4.0(71.6)	0.5(68.1)
United States, SEER: Black	5.1	50.3	141.3	−19.9	22.7	44.2	−6.9	−1.7(62.0)	−7.0(63.4)	2.6(65.9)	−2.9(62.7)	1.6(67.4)	−0.7(65.3)
United States, SEER: White	5.1	31.1	75.6	−18.1	−24.8	23.1	−14.6	−0.2(65.8)	−5.8(66.6)	3.4(70.2)	−2.2(66.6)	3.2(71.3)	−0.7(67.6)
Denmark	5.6	43.1	193.8	−8.7	32.0	−4.0	−35.4	0.4(67.1)	−4.0(69.9)	1.7(69.6)	−0.2(69.6)	1.9(70.7)	−1.9(68.4)
Finland*	6.6	59.7	287.2	−23.4	71.2	55.4	−39.7	1.7(67.3)	−3.5(69.5)	2.8(69.7)	0.9(68.2)	2.0(70.4)	0.8(68.6)
Germany, Saarland	6.6	52.3	198.0	7.4	49.0	66.4	−21.4	2.7(67.0)	6.7(69.8)	2.8(68.3)	1.0(68.5)	2.2(69.5)	1.8(69.0)
Norway	6.4	44.7	123.4	19.1	34.9	10.8	−55.9	0.1(67.5)	−4.5(69.4)	2.8(70.2)	0.7(70.1)	1.6(71.4)	−0.9(70.0)
United Kingdom, England, NW	6.7	37.4	228.6	−27.9	47.8	−15.0	−49.4	3.2(68.6)	−1.7(71.1)	4.6(71.4)	2.5(69.8)	6.3(73.1)	4.0(72.1)
Australia, NSW	7.1	79.5	303.4	−7.6	46.4	−13.4	−14.5	2.8(66.7)	−4.9(67.6)	4.3(70.7)	3.0(68.4)	6.0(73.3)	1.6(69.0)
LMIC	LE shift (years)	Crude rate (% change)						Average age shift (years)					
		All sites	Lung	Liver	Stomach	Prostate	Colo-rectum	All sites	Lung	Liver	Stomach	Prostate	Colo-rectum
Brazil, Goiania	9.9	208.8	67.6	462.5	9.5	945.7	258.1	4.6(62.4)	4.0(65.5)	2.0(62.7)	1.9(64.0)	−6.3(67.6)	7.4(62.3)
Colombia, Cali	5.5	77.4	−7.1	228.6	−7.6	282.5	216.3	3.6(63.1)	3.9(69.0)	19.7(68.9)	0.9(64.7)	−0.7(69.9)	3.2(63.4)
Costa Rica	4.5	58.7	14.3	84.6	−23.9	283.1	137.0	4.4(62.5)	3.8(68.0)	11.9(65.1)	1.8(66.6)	−3.2(69.6)	3.1(64.2)
Ecuador, Quito	8.4	86.9	65.1	166.7	17.0	309.0	128.6	2.6(61.5)	−0.6(68.0)	6.0(66.1)	0.9(65.1)	−2.4(70.6)	2.5(64.1)
China, Hong Kong	7.2	32.0	15.4	10.1	0.0	649.2	125.6	6.1(66.3)	4.9(69.5)	4.9(63.1)	4.5(68.7)	0.0(71.9)	4.5(68.0)
China, Shanghai	6.9	66.6	46.0	187.0	−12.3	1726.3	207.6	5.0(67.2)	3.8(69.2)	4.5(63.5)	4.0(68.3)	4.7(75.1)	7.5(68.6)
India, Chennai	9.8	75.0	110.7	168.8	10.1	400.0	203.8	5.1(56.7)	5.3(60.9)	7.8(60.4)	3.6(59.0)	4.1(70.3)	4.6(58.5)
Philippines, Manila	3.8	2.6	−16.7	−14.5	−52.6	72.9	69.5	2.5(56.4)	3.7(62.2)	4.2(57.5)	1.0(61.0)	0.0(69.1)	1.2(59.2)
Thailand, Chiang Mai	5.4	91.6	95.5	246.6	13.0	500.0	241.9	4.9(61.3)	6.9(65.5)	5.9(59.4)	4.0(62.8)	0.7(71.9)	5.3(61.4)
Uganda**	9.2	−7.3	−38.5	5.6	−6.3	68.4	0.0	5.3(44.1)	−1.5(49.8)	−1.0(44.9)	5.1(55.0)	2.0(68.0)	−1.4(51.7)

LE: life-expectancy; *: rates were CIV volume VI vs. X; **: rates were volume VII vs. XI.

Table 5 Transition in life expectancy, crude rate and average age at diagnosis (1983–87 vs. 2008–12): HIC vs. LMIC, females

		Crude rate (% change)						*Average age shift (years)*					
HIC	*LE shift (years)*	*All sites*	*Breast*	*Colo-rectum*	*Lung*	*Corpus uteri*	*Ovary*	*All sites*	*Breast*	*Colo-rectum*	*Lung*	*Corpus uteri*	*Ovary*
Canada, BC	3.7	31.2	33.2	6.1	60.4	40.0	−17.4	2.1(65.7)	0.6(62.6)	1.2(71.1)	4.5(70.4)	−1.4(63.1)	3.2(64.6)
Canada, Ontario	3.7	31.4	36.0	−3.3	65.9	38.9	−1.3	0.7(63.5)	0.1(61.3)	0.1(69.5)	4.4(69.3)	−0.6(62.9)	0.6(61.2)
United States, SEER: Black	2.8	59.2	72.8	11.4	71.3	128.7	20.8	0.3(61.0)	0.9(58.5)	−2.3(64.4)	−3.7(66.6)	−2.7(62.0)	1.3(60.4)
United States, SEER: White	2.8	24.1	19.5	−27.9	44.3	58.7	−7.6	−0.1(64.3)	−0.3(62.3)	−1.8(69.7)	4.9(70.7)	−1.8(62.9)	1.7(63.6)
Denmark	3.7	35.3	67.6	13.8	100.8	8.1	−16.9	0.3(65.2)	0.8(62.6)	−0.2(71.1)	3.2(68.8)	2.0(67.2)	1.9(65.2)
Finland*	4.7	42.3	82.6	33.9	84.1	58.7	17.9	0.6(65.7)	0.1(61.4)	2.2(70.4)	0.4(69.8)	2.7(67.0)	2.5(64.3)
Germany, Saarland	5.3	38.8	75.6	6.1	243.4	−3.0	2.4	0.6(66.4)	1.6(63.2)	2.4(72.2)	−0.1(66.5)	0.6(66.8)	4.2(67.5)
Norway	3.8	32.2	37.9	30.4	204.9	56.7	−20.1	0.6(65.8)	−2.6(61.5)	1.0(71.9)	3.2(69.3)	2.9(67.0)	2.1(64.5)
United Kingdom, England, NW	4.8	34.3	60.7	2.2	59.0	131.1	2.6	−0.1(66.6)	−0.2(62.7)	−0.5(71.6)	3.7(71.8)	0.6(66.2)	2.1(65.4)
Australia, NSW	5.0	55.4	78.7	28.6	121.6	72.3	8.8	2.7(64.5)	1.3(60.8)	2.1(69.9)	4.4(69.8)	1.0(64.1)	3.2(63.5)
LMIC	LE shift (years)	Crude rate (% change)						Average age shift (years)					
		All sites	Breast	Colo-rectum	Lung	Cervix uteri	Stomach	All sites	Breast	Colo-rectum	Lung	Cervix uteri	Stomach
Brazil, Goiania	10.5	82.5	159.4	245.9	131.5	−39.4	−7.1	3.8(56.7)	1.5(55.3)	5.0(61.8)	3.3(63.9)	−0.3(51.6)	2.6(60.8)
Colombia, Cali	5.8	48.8	109.1	174.2	59.0	−42.7	0.0	3.7(59.3)	3.1(57.4)	2.4(64.3)	5.7(68.7)	0.8(52.3)	−0.5(65.0)
Costa Rica	4.0	67.2	144.3	131.7	46.7	−22.2	0.7	2.1(56.7)	1.8(57.3)	9.6(64.4)	2.3(67.2)	−4.0(47.4)	−0.2(65.2)
Ecuador, Quito	9.5	58.4	124.7	130.8	138.5	−20.9	3.3	−2.4(53.2)	0.9(56.6)	4.7(64.5)	−1.1(66.0)	2.7(54.1)	−1.0(64.1)
China, Hong Kong	6.8	31.4	145.4	300.8	3.9	−44.4	−15.9	1.9(61.9)	−0.9(55.8)	4.1(69.1)	2.2(69.6)	−0.4(54.9)	3.3(68.1)
China, Shanghai	6.8	104.8	197.7	159.5	94.9	57.4	−0.3	4.2(64.6)	3.9(58.9)	8.4(69.8)	4.4(69.9)	−12.9(52.1)	5.7(68.8)
India, Chennai	11.8	34.4	143.4	163.6	355.6	−49.1	21.7	5.3(54.7)	4.0(54.5)	2.5(58.0)	5.5(58.6)	6.3(56.3)	3.8(57.0)
Philippines, Manila	5.4	28.2	60.8	69.0	17.3	−12.2	−40.5	2.5(53.8)	2.6(54.0)	2.7(60.6)	2.9(62.7)	1.4(51.6)	1.8(62.1)
Thailand, Chiang Mai	6.7	75.5	320.6	210.9	62.9	33.3	30.2	4.2(57.8)	1.9(53.4)	4.0(61.8)	8.5(66.4)	4.1(52.2)	2.3(58.6)
Uganda**	8.7	7.2	34.2	43.8	166.7	17.3	−20.0	5.8(43.1)	2.9(46.9)	0.7(52.3)	14.9(56.8)	2.7(46.5)	9.6(61.0)

LE: life-expectancy; *: rates were CI5 volume VI vs. X; **: rates were volume VII vs. XI.

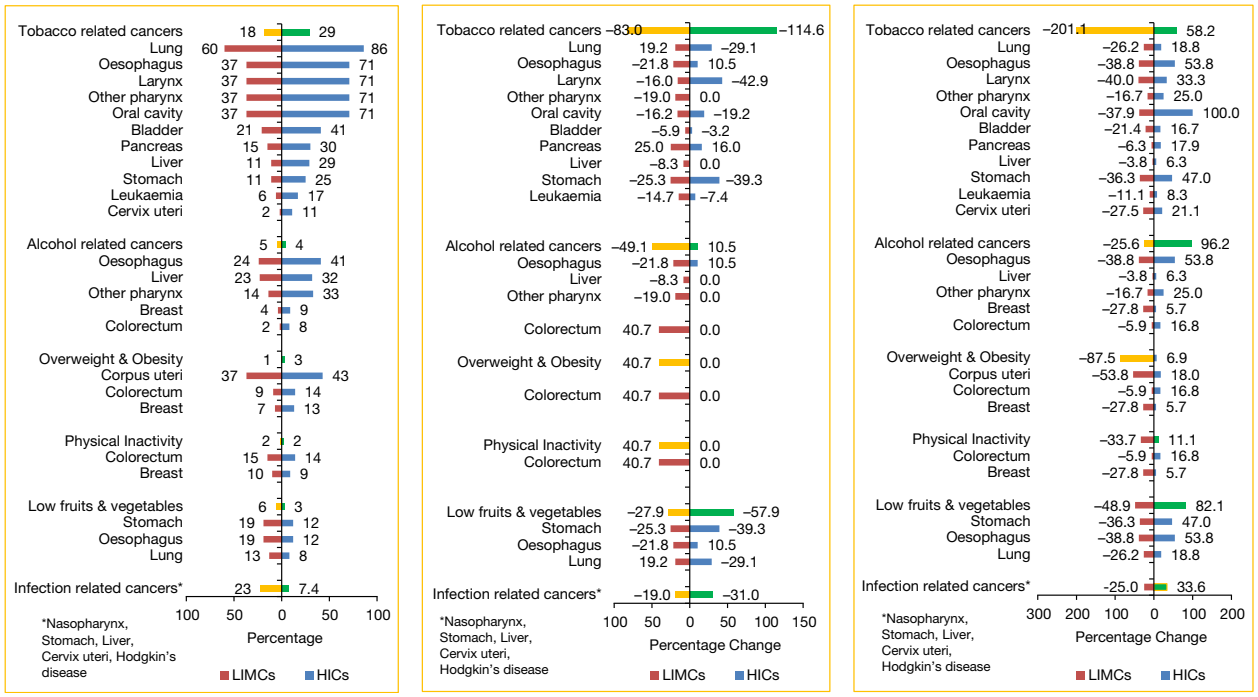

Fig. 5 Population attributable fraction of cancers according to risk factors (tobacco, alcohol, overweight, obesity, physical inactivity, low fruits, and vegetables and infection in 2011 and percentage changes (1983–87 vs. 2008–12) in the respective cancers by gender.

Transition in cigarette smoking and smokeless tobacco and related cancers

In the past 30 years, TRCs declined among males in both HICs (% change: 115%) and LMICs (% change: −83%). It is counteracted by a worrisome increase in among females in HICs (% change: 58%) (Fig. 5B and C). Due to public health effort during the past three to four decades, tobacco prevalence has been declined in many countries (Fig. 6), but still it is the highest risk factor for many cancers. APC for TRCs such as lung, oral cavity, other pharynx, larynx, esophagus, and bladder cancers with a high PAF due to tobacco and APC for these sites pooled, were estimated for the selected HICs and LMICs by gender (Table 6).

Among males, the pooled APC (lung, oral cavity, other pharynx, larynx, esophagus, and bladder) for TRCs, declined, significantly in Canada (Ontario), Denmark, Finland, Germany, United Kingdom (England), and Australia (New South Wales). All the major TRC sites (lung, oral cavity, other pharynx, and larynx) with high PAF (Fig. 5A) due to tobacco use, declined significantly among males in the selected HICs (Table 6). These trends clearly reflect the decline in cigarette smoking since early 80s in these countries (Fig. 6A). Contrary to this, the pooled APC for TRC in females showed an increasing trend with significance in Norway, Germany, Denmark, Finland, United Kingdom, and Australia. However, lung, other pharynx, oral cavity, and larynx declined in all the selected HICs significantly in Canada, United States, United Kingdom, and Australia (Table 6). Decline in oral cavity cancer is due to the encouraging decline in the use of smokeless tobacco use. Even though smoking prevalence declined (Fig. 6) in all the selected HICs, increased incidence of bladder cancer was observed in Norway (significant among males), Finland and Germany (Table 6), which could be due to incidental detection.

The incidence of esophageal cancer did not decline but actually increased significantly in Norway, Denmark, United Kingdom, and Australia. The interpretation of these trends is unclear given that the two types of esophageal cancer [squamous cell carcinoma (SCC) and adenocarcinoma (AC)] have very distinct patterns and risk factors as SCC is more smoking-related. Since the data do not distinguish between the two histological types of esophageal cancer, trends are presented for all esophageal cancers combined and this could be the reason for not being observed a declining trend for this disease.

Pooled TRC declined in both genders among the selected LMICs significantly in Colombia (Cali), Costa Rica, China (Hong Kong and Shanghai), Philippines (Manila), and Thailand (Chiang Mai). In contrast to this, oral cavity, other pharynx, and bladder cancers increased significantly among males in Brazil. Colombia, Philippines, and Thailand showed declining trend in most of the TRCs, with significant decline in lung and esophagus in both genders.

Alcohol

PAF due to alcohol and related cancers

Consistent use of alcohol is linked to other pharynx, breast, esophagus (SCC), liver, colo-rectum. Unlike tobacco, the estimated PAF due to alcohol for all related cancers (ARCs) together in both HICs and LMICs is very low. PAF due to alcohol for ARCs of individual

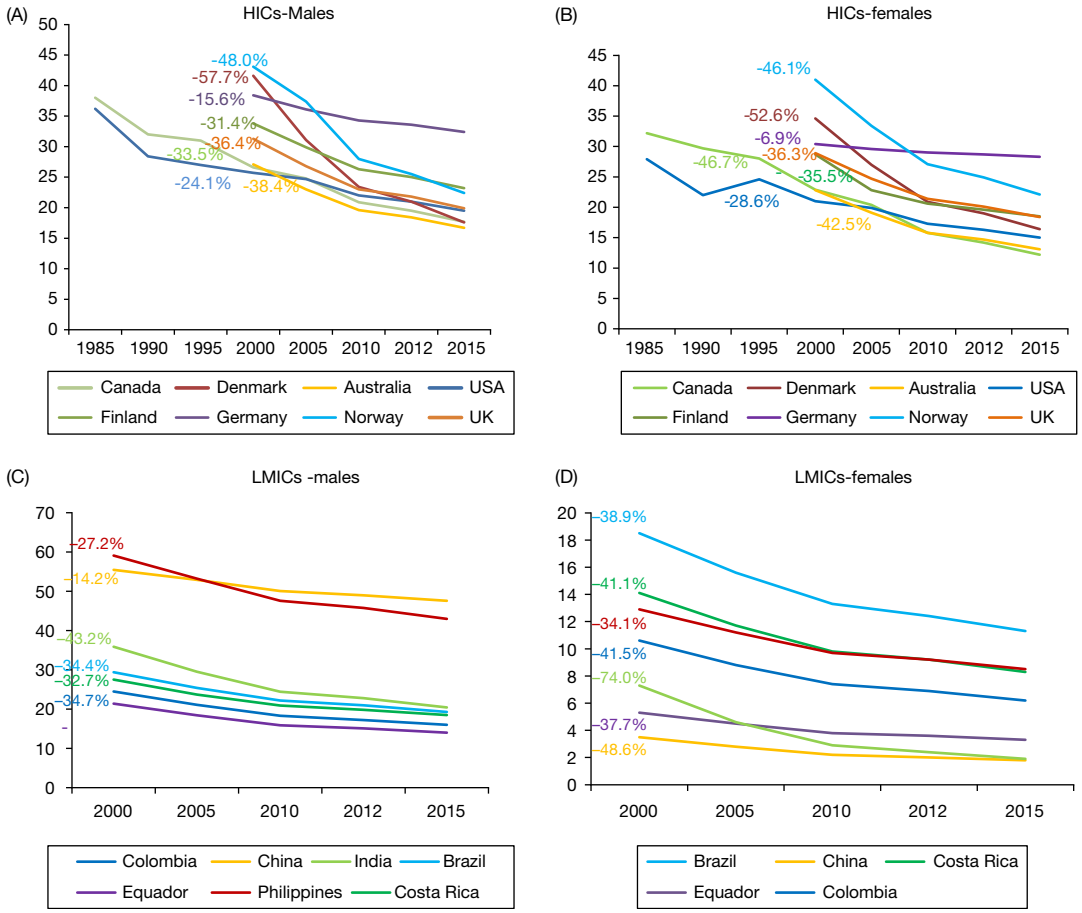

Fig. 6 Transition in tobacco prevalence (% change) (1985–2014) in selected HICs and LMICs by gender.

sites between HICs and LMICs varied markedly, the PAF for esophagus in HICs is 41% and in LMICs is 60%, for liver in HICs is 32%, in LMICs 23% and other pharynx in HICs is 33% and in LMICs is 14% (Fig. 5A).

Transition in alcohol consumption and related cancers

The percentage change in ARCs decreased in both HICs and LMICs among males, with a worrisome increase among females mainly in HICs (% change: 96%) from 1983–87 to 2008–12. ARCs such as esophagus, other pharynx and breast declined in LMICs in both genders, whereas these sites showed an increasing trend among females mainly in HICs [% change from 1983–87 to 2008–12 for esophagus: 96% and other pharynx: 25%]. Increased change (%) for colo-rectal cancers was observed among females in HICs (17%) and among males in LMICs (41%) (Fig. 5B and C).

Almost all the country/region showed significantly declining trend in the pooled ARCs in both HICs among males except Denmark, United Kingdom (England NSW) and Brazil (Goiania). However among females, in all the selected European countries (Denmark, Germany, Norway, United Kingdom, England) and Australia in HICs and Brazil (Goiania), Costa Rica and Uganda (Kyadondo) in LMICs showed significantly increasing trend in the pooled ARCs (Table 7). Significantly, high increasing trend, for liver cancer among males in Canada (BC), Canada (Ontario), United States (SEER, Black and White), Germany (Saarland), Australia, Brazil, Colombia (Cali), Ecuador (Quito), and India (Chennai) was observed. Prevalence of alcohol consumption has increased drastically in LMICs (Fig. 7B). Among females, significantly increasing trend in the ARCs was observed in all the selected European countries (Denmark, Finland, Germany, Norway, United Kingdom, England), Australia, Brazil (Goiania), Costa Rica, and Uganda (Kyadondo) (Table 7). APC related to colo-rectum and breast cancers will be provided in the physical inactivity session.

Overweight and Obesity

PAF due to overweight and obesity and related cancers

Changing dietary patterns are thought to be the contributing to the risk in overweight and obesity. Increasing obesity rates in HICs are confirming the epidemiological transition as the epidemic leads to an increase in the risk of many cancers such as corpus uteri, colo-rectum, breast and esophagus (adenocarcinoma). The picture is more nuanced in LMICs, where there are signs of a protracted

Table 6 Transition (average annual percent change, APC) of tobacco-related cancers (TRC) in selected HICs and LMICs by gender

	TRC			*Lung*			*Oral cavity*			*Other pharynx*			*Larynx*			*Esophagus*			*Bladder*		
	APC	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*	
HICs											*Males*										
Canada, BC	−1.4[a]	−1.7	−1.0	−2.3[a]	−2.5	−2.0	−2.0[a]	−3.8	−0.1	−4.8[a]	−7.1	−2.4	−3.1[a]	−3.7	−2.5	0.1	−0.5	0.8	2.3	−0.3	5.1
Canada, Ontario	−2.3[a]	−2.5	−2.0	−2.4[a]	−2.7	−2.0	−2.5[a]	−3.5	−1.5	−3.8[a]	−6.9	−0.6	−3.0[a]	−3.2	−2.9	0.2	−0.4	0.9	−0.7	−3.6	2.4
USA, SEER: Black	−1.1	−5.8	3.9	−2.5[a]	−2.9	−2.1	−3.5[a]	−4.6	−2.5	−5.0[a]	−6.9	−3.1	−2.5[a]	−4.3	−0.7	−5.2[a]	−5.8	−4.6	−4.0[a]	−6.7	−1.2
USA, SEER: White	−0.3	−4.2	3.9	−2.0[a]	−2.4	−1.6	−1.1[a]	−1.7	−0.5	−4.3[a]	−7.4	−1.2	−2.3	−5.7	1.2	1.2[a]	0.7	1.8	−1.9[a]	−3.7	−0.1
Denmark	−0.8[a]	−1.0	−0.6	−1.2[a]	−1.6	−0.8	−0.2	−0.9	0.5	1.9	−1.4	5.3	−1.0[a]	−1.7	−0.3	2.1[a]	1.0	3.2	−1.4	−3.5	0.7
Finland	−2.4[a]	−2.7	−2.0	−3.1[a]	−3.6	−2.5	−1.1[b]	−2.3	0	−1.1	−3.3	1.1	−2.8[a]	−3.4	−2.1	0.4	−0.2	0.9	0.2	−0.8	1.3
Germany, Saarland	−1.1[a]	−1.9	−0.3	−1.4[a]	−2.1	−0.7	−1.8[a]	−2.0	−1.6	−0.3	−2.1	1.5	−1.8[a]	−2.9	−0.7	−0.6	−2.2	0.9	1.0	−0.1	2.1
Norway	0.1	−0.4	0.6	0.2	−0.1	0.6	−1.2[a]	−2.2	−0.1	−2.9[a]	−4.2	−1.7	−1.4[a]	−2.1	−0.6	1.5[a]	0.8	2.2	2.9[a]	0.9	4.9
UK, England, NW	−1.5[a]	−1.8	−1.2	−2.4[a]	−3.0	−1.9	2.1[a]	1.5	2.7	−0.7	−3.9	2.6	−0.9	−2.1	0.2	1.6[a]	1.3	1.8	−1.4	−4.6	1.8
Australia, NSW	−1.7[a]	−2.1	−1.4	−1.9[a]	−2.2	−1.5	−0.6	−1.7	0.5	−4.5[a]	−6.7	−2.1	−2.6[a]	−3.2	−2.0	0.9[a]	0.5	1.2	−2.4[a]	−3.1	−1.6
LMICs																					
Brazil, Goiania	−7.2	−19.6	7.0	1.2	−1.0	3.4	2.9[a]	2.2	3.5	2.1	−1.0	6.4	1.2	−0.4	2.9	0.5	−1.2	2.3	3.2[a]	1.9	4.5
Colombia, Cali	−1.7[a]	−2.8	−0.6	−2.1[a]	−3.4	−0.7	−0.8	−3.2	1.7	−5.2	−13.4	3.8	−0.8	−2.2	0.6	−2.8[a]	−5.3	−0.2	0.5	−1.9	2.9
Costa Rica	−2.0[a]	−3.0	−1.0	−1.8[a]	−3.2	−0.3	−3.2	−7.2	0.9	−6.0[a]	−9.2	−2.6	−1.5[a]	−2.6	−0.5	−3.4[a]	−5.0	−1.7	0.2	−1.5	2.0
Ecuador, Quito	−0.6	−1.4	0.2	−0.5	−1.6	0.6	−0.8	−3.7	2.3	0	−0.4	0.4	−0.5[a]	−2.9	−0.7	−3.4[a]	−4.9	−1.8	1.0	−0.1	2.1
China, Hong Kong	−2.9[a]	−3.3	−2.5	−2.3[a]	−2.8	−1.8	−1.4[a]	−2.2	−0.5	−3.1[a]	−4.8	−1.4	−4.6[a]	−5.0	−4.1	−4.5[a]	−4.8	−4.1	−3.8[a]	−4.9	−2.6
China, Shanghai	−1.4[a]	−2.6	−0.2	−1.4[a]	−2.6	−0.1	−0.3	−2.2	1.7	0.9	−3.7	5.6	−1.1	−2.6	0.5	−3.8[a]	−5.3	−2.3	−0.3	−2.6	2.0
India, Chennai	0.1	−1.2	1.4	1.1	−0.6	2.8	0.3	−0.9	1.6	−1.1	−3.1	0.8	0	−0.6	0.6	−1.1	−3.3	1.2	0.2	−3.8	4.3
Philippines, Manila	−1.6[a]	−3.0	−0.1	−1.5[a]	−2.9	−0.1	−4.0[a]	−6.0	−1.9	−4.4[a]	−7.0	−1.7	−1.3	−3.9	1.4	−1.2[b]	−2.3	0	−2.1	−6.1	2.1
Thailand, Chiang Mai	−1.4[a]	−2.6	−0.1	−0.3	−1.2	0.6	−3.5[a]	−5.8	−1.1	−7.7[a]	−10.0	−5.3	−4.9[a]	−6.5	−3.3	−3.0	−5.9	0.1	−0.3	−0.9	0.3
Uganda, Kyadondo	0.6	−0.8	2.1	−1.4	−4.3	1.7	4.3[a]	1.9	6.7	1.0	−6.6	9.2	5.5[a]	3.4	7.7	0.7	−1.7	3.1	0.2	−1.4	1.9
HICs											*Females*										
Canada, BC	0	−0.4	0.4	0.3	−0.4	1.1	−1.6[b]	−3.2	0	−5.6[a]	−8.7	−2.4	−2.3	−5.1	0.6	−0.1	−0.9	0.7	1.9	−1.5	5.4
Canada, Ontario	0.2	−0.4	0.9	0.7	−0.1	1.4	−1.6[a]	−2.6	−0.5	−5.7[a]	−10.3	−0.8	−2.9[a]	−3.9	−1.9	−0.8[b]	−1.6	0	−2.1[a]	−3.0	−1.2
USA, SEER: Black	1.0	−3.9	6.3	0.2	−0.8	1.1	−2.6[a]	−3.0	−2.1	−4.7[a]	−7.6	−1.7	−2.6[a]	−4.0	−1.2	−3.8[a]	−5.0	−2.7	−0.4	−1.1	0.2
USA, SEER: White	1.0	−3.0	5.3	1.9[a]	1.3	2.9	−1.4[a]	−2.3	−0.5	−5.6[a]	−9.1	−2.0	−2.4[b]	−3.1	−1.7	−0.5[a]	−0.9	−0.1	−0.2	−0.7	0.2
Denmark	1.5[a]	1.0	1.9	1.9[a]	1.5	2.4	1.4	0.6	−2.2	1.6	−1.8	5.2	−1.0[a]	−1.4	−0.7	2.1[a]	1.5	2.8	−0.3	−1.8	1.2
Finland	0.5[a]	0.1	1.0	1.9[a]	1.6	2.2	2.1	−0.7	5.0	−1.2	−7.2	5.3	−1.8[a]	−3.5	−0.1	−2.8[a]	−4.3	−1.3	0.2	−1.3	1.7
Germany, Saarland	3.3[a]	2.3	4.3	4.7[a]	3.3	6.0	2.6[a]	1.0	4.4	2.2	−0.1	4.5	0.9	−0.8	2.6	2.0	−1.1	5.2	0.9	−2.7	4.7
Norway	2.8[a]	2.2	3.4	4.0[a]	3.1	4.9	−0.4	−1.4	0.6	−1.7	−5.5	2.2	1.9	−1.6	5.4	1.7[a]	0.7	2.7	1.0	−0.4	2.5
UK, England, NW	0.7[a]	0.4	1.0	1.0[a]	0.5	1.4	3.6[a]	2.8	4.4	−2.7[a]	−5.3	−0.1	−0.2	−1.0	0.6	0.4	−0.3	1.1	−1.1	−4.1	2.0
Australia, NSW	0.4[b]	0	0.9	1.8[a]	1.4	2.2	−1.4	−4.0	1.3	−6.0[a]	−8.6	−3.3	−2.0[a]	−3.1	−0.9	−1.1[a]	−1.8	−0.4	−2.8	−3.6	5.2
LMICs																					
Brazil, Goiania	−9.5	−24.4	8.3	1.7	−1.1	4.6	2.1	−0.1	4.4	0.5	−4.3	5.4	−0.2	−2.9	2.6	0.5	−1.7	2.6	1.9	−1.3	5.2
Colombia, Cali	−1.4	−2.0	0.7	−0.7[a]	−1.2	−0.2	−1.6	−3.8	0.6	−7.2[a]	−12.6	−1.6	−2.1	−4.9	0.8	−2.2	−4.8	0.5	−2.1	−3.6	0.7
Costa Rica	−1.3[a]	−2.3	−0.3	−0.6	−1.5	0.4	−2.4[b]	−4.8	0	−7.1[a]	−13.3	−0.4	−1.8	−7.9	4.7	−3.8[a]	−6.9	−0.6	−0.3	−1.4	3.9
Ecuador, Quito	1.3	−0.3	3.0	2.1[a]	0.4	3.4	0.9	−5.1	7.2	−0.7	−9.4	9.0	0.4	−3.7	4.7	0.5	−3.2	4.3	1.2	−1.4	3.9
China, Hong Kong	−2.4[a]	−2.8	−1.9	−1.9[a]	−2.4	−1.5	−0.3	−2.2	1.6	−6.9[a]	−10.6	−3.0	−7.5[a]	−9.5	−5.5	−4.9[a]	−6.2	−3.6	−5.5	−7.4	1.6
China, Shanghai	−0.7[a]	−1.1	−0.3	0.2	−0.3	0.7	−0.7	−1.6	0.2	−2.5	−3.2	−1.8	−5.8[a]	−7.7	−4.0	−5.9[a]	−7.1	−4.7	0.8	−0.1	3.5
India, Chennai	−1.0	−2.2	0.1	3.9[a]	2.1	5.8	−2.1[a]	−3.6	−0.6	−1.1	−2.8	0.6	−0.3	−2.3	1.8	−2.1[a]	−3.3	−0.9	0.4	−2.5	0.6
Philippines, Manila	−2.3[a]	−3.0	−1.6	−1.0[a]	−1.8	−0.3	−6.8[a]	−8.7	−4.9	0	−0.2	0.3	−3.9[a]	−6.7	−1.1	−5.0[a]	−6.7	−3.2	−0.5	−1.6	0.6
Thailand, Chiang Mai	−1.9[a]	−2.7	−1.1	−1.0[a]	−1.8	−0.3	−4.9[a]	−7.0	−2.7	0	−0.3	0.5	−7.9[a]	−9.9	−5.2	−7.0[a]	−9.9	−4.1	−1.6[a]	−2.7	−0.4
Uganda, Kyadondo	1.3	−0.3	2.9	1.1[a]	0.4	1.9	−0.8	−3.1	1.5	0	−4.3	0.4	−7.8[a]	−15	−0.1	1.1	−1.0	3.2	1.1	−0.6	20.6

[a]Statistically significant at 5% level.

[b]Borderline significance.

Table 7 Transition (average annual percent change, APC) of alcohol-related cancers (ARCs) in selected HICs and LMICs by gender

	ARC, males			*Liver, males*			*Colo-rectum, males*			*ARC, females*			*Liver, females*			*Colo-rectum, females*			*Female breast*		
	APC	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*	
HICs																					
Canada, BC	−0.3	−0.8	0.3	3.1[a]	2.1	4.1	−0.3	−0.8	0.2	−0.2	−0.7	0.2	1.8[a]	1.0	2.7	−0.6	−1.4	0.3	−0.1	−0.8	0.6
Canada, Ontario	−0.6[a]	−0.8	−0.4	3.5[a]	2.7	4.4	−0.6[a]	−1.0	−0.2	0	−0.3	0.4	3.1[a]	1.5	4.7	−0.8[a]	−1.2	−0.3	0.3	−0.3	0.9
USA, SEER: Black	−0.6	−1.2	0.1	4.4[a]	3.6	5.3	−0.6[b]	−1.0	0	0.1	−0.1	0.3	2.7[a]	1.5	4.0	−0.7[b]	−1.4	0	0.5[a]	0.3	0.8
USA, SEER: White	−1.4[a]	−1.8	−1.1	4.6[a]	4.4	4.8	−1.8[a]	−2.3	−1.2	−0.2	−0.6	0.1	2.8[a]	2.0	3.6	−1.3[a]	−1.8	−0.8	0.1	−0.4	0.5
Denmark	0	−0.4	0.5	0.9	−0.3	2	0.6[a]	0.3	0.9	1.1[a]	0.7	1.5	−0.6	−1.7	0.4	0.4	−0.1	0.8	1.4[a]	0.9	1.9
Finland	0.1	−0.5	0.3	1.6	−0.7	3.9	1.0[a]	0.7	1.2	1.6[a]	1.0	2.2	0.1	−2.4	2.8	0.5[a]	0.2	0.9	2.0[a]	1.2	2.2
Germany, Saarland	−0.3	−1.2	0.6	3.4[a]	2.2	4.5	0.4	−1.1	2.0	1.1[a]	0.4	1.9	2.6[a]	1.3	3.9	−0.4	−1.9	1.0	1.7[a]	1.1	2.2
Norway	−0.1	−0.4	0.2	1.3	−0.6	3.2	0.9[a]	0.8	1.0	1.1[a]	0.5	1.8	−4.9	−14.0	5.2	1.0[a]	0.6	1.3	1.6[a]	0.9	2.3
UK, England, NW	0.1	−0.2	0.3	0.1	−0.2	0.3	0.7[a]	0.4	1.1	1.2[a]	0.8	1.6	4.1[a]	2.8	5.4	0.2	−0.3	0.7	1.5[a]	0.8	2.2
Australia, NSW	−0.2	−0.5	0.1	5.6[a]	4.6	6.5	0.1	−0.4	0.6	1.0[a]	0.4	1.7	6.2[a]	4.5	7.9	0.2	−0.1	0.5	1.4[a]	0.5	2.4
LMICs																					
Brazil, Goiania	0.9	−1.5	3.3	5.3[a]	1.2	9.6	4.5[a]	1.6	7.4	2.6[a]	1.6	3.6	0	−5.1	5.4	3.4[a]	2.2	4.7	2.5[a]	1.6	3.5
Colombia, Cali	−1.4[a]	−2.0	−0.8	3.1[a]	2.1	4.1	2.8[a]	1.4	4.3	1.4[a]	0.5	2.3	2.7[a]	1.5	3.9	2.3[a]	1.2	3.5	1.3[a]	0.1	2.4
Costa Rica	−1.8[a]	−2.7	−0.8	0.6	−1.8	2.9	2.1[a]	1.5	2.7	1.7[a]	1.4	1.9	0.9	−0.8	2.5	1.7[a]	1.2	2.1	2.0[a]	1.5	2.5
Ecuador, Quito	−0.2	−1.0	0.7	2.6[a]	0.9	4.2	1.9[b]	0	3.8	1.6[a]	0.7	2.5	1.3	−1.3	3.9	1.5[a]	0.2	2.7	1.8[a]	0.8	2.8
China, Hong Kong	−0.2[a]	−0.4	−0.1	−2.0[a]	−2.4	−1.5	0.6[a]	0.2	1.0	0.6[a]	0.5	0.8	−1.8[a]	−2.9	−0.8	0	−0.6	0.5	1.9[a]	1.5	2.4
China, Chennai	0.2	−0.6	0.9	2.2[a]	0.6	3.9	2.9[a]	1.3	4.4	1.4[a]	0.8	2.0	2.9	−0.7	6.6	2.2[a]	1.1	3.2	2.2[a]	1.5	2.9
Philippines, Manila	0.2	−0.6	0.9	−1.2[a]	−2.2	−0.3	1.5[a]	0.4	2.6	0.7	−0.3	1.7	4.7	−4.7	15.1	0.9	−0.5	2.4	0.6	−0.2	1.5
Thailand, Chiang Mai	−0.9	−1.8	0.1	2.1	−0.3	4.6	2.5	−0.2	5.3	2.7[a]	1.9	3.5	7	−2.6	17.5	2.2[a]	0.5	3.9	3.4[a]	2.5	4.3
Uganda, Kyadondo	1.8[a]	0.5	3.0	0.9	−1.7	3.5	0.4[a]	0.1	0.7	2.0[a]	0.8	3.3	7[a]	0.5	13.9	2.4[a]	0.3	4.5	1.9[a]	0.3	3.5

[a]Statistically significant at 5% level.
[b]Borderline significance.

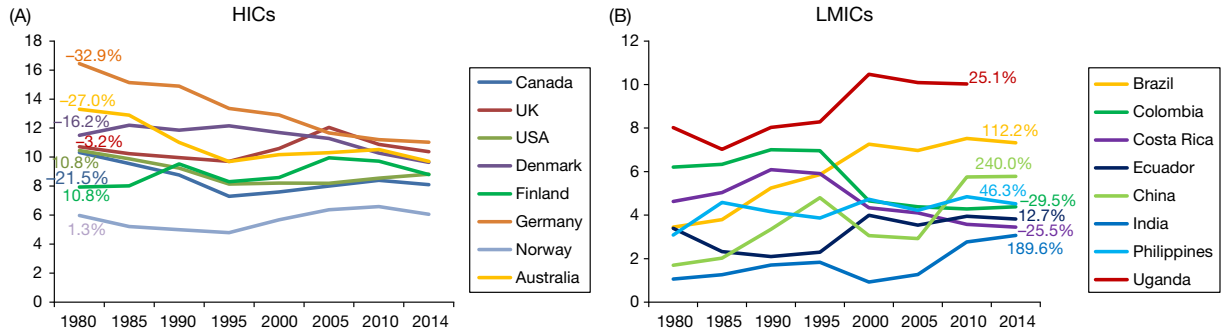

Fig. 7 Transition in alcohol prevalence (% change) (1980-2014) in selected HICs and LMICs.

transition in the burden of cancer due to increased rates in over weight and obesity. The estimated PAF of related cancers (corpus uteri, colo-rectum and breast) due to overweight and obesity in HICs and LMICs is minimal. The PAF for corpus uteri cancer due to overweight and obesity is 43% in HICs and the same in LMICs is 37% (Fig. 5A).

Transition in the prevalence of overweight and obesity and related cancers

Increased % change in overweight and obesity-related cancers (OORCs) (colo-rectum, breast and corpus uteri) was observed in HICs in both genders (Fig 5A and B). As esophagus (adenocarcinoma), could not be separated, not included for the pooled analysis. APC for OORCs due to overweight and obesity were estimated for the selected HICs and LMICs (Table 8). Most of the European countries showed significantly increasing trend in the pooled OORCs in both gender [Denmark, Finland, Germany (Saarland), Norway, United Kingdom (England NSW), and Australia]. All the selected LMICs showed increasing trend in both gender (except Philippines (Manila) females and Thailand (Chiang Mai) males). The APCs were >2% in most of the LMICs [Brazil (Goiania), Colombia (Cali), Costa Rica and Ecuador (Quito), China (Shanghai), and India (Chennai)]. Increase in the OORCs is due to the increase in the prevalence in overweight and obesity during the past two to three decades in many countries (Fig. 8). As SCC and AC esophagus could not be separated, excluded in the estimation of pooled APC due to OORC.

Significantly increasing trend for corpus-uteri cancer in many selected HICs and LMICs was observed [Canada (BC and Ontario), United States (SEER, Black and Whites), Germany (Saarland), Australia, Brazil (Goiania), Colombia (Cali), Ecuador (Quito), and India (Chennai)] (Table 8).

Physical Inactivity

PAF due to physical inactivity and related cancers

Physical activity is a key determinant of energy expenditure and thus fundamental to energy balance and weight control. Rapid technological advancements has occurred hand in hand with widespread urbanization, the rise of more service-based economies, significant changes to transportation system and new opportunities for sedentary leisure pursuits (e.g., television, mobile phone, and other electronic media). Physical inactivity is strongly linked to colo-rectal and breast cancers. The estimated PAF of related cancers due to physical inactivity in both HICs and LMICs is 2%. The PAF for colo-rectum due to physical inactivity is 14% in HICs and 15% in LMICs, the same due to breast is 9% in HICs and 10% in LMICs (Fig. 5A).

Transition in the prevalence of physical inactivity and related cancers

Percentage change in rates between the period 1983–87 and 2008–12 among females in HICs are 11% and among males in LMICs are 41% (Fig. 5B and C). Physical inactivity-related cancers (PIRCs) (colo-rectum and breast) increased in both HICs (except in Canada and United States, males) and LMICs in both genders (Table 8).

Most of the European countries showed significantly increasing trend in the pooled PIRCs in both gender (Denmark, Finland, Norway, United Kingdom (England NSW), and Australia. All the selected LMICs showed increasing trend in females. The significant APCs were in Brazil, Colombia, Costa Rica, Ecuador, China Hong Kong, China (Shanghai), India, Thailand, and Uganda.

Increase in the PIRCs is due to the increase in the prevalence physical inactivity during the past two to three decades in many countries. The prevalence of insufficient physical activity rose according to the level of income. HICs had more than double the prevalence compared to LMICs for both men and women, with 41% of men and 48% of women being insufficiently physically active in HICs as compared to 18% of men and 21% of women in LMICs (Fig. 9).

Nutrition

Nutritional transition is characterized by a shift away from indigenous staple grains, local legumes, fruits and vegetables and limited foods of animal origin in favor of more animal based food products and processed food high in saturated fats and sugar. Low-intake of fruits and vegetable-related cancers include esophagus (SCC), stomach, and lung.

Table 8 Transition (average annual percent change, APC) of obesity, physical inactivity and low intake of fruits and vegetables-related cancers (ARCs) in selected HICs and LMICs by gender

	Obesity						*Physical inactivity*						*Low fruits and vegetables*						*Infection*					
	Males			*Females*			*Males*			*Females*			*Males*			*Females*			*Males*			*Females*		
	APC	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*		*APC*	*95% CI*	
												HICs												
Canada, BC	−0.3	−0.8	0.2	−0.2	−0.7	−0.3	−0.3	−0.8	0.2	−0.3	−0.7	0.2	−2.1[a]	−2.3	−1.9	0	−0.5	0.6	0	−0.4	0.3	−0.3[a]	−0.4	−0.2
Canada, Ontario	−0.6[a]	−1.0	−0.2	0.1	−0.2	0.3	−0.6[a]	−1.0	−0.2	0	−0.4	0.4	−2.1[a]	−2.4	−1.9	0.3	−0.2	0.8	0.1	−0.2	0.3	−1.1	−2.9	0.7
USA, SEER: Black	−0.6[b]	−1.0	0	0.6	−0.2	1.3	−0.6[b]	−1.0	0	0.6	−0.2	1.3	−1.1	−6.0	3.9	1.3	−3.7	6.6	1.3	−0.2	2.9	−0.2	−0.8	0.5
USA, SEER: White	−1.8[a]	−2.3	−1.2	0	−0.7	0.7	−1.8[a]	−2.3	−1.2	0	−0.7	0.7	−0.5	−4.5	3.7	1.2	−2.9	5.6	0.6[a]	0.1	1.0	0	−0.4	0.3
Denmark	0.6[a]	0.3	0.9	0.9[a]	0.5	1.3	0.6[a]	0.3	0.9	1.1[a]	0.7	1.5	−1.0[a]	−1.5	−0.6	1.4[a]	1.1	1.7	0	−1.0	1.1	−1[a]	−1.6	−0.3
Finland	1.0[a]	0.7	1.2	1.6[a]	0.9	2.2	1.0[a]	0.7	1.2	1.7[a]	1.0	2.4	−3.1[a]	−3.7	−2.6	−0.8[b]	−1.6	0	−0.9[a]	−1.6	−0.3	−0.8[a]	−1.1	−0.4
Germany, Saarland	0.4	−1.1	2.0	0.9[a]	0.1	1.6	0.4	−1.1	2.0	1.1[a]	0.3	1.8	−1.6[a]	−2.2	−0.9	1.7[a]	1.1	2.3	−0.2	−0.6	0.1	−0.6[a]	−0.9	−0.4
Norway	0.9[a]	0.8	1.0	1.4[a]	0.9	1.9	0.9[a]	0.8	1.0	1.4[a]	0.9	1.9	−0.5[a]	−0.7	−0.3	2.1[a]	1.8	2.4	−0.8[a]	−1.2	−0.4	−0.8[a]	−1.2	−0.4
UK, England, NW	0.7[a]	0.4	1.1	1.4[a]	1.0	1.8	0.7[a]	0.4	1.1	1.2[a]	0.8	1.6	−2.1[a]	−2.5	−1.7	0.2	−0.2	0.7	−0.5[a]	−0.9	−0.1	−1.2[a]	−1.7	−0.6
Australia, NSW	0.1	−0.4	0.6	1.1[a]	0.5	1.6	0.1	−0.4	0.6	1.0[a]	0.4	1.7	−1.6[a]	−2.0	−1.3	0.9[a]	0.6	1.3	0.7[a]	0.2	1.1	−0.6[a]	−1.0	−0.2
												LMICs												
Brazil, Goiania	4.5[a]	1.6	7.4	2.7[a]	1.6	3.8	4.5[a]	1.6	7.4	2.8[a]	1.8	3.8	0.5	−1.5	2.5	0.4	−1.9	2.7	0.2	−2.2	2.5	−2.7[a]	−5.1	−0.2
Colombia, Cali	2.8[a]	1.4	4.3	1.3[a]	0.4	2.2	2.8[a]	1.4	4.3	1.5[a]	0.5	2.5	−2.1[a]	−3.1	−1.1	−1.8[a]	−2.7	−0.9	−0.7[b]	−1.5	0	−2.3[a]	−3.1	−1.4
Costa Rica	2.1[a]	1.5	2.7	1.9[a]	1.5	2.4	2.1[a]	1.5	2.7	1.9[a]	1.6	2.2	−3.1[a]	−4.4	−1.7	−2.0[a]	−3.0	−0.9	−2.4[a]	−3.6	−1.2	−1.9[a]	−2.6	−1.2
Ecuador, Quito	1.9[b]	0	3.8	1.6[a]	0.7	2.5	1.9[b]	0	3.8	1.7[a]	0.8	2.6	−1.5[a]	−2.5	−0.4	−1.0	−2.3	0.3	−0.2	−1.3	0.8	−1.2[a]	−2.1	−0.2
China, Hong Kong	0.6[a]	0.2	1.0	1.3[a]	1.1	1.5	0.6[a]	0.2	1.0	1.2[a]	1.0	1.3	−2.6[a]	−2.9	−2.3	−2.3[a]	−2.6	−1.9	−2.3[a]	−2.6	−2.1	−2.8[a]	−3.7	−1.9
China, Shanghai	2.0[a]	0.9	3.1	2.4[a]	1.5	3.3	2.0[a]	0.9	3.1	2.3[a]	1.5	3.1	−2.3[a]	−3.5	−1.2	−1.4[a]	−1.9	−1.0	−2.3[a]	−3.2	−1.3	−1.4[a]	−1.8	−1
India, Chennai	2.9[a]	1.3	4.4	2.2[a]	1.6	2.9	2.9[a]	1.3	4.4	2.2[a]	1.5	2.8	−0.5	−1.7	0.7	−0.5	−1.2	0.3	−0.2	−0.8	0.5	−2.8[a]	−3.1	−2.5
Philippines, Manila	1.5[a]	0.4	2.6	0.8	−0.2	1.9	1.5[a]	0.4	2.6	0.7	−0.3	1.7	−1.8[a]	−3.0	−0.6	−1.8[a]	−2.6	−1.1	−1.7[a]	−2.7	−0.7	−1.7[a]	−2.3	−1.1
Thailand, Chiang	2.5	−0.2	5.3	2.9[a]	2.0	3.9	2.5	−0.2	5.3	3.0[a]	1.8	4.2	−0.8	−2.0	0.4	−1.3[a]	−1.9	−0.7	1.1	−0.6	2.9	−0.4	−0.9	0.2
Uganda, Kyadondo	0.4[a]	0.1	0.7	2.2[a]	0.7	3.6	0.4[a]	0.1	0.7	2.0[a]	0.5	3.4	0.3	−0.9	1.4	2.1	−0.4	4.7	1.6[b]	0	3.1	1.6[a]	0.4	2.7

[a]Statistically significant at 5% level.

[b]Borderline significance.

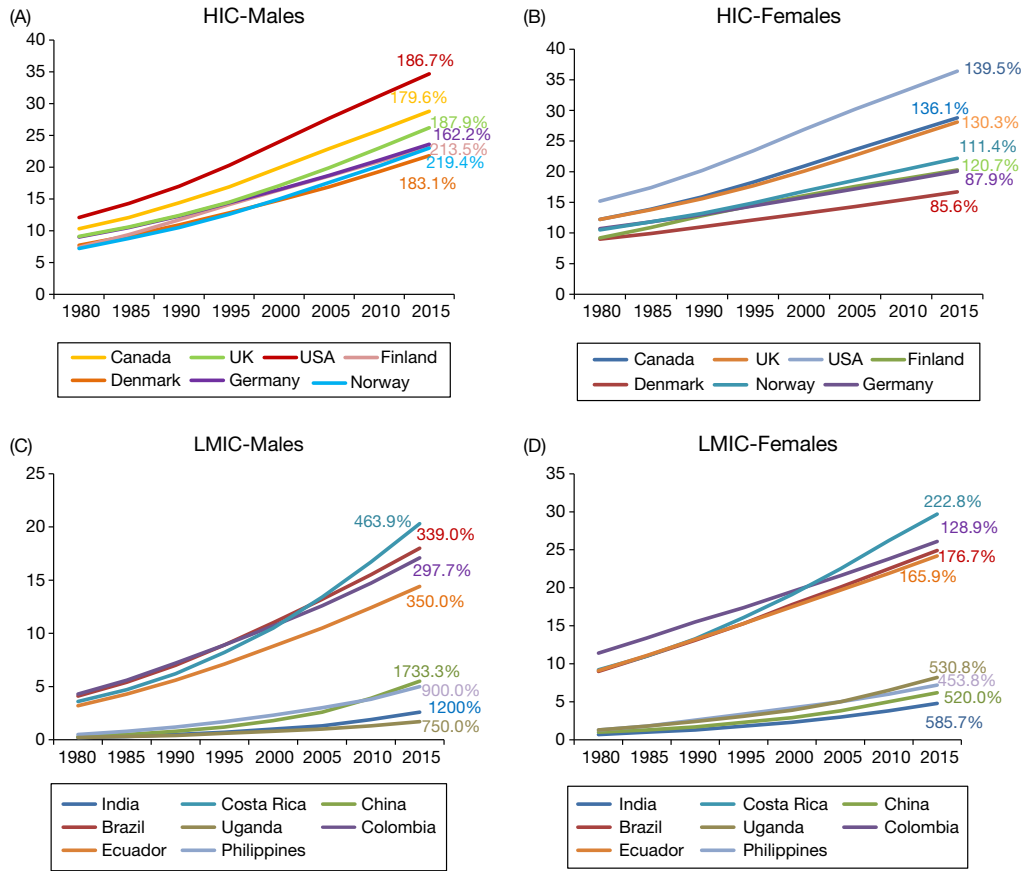

Fig. 8 Transition in overweight and obesity prevalence (% change) (1980–2015) in selected HICs and LMICs by gender.

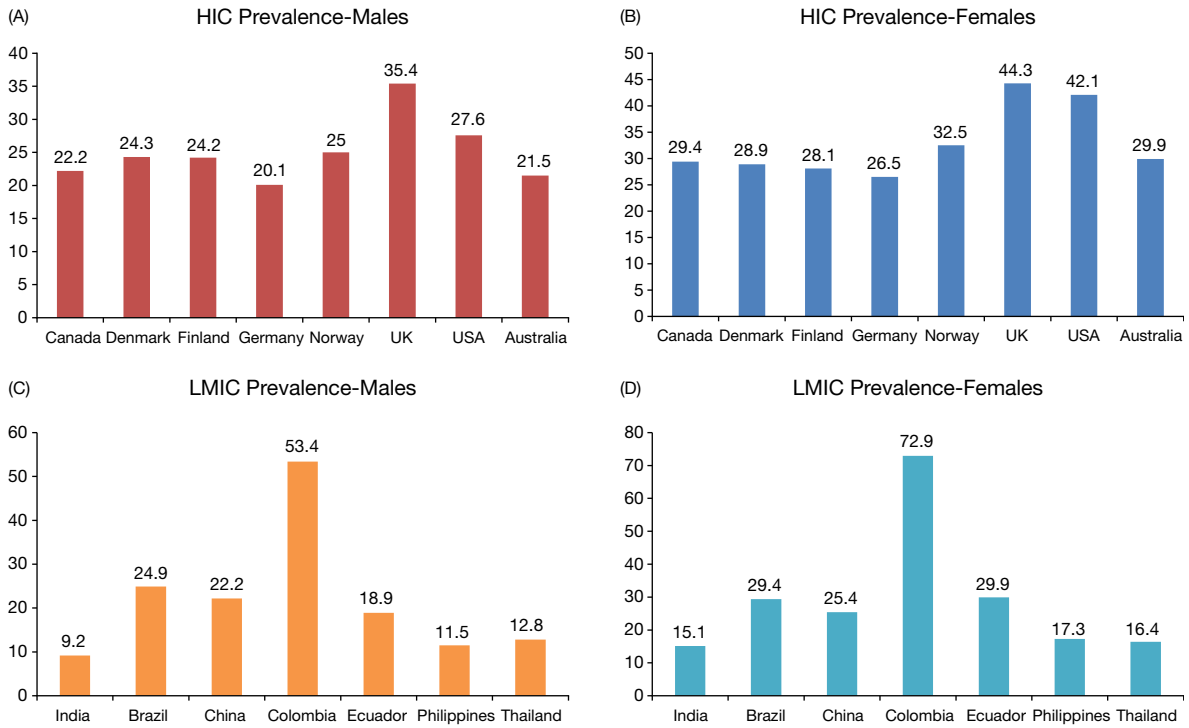

Fig. 9 Prevalence in physical inactivity (2010): selected HICs and LMICs by gender.

PAF due to low intake of fruits and vegetables and related cancers

The estimated PAF for related cancers (esophagus, stomach and lung) due to low intake of fruits and vegetables for both stomach and esophageal cancers are 19% in LMICs and the same in HICs is 12% and the same for lung is 13% in LMICs and 8% in HICs (Fig. 5A).

Transition in the prevalence and related cancers

Low intake of fruits and vegetables-related cancers (FVRCs) declined in both HICs and LMICs in males and among females in LMICs. Percentage changes in rates between the period 1983–87 and 2008–12 are in males −58% in HICs, −28% in LMICs in females, −49% in females (Fig 5B and C). Pooled FVRCs (included stomach, esophagus and lung) declined significantly in most of the HICs and LMICs in both gender (Table 8). Decline in the FVRCs are due to the increased consumption in the prevalence of fruits and vegetables during the past two to three decades in many countries.

Infection

PAF due to Low Intake of Fruits and Vegetables and Related Cancers

Infection-related cancers include mainly cervix-uteri, liver, Hodgkin's disease, nasopharynx, stomach, and Kaposi's sarcoma. The established risk factors for cervix uteri is infection with the human Papilloma virus (HPV), transmitted through sexual intercourse, for liver cancer, chronic liver infection with hepatitis B or hepatitis C virus, for Hodgkin's disease, infection with the Epstein-Barr virus (EBV) or the human immune deficiency virus (HIV), for nasopharyngeal carcinoma, infection with the EBV and for stomach cancer, infection with helicobacter pylori. The estimated PAF due to infection for related cancers (cervix-uteri, liver, Hodgkin's disease, nasopharynx, stomach, and Kaposis sarcoma) is much higher in LMICs (23%) than the HICs (7.4%) (Fig. 5B and C).

Transition in the prevalence of infection and related cancers

Infection-related cancers (IRCs) declined in both HICs and LMICs in males and among females in LMICs. Percentage changes in rates between the period 1983–87 and 2008–12 are −31% in males in HICs, and in LMICs −19% in males and −25% in females (Fig. 5B and C). Pooled IRCs (nasopharynx, stomach, liver, cervix uteri, and Hodgkin's disease) declined significantly in most of the HICs and LMICs in both gender (Table 8). Decline in the IRCs could be due to the decline in the prevalence of various infections in many countries through the vaccination programs and public health effort during the past two to three decades.

In summary, transition in economic status and life-style modification happened in many countries reflected reduction in infection-related cancers, but there is a drastic increase in cancer burden due to epidemiologic transition. Among males in HICs have experienced a decrease in tobacco prevalence and related cancers, but among females in HICs and males in LMICs, such cancers are emerging as its prevalence has not been declined much. In addition to this, the increased prevalence of alcohol consumption, overweight, obesity and physical inactivity in HICs and in many LMICs, the burden of cancer has been increased with higher rate of increase in LMICs.

Increased use of imaging and other sensitive techniques in various fields of medicine, has led to an increased frequency of incidental cancer lesions. The following session presents transition of cancer due to changes in the detection practices.

Transition in the Cancer Detection

Many cancers such as prostate, colo-rectum, breast, cervix-uteri, oral cavity can be detected in early stages. For example, widespread use of prostate-specific antigen (PSA) test, and the rapid increase in the incidence of prostate cancer in early stages; more sensitive techniques to detect small thyroid tumors, before clinical signs are present and the drastic increase in thyroid cancers, high-sensitivity fecal occult blood tests (FOBT) and increase in the incidence of colo-rectal cancers in early stages, Pap-smear test for detection of cervix-uteri cancers in pre-malignant stages. There is wide difference in the cancer detection practices between HICs and LMICs. GLOBOCAN series indicated that mortality rates for all cancers are only 8%–15% higher in more developed countries, even though the incidence rates are more than four times higher in HICs than LMICs. This disparity is primarily due to the differences in the in-equalities in the detection and survival between HICs and LMICs.

There is a reduction in the MIR values for several early detectable cancers in both HICs and LMICs in 2012 compared to 1990, but the values in LMICs are much higher than HICs (Fig. 10), which indirectly indicated that the proportion of late stage at diagnosis is much higher in LMICs.

Conclusion

Burden of cancer is increasing worldwide and over the next few decades, the majority of the cancer burden will occur in LMICs, as these regions/countries continue to experience rapid changes in urban environment, technological advancement, industrialization and material prosperity. Transition in economic status and life-style modification happened in many countries reflect reduction in infection-related cancers, but there is a drastic increase in cancer burden due to epidemiologic transition. HICs among males have experienced a decrease in tobacco prevalence and related cancers, but not much among females in HICs and males in LMICs. The

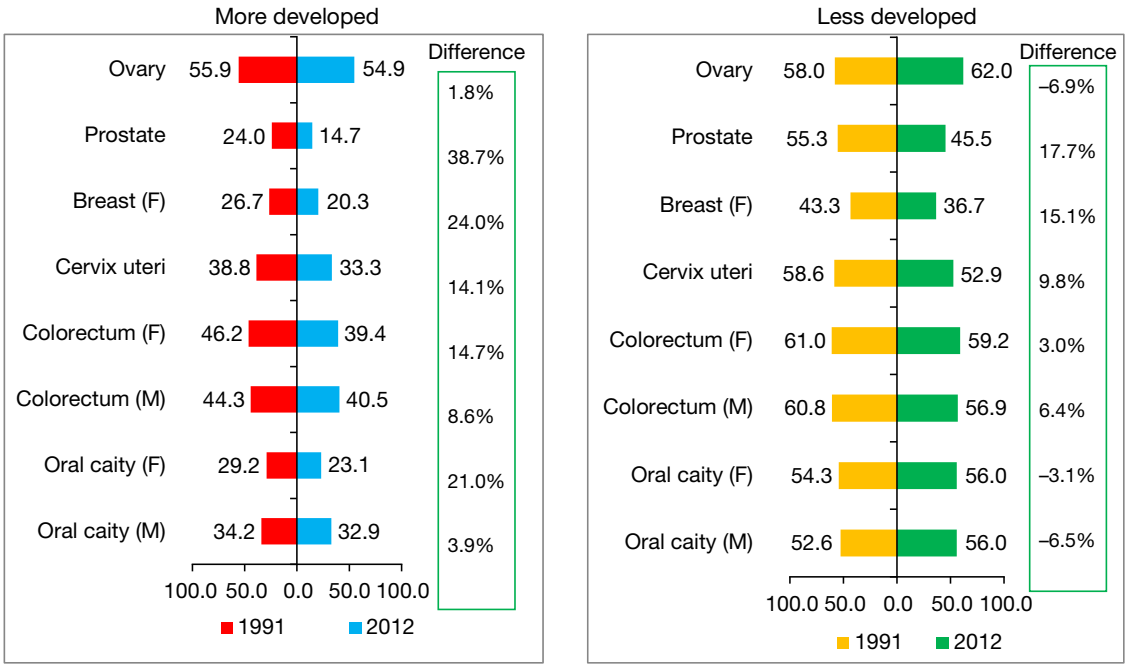

Fig. 10 Transition in mortality-to-incidence ratio (1990 vs. 2012).

future burden of cancers will already expect to be large due to demographic effects. In addition to this, with the tobacco epidemic, increase in alcohol consumption, increased prevalence in overweight, obesity and physical inactivity, the burden will be very heavy with higher rate of increase in LMICs. Continued public health efforts for the increasing prevalence in overweight, obesity, physical inactivity and alcohol consumption in addition to the tobacco prevention and control are needed especially among females to reduce the cancer burden.

Higher MIR values for several cancers in LMICs indicated that the proportion of late stage at diagnosis is much higher in LMICs. For equitable access to cancer treatment for patients in less-developed nations, proper system should be developed to ensure that the patients complete treatment and are on regular follow-up.

A substantial portion of cancer cases and deaths could be prevented by broadly applying effective prevention measures and the use of early detection tests. It is high-time that cancer should be included as a notifiable disease in all countries even in LMICs and the burden in terms of both incidence and mortality needs to be assessed more accurately. Region/country-wise relevant "tool kit" has to be set up for down-staging and effective cancer control program.

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See also: Cancer Disparities. Cancer in Sub-Saharan Africa. Cancer in the Middle East.

References

Baan, R., et al. WHO International Agency for Research on Cancer Monograph Working Group, 2007. Carcinogenicity of alcoholic beverages. Lancet Oncology 8, 292–293.

Bray F, Colombet M, Mery L, Piñeros M, Znaor A, Zanetti R and Ferlay J, Cancer incidence in five continents, Vol. XI (electronic version). Lyon: International Agency for Research on Cancer. http://ci5.iarc.fr, 2017.

Connor, J., 2017. Alcohol consumption as a cause of cancer. Addiction 112 (2), 222–228.

Curado, M.P., Edwards, B., Shin, H.R., Storm, H., Ferlay, J., Heanue, M., 2007. Boyle cancer incidence in five continents, vol. IX. IARC Scientific Publication No. 160.

Danaei, G., Vander Hoorn, S., Lopez, A.D., Murray, C.J., Ezzati, M., 2005. Causes of cancer in the world: Comparative risk assessment of nine behavioural and environmental risk factors. Lancet 366, 1784–1793.

de Martel, C., Ferlay, J., Franceschi, S., Vignat, J., Bray, F., Forman, D., Plummer, M., 2012. Global burden of cancer attributable to infections in 2008: A review and systematic analysis. Lancet Oncology 13 (6), 607–615.

de Martel, C., Maucort-Boulch, D., Plummer, M., 2015. Franceschi worldwide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma. Hepatology 62, 1190–1200.

de Martel, C., Plummer, M., Vignat, J., Franceschi, S., 2017. Worldwide burden of cancer attributable to HPV by site, country and HPV type. International Journal of Cancer 141, 664–670.
Ezzati, M., Lopez, A.D., Rodgers, A., Vander Hoorn, S., Murray, C.J., 2002. Selected major risk factors and global and regional burden of disease. Lancet 360, 1347–1360.
Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11. Lyon, France: International Agency for Research on Cancer, 2013.
Forman D, Bray F, Brewster DH, et al. Cancer incidence in five continents, Vol. X, IARC Scientific Publication No. 164, 2014.
Gilmore, J. Report on smoking prevalence in canada, 1985 to 1999 (Statistics Canada, Catalogue 82F0077XIE), 2000.
Giovino, G.A., 2002. Epidemiology of tobacco use in the United States. Oncogene 21, 7326–7340.
Hamajima, N., et al. Collaborative Group on Hormonal Factors in Breast Cancer, 2002. Alcohol, tobacco and breast cancer—Collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. British Journal of Cancer 87, 1234–1245.
International agency for research on cancer (IARC). IARC monographs on the evaluation of the carcinogenic risks to humans, Supplement. Overall evaluations of carcinogenicity: An updating of IARC monographs, Vols 1–42. Lyon: IARC, 357-61, 1987.
International agency for research on cancer (IARC). IARC handbooks of cancer prevention, body fatness and cancer Vol. 16: Lyon, IARC, 2016.
Oyebode, O., Gordon-Dseagu, G., Walker, A., Mindell, J.S., 2014. Fruit and vegetable consumption and all-cause, cancer and CVD mortality: Analysis of Health Survey for England data. Journal of Epidemiology and Community Health 68, 856–862.
Parkin, D.M., 2011. Cancers attributable to infection in the UK in 2010. British Journal of Cancer 105 (Suppl. 2), S49–S56.
Parkin DM, Muir CS, Whelan SL, Gao Y-T, Ferlay J, Powell J Cancer incidence in five continents, Vol. VI, IARC Scientific Publication No. 1201992.
Parkin, D.M., Pisani, P., Ferlay, J., 1999. Global cancer statistics. CA: A Cancer Journal for Clinicians 49, 33–64.
Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DB, Cancer incidence in five continents, Vol. VIII IARC Scientific Publication No. 155, 2002.
Parkin DM, Whelan, SL, Ferlay J, Raymond L, Young J Cancer incidence in five continents, Vol. VII, IARC Scientific Publication No. 143, 1997.
Plummer, M., de Martel, C., Vignat, J., et al., 2016. Global burden of cancers attributable to infections in 2012: A synthetic analysis. The Lancet Global Health 4, e609–e616.
Plummer, M., Franceschi, S., Vignat, J., Forman, D., de Martel, C., 2015. Global burden of gastric cancer attributable to Helicobacter pylori. International Journal of Cancer 136, 487–490.
Schmid, D., Leitzmann, M.F., 2014. Association between physical activity and mortality among breast cancer and colorectal cancer survivors: a systematic review and meta-analysis. Annals of Oncology 25 (7), 1293–1311.
US Department of Health and Human Services. The health consequences of smoking: A report of the Surgeon General, Atlanta: US Department of Health and Human Services, Centres for Disease Control and Prevention, National Centre for Disease Control and Prevention and Health Promotion, Office on Smoking and Health, 2004.

Further Reading

Parkin, D.M., Boyd, L., Walker, L.C., 2011. The fraction of cancer attributable to lifestyle and environmental factors in the UK in 2010. British Journal of Cancer 105, S77–S81.

Relevant Websites

http://apps.who.int/gho/data/node.main.688 - WHO. Life expectancy and Healthy life expectancy. Data by country
https://data.worldbank.org - World Bank Open Data.

Cancer in Sub-Saharan Africa

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Cancer in Sub-Saharan Africa

Sub-Saharan (SS) Africa consists of all African countries that are fully or partially located south of the Sahara Desert. It includes 48 countries and, in 2016, its population consisted of almost 1 billion people, of which 47% live on less than $1.25 a day. However, the United Nations predicts that, in 2050, it will be between 1.5 and 2 billion people.

Burden of Cancer

In SS Africa, the overall incidence of cancer (all cancers, excluding nonmelanoma skin cancer) is in general much lower than in more developed regions; among the latter, the estimated incidence rates in 2012 were nearly ninefold higher in men and fivefold higher in women. This primarily reflects differences in countries' age distributions, with SS Africa presenting a lower proportion of the population in the older age-groups, among whom most cancers tend to be more frequent. However, even the age-standardized rates, using the world standard population (ASIR-W), were more than twice higher in more developed regions. These differences are depicted in Fig. 1. The overall cancer incidence is also heterogeneous among SS African countries; for example, in South Africa, the incident rates (per 100,000) were 148.7 in men and 156.5 in women, yet they were below 60 in several other countries, such as Angola, Djibouti, or Tanzania.

Limited access to diagnosis contributes to lower incidence rates in many SS African settings due to underdiagnosis. This is illustrated by a report of a steep increase in the incidence of stomach cancer in rural Kenya soon after an endoscope became available, or by the fact that the 2006 report of the population-based registry of Beira, in Mozambique, referred no cases of prostate cancer. Further, overdiagnosis of breast and prostate cancers is substantially lower than in developed countries, since mammography screening and prostate specific antigen (PSA) testing are available to a much lesser extent in SS Africa, and are also unevenly distributed throughout the region. Risk factors such as tobacco smoking, obesity or low parity have remained infrequent in many SS

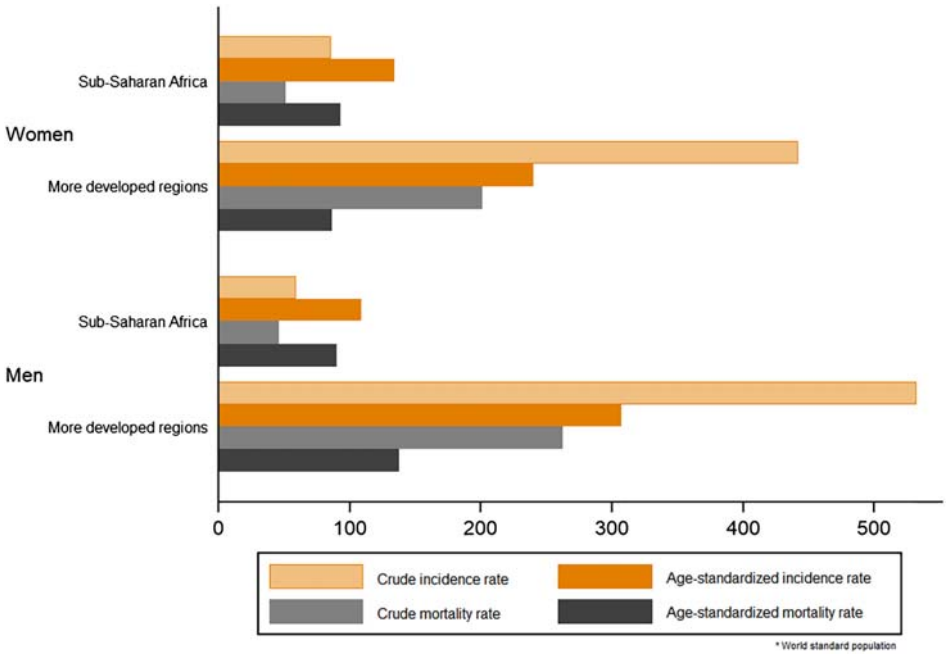

Fig. 1 Incidence and mortality rates in Sub-Saharan Africa and more developed regions. From Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D., Bray, F. (2013). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr, accessed on 30/01/2018.

African regions, contributing to the low incidence of some of the cancers that account for a large proportion of cases in developed countries. In SS Africa and in more developed regions, the ASIR-W (per 100,000) for lung cancer among men were 4.8 and 44.7, respectively, for breast cancer among women were 33.8 and 73.4, respectively, and for colorectal cancer in both sexes were 5.8 and 29.2, respectively. Conversely, infection-related cancers are frequent in SS Africa; for example, for Kaposi sarcoma among men, the ASIR-W (per 100,000) were 7.2 in SS Africa and 0.3 in more developed regions, the corresponding figures for cervical cancer were 34.8 and 9.9, and for liver cancer, both sexes, were 7.6 and 5.4.

Taken together, these factors contribute to explain the lower cancer rates in SS Africa, but also the distinct patterns of occurrence of different cancers compared with other regions. In SS Africa, it was estimated that, in 2012, the most frequent were prostate and liver cancers and Kaposi sarcoma among men, and breast, cervical and liver cancers among women. In more developed regions, the leading cancers were prostate, lung and colorectal cancers among men, and breast, colorectal and lung cancers among women. In SS African countries, in men, prostate and liver cancers and Kaposi sarcoma were the most frequent in 23, 12, and 6 countries, respectively. Among women, cervical (28 countries) and breast (20 countries) cancers were the most frequent (**Fig. 2**).

In SS Africa, low priority is usually given to cancer prevention and control, because other conditions may constitute more immediate threats to public health. However, the absolute number of cases of cancer diagnosed and requiring treatment is currently high in SS Africa—over 600,000 cases were estimated in 2012—and in many settings, the health systems are unable to promote early diagnosis or to provide appropriate care to these patients, as will be discussed in more detail along this article. This leads to cancer patients' low survival, as shown in some of the few population-based estimates available from SS African regions (**Table 1**). Compared to the incidence gap, this results in much smaller differences in mortality estimates between SS African countries and more developed regions. Among men, the age-standardized mortality rates, world standard population (ASMR-W), were higher in more developed regions by only 50%, whereas among women the ASMR-W were actually nearly 10% higher in SS Africa. For cervical cancer, the estimated ASMR-W was nearly sevenfold higher in SS Africa, whereas for the ASIR-W the ratio was 3.5.

Disability adjusted life years (DALYs) are computed by combining the number of years of life lost (YLLs) due to premature mortality in the population and the years lost due to disability (YLDs) in people living with cancer, and may be interpreted as the years of "healthy" life lost. Therefore, the low survival of cancer patients results in a small contribution of YLDs to this more comprehensive indicator of the burden of cancer. For 2016, the Institute of Health Metrics estimated an overall number of nearly 16.5 million DALYs in SS Africa, from which just over 200,000 were from YLDs (1.2%). However, for high-income countries, the YLDs accounted for 4.4% of the DALYs; this gap is wider when considering cancers with higher potential survival, such as female breast (2.0% vs. 8.8%) or prostate (2.8% vs. 14.8%) cancers.

Mortality trends in more developed countries have been characterized by declining age-standardized rates, reflecting improvements in living conditions, specific preventive measures to decrease the exposure to risk factors, vaccination, earlier detection of cancer and improved access to effective treatments. This also shows the potential and points to strategies for controlling cancer in SS Africa. Yet, this will be challenging due to regional specificities of the epidemiology of cancer and its risk factors, the shortage of financial and human resources to tackle cancer, competing priorities in already overburdened health systems, and the need to tailor prevention and control measures to the SS African setting. Despite the progress that has been observed in more developed regions, the growth and aging of the population have contributed to an increasing absolute number of cancer deaths in most countries, showing that advances in prevention and control remain insufficient to overcome the impact of demographic changes in cancer mortality. This will bring additional difficulties to the prevention and control of cancer in SS Africa, since its population is expected to increase substantially in absolute numbers and average age over the next decades. Taking into account only the expected demographic changes, the GLOBOCAN projections for 2035 show 100% increases in the number of incident cancers and cancer deaths, in relation to 2012.

Monitoring the Burden of Cancer

The currently high frequency of cancer and its projected variation in SS Africa emphasize the importance of monitoring its burden and management in the region. Cancer registries are essential for this purpose, but in SS Africa these dedicated structures are often unavailable, or do not produce quality data. In the context of *Cancer Incidence in Five Continents*, volume X, it was estimated that 10.5% of the population was covered by population-based cancer registries with a completeness of case finding equal to or higher than 70%, and only four registries met the standards to be cited in this report, corresponding to less than 2% of the total population. This also results in scarce population-based survival estimates, as depicted in **Table 1**.

The GLOBOCAN project provides incidence and mortality estimates for most SS African countries, based on the best sources available for each country and sometimes on data from neighboring countries or registries in the same area. However, 19 countries had no incidence data, and only three had high quality regional data, with a coverage lower than 10%. Mortality data was only available in three countries, with complete vital registration classified as low or medium quality.

Although it is not surprising that low priority may be given to demanding cancer registration activities, when specialized human resources are lacking and financial capacity is limited, accurate population-based data are difficult to obtain when there is limited access to cancer health care services by the population and when cancer mortality statistics are not reliable. In these settings, hospital-based registries are important sources of data for population-based registries, and may support the evaluation of cancer management in each hospital, towards an efficient use of the available resources.

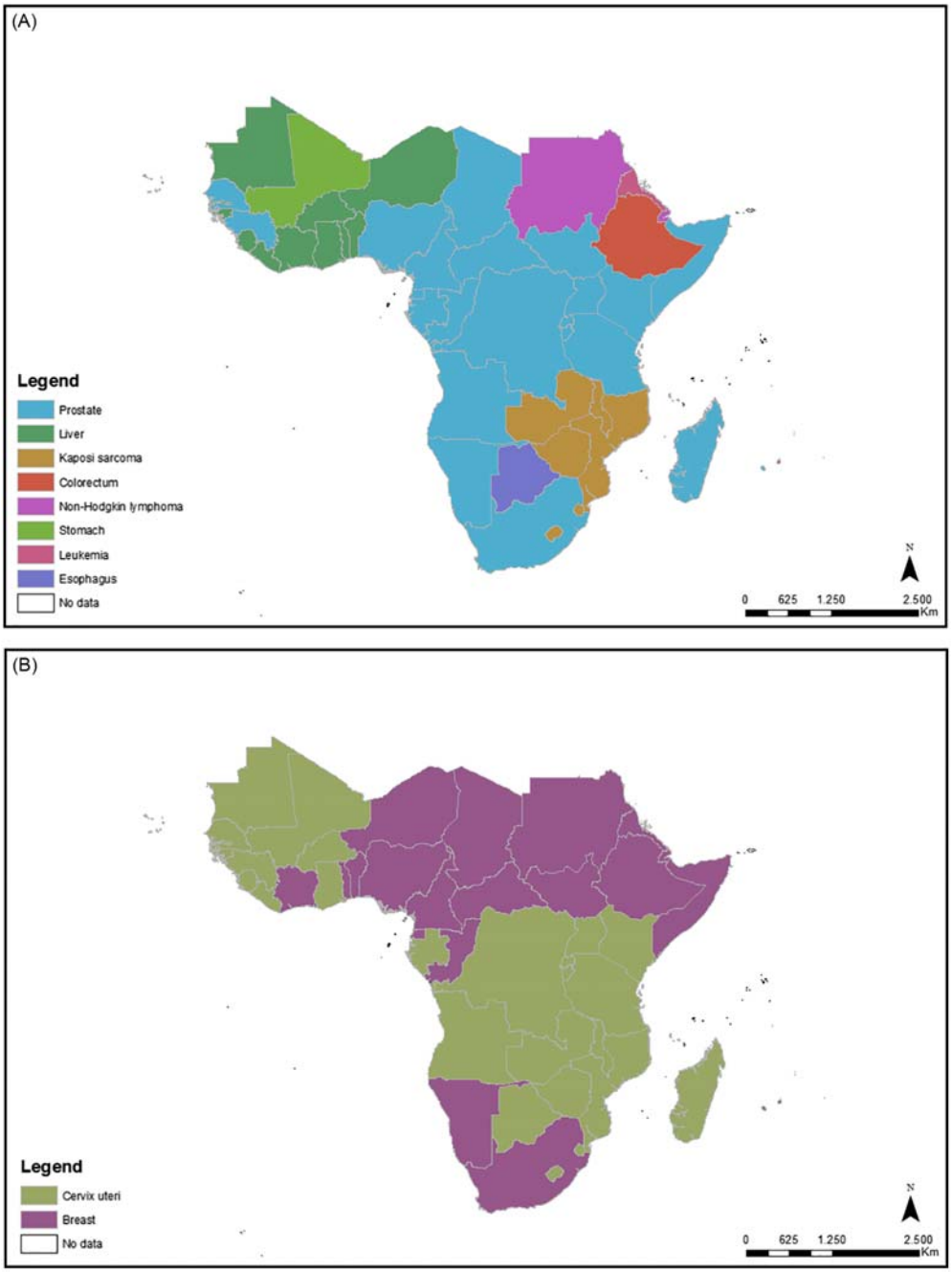

Fig. 2 Cancers with the highest absolute number of cases in Sub-Saharan African countries, among men (A) and women (B). From Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D., Bray, F. (2013). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr, accessed on 30/01/2018.

Cancer Prevention and Screening

The burden of some of the most frequent cancers in SS Africa can be reduced through decreasing the exposure to risk factors, vaccination, or early diagnosis and treatment. In fact, there is robust evidence on the causes of cancer and interventions that have proven effective for both preventing and reducing its morbidity and mortality through screening. Many SS African countries have already started to develop cancer prevention programs, often with foreign help. However, these efforts are challenged by competing problems, such as the human immunodeficiency virus (HIV) and malaria infections, and limited resources. This has a potential impact both on the perception of cancer as a major health problem and on the allocation of resources for its prevention and control. Additionally, adapting to local culture and traditions constitute a great challenge to the implementation of cancer prevention strategies.

Table 1 Age-standardized survival (95% confidence intervals), by country and type of cancer

	Country						
Cancer	*Mali (Bamako)*	*Mauritius*	*Nigeria (Ibadan)*	*South Africa (Eastern Cape)*	*The Gambia*	*Uganda (Kampala)*	*Zimbabwe (Harare)*
Breast	13.6 (0.0–30.1)[a] 0.0 (0.0–0.0)[b]	83.6 (75.9–91.3)[c]	98.8 (95.6–100.0)[c] 97.5 (89.9–100.0)[b]	71.5 (51.4–91.6)[d] 53.0 (23.4–82.7)[e] 32.0 (23.3–40.7)[c] 40.1 (30.7–49.6)[b]	9.5[f] 11.9 (0.0–24.7)[d]	36.1[f]	54.8[f]
Cervix uteri		80.8 (76.0–85.6)[c]	93.6 (83.7–100.0)[a] 58.6 (46.5–70.7)[c] 49.8 (36.5–63.1)[b]	37.0 (24.7–49.4)[d] 70.7 (56.7–84.7)[e] 40.2 (32.2–48.1)[c] 37.1 (31.4–42.9)[b]	22.9[f] 19.5 (11.0–28.0)[d]	18.7[f]	44.1[f]
Colon		65.9 (56.7–75.1)[c] 57.9 (48.5–67.2)[b]	41.2 (16.9–65.6)[c] 17.4 (0.1–34.8)[b]	31.9 (10.7–53.1)[c] 12.3 (4.3–20.2)[b]		6.2[f]	24.9[f]
Eye						35.7[f]	69.9[f]
Kaposi Sarcoma						21.9[f]	4.4[f]
Leukemia (adults)		57.2 (37.4–76.9)[c]	82.7 (59.8–100.0)[g]				
Liver		52.6 (28.9–76.3)[c] 17.0 (2.6–31.3)[b]		10.2 (0.0–22.9)[g] 27.1 (0.0–57.1)[e] 0.0 (0.0–0.0)[b]	2.6[f] 4.5 (0.2–8.8)[d]	1.1[f]	3.0[f]
Lung		31.7 (25.9–37.6)[c] 20.4 (13.2–27.7)[b]		16.7 (0.8–32.5)[c] 15.0 (6.1–24.0)[b]	49.8[f] 30.0 (3.6–56.4)[d]	0.0[f]	16.0[f]
Non-Hodgkin lymphoma					22.5[f]	26.1[f]	31.4[f]
Esophagus		28.1 (14.6–41.5)[b]		12.1 (0.0–27.0)[e] 19.2 (12.0–26.4)[c] 18.0 (12.6–23.4)[b]		4.6[f]	9.2[f]
Ovary		79.7 (69.6–89.8)[c]	59.4 (24.9–93.9)[c] 49.1 (33.8–64.4)[b]	90.9 (67.8–100.0)[g] 82.5 (42.5–100.0)[e] 81.0 (58.8–100.0)[c] 67.8 (47.4–88.2)[b]		10.2[f]	39.1[f]
Pancreas		24.5 (11.4–37.6)[b]		21.8 (0.0–49.5)[b]			
Prostate		61.8 (54.1–69.4)[c] 63.5 (54.7–72.4)[b]	97.4 (89.5–100.0)[a] 73.9 (50.2–97.6)[c] 58.7 (40.1–77.2)[b]	85.3 (60.0–100.0)[a] 77.6 (55.0–100.0)[g] 38.6 (25.1–52.0)[c] 37.8 (25.5–50.1)[b]		45.6[f]	
Rectum		83.6 (75.0–92.1)[c] 72.9 (62.7–83.0)[b]	25.9 (0.0–53.7)[c] 16.9 (0.0–37.8)[b]	19.9 (0.0–46.4)[c] 9.1 (0.0–21.5)[b]		7.2[f]	66.3[f]
Stomach		44.3 (36.8–51.7)[c] 25.7 (18.0–33.3)[b]		25.0 (6.1–44.0)[c] 25.6 (7.2–43.9)[b]	4.0[f] 0.3 (0.0–1.0)[d]	0.0[f]	18.6[f]

From Allemani, C., Weir, H. K., Carreira, H., Harewood, R., Spika, D., Wang, X. S., et al. (2015). Global surveillance of cancer survival 1995–2009: Analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet* **385**(9972), 977–1010. Allemani, C., Matsuda, T., Di Carlo, V., Harewood, R., Matz, M., Nikšić, M., et al. (2018). Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): Analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. *Lancet* **391**(10125), 1023–1075. Sankaranarayanan, R., Swaminathan, R., Lucas, E. (2011). *Cancer survival in Africa, Asia, the Caribbean and Central America (SurvCan)*. IARC Scientific Publications, Vol. 162, ISBN 978-92-832-2162-3. Lyon: International Agency for Research on Cancer.

[a]Age-standardized (International Cancer Survival Standard weights) 5-year net survival for women diagnosed between 1995 and 2004.

[b]Age-standardized (International Cancer Survival Standard weights) 5-year net survival for women diagnosed between 2010 and 2014.

[c]Age-standardized (International Cancer Survival Standard weights) 5-year net survival for women diagnosed between 2005 and 2009.

[d]Age-standardized (International Cancer Survival Standard weights) 5-year net survival for women diagnosed between 1995 and 1999.

[e]Age-standardized (International Cancer Survival Standard weights) 5-year net survival for women diagnosed between 2000 and 2004.

[f]Age-standardized (Cancer standard population) 5-year relative survival for women diagnosed between 1993 and 1997.

[g]Age-standardized (International Cancer Survival Standard weights) 5-year net survival for women diagnosed between 1995 and 2009.

Reducing Exposure to Risk Factors

Tobacco consumption increases the risk of developing different types of cancer, such as lung, head and neck or esophageal cancers, among others. Many of the tobacco-related cancers are not among the most frequent in SS Africa, but there is a large potential for an increase in the prevalence of smoking and in the frequency of these cancers. Tobacco consumption is currently one of the most targeted risk factors throughout Africa. National level legislation is already in place in some countries, such as Gambia, Uganda, Nigeria or Kenya. They comprise prohibition of smoking in public or working places, banning of tobacco advertising, restriction of sales to minors, among others. The World Health Organization (WHO) also established a Centre for Tobacco Control in Africa in 2011 that is currently focusing on policies, legislation and regulations in several countries, namely Botswana, Ethiopia, Gambia, Gabon, and Niger. Moreover, private nongovernmental organizations (NGOs) developed antitobacco campaigns targeting different communities. However, tobacco control can prove difficult in Africa due to its role as an income provider for some communities, where tobacco leaf farming is still promoted as a way to reduce poverty, leading to increased consumption. Traditional tobacco products, other than manufactured cigarettes, are also frequently consumed in some settings, and may require more specific interventions for its control.

The amount of alcohol consumed in SS Africa is the second highest in the world (19.5 L of pure alcohol per year) and this is expected to increase. Alcohol is one of the leading causes of death in SS Africa, and is also related to the development of different cancers, namely esophageal, and head and neck cancers. Alcohol consumption depends on social influence and cultural changes that are closely linked to globalization, such as the growth of urban areas, industrialization, and marketing. Community norms and beliefs towards alcohol, neighborhood characteristics and peer effect are also determinants of drinking patterns, mostly in adolescents and young adults. The lack of specific governmental policies aiming to reduce alcohol consumption, the absence of community education and the increasing exposure to marketing of alcoholic beverages may contribute to increased consumption. However, countries such as Malawi, Equatorial Guinea or Zambia have already invested in political and regulatory changes with favorable outcomes in reducing consumption levels. Policy changes encompass, among others, raising taxes on alcohol, controlling advertising and enforcing drink-driving laws.

Exposure to mycotoxins through contaminated food is an established risk factor for liver (aflatoxins) or esophageal cancer (fumonisins), which are among the most frequent in SS Africa. Environmental conditions such as high levels of humidity and temperature favor the development of fungus responsible for the production of these toxins. Moreover, the disruption of the food preservation chain and constrained economic conditions increase the consumption of such products. Harvest practices, transportation and product processing are already regulated, in some countries, to help reduce contamination and exposure. However, a high portion of the SS African population is believed to still be exposed to contaminated food.

Cancers caused by infectious agents, such as cervical cancer (human papillomavirus, HPV), liver cancer (hepatitis B virus, HBV/hepatitis C virus, HCV), or Kaposi Sarcoma (herpesvirus 8, HHV8), are among the most common in Africa. HIV coinfection further increases the risk of developing these and other cancers by impairing immune response. Thus, reducing the risk of infection with these agents is an important step for cancer prevention. Strategies based on healthcare regulation and education of healthcare personnel are currently the focus of the WHO and various NGOs across multiple SS African countries. These include changing high-risk behaviors, by promoting measures such as the use of barriers to prevent exposure to body fluids, hands hygiene, prevention of injuries, screening of blood products and sterilization of medical equipment. Furthermore, community education plays a major role in preventing infections, mostly HIV and HPV, by reducing high-risk sexual behaviors. Some community educational programs encourage abstinence, monogamy and condom use. Circumcision may reduce high-risk HPV infection and transmission, and the WHO has proposed a target rate of 80% circumcision in men in low-income countries as the fallout of different randomized controlled trials. Sociocultural and healthcare barriers are being addressed in order to achieve high circumcision rates and several programs are currently in place to achieve this goal. In fact, the Joint United Nations Programme on HIV/AIDS (UNAIDS) defined 14 priority countries to scale-up circumcision. However, in some of those countries such as Lesotho, Malawi, Namibia, Rwanda, and Zimbabwe, the prevalence of circumcised men remains below 35%. Finally, in HIV infected patients, the use of highly active antiretroviral therapy has been shown to reduce the incidence of cancer. While this is true for some types of cancer such as Kaposi sarcoma, there is still insufficient evidence to establish a clear association between HIV treatment and the reduction of other cancers, like cervical tumors.

In SS Africa, *Schistosoma haematobium* is an important risk factor for bladder cancer. It ranks as the second most common parasitic infection in the region and 11.7 million people were treated for schistosomiasis in 2008. It is considered a neglected tropical disease and is estimated to cause over half a million deaths per year in SS Africa. *Schistosoma* can be transmitted by bathing in larvae infested water and the deposition of parasitic ova in the bladder induces severe inflammation leading to carcinogenesis and the development of squamous cell carcinoma. There are effective drugs for eliminating *Schistosoma* and preventing cancer, such as praziquantel. Therefore, there are several NGOs currently improving treatment coverage in order to reduce the burden of this infection in SS Africa.

Globalization is responsible for several changes in the exposure to risk factors for cancer. Obesity is also now being considered an important risk factor among countries in SS Africa, mostly in urban areas, where the intake of highly caloric food and physical inactivity is increasing. Paradoxically, increasing levels of obesity are side-by-side with severe undernutrition and stunting. Overweight and obesity incidence in adults reaches 40% in some countries, such as Burundi, alongside a 50% prevalence of stunting. Thus, healthcare interventions may also be needed in SS Africa to prevent and control weight gain and obesity, at least in some subsets of the population.

Other risk factors that are common in high-income countries are becoming more frequent in low-income countries. Younger menarche, later age at pregnancy and low parity are known risk factors for breast cancer that are clearly increasing in low-income settings.

Vaccination

There are currently two vaccines known to reduce cancer incidence: HPV vaccine and HBV vaccine. The Global Alliance for Vaccines and Immunization (GAVI), a global vaccine alliance, working closely with the United Nations Children's Fund (UNICEF), is responsible for improving access to vaccines in low-income countries.

Vaccination against HPV proved to be highly effective in preventing the development of high-grade premalignant cervical lesions that lead to cervical cancer. Currently, several HPV vaccines are available, protecting against different high-risk HPV subtypes. They also protect against some low-risk HPV subtypes. In SS Africa, HPV vaccines are usually administered to girls aged 9–14 years, using different delivery strategies, such as school based vaccination, routine immunization in conjunction with national vaccination plans, or in coordination with other healthcare programs. GAVI started several demonstration projects in different countries for HPV vaccination before its definitive inclusion in the national vaccination plan, first in Kenya, in 2013, and then in countries such as Rwanda, Cameroon, Gambia, Kenya, Malawi, Mozambique, São Tomé & Principe, among others. The price of HPV vaccine is decreasing, which enables a potential increase in vaccination, along with the possibility of including preadolescent boys in vaccination programs. Male vaccination proved to be cost-effective and is capable of further interrupting HPV transmission. Moreover, it may offer protection against other HPV-related cancers, such as head and neck or anal cancers.

A vaccine against HBV is available since the 1980s, but the world's population coverage by this vaccine is still expanding. The WHO recommends a three-dose vaccination schedule, with the first dose being administered within 24 h of birth and the other two at least 4 weeks apart. At a global level, the three-dose HBV vaccination coverage is estimated to be 84%, and in SS African increased from 5% in the year 2000 to an estimated 76% in 2015. This ranges broadly throughout different countries in the region, starting at 16% in Equatorial Guinea and reaching 98% in Rwanda and other countries. Thus, further progress must be made in many SS African countries in order to improve vaccination coverage and control of HBV infection.

Screening

Cancer screening may contribute to reduce the incidence and mortality of cancer, by diagnosing and treating premalignant lesions, or to promote timely treatment of malignant lesions with reduced morbidity. The first is best depicted by cervical cancer screening and the second by breast cancer screening. The potential benefits of cancer screening in resource constrained countries has been thoroughly discussed but even if the screening tests are available, most SS African countries still lack trained healthcare personnel and technical capability to diagnose and treat suspected cancers. Cervical and breast cancers screening are the most frequently available in SS Africa, reflecting the fact that these have been studied in resource constrained settings, mostly after adapting strategies and methods to the available human and financial resources, and cultural characteristics.

Cervical cancer screening

Despite the increasing coverage of HPV vaccination in SS Africa, scarce resources and failure to complete full schedules make screening an essential tool for prevention and control of cervical cancer. Screening with the Papanicolaou (Pap) test contributed greatly to the reduction in cervical cancer mortality in developed countries. However, cytology testing is expensive, and requires highly trained professionals and complex logistics. Furthermore, screening with the Pap test requires a follow-up visit for treatment, which is frequently impractical in resource constrained countries. Therefore, "test & treat" strategies try to obviate these difficulties by diagnosing and treating in the same visit, and they are currently proposed by the WHO for low-income settings. Test can be accomplished with visual inspection with acetic acid (VIA), digital cervicography, HPV test or with a combination of methods (e.g., HPV test and visual inspection). Treatment can be provided using cryotherapy. This approach was shown to be cost-effective, safe and culturally adapted. Other approaches are being evaluated to improve screening uptake in different cultural settings. Self-sample collection by women has proved to be reliable and reproducible, and urinary detection of HPV is being perfected. Also, recently developed rapid and portable HPV detection tests may contribute for increasing screening coverage.

Breast cancer screening

Mammography-based screening was shown to reduce breast cancer mortality in developed regions, and organized screening programs are available in many countries, with varying coverage. However, the logistics leading to a successful detection, diagnosis and treatment of breast cancer constitute a highly complex multidisciplinary undertake. Breast screening programs are expensive and require infrastructures that are often unavailable in low-income countries. This has led to a simplified screening approach based first on awareness and education, then on self-examination of the breast and finally on clinical examination of the breast. Currently, there is no screening program based exclusively on self or clinical examination, but several programs are including these methods to increase awareness for breast cancer and screening uptake. Also, randomized controlled trials showed an increase in the detection of invasive breast cancer using clinical breast examination screening. Nevertheless, none of these strategies proved to be effective in reducing breast cancer mortality and randomized trials are underway.

Cancer Diagnosis

Cancer diagnosis requires clinical evaluation of patients through various procedures, such as imaging, clinical laboratorial tests, and microscopic examination of cells and tissue samples (cytology or histopathology). The availability of resources needed for cancer diagnosis varies across SS Africa, and diagnostic capacity is often suboptimal, mainly due to the lack of adequate pathology capacity and infrastructure, as well as low quality of these services. This situation leads to delays in diagnosis and treatment, inadequate treatment strategies and follow-up, with increased morbidity and mortality.

Pathology

All patients should have a pathologic diagnosis before cancer treatment, and both anatomic and clinical pathology are crucial for its control. Cancer screening and diagnosis, including the identification of cancer type, grading and staging, as well as guidance of surgical resections and evaluation of the effectiveness of treatment depend on pathology investigation. The accuracy of cancer registration data also relies to a large extent on the quality of the pathological diagnoses.

Histopathology and cytology, such as fine needle aspiration cytology (FNAC), are needed for microscopic diagnosis. Complementary methods, namely immunohistochemistry, flow cytometry and molecular pathology, are used for phenotyping tumors (including leukemia and lymphomas), and for the demonstration of genetic alterations. These results may be used for a finer characterization of tumors, leading to a better definition of treatment and prognosis.

The availability of pathology providers and services varies widely among SS African countries. The number of pathologists per inhabitant is less than 1:500,000 (Fig. 3), which is about one tenth of the observed in high-income-countries (about 1:15–20,000) and some countries have no pathologists at all. Therefore, the number of pathologists and technicians is clearly insufficient for the provision of adequate services. Moreover, only a few countries have more than 50% of cancers with a pathologic diagnosis and, when available, the turnaround time of diagnosis can reach 1 month.

In places where pathology services are available, the quality of diagnosis is further limited by factors such as lack of maintenance of equipment, inadequate supply chain of quality reagents, inadequate specimen fixation and transportation from the operating room to pathology services, and absence of quality control programs. Immunohistochemistry and molecular testing are available in just over half and in less than 20% of SS African countries, respectively. Consequently, and as an example, the study of hormone receptors in breast cancer, which is fundamental to its management, is limited or absent in many countries. The African Strategies for Advancing Pathology (ASAP) Group, created in 2014, generated a 5-year strategic plan aiming to increase and improve pathology services in SS Africa, including advocacy for pathology, education and training of pathologists, as well as building, strengthening and maintaining operational laboratories of pathology. Local strategies for improving pathology in a specific country or site must also be encouraged.

In the last decades, FNAC has gained importance as an alternative pathological method for cancer diagnosis in low-income countries. For example, in Mozambique, in 2009–10, 57% of all diagnoses of pediatric cancers in the Department of Pathology of the Maputo Central Hospital were based on FNAC. In other series, when considering Burkitt lymphoma alone, FNAC was

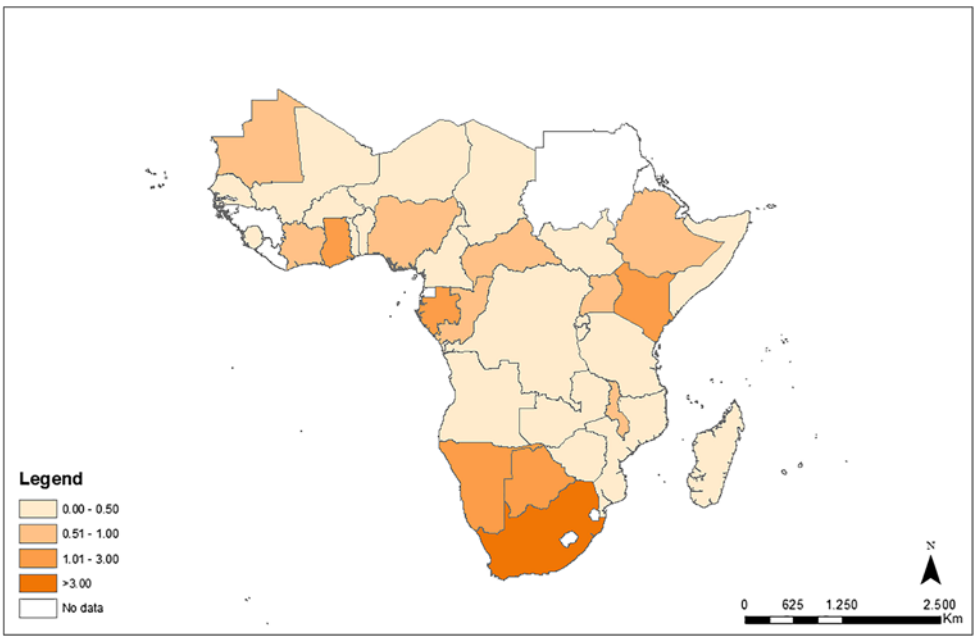

Fig. 3 Total density of pathologists per million population in 2011–13, in Sub-Saharan Africa. From African Strategies for Advancing Pathology [Internet]. Available from: http://www.pathologyinafrica.org/, accessed on 30/01/2018.

responsible for 82% of the diagnoses. Although core biopsy with histology is the preferred method for the preoperative diagnosis of breast cancer, FNAC is widely used for the initial diagnosis of palpable breast cancer in many parts of SS Africa, when core biopsy and histology are not available. Most breast cancer patients present with advanced disease and mastectomy is done many times on the basis of FNAC results, when triple assessment (clinical, radiological, and cytological findings) is conclusive for malignancy. FNAC can be conducted faster and at a lower cost than conventional histology, and with only minimal laboratory pathology infrastructures. However, diagnostic accuracy varies widely with the technique used, the specific site being sampled, and the expertise of the personnel collecting the sample and reading the slide. Samples may have poor quality when the technique is done by a person that only performs FNAC occasionally, but the proportion of inadequate samples drops from 15%30% to 12% when FNAC is performed by a trained cytopathologist. Therefore, when used appropriately and with well-trained personnel, it constitutes a quick and accurate tool for diagnosis and follow-up of cancer.

Clinical Diagnosis

Although the gold-standard for cancer diagnosis is based on tissue sample evaluation, due to the limited access to pathologic diagnosis, in some cases of specific cancers with high prevalence, treatment may start with clinical diagnosis only. Examples include bulky cutaneous Kaposi sarcoma associated with HIV, and some hepatocellular carcinomas that are diagnosed based only on ultrasonography in combination with clinical presentation and laboratorial tests. Additionally, and as already mentioned, cervical premalignant or malignant lesions can be visualized directly using acetic acid and treated with cryotherapy during the same visit. Clinical diagnosis of certain highly prevalent cancers do not require specialized personnel, are less dependent on laboratory facilities and are easier to perform.

Imaging

In the last few decades, imaging became an important tool for diagnosis, treatment planning and follow-up of patients with cancer. Currently, there are many methods available, such as conventional X-rays, ultrasound, mammography, endoscopies, computerized tomography (CT) scans, magnetic resonance imaging (MRI) and nuclear medicine, used for different purposes, namely diagnosis, staging, evaluation of response to treatment and detection of relapses.

After conventional X-rays, that are still important to detect bone tumors in many SS African countries, ultrasonography is the second most used imaging method. It is useful for the initial investigation of signs and symptoms, especially to detect and localize neoplastic lesions in the abdomen and thyroid. It is also useful to guide core needle biopsies or FNAC, and for staging and follow-up. However, its use is limited due to the lack of trained personnel.

Most of the other imaging methods, such as CT scans, MRI and nuclear medicine are scarcely available in many SS African countries. But even in countries which have this equipment, radiologists are often not adequately trained on the diagnosis and follow-up of cancer lesions, namely in the assessment of response to cancer treatment. Additionally, access to endoscopy services, which are extremely important for early detection of esophageal cancer, is also limited. Finally, the low number of mammography units in many SS African countries (Fig. 4) is a barrier for the implementation of breast cancer screening through this method. Alternatively, as mentioned, in some countries, screening is being based on clinical examination of the breast.

Treatment of Cancer

Conceptually, the management of cancer includes the treatment of local disease with surgery, radiation therapy, or both, and the treatment of systemic disease with cytotoxic chemotherapy, endocrine therapy, targeted therapy, immunotherapy or combinations of these. The choice of treatment strategy is based on the tumor extent/location and biology (pathology including biomarkers and gene expression) as well as on the age and general health status of the patient, and personal preferences. However, in SS Africa, the availability of surgery, radiation and systemic therapy varies widely between and within countries. Moreover, many patients are not managed in a multidisciplinary manner, being referred from surgeon to medical oncologist or radiation oncologist, without a proper discussion in a multidisciplinary tumor board. This impairs the achievement of the best possible treatment for the individual patient, adds delays in the patient care pathway and increases the chance of discontinuing care, all of which compromise survival outcomes. Moreover, access to different forms of treatment depends on patients' socioeconomic status.

Surgery

In the most developed countries, surgical treatment of solid tumors has evolved substantially in the last decades, which may be related to the combination with neoadjuvant and adjuvant chemotherapy, targeted therapy and radiotherapy, as well as the development of techniques such as the sentinel-node biopsy and laparoscopy. Therefore, surgery is now much less aggressive in most tumors (e.g., breast-conservation surgery, partial nephrectomy), allowing for a preservation of organ function, with decreased morbidity and mortality, and a benefit in the quality of life of cancer survivors. At the same time, the training of most surgeons on basic cancer principles (e.g., systematic lymphadenectomy in certain cancers and achievement of clear margins in most tumors), has led to an improvement in survival.

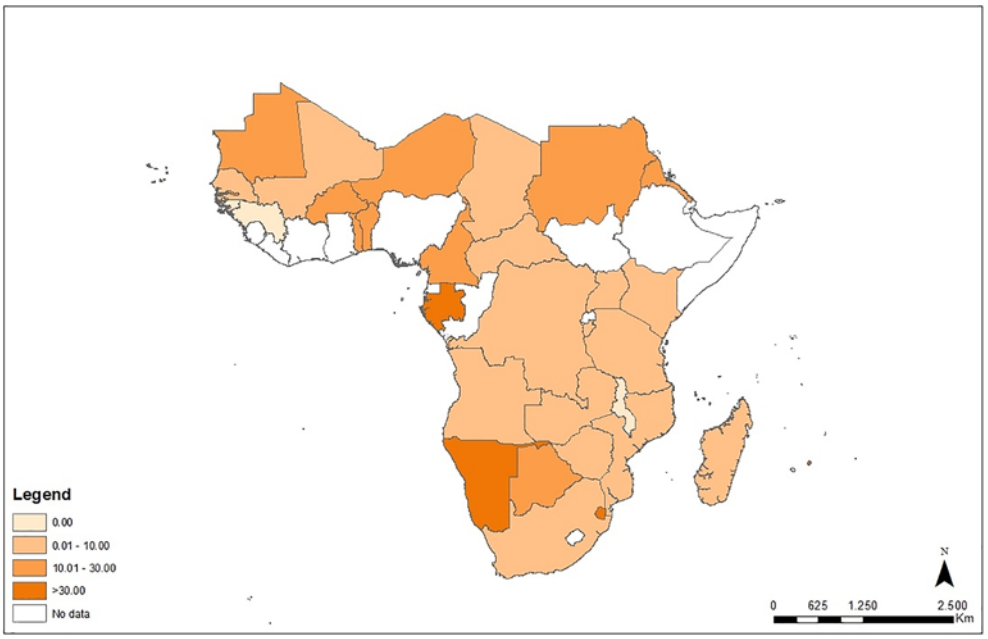

Fig. 4 Total density of mammography units per million females aged from 50 to 69 years old in 2014, in Sub-Saharan Africa. From Global Health Observatory (GHO) data [Internet]. Geneva: World Health Organization. Available from: http://www.who.int/gho/en/, accessed on 30/01/2018.

However, in SS Africa, surgery is still highly centered in cases of trauma and acute conditions. Therefore, surgical training is usually more focused on these situations and not so much on the performance of adequate oncological resections with clear margins, the achievement of a broad lymphadenectomy or the need to send the surgical specimen for proper fixation for histological assessment. Additionally, in some SS African countries, due to the absence of a proper cancer diagnosis prior to surgery and/or lack of awareness of some types of tumors, cancer surgeries may sometimes be performed by nonphysicians, who do not have the expertise to carry out these types of complex surgeries. On top of that, many of the organ-sparing approaches are also not possible due to the lack of trained cancer surgeons in organ-sparing approaches and the absence of radiotherapy. Another problem is that patients frequently present with extensive local tumors that are not suitable for surgery due to the absence of effective neoadjuvant therapies, such as radiotherapy and/or systemic treatment (e.g., trastuzumab in HER2-positive breast cancer).

As an example, currently, in Western Europe ~60%–80% of newly diagnosed breast cancers are amenable to breast conservation (wide local excision and radiation therapy). However, in low-income countries, mastectomy is still the most common intervention for breast cancer—in SS Africa, 85% of the surgeries are mastectomies. Even so, in a retrospective study from Nigeria, only 35% patients underwent mastectomy, as many of the patients presented with inoperable local or metastatic tumors at diagnosis. Also, in Rwanda, from 145 patients receiving breast cancer care, only 48% underwent mastectomy. This is in contrast with Europe, in which over 90% of women receive surgical treatment.

Radiation Therapy

Radiation therapy is an important component of cancer control programs. Given that cervical, breast, prostate, esophageal, and head and neck cancers are among the most common types of cancer in SS Africa, radiotherapy plays a very important control as a radical as well as an adjuvant therapy.

However, according to the WHO, in 2013, only 20 countries were known to have radiotherapy, and these facilities were concentrated in the southern and northern states of the region (Fig. 5). According to a survey conducted by The International Atomic Energy Agency in 2010, brachytherapy resources (high-dose rate or low-dose rate) were only available in 20 countries as well. Additionally, access can be difficult even when radiation therapy facilities are available due to the large number of people covered by each of the centers. This leads to long patient waiting lists, which results in large numbers of patients referred for radiation therapy not being treated in due time. Also, due to the out of pocket expense of traveling daily to the radiotherapy facility, some patients have a low treatment compliance, which undermines its effectiveness, especially in very radiosensitive cancers, such as head and neck tumors.

Systemic Treatment

Systemic therapy can be neoadjuvant, adjuvant or palliative, contributing to substantial improvements in survival and symptom burden, depending on its intended use. A good example is breast cancer, in which more than half of the women with localized cancer who would otherwise die of metastatic breast cancer remain disease-free when treated with the appropriate systemic regimen.

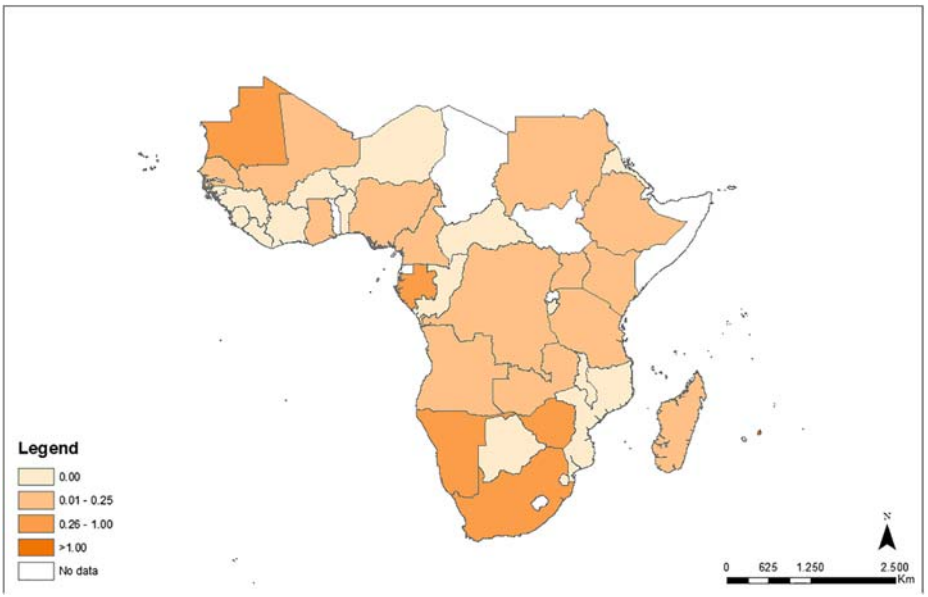

Fig. 5 Total density of radiotherapy units per million population in 2013, in Sub-Saharan Africa. From Global Health Observatory (GHO) data [Internet]. Geneva: World Health Organization. Available from: http://www.who.int/gho/en/, accessed on 30/01/2018.

However, there are multiple problems regarding the delivery of systemic treatment in SS Africa. For example, the safe administration of systemic chemotherapy, which is crucial to improve outcomes for a number of common cancers (e.g., colon, breast, cervical, and head and neck), depends on the availability of drugs, infrastructure for drug administration, and well-trained medical and nonmedical staff. Unfortunately, adequate infrastructures and trained staff are scarce in low-resource countries, and treatments are often interrupted due to the irregular supply of systemic drugs. The effectiveness of chemotherapy protocols can be further impaired by inadequate management of toxicity due to limited access to support treatments, including blood transfusions, granulocyte-colony stimulating factors, antibiotics and antiemetics.

There are examples of programs trying to overcome the absence of specialists and specialty centers in SS Africa. As an example, since 2014, the Gulbenkian Foundation has been providing support for Mozambican medical and nonmedical staff to be trained in Portugal in different cancer areas (surgery, radiation therapy, medical oncology, oncological nursing and pathology, among others) during short-term fellowships, allowing for an effective education in multidisciplinary cancer care. This has led to the implementation of new surgical cancer techniques in Mozambique and to the creation of the first multidisciplinary tumor board in the country. Also, an international partnership of Partners in Health and the Dana-Farber Cancer Institute, Harvard Medical School, and Brigham and Women's Hospital, is working in rural Malawi and Rwanda. They are operating health centers and hospitals in rural districts, supporting and training local physicians and nurse teams on the delivery of chemotherapy to patients with multiple cancers, including breast, cervical, rectal, head and neck, Hodgkin's and non-Hodgkin lymphoma, and Kaposi sarcoma.

However, the difficulty lies in scaling up these projects to a national level. One of the reasons is that the cost of present chemotherapeutic agents for all patients with cancer exceeds the healthcare budgets of low-resource countries. Therefore, many SS African countries cannot ensure that the drugs in the WHO essential drug list for cancer and newer drugs are available. This also leads to out-of-pocket payments that are unaffordable to most patients, leading to treatment nondelivery or interruption. Besides, patients that progress during first-line chemotherapy, which is usually limited to the available drugs and may not include the best possible treatment, have even more limited options for further treatment.

Regarding endocrine therapy (e.g., in breast cancer), the absence of advanced pathology services to establish hormone-receptor status continues to obstruct appropriate decision making for this treatment. However, the potential savings with the selective use of endocrine therapy are expectedly greater than the costs of hormone-receptor testing, so it would be important to develop it instead of carrying on with the blind prescription of endocrine therapy. In terms of breast cancer, tamoxifen is accessible in most countries and, in some of them, it is free of charge. Yet, newer agents, like aromatase inhibitors are mostly not available in public hospitals.

With respect to targeted therapy, in several SS African countries, agents such as trastuzumab or rituximab are not available due to their high price, even if they are part of the WHO essential drug list. However, biosimilars are already available in countries like India at a reduced cost, so they could be introduced in Africa in the near future, helping in the reduction of cancer mortality.

The development of guidelines for drug selection and administration would be very important to ensure that all patients with cancer receive the best available drugs in appropriate and therapeutic doses. For example, guidelines regarding cervical cancer treatment in limited-resource settings were issued in 2016 by the American Society of Clinical Oncology, to help with the optimization of clinical outcomes. However, it would also be important to have the technology for a better characterization of each tumor, as it could lead to a more personalized and effective treatment—for example, use of platinum drugs, that are cheap and usually available, and avoidance of endocrine therapy in triple negative breast cancer.

Clinical Trials

Clinical trials are an essential part of cancer research efforts, enable patients to have access to more treatment options and expand the possibilities of use of each drug. However, in SS Africa, the number of cancer clinical trials is still very low: in a recent review of randomized controlled trials in breast cancer registered on ClinicalTrials.gov ($n = 1099$ trials), only 6.3% occurred in one or more low- and middle-income country. Besides, among the 44 SS African countries, studies were only being conducted in Nigeria, Kenya, Ghana and South Africa; the latter had 91% of the 54 studies taking place in the region.

This is worrisome because it is paramount to undertake cancer clinical trials in SS Africa, in order to: (a) develop new drugs and treatments for cancers that are less frequent in the West, but predominant in SS Africa (e.g., esophageal cancer); (b) study diseases that are frequent in the West, but have a different presentation and course in SS Africa (e.g., higher proportion of triple negative breast cancer compared to Europe); and (c) develop more affordable ways of delivering cancer care in SS Africa (e.g., hypofractionated schemes of radiotherapy in prostate cancer).

Patients with HIV

The prevalence of HIV in SS Africa is highly variable among countries, but can be as high as 37% of the population (in urban women in Swaziland), according to the WHO. Furthermore, more than 70% of the global burden of HIV infection is in SS Africa. This is important because, since the discovery of HIV, it is known that infected patients have a higher risk of developing cancer, mainly due to the immunosuppression induced by the virus. When some of these cancers are diagnosed in an HIV-positive patient, it means that the patient has developed the stage of HIV infection called "acquired immune deficiency syndrome" (AIDS). Therefore, these cancers are called "AIDS-defining malignancies" and include Kaposi sarcoma, primary central nervous system lymphoma, peripheral intermediate- and high-grade B-cell non-Hodgkin lymphoma, and cervical cancer. HIV-positive patients also have an increased risk of developing other cancers, such as Hodgkin lymphoma or lung cancer, but these are considered non-AIDS defining malignancies. Meanwhile, due to the scale-up of highly active antiretroviral therapy (HAART) in SS Africa, HIV infection is turning into a chronic condition, with aging and growth of HIV-infected populations. Reports from countries like Brazil and United States show a decline in AIDS-defining malignancies and an increase in non-AIDS defining malignancies.

Yet, despite the high burden of HIV in SS Africa and its relation with cancer, HIV infection status is often missing for cancer patients, which is troublesome since HIV infection also influences cancer treatment and prognosis. In 2017, among a series of 1137 cancer patients from Nigeria, the HIV status was not recorded for nearly one-third of the patients, and 32% of the remaining were HIV-positive.

Regarding survival, data in HIV-positive cancer patients comes mainly from American or European series, and they all show that these patients have worse cancer-specific survival compared to those not infected. In a very large American series, even after adjustment for stage and cancer treatment, HIV-positivity was associated with higher cancer-specific mortality for colorectal cancer (hazard ratio [HR] 1.40, 95% CI 1.09–1.80), lung cancer (HR 1.28, 95% CI 1.14–1.44), melanoma (HR 1.93, 95% CI 1.14–3.27) and breast cancers (HR 2.64, 95% CI 1.86–3.73). This could be attributed to the effect of immunosuppression (as we nowadays know that an intact immune system is essential for the effectiveness of anticancer drugs) and to suboptimal therapy due to increased toxicity and higher rates of infection. On the contrary, there are known pharmacological interactions between HAART and common chemotherapy drugs, such as alkylating agents, taxanes, and anthracyclines. There are also interactions with targeted agents, like imatinib or erlotinib. Therefore, HIV-positive patients are at risk of overlapping toxicity, namely hematological, which can compromise treatment. As an example, in a case-control study in the United States (with 52 HIV-positive and 104 HIV-negative breast cancer patients), the HIV-positive women needed dose reductions and/or had chemotherapy delays due to toxicity more often (56% vs. 30%; $P = .03$). Therefore, in order to avoid immunosuppression-related infections, CD4+ cell count should be closely monitored and the use of prophylactic G-CSF/GM-CSF is strongly recommended following chemotherapy. However, these drugs may not be available in all SS African clinical centers.

Additionally, even in clinical trials regarding cancers with higher risk in HIV-positive patients (like lung or cervical cancer), these patients are usually excluded, so data regarding the efficacy and safety of new cancer therapies in HIV-positive patients is scarce. Therefore, the creation of specific guidelines to cancer treatment and management of complications in HIV-infected patients in low-resource settings, such as SS Africa, is essential in order to maintain treatment intensity and achieve the best possible survival outcomes.

Palliative Care

In SS Africa, supportive care, which broadly includes the treatment of side effects, palliative care and end-of-life care, is still an emerging specialty. Even if palliative care is now delivered in nearly 50% of SS African countries, still less than 5% of people in need receive it. This is extremely worrisome, especially because about 80% of cancer patients in SS Africa are diagnosed at advanced stages of the disease, and in many of these patients, palliative care is often the only choice of treatment.

One of the core components of palliative care is pain control. However, there are a number of barriers to the use of inexpensive opioid analgesics in SS Africa, including legal and regulatory restrictions, inadequate training of health care providers, concerns about abuse and cultural misperceptions about pain. For instance, a survey directed to medical doctors, conducted in a University Hospital in Nigeria, showed that only 40% of the respondents routinely conducted pain assessments among cancer patients, only 20% used strong opioids and over 90% of the respondents had no formal training on pain management.

According to an American Cancer Society/African Organization for Research and Training in Cancer (AORTIC) report, in 2008, the actual procurement of morphine and equivalent opioids reported by SS African governments to the International Narcotics Control Board corresponded only to about 10% of the quantity needed just for the terminal months of cancer and HIV-positive patients. This means that the vast majority of cancer patients in SS Africa do not have access to opioid treatment.

However, there are some inspiring examples, such as Kenya and Uganda that have been pointed as leaders in palliative care development in SS Africa. Through a strategy based on education and training of health care providers, improved access to opioid medications, better professional and public attitudes towards palliative care, integration of palliative care into national health care systems, and palliative care research, these countries have incorporated palliative care as part of their mainstream health service. An interesting strategy was the involvement of lay community volunteer workers, who advocate for palliative care in the community and help to clarify the myths related with the process of dying. Also, education programs were offered not only to health professionals, but also to community volunteer workers, spiritual caregivers and traditional healers.

While governments should take steps towards an easier and more comprehensive access to palliative care for their cancer patients, NGOs can lobby to put palliative care on the public agenda, and also to help on the design and implementation of palliative care programs on a local and national level.

Prospective Vision

As explained above, cancer control in SS Africa is not an easy task, due to the increasing incidence of cancer, and lack of proper healthcare networks for diagnosis and treatment. However, as this burden is growing at a fast pace, new solutions will have to be provided. First, vaccination programs, covering HBV and HPV are fortunately expanding and that could substantially decrease the burden of liver and cervical cancer, respectively, as well as head and neck cancer. Second, even if legislation control over known risk factors like tobacco and alcohol is still in its beginning, it is predictable that it will tighten in the near future, which would also be valuable for cancer control. However, "western" risk factors such as obesity are becoming more frequent and, even with educational strategies, will be hard to overcome. Third, regarding screening, diagnosis and treatment, a change of mind-set will have to occur: as it is not feasible to implement strategies used in developed countries, due to their complexity and cost, new ways of delivering cancer care will have to be pursued. For that, clinical trials in SS Africa regarding new strategies for screening (like clinical breast examination) and diagnosis (such as portable HPV detection tests) should be carried out, in order to assess their effectiveness, cost and application in SS African health systems, as well to adapt them to local habits. At the same time, trials regarding new treatment strategies with old or new drugs and combinations are also urgently needed, as a way to improve survival outcomes. Investing in strengthening pathology for early detection and diagnosis with quality is crucial. Also, a better organization of drugs' supply chain, and the use of generic drugs and biosimilars could decrease the costs and improve the delivery of systemic treatment. Finally, it is essential to train a whole new generation of physicians, nurses and technicians on cancer principles and care, in order to improve their expertise in the delivery of care and, at the same time, raise awareness for this growing problem. This would be an important step for the change of attitudes in SS Africa regarding the importance of cancer prevention and care.

See also: Cancer Disparities. Cancer in the Middle East. Cancer in Populations in Transition.

Further Reading

Abdel-Wahab, M., Bourque, J.M., Pynda, Y., et al., 2013. Status of radiotherapy resources in Africa: An International Atomic Energy Agency analysis. The Lancet Oncology 14 (4), e168–75.

African Strategies for Advancing Pathology Group Members, 2015. Quality pathology and laboratory diagnostic services are key to improving global health outcomes: Improving global health outcomes is not possible without accurate disease diagnosis. American Journal of Clinical Pathology 143 (3), 325–328.

American Cancer Society, 2011. Cancer in Africa. American Cancer Society, Atlanta.

Coghill, A.E., Shiels, M.S., Suneja, G., Engels, E.A., 2015. Elevated Cancer-specific mortality among HIV-infected patients in the United States. Journal of Clinical Oncology 33 (21), 2376–2383.

Ferreira-Borges, C., Esser, M.B., Dias, S., Babor, T., Parry, C.D., 2015. Alcohol control policies in 46 African countries: Opportunities for improvement. Alcohol and Alcoholism 50 (4), 470–476.

Finocchario-Kessler, S., Wexler, C., Maloba, M., et al., 2016. Cervical cancer prevention and treatment research in Africa: A systematic review from a public health perspective. BMC Women's Health 16, 29.

Harford, J.B., 2015. Barriers to overcome for effective cancer control in Africa. The Lancet Oncology 16 (8), e385–93.

Jemal, A., Bray, F., Forman, D., et al., 2012. Cancer burden in Africa and opportunities for prevention. Cancer 118 (18), 4372–4384.

McCormack, V.A., Boffetta, P., 2011. Today's lifestyles, tomorrow's cancers: Trends in lifestyle risk factors for cancer in low- and middle-income countries. Annals of Oncology 22 (11), 2349–2357.

Menon, S., Rossi, R., Zdraveska, N., et al., 2017. Associations between highly active antiretroviral therapy and the presence of HPV, premalignant and malignant cervical lesions in sub-Saharan Africa, a systematic review: Current evidence and directions for future research. BMJ Open 7 (8), e015123.

Nelson, A.M., Milner, D.A., Rebbeck, T.R., Iliyasu, Y., 2016. Oncologic care and pathology resources in Africa: Survey and recommendations. Journal of Clinical Oncology 34 (1), 20–26.

Sasco, A.J., Jaquet, A., Boidin, E., et al., 2010. The challenge of AIDS-related malignancies in sub-Saharan Africa. PLoS One 5 (1), e8621.

Stefan, D.C., 2015. Cancer Care in Africa: An Overview of Resources. Journal of Global Oncology 1 (1), 30–36.
Vanderpuye, V., Grover, S., Hammad, N., PoojaPrabhakar, S.H., Olopade, F., et al., 2017. An update on the management of breast cancer in Africa. Infectious Agents and Cancer 12, 13.
World Health Organization, 2014. Comprehensive cervical Cancer control. A guide to essential practice, 2nd ed. WHO, Geneva.

Relevant Websites

http://www.aortic-africa.org/page_02_Home.php —AORTIC (African Organization for Research and Training in Cancer).
http://www.gavi.org/ —GAVI Alliance.
http://www.healthdata.org/ —Institute for Health Metrics and Evaluation (IHME).
http://www.who.int/cancer/en/ —WHO Cancer Control webpage.
http://globocan.iarc.fr/Default.aspx —WHO/International Agency for Research on Cancer (IARC) GLOBOCAN 2012.
http://ci5.iarc.fr/Default.aspx —WHO/IARC Cancer incidence in five continents.
http://survcan.iarc.fr/ —WHO/IARC Cancer survival in Africa, Asia, the Caribbean and Central America.

Cancer in the Middle East

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Glossary

Age-standardized death rate (ASDR) A weighted average of the age-specific mortality rates per 100,000 persons, where the weights are the proportions of persons in the corresponding age groups of the WHO standard population.

Area under the curve (AUC) for screening test Represents an overall indication of the diagnostic accuracy of a ROC curve. AUC values closer to 1 indicate the screening measure reliably distinguishing diseased and nondiseased subjects, whereas values at 0.50 indicate that the predictor's diagnostic accuracy is no better than chance.

Disability-adjusted life years (DALYs) A measure of overall disease burden, expressed as the number of years lost due to ill health, disability, or early death. It was developed as a way of comparing the overall health and life expectancy of different countries.

Early diagnosis The early identification of cancer in patients who have symptoms of the disease.

Incremental cost The increase in total costs resulting from an increase in production or other activity. For instance, if a company's total costs increase from $320,000 to $360,000 as the result of increasing its machine hours from 8000 to 10,000, the incremental cost of the 2000 machine hours is $40,000.

Primary prevention It is concerned with preventing the onset of disease; it aims to reduce the incidence of disease. It involves interventions that are applied before there is any evidence of disease or injury.

Relative risk The ratio of the probability of an event occurring (for example, developing a disease, being injured) in an exposed group to the probability of the event occurring in a comparison, nonexposed group.

Screening Identification of unrecognized (preclinical) cancer or precancerous lesions in an apparently healthy target population.

Epidemiology of Cancer in the Middle East

Cancer is a global health problem. The growing burden of cancer includes increasing global trend of 1% every year. Low- and middle-income countries report more than two-thirds of cancer mortality all over the world. Cancer kills one-quarter of a million people each year in the WHO Eastern Mediterranean Region alone. According to GLOBOCAN (global cancer observatory) cancer incidence and mortality data, in Eastern Mediterranean region in 2012, the overall age-standardized cancer incidence rate was almost 25% higher in men than in women. Cancer incidence rates among males were 119.5 cases per 100,000 with a corresponding mortality rate of 90 per 100,000 males. The incident cancer cases in females were 126.2 per 100,000 (79.4 deaths/100,000). Using age-standardized incidence rate from all ME countries, lung, bladder, and prostate cancers recorded the highest incidence in males (Table 1) while breast cancer is the commonest type in females (Table 2). Country-specific cancer incidence among males revealed the highest rate being stomach in Iran, Afghanistan, Colorectum in Saudi-Arabia, lymphoma in Sudan and Djibouti, and liver cancer in Egypt (Table 3)

An overview of age-standardized death rates from World health organization (WHO) mortality data 2000–2014 for selected Middle East (ME) countries revealed; a steady high mortality pattern of both sexes for some countries and prominent rising trend for others. The highest cancer mortality among men is reported in Israel, Cyprus, and Egypt, also high mortality but insufficient data is in Turkey (Fig. 1). Females' breast cancer mortality is the highest in Israel and Egypt (Fig. 2).

Despite advances in cancer treatment, a minimal decline in cancer mortality along the recent years in the ME. Many cases are diagnosed at a late stage with subsequent poor prognosis and lower survival than the European region. The proportion of mortality to total incident cases of both genders is greater in the WHO Eastern Mediterranean region than the WHO European region as well as the European Union and USA (Fig. 3).

Risk Factors for Cancer in the ME

It is crucial to illustrate the possible causes and prominent risk factors that contribute to cancer in developing countries so that preventive strategies and cancer control programs can be properly allocated.

Tobacco Smoking and Alcohol

Tobacco is responsible for 5 million deaths worldwide every year. About 80% of one billion smokers live in the low-middle income countries where tobacco has harmful health effects and deaths are the heaviest. Waterpipe smoking (Shisha) is popular in the ME

Table 1 WHO Eastern Mediterranean countries (2012): estimated cancer incidence, all ages (male)

Cancer	*Numbers*	*Crude rate*	*ASR (W)*	*Cumulative risk*
All cancers excl. Nonmelanoma skin cancer	261,574	89.2	125.9	13.39
Bladder	22,471	7.7	11.7	1.40
Brain, nervous system	10,973	3.7	4.6	0.46
Colorectum	18,908	6.4	9.2	1.04
Gallbladder	2016	0.7	1.0	0.12
Hodgkin lymphoma	4888	1.7	1.8	0.16
Kaposi sarcoma	533	0.2	0.3	0.03
Kidney	5679	1.9	2.7	0.30
Larynx	8934	3.0	4.6	0.56
Leukaemia	13,064	4.5	5.2	0.47
Lip, oral cavity	11,165	3.8	5.2	0.59
Liver	19,018	6.5	9.7	1.21
Lung	26,195	8.9	13.5	1.65
Melanoma of skin	1429	0.5	0.7	0.07
Multiple myeloma	2595	0.9	1.3	0.16
Nasopharynx	2549	0.9	1.1	0.11
Non-Hodgkin lymphoma	15,279	5.2	6.8	0.73
Oesophagus	8284	2.8	4.2	0.49
Other pharynx	2823	1.0	1.4	0.17
Pancreas	4688	1.6	2.4	0.29
Prostate	21,294	7.3	11.5	1.35
Stomach	14,867	5.1	7.5	0.85
Testis	2854	1.0	1.0	0.08
Thyroid	2832	1.0	1.2	0.13

Crude and age-standardized rates per 100,000.
Cumulative risk [0–74], percent.
GLOBOCAN 2012, IARC – 29.3.2017.

Table 2 WHO Eastern Mediterranean countries (2012): estimated cancer incidence, all ages (female)

Cancer	*Numbers*	*Crude rate*	*ASR (W)*	*Cumulative risk*
All cancers excl. Nonmelanoma skin cancer	287,468	102.8	131.5	13.29
Bladder	5872	2.1	3.0	0.35
Brain, nervous system	8324	3.0	3.6	0.36
Breast	97,530	34.9	43.8	4.56
Cervix uteri	13,179	4.7	6.0	0.64
Colorectum	15,589	5.6	7.4	0.82
Corpus uteri	7997	2.9	3.9	0.47
Gallbladder	3414	1.2	1.7	0.20
Hodgkin lymphoma	3190	1.1	1.2	0.10
Kaposi sarcoma	268	0.1	0.1	0.01
Kidney	3542	1.3	1.6	0.17
Larynx	1421	0.5	0.7	0.08
Leukaemia	9420	3.4	3.9	0.36
Lip, oral cavity	8806	3.1	4.2	0.48
Liver	9113	3.3	4.6	0.55
Lung	7538	2.7	3.7	0.43
Melanoma of skin	1292	0.5	0.6	0.07
Multiple myeloma	1765	0.6	0.9	0.11
Nasopharynx	1160	0.4	0.5	0.05
Non-Hodgkin lymphoma	10,797	3.9	4.9	0.53
Oesophagus	7260	2.6	3.6	0.42
Other pharynx	1639	0.6	0.8	0.09
Ovary	11,666	4.2	5.3	0.58
Pancreas	3408	1.2	1.7	0.20
Stomach	8449	3.0	4.1	0.47
Thyroid	9205	3.3	3.8	0.37

Crude and age-standardized rates per 100,000.
Cumulative risk [0–74], percent.
GLOBOCAN 2012, IARC – 29.3.2017.

Table 3 Adult risk factors and overview of cancer monitoring and surveillance in Middle East countries

	Commonest cancer incidence types		*Adult risk factors*					*Cancer plans, monitoring, and surveillance*				
Country	*Male*	*Female*	*Current tobacco smoking (2011)*	*Total alcohol per capita consumption, in liters of pure alcohol (2010)*	*Physical inactivity (2010)*	*Obesity (2014) Male% Female%*	*Household solid fuel use (2012)*	*an operational cancer policy/ strategy/ action plan*	*Has a cancer registry*	*Scope of cancer registry*	*Coverage of cancer registry*	*Last year of the data for cancer registry*
Afghanestan	Stomach	Breast	–	1.2 M 0.1 F	–	1.5% M 3.3% F	81%	No	No	–	–	–
Bahrain	Lung	Breast	34.9% M 7.6% F	2.7 M 1 F	–	29.7% M 41.3% F	–	Yes	Yes	Hospital-based	National	2012
Cyprus	Prostate	Breast	41.4% M 18.4% F	12.5 M 5.7 F	29.3% M 41.5% F	22.3% M 26.8% F	–	Yes	Yes	Population-based	National	2009
Dibouti	Non-Hodgkin lymphoma	Breast	–	2.4 M 0.3 F	–	5.1% M 12.0% F	–	No	No	–	–	–
Egypt	Liver	Breast	46.5% M <1% F	0.7 M 0.0 F	23.4% M 38.6% F	19.4% M 36.0% F	–	No	Yes	Population-based	Subnational	2009
Iran	Stomach	Breast	26.4% M <1% F	1.7 M 0.3 F	22.3% M 41.6% F	19.3% M 30.6% F	–	Yes	Yes	Population-based	Subnational	2010
Iraq	Lung	Breast	30.8% M 4.5% F	0.9 M 0.1 F	49.6% M 43.1% F	15.3% M 27.3% F	–	Yes	Yes	Hospital-based	National	2009
Israel	Prostate	Breast	34.9% M 16.6% F	4.0 M 1.7 F	–	23.7% M 27.8% F	–	Yes	Yes	Population-based	National	2010
Jordan	Colorectal	Breast	46.9% M 5.5% F	5.5% M 0.2% F	12.7% M 11.4% F	21.0% M 35.6% F	–	Yes	Yes	Population-based	National	2010
Kwait	Colorectal	Breast	35.1% M 3.5% F	0.2 M 0.0 F	48.3% M 62.8% F	34.8% M 43.5% F	–	No	Yes	Population-based	National	2010
Lebanon	Prostate	Breast	42.7% M 21.9% F	3.9 M 0.8 F	43.7% M 34.2% F	26.1% M 35.7% F	–	DK	Yes	Other	National	2007
Libya	Lung	Breast	44.7% M <1% F	0.1 M 0.0 F	31.0% M 42.3% F	25.8% M 38.0% F	–	No	No	–	–	–
Morocco	Lung	Breast	32.1% M 2.4% F	1.9 M 0.1 F	–	15.6% M 27.6% F	–	Yes	Yes	Population-based	Subnational	2007
Oman	Colorectal	Breast	12.8% M <1% F	1.2 M 0.4 F	–	22.7% M 33.5% F	–	No	Yes	Population-based	National	2010
Pakistan	Mouth and oropharynx	Breast	37.6% M 7.4% F	0.1 M 0.0 F	18.5% M 29.7% F	3.3% M 6.4% F	–	No	No	–	–	–

(Continued)

Table 3 Adult risk factors and overview of cancer monitoring and surveillance in Middle East countries—cont'd

	Commonest cancer incidence types		*Adult risk factors*					*Cancer plans, monitoring, and surveillance*				
Country	*Male*	*Female*	*Current tobacco smoking (2011)*	*Total alcohol per capita consumption, in liters of pure alcohol (2010)*	*Physical inactivity (2010)*	*Obesity (2014) Male% Female%*	*Household solid fuel use (2012)*	*an operational cancer policy/ strategy/ action plan*	*Has a cancer registry*	*Scope of cancer registry*	*Coverage of cancer registry*	*Last year of the data for cancer registry*
Qatar	Colorectal	Breast	–	1.8 M 0.4 F	29.9% M 46.9% F	38.9% M 47.8%	–	Yes	Yes	Population-based	National	2008
Saudi Arabia	Colorectal	Breast	37.9% M <1% F	0.3 M 0.1 F	52.1% M 52.1% F	29.5% M 39.5% F	–	Yes	Yes	Population-based	National	2008
Somalia	Prostate	Breast	–	0.9 M 0.1 F	–	1.8% M 6.0% F	–	No	No	–	–	–
Sudan	Non-Hodgkin lymphoma	Breast	–	4.8 M 0.6 F	–	3.6% M 9.6% F	–	Yes	Yes	Population-based	Substantial	2010
Syria	Lung	Breast	–	2.3 M 0.1 F	–	15.9% M 27.5% F	–	Yes	Yes	Hospital based	National& Substantial	2009
Tunisia	Lung	Breast	52.1% M 11.1% F	3.0 M 0 F	18.6% M 26.6% F	20.2% M 33.9% F	–	No	Yes	Population-based	Substantial	2004
Turkey	Lung	Breast	42% M 13% F	4.4 M 4.4 F	27.1% M 37.1% F	22.6% M 35.9% F	–	Yes	Yes	Population-based	National	2008
UAE	Colorectal	Breast	–	5.5 M 0.8 F	27.0% M 39.4% F	31.6% M 41.2% F	–	No	Yes	Population-based	National	2012
Yemen	Leukaemia	Breast	35% M 10.8% F	0.4 M 0.1 F	–	9.1% M 19.4% F	–	No	Yes	Hospital based	National	2011

– Data not available.

M: Males.

F: Females.

Source: WHO Cancer country profiles (http://www.who.int/cancer/country-profiles/en/).

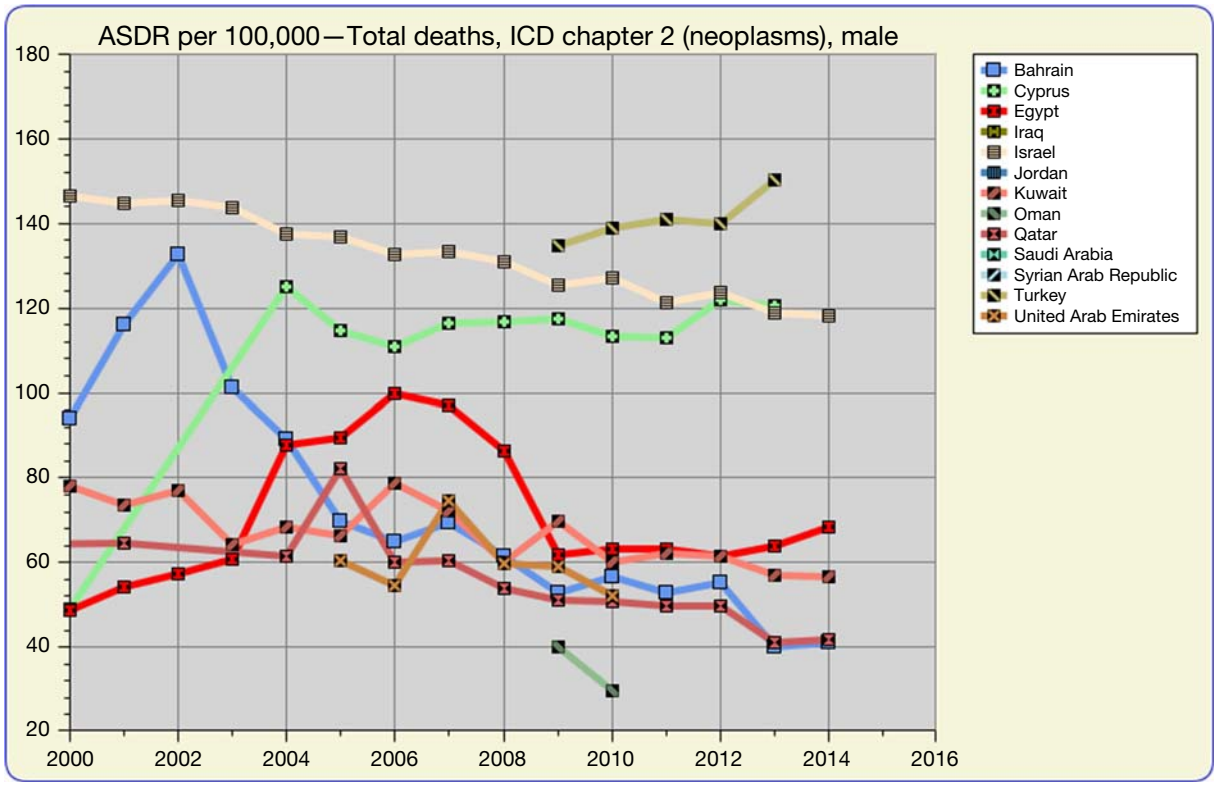

Fig. 1 ASDR per 100.000 total deaths, male.

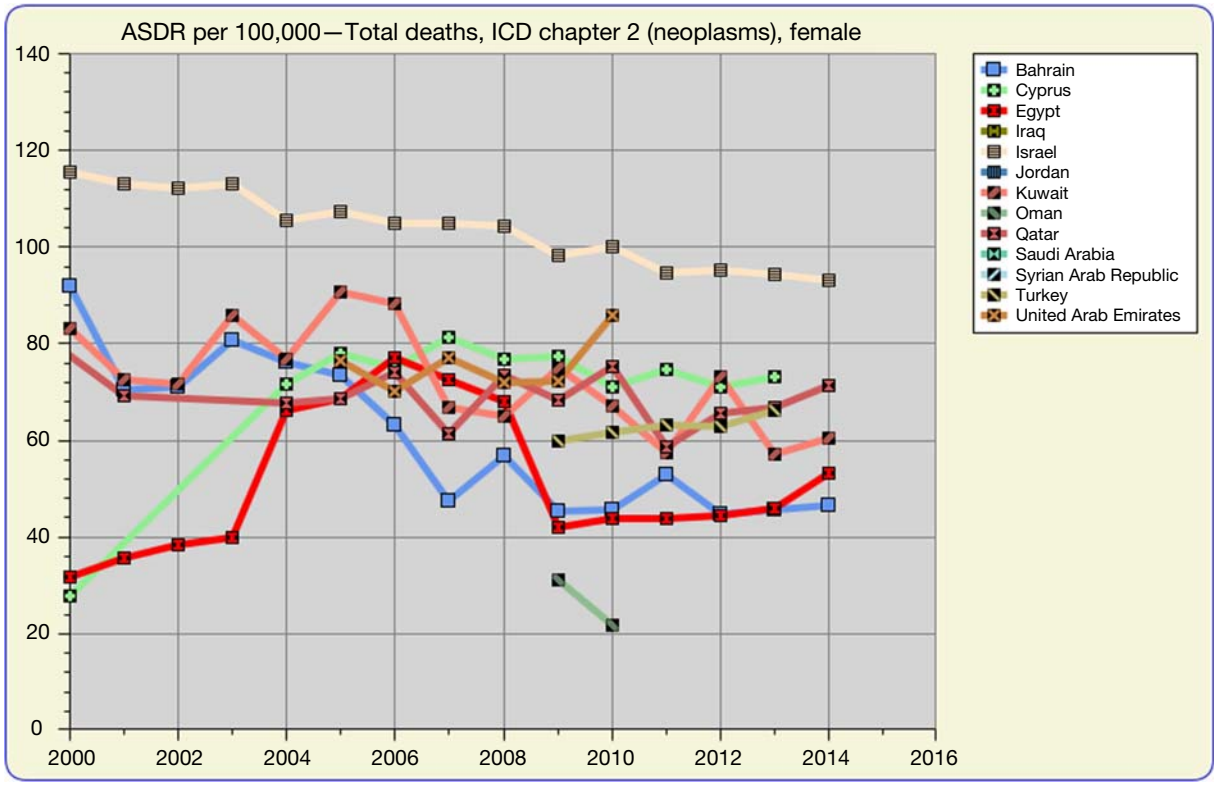

Fig. 2 ASDR per 100.000 total deaths, female.

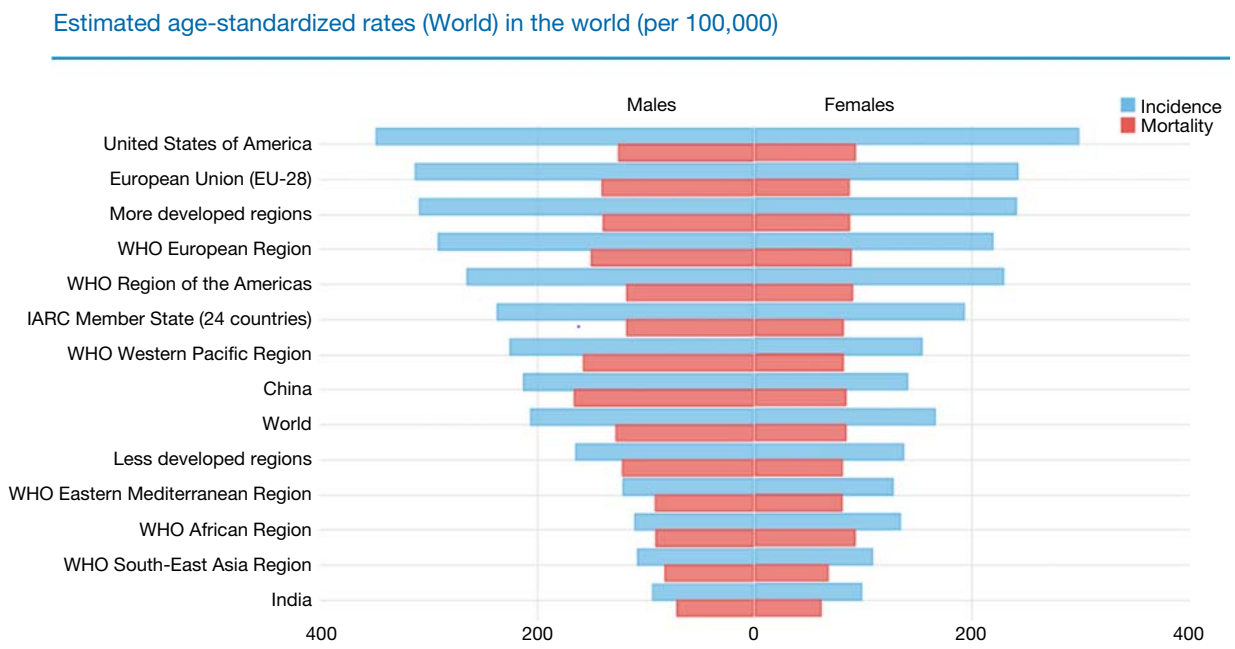

Fig. 3 Estimated age-standardized rates per 100.000 in the world.

not only among elderly but also in youth, university students, and even high-school-aged children. Daily waterpipe smoking produced a 24-h urinary cotinine level that is equivalent to smoking of 10 (95% CI: 7–13) cigarettes/day. Occasional waterpipe smoking (one session of waterpipe use during a 4-day period) produced a 24-h urinary cotinine level equivalent to smoking of two cigarettes in one pipe session. Active tobacco smoking has been considered as a strong risk factor for lung cancer, with an average risk ratio (RR) of 15–30. However, One fourth of lung cancer cases are not attributable to active smoking. The association also between second-hand smoking (i.e., environmental tobacco smoke) and lung cancer was established for all histological types of lung cancer: 1.26 (95% CI: 1.10–1.44) for adenocarcinoma, 1.41 (95% CI: 0.99–1.99) for squamous cell carcinoma, 1.48 (95% CI: 0.89–2.45) for large cell lung cancer, and 3.09 (95% CI: 1.62–5.89) for small cell lung cancer. Bladder cancer, which reports the second common incident cancer type among males in The ME, is proved to be triggered by smoking with a causal association between bladder cancer and current (RR 3.14) or former (RR 1.83) cigarette smoking, pipe (RR 1.9), or cigar (RR 2.3) smoking. Exposure to tobacco smoke causes a sharp drop in oral peroxidase (OPO) activity, an enzyme in the saliva resulting in increased incidence of oral cancer. Tobacco smoke was more likely to overexpress p53 that is the most frequently mutated gene in human oral cancer. WHO revealed the prevalence of current tobacco smoking (2011) to be the highest in Tunisia, Jordan, and Egypt for men and Lebanon, Israel, and Cyprus for women. In Jordan, Narghile (waterpipe) smoking was significantly associated with oral cancer at a younger age (45 years). A few studies from the ME illustrated the association between excessive alcohol intake and cancer risk. WHO reported low alcohol intake in most of ME countries with a relative increase in Cyprus, Jordan, UAE, and Turkey (Table 3). Excessive alcohol drinking is reported to be associated with increased incidence of oral cancer in Jordan (17%) and Sudan (10%).

Obesity, Unhealthy Lifestyle, and Environmental Factors

Many countries of the ME showed a rising pattern of cancer due to changes in the dietary habits. These countries could be divided into high- and low-income countries. The dietary habits of dates, milk, excess fruit and vegetables intake were replaced with an excess intake of energy-dense foods rich in fat and free sugars and deficient in complex carbohydrates, with daily energy intake exceeding 3000 kcal/per capita especially in high income Arab countries (e.g., Bahrain, Kuwait, Oman, Qatar, Saudi Arabia, and United Arab Emirates) resulting in overnutrition. In low-income Arab countries, contaminated food with carcinogens as well as the unhealthy life style, obesity, and lack of physical activity contribute significantly to increased cancer incidence (Table 3). Higher dietary inflammatory index (DII) was associated with increased odds of colorectal cancer among Jordanian population ($OR_{continuous\ DII} = 1.45$, 95% CI: 1.13, 1.85). Food contamination by mycotoxins has been recognized as a public health threat. Aflatoxin B1 (AFB1), Aflatoxin M1 (AFM1), as well as Ochratoxin A (OTA) and Deoxynivalenol (DON) are considered potent toxic mycotoxins. AFB1 is the strongest carcinogen and chronic exposure to AFB1 was considered to increase the risk of liver cancer especially when associated with hepatitis B or C. AFB1 has been classified as "carcinogenic to humans" (group 1) by the International Agency for Research on Cancer. The carcinogenicity of AFB1 is 10 times more than that of AFM1. Four mycotoxins (AFB1, AFM1, OTA, DON) were evaluated in diet of adult Lebanese urban population. Average and excessive consumer exposure estimates (p95) were calculated and compared with appropriate

toxicological reference values (TRVs) of these myotoxins. Average dietary exposure levels to OTA and DON represented 29.9% and 156.8% of the respective TRVs, with the p95 exposure estimates approaching or exceeding the TRVs for these mycotoxins (95.1% and 355.8%, respectively). Average dietary exposure to AFB1 and AFM1 was associated with increased cancer risk to 0.0527–0.0545 cases/100,000 persons/year and a population risk of 0.0018–0.0027 cases/100,000 persons/year, respectively. Polycyclic aromatic hydrocarbons (PAHs) and their derivatives are also associated with many cancer types including prostate cancer which reports the highest incidence in Israel and the second incident type in Turkey. The adult population in Israel is widely exposed to PAHs. The Urinary concentrations of all PAHs were significantly higher among current smokers and increased significantly with smoking frequency. Dermal and inhalation exposures to PAHs were also assessed in the Turkish population. >99% for inhalation and 28% for dermal exposure route exceeded the acceptable level in the Turkish population.

Infection

In 2012, 15% of cancer is attributable to infection. Many types of infections can cause cancer, human papilloma virus (HPV), *Helicobacter pylori*, hepatitis B, C viruses, and Epstein-Barr virus (EBV).

Human papilloma virus

Recent studies proved the contribution of HPV, the most common sexually transmitted infection, not only to the progression of cervical cancer but also to head and neck, breast and colorectal cancer, with roughly 30%–80% of these cancer types are positive for high-risk HPV infection. HPV include onco proteins (e.g., E5, E6, and E7) induce cellular alteration and lead to carcinogenesis. Recent evidence showed a prevalence of ~50%–90% of HPV in cases of cervical cancer in ME countries. Studies from Syria, Turkey, and Tunisia on women with breast cancer concluded the presence of HBV types 16, 18, 31, 33, and 35 and their role in initial and progression of cancer.

Hepatitis C, B viruses (HCV, HBV) and human immunodeficiency virus

HCV reports high prevalence in Northern Africa, which is highly notable in Egypt that recorded the highest prevalence of HCV worldwide (15%–20%) especially after massive treatment of Schistosomiasis. Also, high rates were reported in Pakistan. Many studies addressed the association between HCV and hepatocellular carcinoma. In 2010, worldwide deaths associated-HCC reached 77% from HBV/HCV-linked HCC. Meta-analyses were conducted and proved the association between HCV and hepatocellular carcinoma; HCV and HBV coinfection was determined as independent risk factor for HCC occurrence. In Egypt, Prevalence of HBV and HCV is 6.7% and 13.9% among patients without HCC, and 25.9% and 78.5% among HCC patients. The estimated pooled OR was higher in regions where HCV is predominant; OR 16.8 (95%: 11.9–24.1) in Mediterranean countries. Cohort and case control studies were also conducted and proved the association between HCV and HCC after taking into account the confounding effect of smoking and alcohol consumption. Hepatitis C virus genotype 4 is the most prominent in Arab countries of the ME including Egypt, Iraq, Saudi Arabia, and Syria with rates of 86% (95% CI: 85%–88%), 60% (95% CI: 56%–64%), 56% (95% CI: 54%–55%), and 57% (95% CI: 54%–61%), respectively. Hepatitis C virus genotype 1 is the most prevalent in non-Arab countries in the ME region including Turkey, Iran, Cyprus, and Israel. A Joint epidemiology of HIV and HCV infections was pronounced in the ME region especially among injecting drug users,***[29] HCV infection could predict HIV prevalence especially low-level HIV epidemic $RR_{HCV/HIV}$ 16.3 (95% CI: 11.5–23.1) rather than emerging epidemic $RR_{HCV/HIV}$ 3.8 (95% CI: 3.1–4.7) or established HIV epidemic $RR_{HCV/HIV}$ 2.8 (95% CI: 2.1–3.6). HIV epidemic was high among injecting drug users in Egypt, Lebanon, Palestine, Saudi Arabia, and Syria, and less in Afghanistan, Morocco, and Tunisia.

Epstein-Barr Virus (EBV) and H. pylori

Iran reported the highest incidence of gastric carcinoma in the ME. However, EBV-Gastric Carcinomas (EBV-GCs) occur in 3% of the gastric carcinomas in Iran and most of gastric carcinoma are EBV negative. The causal association could be proved by virus predisposition in neoplastic cells and colonization in tumor cells by in situ hybridization of EBV-encoded small RNA-1(EBER-1), but not in surrounding epithelial cells. The most commonly expressed latent proteins of EBV are EBNA1 (98.1%) and LMP2A (53.8%) which differ from the standard pattern of EBV proteins transcription in cases of gastric carcinomas. So, more researches are needed to explain EBV-associated carcinogenesis. Nasopharyngeal carcinoma (NPC) is rare cancer in ME, but the geographical variation of this cancer is due to combined Epstein-Barr virus (EBV) infection, genetic predisposition, and environmental factors including diet. Breast cancers showed a positive EBV genome in 40.0% of cases in Algeria, and 32.8% of cases from Tunisia. The incidence of non-Hodgkin's lymphoma (NHL) caused by EBV is increasing in North Africa especially Burkitt's lymphoma where EBV could be detected in almost all cases but less frequently observed in sporadic lymphoma. The risk of NHL in human immunodeficiency virus-seropositive (HIV+) subjects is also double that of HIV-seronegative (HIV−) individuals. Regarding *H. pylori* infection, the cytotoxin associated gene A (CagA) protein is one of *H. pylori* virulent proteins associated with atrophic gastritis, peptic ulcer, and gastric carcinogenesis. The distribution of this protein is high in Turkey, Iran, Iraq, and very low in Egypt. The Vacuolating cytotoxin activity (VacA) is another *H. pylori* virulence factor that is associated with gastric cancer and gastric ulcer in Iran and Iraq.

Preventive Strategies and Cancer Control

Core components for comprehensive cancer control include prevention, screening, early diagnosis, treatment, and palliative care.

Cancer Primary Prevention Policies

While primary prevention presents the optimal solution for cancer control based upon knowing the causative agents, many preventive measures are not suitable for certain cancer types. It is important to consider the available local evidence and availability of country-specific resources.

Tobacco smoking and alcohol

Low-middle income countries are fighting with tobacco dependence in the ME. By 2030, death forecast reveals that these countries will suffer from 80% of tobacco deaths. Collaborative efforts should be exerted to apply the consensus smoking cessation guidelines considering the limited available resources. The guideline documents involve the following domains for tobacco control:

Policies for tobacco prevention

Six policies are highly cost-effective and suitable for ME countries to reduce tobacco demands:

- Higher taxes on cigarettes and other tobacco products
- Bans/restrictions on smoking in public and workplaces
- Comprehensive bans on advertising of all tobacco products
- Better consumer awareness and information
- Large warning labels on all tobacco products
- Support programs for smoking cessation

Support programs for treating tobacco dependence

A program for treating tobacco dependence includes psychological, behavioral, and pharmacological therapies. Clinicians should consider that tobacco smokers may persist for several years till achieving the required outcome and they may pass through several cycles of attempts, remission, and relapse. Health care providers could use either the individualized approach by using the most effective treatment strategy or the combined approach of either counseling with medications or several types of therapies. Two forms of counseling are effective: practical counseling (problem solving/skills training) and social support delivered as part of treatment. The combination of counseling and medication is more effective than either alone. Brief interventions are also effective especially in low-middle income countries as could reach large population within short period (10 min) of time. The 5 A model is an example of these interventions that can be conducted in the primary care settings (**Fig. 4**). It consists of ask, advise, assess, assist, and arrange for the treatment of tobacco dependence. Another approach is the algorithm derived from the New Zealand Ministry of Health guidelines. The following are summaries of the recommendations that could be adapted in the ME:

- Ask about smoking status.
- Give positive feedback with regular checkup for nonsmokers.
- Advice smokers to quit tobacco use.
- If the smoker has a smoking-related disease, advice could be linked to the related condition to encourage quitting.
- The smoking status should be checked every one year for those willing to quit to ensure cessation.
- If primary health care worker could not provide support, he should refer the patients to the appropriate smoking cessation services.
- Support of smoking cessation services involves setting of cessation date, arranging of follow-up visits especially at the start of cessation, and providing the suitable medications. First-line medications reliably increase long-term smoking abstinence rates:
  - Bupropion (sustained release, usual duration up to 14 weeks)
  - Nicotine gum (usual duration 6–14 weeks)
  - Nicotine inhaler (usual duration up to 6 months)
  - Nicotine nasal spray (usual duration up to 6 months)
  - Nicotine patch (usual duration 6–14 weeks)
  - Varenicline (usual duration up to 14 weeks)

Updated data from ME are needed to describe the observable new rising trend of alcohol intake. WHO promotes public policies worldwide that can be implemented in the ME to reduce the burden of harmful use of alcohol:

- Regulating the marketing of alcoholic beverages (in particular to younger people);
- Restricting availability of alcohol;
- Reducing demand through taxation and pricing mechanisms;
- Raising the public awareness of different health problems caused by excessive use of alcohol and ensuring support for effective alcohol policies;

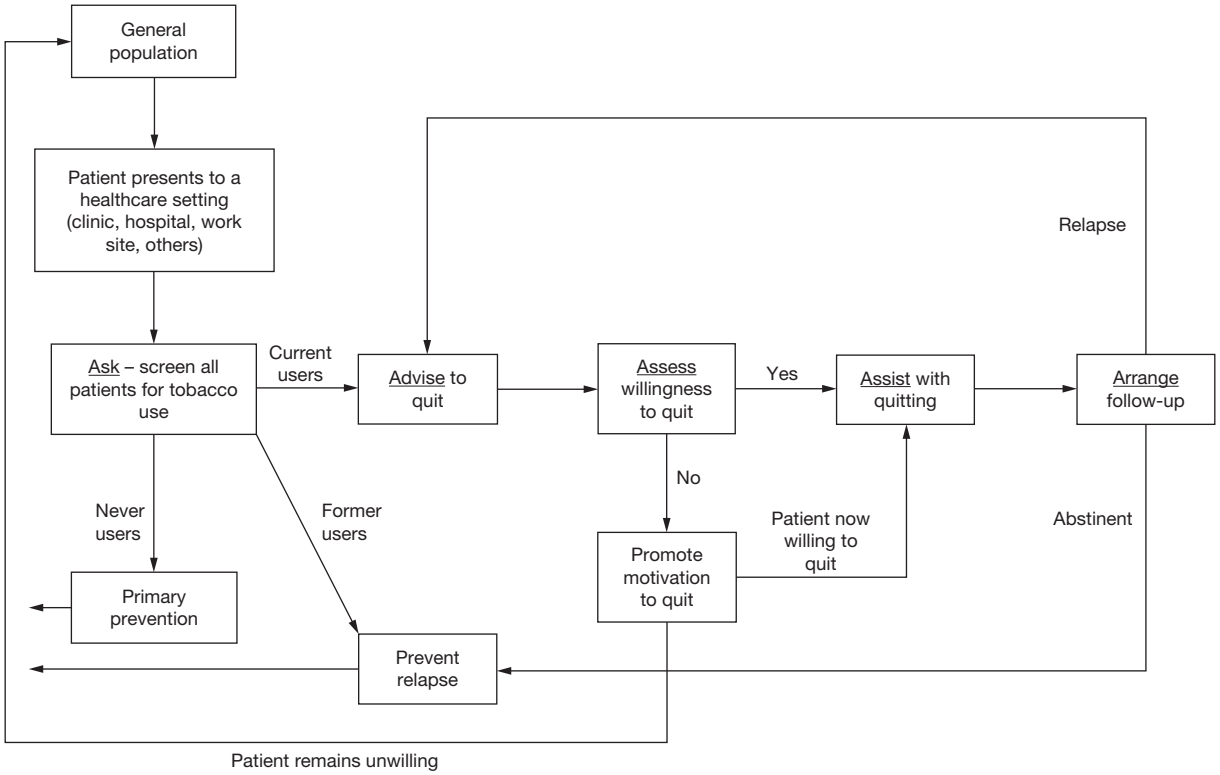

Fig. 4 The 5 A model for treating tobacco dependence.

- Providing accessible and affordable treatment for alcohol use-associated disorders; and
- Implementing screening programs for hazardous and harmful drinking in health services.

Obesity, unhealthy lifestyle, and environmental factors

Although cancer incidence in the Non-European Mediterranean region is still now less than that reported in the European Union, projection of the next two decades denotes a sharp rising trend which could be attributed to insufficient effective cancer prevention strategies and control programs together with population aging and worsening of dietary and environmental habits. Health care professional should apply the appropriate counseling for high-risk population.

Infection

Human papilloma virus

Control of HPV infection can reduce the incidence of cervical as well as other cancer types as breast cancer that was proven by two metaanalyses and a study from the ME. Vaccination for human papilloma is highly effective, safe, and licensed by national control authorities in developed countries. However, vaccines are not easily available in most of developing countries in the ME (**Table 4**). Some countries even lack the appropriate investigations for detection of high-risk HPV besides the expensive vaccination programs that are not affordable in many ME countries. Synthesis of population and epidemiologic data for 20 countries in the Extended Middle East and North Africa (EMENA) was done to study the effect of vaccination in preadolescent girls estimated by averted cervical cancer cases and deaths, disability-adjusted life years (DALYs) and cost-effectiveness ratios (I$ [international dollars] per DALY averted). When the cost per vaccinated girl exceeded I$50 in Lebanon and Turkey and I$150 in Algeria, the vaccine was not cost-effective. It was affordable only in five countries when its cost was I$200. Two available vaccines are available according to HPV type; Gardasil® for HPC types 6, 11, 16, and 18 and Cervarix for HPV types 16 and 18. However, these vaccines can protect against only 70% of HPV infection and not effective for high-risk HPV virulent strains that trigger various types of cancer. The United Arab Emirates health system was the first to introduce HPV free of charge vaccine for high school girls. One of the best preventive measures in addition to sexual health education is the development of vaccine covering the most frequent 9 types of HPV that cause carcinogenesis (6, 11, 1, 18, 31, 33, 45, 52, and 58) but not yet implemented in the high-risk countries of ME.

Hepatitis C virus

Egypt represents the highest burden of Hepatitis C virus in the ME. However, the prevalence of HCV was reduced by 30% from 2008 to 2015. Strategies to eliminate HCV subsequently reduced the incidence of HCC. Recently, the National Committee for Control of

Table 4 Cancer primary prevention policies and strategies

	Tobacco control						*Obesity prevention and control*	*Physical inactivity prevention*	*Harmful use of alcohol prevention*	*National immunization*		
Country	*Operational policy, strategy or action plan*	*Smoke-free legislation (number of smoke free public places)*	*Tobacco dependence treatment (NRT and/or some cessation services)*	*Warning labels (no/yes)*	*Bans on advertising, promotion and sponsorship*[a]	*Tobacco taxes (% of tax in retail price)*	*Presence of an operational policy, strategy or action plan*			*HPV vaccine schedule*	*Hepatitis B vaccine schedule*	*Hepatitis B vaccine coverage, infants*
Afghanestan	No	3–5	Yes	No	Yes	≤25	No	No	No	–	–	71%
Bahrain	Yes	Up to two	Yes	Yes	Yes	≤25	Yes	Yes	Yes	–	Birth, + 1 month	99%
Cyprus	**Yes**	Six to seven	Yes	Yes	Yes	>75%	Yes	DK	DK	–	2, + 4, + 8 to 12 months	96%
Dibouti	**No**	Six to seven	Yes	Yes	Yes	26%–50%	No	No	No	–	Birth	82%
Egypt	**Yes**	Three to five	Yes	Yes	Yes	51%–75%	No	No	No	–	months, + 4 months,+ 6 months	97%
Iran	Yes	All public places completely smoke-free	Yes + national quit line	Yes	Yes	≤25%	Yes	Yes	Yes	–	Birth, + 2, + 6 months	99%
Iraq	Yes	Up to two	Yes + national quit line	No	Yes	≤25	Yes	Yes	DK	–	Birth	66%
Israel	No	Up to two public places	Yes + national quit line	Yes	Complete absence of ban	>75%	No	No	Yes	13 years, + 2 months, + 6 months	Birth, + 1, + 6 months	98%
Jordan	Yes	Three to five	Yes + national quit line	Yes	Yes	>75%	Yes	Yes	Yes	–	3, + 4, + 5 months	98%
Kwait	No	Six to seven	Yes + national quit line	Yes	Yes	≤25%	No	No	No	–	Birth	99%
Lebanon	Yes	all	Yes	Yes	Yes	26%–50%	DK			–	Birth	81%

Libya	No	all	Yes	No or small size	Yes	≤25%	No	No	No	15 years (×3)	Birth	98%
Morocco	Yes	Three to five	Three to five	No or small size	Yes	51%–75%	No	No	No	–	Birth	99%
Oman	Yes	Data not recognized	Yes	Yes	Complete absence of ban	≤25%	Yes	Yes	No	–	Birth	97%
Pakistan	No	all	Yes	Yes	Complete absence of ban	51%–75%	No	No	No	–	–	72%
Qatar	Yes	Up to two	Yes	Yes	Yes	≤25%	Yes	Yes	Yes	–	Birth	99%
Saudi Arabia	Yes	Six to seven	Yes	Yes	Complete absence of ban	≤25%	Yes	Yes	No	–	Birth	98%
Somalia	No	Up to two	None	No or small size	Complete absence of ban	≤25%	No	No	No	–	–	34%
Sudan	Yes	Up to two	None	No or small size	Yes	51%–75%	Yes	Yes	No	–	–	98%
Syria	Yes	Six to seven	Yes	No or small size	Yes	51%–75%	No	No	No	–	Birth, + 2, + 6 months	71%
Tunisia	Yes	Up to two	Yes	No or small size	Yes	>75%	No	No	No	–	Birth	98%
Turkey	Yes	All	Yes + national quit line	Yes	Yes	>75%	Yes	Yes	No	–	Birth, + 1, + 6 months	97%
UAE	No	Three to five	Yes + national quit line	Yes	Yes	≤25%	No	No	No	–	Birth, + 2, + 6 months	94%
Yemen	No	Up to two	Yes	Yes	Yes	51%–75%	No	No	No	–	–	88%

DK: Country responded "don't know".

– Data not available.

[a]Ban on national television, radio and print media.

Source: WHO Cancer country profiles (http://www.who.int/cancer/country-profiles/en/).

Viral Hepatitis (NCCVH) was established to provide a novel model of care (MOC). The mission of MOC is to provide national treatment programs supplying antiviral therapy either at very minimum cost or totally free of charge. Egypt has succeeded to negotiate in reducing the price of sofosbuvir with previous commitment in treatment by Peg-interferon/RBV. Preventive strategies were provided to raise public awareness especially the high-risk groups to further minimize the risk of HCV transmission, improve infection control, ensure safe injection practices, and adopt safe blood products. The MOC activity also establishes the practice of adapted national guidelines for chronic HCV treatment as supplied by national expert hepatologists and training of health care professionals for efficient counseling of chronic HCV patients. The national guidelines were adapted from guidelines for screening and management of patients with Hepatitis C virus. It involves stepwise approach for screening, diagnosis, and treatment of HCV infection and follow-up of patients to prevent hepatocellular carcinoma.

- *Screening for HCV*: Patients on haemodialysis, blood/tissue donors, and health care professional who are exposed to procedures should be tested for HCV. Screening also includes patients with persistently elevated alanine aminotransferase, injecting drug users, patients diagnosed with HIV infection, patients with medical and dental treatment or those making tattoo or piercing in ME countries known to be endemic with HCV, children born to HCV-infected mothers and finally, household contact with HCV-infected patients.
- *HCV diagnostic testing*: It should be tested on serum or plasma where possible and HCV genotyping should be undertaken if antiviral therapy is being considered. If health care professionals are suspected of percutaneous exposure to infection, they should be tested for HCV ribonucleic acid (RNA) at 6, 12, and 24 weeks and anti-HCV testing should be considered at 12 and 24 weeks.
- *Treatment of acute infection*: Patients require close monitoring, both clinical and laboratory for the first 3 months so that the infection may be resolved spontaneously. They should be offered IFN therapy either pegylated IFN or nonpegylated IFN for 24 weeks regardless the genotype.
- *Treatment of chronic HCV infection*: All patients should be treated with antiviral therapy and sustained virological response should be a marker for viral clearance. The selection of antiviral therapy is based on the genotype:

– Genotype 1

Treatment: Naïve and treatment-experienced patients should be treated with pegylated IFN, weight-based ribavirin, and a protease inhibitor as triple therapy. The duration of therapy varies depending on early viral response at 12 weeks. The treatment should continue until 48 weeks; those with still positive virus RNA at 24 weeks, should discontinue the treatment.

– Genotype 4

Patients with HCV genotype 4 infections: Standard treatment should be 48 weeks of pegylated IFN and weight-based ribavirin.

Caution should be taken to avoid adverse effects of interferon (IFN) and ribavirin therapy. They should not be prescribed in pregnant women and in patients with psychiatric illnesses.

Hepatitis B virus

Hepatocellular carcinoma is caused by HBV in most ME countries with the exception of Egypt and Pakistan. Prevention of HBV involves three levels: primary prevention by effective HBV vaccination for healthy population, secondary prevention through hepatitis B antiviral agents, and tertiary prevention to prevent infection recurrence. Hepatitis B vaccine is safe and very cost-effective as licensed and incorporated by WHO into expanded program immunization (EPI) worldwide with wide coverage in the low-middle income countries (>90% response rate in nonimmunocompromized subjects). It is available in three doses that significantly reduce the prevalence of HBsAg in children and young adults (**Table 4**). WHO recommends a birth dose vaccine given to all newborns within the 24 h after birth. Although the birth dose vaccine significantly reduced HBV infection by about 70% in infants born to highly infectious mothers, not all African countries adopt it; even these counties face difficulties in implementing the vaccine. Some Countries didn't not yet integrate it. This may be due to the minimal estimated perinatal transmission and lack of sufficient evidence and clinical trials conduction for it in pregnant women in the African countries (**Table 4**).

The following strategies are needed for better control of both HCV and HBV infections and decrease HCC incidence:

- *Operational research programs*: Epidemiological surveys and population-based screening programs are needed to assess research priorities about risk factors of transmission and to determine cost-effective approaches for screening and treatment. Unfortunately, most of the screening programs in Africa are expensive and are not of considerable quality. The PROLIFICA (Prevention of Liver Fibrosis and Cancer in Africa) program is a screening regimen that was conducted in Gambia, not in the ME. It is concluded that only 1% of positive HBs antigen in the adult previously tested and aware of their diagnosis. Though the PROLIFICA program estimated around 50% of HBV infection in the Gambia was attributed to vertical mother to infant transmission, only 3% of infants received the birth dose HBV vaccine due to recognized social and financial barriers that are similar to the situation in many ME countries.
- *Improving screening and early treatment of infected patients*: WHO facilitates the selection criteria for infected patients who need early treatment in resources constrained countries: (a) A patient who is HBsAg positive and has cirrhosis and (b) patients with HBsAg who are over 30 and have persistently raised alanine aminotransferase (ALT) levels. Policy makers should focus on resource allocation for treatment, negotiation with generic drugs production companies to supply drugs at lower prices. Treatment of

HBV is challenging in the ME. Proper screening and treatment programs are lacking in many ME countries because the cost of the programs are the responsibility of patients and due to their availability in private sector only. Tenovir, the drug of choice for HBV treatment, is supplied by the global funds at a lower price of $50 per patient per year but not for HBV monoinfection and even not licensed by some African countries.

- *Surveillance for HCC in cirrhotic patients*: Cirrhosis is the main causative agent for occurrence of HCC. Regular surveillance interval at 6 months is recommended than annual surveillance to improve the survival of cirrhotic patients and minimize the incidence of HCC. The best-known surveillance option is the liver ultrasound that is available with good accuracy in most of ME countries, but not in the rural areas and it is highly dependent on operator experience. It is imperative to train health care providers on ultrasound as an easy and applicable surveillance system tool.

H. pylori infection

WHO recommends all countries consider screening for *H. pylori* for primary prevention of gastric cancer. The International Agency for Research on Cancer (IARC) *Working Group of international experts suggested population-based* screening for eradication of *H. pylori* which reduce the incidence of gastric cancer by 30%–40%. However, low-middle income countries recognized with high burden of gastric cancer do not properly implement this screening program. The screening program consists of three steps: *First:* culture, histology, rapid urease test (RUT), histopathology, PCR, and a serology test to screen for *H. pylori* in healthy individuals. Second step involves the eradication treatment with antibiotics from a primary care doctor if required, then the final step is retest by urea breath test (UBT), stool antigen test (SAT), and second-line treatment if required. *H. pylori* screening programs are cost-effective. A cost-effectiveness of a *H. pylori* serology-based screening program in New Zealand, a country with relatively high gastric cancer rates, illustrated that the incremental cost for national screening program was US$196 million (95% uncertainty interval [95% UI]: $182-$211 million) with health gains of 14,200 quality adjusted life years (QALYs) (95% UI: 5,100–26,300) and the screening program was more cost-effective by US$8,000 ($3,800-$18,500) per QALY.

H. pylori eradication therapy was proved to protect against the occurrence of gastric carcinoma and reduce its incidence (risk ratio (RR) 0.66; 95% CI: 0.46–0.95). However, in patients with preneoplastic lesions, *H. pylori* eradication measures do not play a significant role for gastric cancer prevention. It is available in many forms: (1) proton pump inhibitor (PPI) dual therapy (PPI plus either amoxicillin or clarithromycin); (2) PPI triple therapy (PPI plus any two of the following: amoxicillin, macrolide, 5-nitroimidazole); (3) histamine 2-receptor antagonist (H2RA) triple therapy (H2RA plus any two of the following: amoxicillin, macrolide, 5-nitro imidazole); (4) bismuth triple therapy (bismuth salt and 5-nitroimidazole with either amoxicillin or tetracycline); (5) bismuth quadruple therapy (as bismuth triple therapy, but with the addition of a PPI); (6) ranitidine bismuth citrate (RBC) dual therapy (RBC plus either amoxicillin or clarithromycin); (7) RBC triple therapy (RBC plus any two of the following: amoxicillin, macrolide, 5-nitroimidazole); (8) clarithromycin monotherapy. Antibiotic resistance is a possible cause of *H. pylori* treatment failure in the ME. The combined levofloxacin, and clarithromycin and esomeprazole-based regimen as first-line triple therapy for *H. pylori* eradication can give better eradication rate with same safety when compared with standard triple therapy (clarithromycin 500 mg twice daily, Amoxicillin 1000 mg twice daily, plus esomeprazole 20 mg twice daily for 7 days). The rapid urease test (RUT) is a popular, easy, and cheap screening test based on the presence of Urease in the *H. pylori*-infected gastric mucosa. It could be easily used in low-middle income countries of the ME. However, the former intake of proton pump inhibitors (PPI) should be taken into account prior to testing to avoid false negative results. Also, More than 10,000 bacterial cells are required to achieve positive test results.

In Iranian population, Gemifloxacin-containing quadruple therapy (amoxicillin 1 g, bismuth 240 mg, and omeprazole 20 mg for 14 days) provides high *H. pylori* eradication rate ($\geq$90% per protocol cure rate) after the failure of first-line standard quadruple therapy (clarithromycin–amoxicillin–bismuth–omeprazole).

Early screening and prevention of EBV could prevent gastric carcinoma, lymphoma, and nasopharyngeal carcinoma. IgA antibody titres to EBV viral capsid antigen (EBV-IgA-VCA) and EBV early antigen (EBV-EA) can be used for the serologic screening of nasopharyngeal carcinoma. However, even after screening, attempts to develop vaccines continue and still under investigations because the success of this vaccine depends on cancer pattern in each country and the cost versus benefit.**Table 4** illustrates the summary of cancer primary prevention policies in all ME countries as adapted from WHO country profiles report.

Cancer Screening and Early Detection

The focus of cancer control is to diagnose cancer in the earliest possible curable stage, thus improving the quality of life and prognosis of cancer patients.

WHO summarized the major elements for cancer early diagnosis into three main domains:

1. *Step 1*: Awareness of cancer symptoms and accessing care
2. *Step 2*: Clinical evaluation, diagnosis, and staging
3. *Step 3*: Access to treatment, including pain relief.

Step 1: Awareness of cancer symptoms and accessing care

The patient should be aware of cancer symptoms (symptom appraisal interval). He or she should be encouraged to overcome fear and cancer stigma, and access primary care. Thus, awareness of symptoms will be translated into health-seeking interval.

Barriers

(a) Poor health literacy
(b) Cancer stigma
(c) Poor access to primary care

Solutions

(a) Empower and engage people and communities
(b) Improve health literacy and reduce cancer stigma
(c) Leadership and governance to improve access to care

Step 2: Clinical evaluation, diagnosis, and staging

This step is known as diagnosis interval. It involves three stages: First, the patient should be evaluated by health care provider to ensure if cancer may be present (diagnostic evaluation); second, the patient receives diagnostic testing for confirmation including clinical, laboratory, and pathological confirmation testing and patients undergo staging to determine cancer spread (diagnostic testing and staging). Finally, if patient confirmed by cancer presence, he or she should be referred for treatment to specialized centers providing safe and effective treatment across ranges of modalities needed (treatment interval).

Barriers

(a) Inaccurate clinical assessment and delays in clinical diagnosis
(b) Inaccessible diagnostic testing, pathology, and staging
(c) Poor coordination and loss to follow-up

Solutions

(a) Improve provider capacity at first contact point
(b) Strengthen diagnostic and pathology services
(c) Develop referral mechanisms and integrated care
(d) Provide supportive counseling and people-centered care

Step 3: Access to treatment

Many patients from LMIC recently diagnosed with cancer do not immediately initiate treatment due to financial, geographic, logistical, and sociocultural barriers.**Table 5** shows how ME countries use cervical, breast, and colorectal cancer screening and early detection strategies at the public primary health care level for early diagnosis of cancer types.

An example of early detection program is the EUROMED CANCER Network project. It aimed to support the non-EU Mediterranean countries about the appropriate cancer screening programs. It illustrates the importance of public health surveillance through a structured questionnaire survey applied to 15 countries (Albania, Algeria, Bosnia and Herzegovina (BiH), Croatia, Egypt, Jordan, UN Interim Administration Mission in Kosovo, Lebanon, Montenegro, Morocco, Palestinian National Authority, Serbia, Syria, Tunisia, and Turkey). Many of them are ME countries. It is concluded that applying a uniform strengthening system for cancer control for all these countries may not be feasible. However, tailored surveillance of early detection programs considering each country's resources should start early with appropriate uniform health information system. One of the project's recommendations is breast cancer (BC) and cervical cancer (CC) screening as the commonest female cancers in the region. The most frequent breast cancer screening test is mammography, either alone or combined with clinical breast examination (CBE), breast self-examination (BSE), or ultrasound. It is feasible and applied in all project's studied countries (**Table 5**). The starting age in BC organized screening is 40–45 years in most countries but in others, screening starts even earlier. The age upper limit is 69 years in most countries, but in Lebanon, it goes up to 75. An interview by structured questionnaire in a multicenter survey among the Qatari women showed that fewer than half of the participants were aware of breast cancer screening recommendations; less than one third performed this screening. Around one-quarter of them stated that they were counseled by their doctors about appropriate BC screening. Most of the participants stated that they were ready for a mammogram appointment based on a recommendation by their health care professional, hence, notifying the important role of heath care providers for women counseling about different cancer screening programs. Many ME countries lack the National Organized Cervical Cancer Screening (NOCCS) program due to the insufficient political support of public health programs, lack of resources, little participation due to cultural factors, and insufficient women knowledge. This subsequently contributes to late detection of cervical cancer. Screening by Pap test could be possible in many countries (**Table 5**). It is usually in use starting at about 20 and ending at 60 years. In Morocco, an alternative to Pap test is visual inspection after application of acetic acid (VIA). Comparison of the cost-effectiveness of different cervical cancer (CC) screening tests in Lebanon using either cytology or HPV testing and at different coverage percentage concluded that 20% coverage by annual cytology testing reduced CC risk by 14% with an incremental cost-effectiveness ratio of I$80,670/year of life saved (YLS). Increased coverage to 50% with 3 and 6 years intervals yielded CC reduction of 26.1% and 21.4%, respectively at lower costs compared to 20% coverage with annual screening. Screening every 5 years with HPV DNA testing at 50% coverage resulted in 23.4% reduction in CC (than cytology at the same frequency) with an incremental cost-effectiveness ratio of I$12,210/YLS. The main implication of this research that repeated cytology covering a small percentage of women may be inefficient, while increased coverage up to

Table 5 Cancer screening early detection strategies and palliative care policies in Middle East countries

Country	Cervical cancer early detection		Breast cancer Early detection		Colorectal cancer Early detection		Cancer treatment and palliative care						
	Cervical cytology (PAP)	Acetic acid visualization (VIA)	Breast palpation/ clinical breast exam (CBE)	Mammogram	Faecal occult blood test or faecal immunological test	Screening by exam or colonoscopy	Radiotherapy	Total high energy teletherapy units/ million inhabitants	Number of radiotherapy centers	Number of radiation oncologists	Chemotherapy (medicines not specified)	Oral morphine (formulation not specified)	Community/ home care for people with advanced stage
	Availability at the public primary health care (PPHC) level (yes/no)										Availability at the public health system(yes/no)		
Afghanistan	No	No	No	No	No	No	No	**0**	**–**	–	No	Yes	No
Bahrain	Yes	No	Yes	Yes	Yes	Yes	Yes	**0**	**1**	8	Yes	Yes	Yes
Cyprus	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**2.6**	**1**	7	Yes	Yes	Yes
Dibouti	No		Yes		No		No	**–**	**–**	–	No	No	No
Egypt	No	Yes		No	Yes	No	No	**0.8**	**34**	237	No	Yes	No
Iran	Yes						Yes	**0.9**	**40**	147	Yes		No
Iraq	No		Yes	No			Yes	**0.2**	**8**	27	Yes	No	No
Israel	Yes	Yes	Yes	Yes	Yes	Yes	Yes	3.4	9	35	Yes		
Jordan	Yes	Yes	Yes	Yes	Yes	No	Yes	0.8	5	28	Yes		No
Kwait	Yes	No	Yes	No	No	No	Yes	1.2	1	–	Yes		
Lebanon	No	No	Yes	Yes	No	No	Yes	1.9	9	15	Yes		No
Libya	No	No	No	No	No	No	Yes	1	4	7	Yes		
Morocco	Yes	Yes	Yes	Yes	No	Yes	Yes	0.4	17	106	Yes	No	No
Oman	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0.6	1	5	Yes	No	No
Pakistan	No	No	No	No	No	No	No	0.1	26	31	No		
Qatar	Yes	No	Yes	No	Yes	No	Yes	0.9	1	3	Yes	Yes	No
Saudi Arabia	No	No	Yes	No	No	No	Yes	0.1	12	55	Yes	DK	DK
Somalia	No	No	Yes	No	No	No	No	–	–	–	No	No	No
Sudan	No	No	No	No	No	No	Yes	0.2	2	19	Yes	No	No
Syria	Yes	NR	Yes	Yes	Yes	Yes	Yes	0.3	2	9	Yes	Yes	DK
Tunisia	No	No	Yes	No	No	No	Yes	1.6	10	26	Yes	No	No
Turkey	Yes	No	Yes	Yes	Yes	Yes	Yes	2	96	501	Yes	No	Yes
UAE	No	No	Yes	Yes	Yes	No	Yes	0.6	3	6	Yes	Yes	No
Yemen	No	No	Yes	No	No	No	Yes	0.1	1	–	Yes	Yes	No

PPHC: public primary health care level.

DK: Country responded "don't know".

NR: Country replied to survey but did not give a response to specific question.

– Data not available.

Source: WHO Cancer country profiles (http://www.who.int/cancer/country-profiles/en/).

50% and extending screening interval are cost-effective. More attention should be paid toward novel HPV screening strategies providing greater CC reduction and more cost-effective than cytology. Colorectal cancer (CRC) screening program adoption is variable. While some countries as Lebanon did not provide any information either optimistic or organized about it, Turkey has a well-organized screening program. The immunochemical faecal occult blood test (i-FOBT) is the most commonly adopted test. In Oman, although CRC screening test could be implemented immediately upon physician's order, an operational cancer policy or action plan does not exist in addition to the lack of official statistics measuring regular uptake of screening services. A survey among Omani physicians and nurses to identify their practice toward CRC screening concluded that a few percentage did not exceed 40% who practice the following for screening:

- Order CRC screening for patients
- Refer patients to get CRC screening
- Health educates patients about CRC screening
- Order genetic testing for patients suspected of inherited susceptibility to CRC
- Health educate patients with suspected inherited susceptibility to CRC and refer them whenever possible for genetic testing

The following recommendations were perceived by Omani physicians and nurses to positively influence the process of CRC screening:

- Availability of system to identify patients eligible for CRC screening services
- Continuing professional education on cancer prevention
- Availability of a specialist in cancer
- Evidence published in scientific journals (somewhat influential)
- Policy on cancer screening

Barriers for implementation of CRC screening test were:

(a) Patients-related barriers

- Fear of finding out that they have cancer.
- Believe screening is not effective.
- Embarrassment or anxiety about screening tests.
- Unawareness of CRC screening tests and when they should be done.
- Patients do not perceive CRC as a serious health problem.
- Culture is not favorable to procedures used for CRC screening.

(b) System-related barriers:

- Screening costs are expensive.
- Long waiting time for screening appointments.
- Lack of hospital policy or protocol on cancer screening.
- Health care providers do not actively recommend CRC screening to patients.
- Shortage of trained health care workers to conduct CRC screening and follow-up with invasive procedures.

One of effective primary prevention tools for HCC is regular follow-up of cirrhotic patients with ultrasound. Early cancer detection measures for screening of HCC are serum alpha-fetoprotein (AFP), but still expensive with poor sensitivity and urinary markers. The urinary metabolite panel, comprising inosine, indole-3-acetate, galactose, and an *N*-acetylated amino acid (NAA), showed an area under ROC curve of 0.88 to differentiate between cases with HCC and only Cirrhosis. The measurement of Alfa-fetoprotein should not be used in isolation for screening or surveillance of the development of HCC in patients with hepatitis C, but, combined with ultrasound every six months and should be confined to patients with cirrhosis. Prostate cancer incidence is relatively low in ME with high incidence in Cyprus, Israel, and Somalia. Screening by total prostate-specific antigen (tPSA) may enhance early detection. A multicentered study from 22 Arab countries of the ME and North Africa investigates the proportion of patients with tPSA $>$ 10 ng/mL who had prostate cancer. Although evidence from Europe and USA denotes around 50% chance of prostate cancer with a high level of tPSA $>$ 10 ng/mL, there was prominent overlap in proportion of high PSA among ME patients with Prostatitis (20.5), benign prostatic hyperplasia (63.8), and prostate cancer (11.2). Absence of indicative tumor marker in light of low sensitivity and specificity of tPSA necessitates close follow-up of patients with high tPSA for a progressive decline in or return to normal PSA values with or without treatment of prostatitis, but certainly after transurethral resection of prostate (TURP) for large symptomatic BPH, to confirm the absence of prostate cancer. Persistent rising of tPSA is preliminary suggestive of prostate cancer and needs further investigation by trans rectal ultrasonography (TRUS) of the prostate and biopsy of suspicious lesions for histological diagnosis.

Palliative Care Policies

Cancer remains one of the most important challenges for the population in the ME. Around 50% of cancer patients consult physicians for the first time at stage 3 or 4. Nothing is left except palliation. Palliative care (PC) is defined according to WHO as "an approach that improves the quality of life (QOL) of patients and their families facing the problem associated with

life-threatening illness, through the prevention and relief of suffering by means of early identification, assessment, treatment of pain and other problems, physical, psychosocial and spiritual."

PC is still in its infancy in many ME countries. Application of cancer care model from western developed to developing countries proves to be unrealistic due to many reasons:

- The dysfunctioning primary health care system: Lack of well-trained family physicians in many countries compels cancer patients to seek treatments at tertiary care centers for minimal symptoms that could be managed by trained local treating physicians.
- Current beliefs that hinder the best cancer care: These include cultural, religious, and family opinions. Fear and stigma associated with cancer let many patients hesitate to seek treatment. Cultural concepts affect the female patients' adherence to treatment and follow-up due to insufficient professional female caregivers. Also, many terminal cases refuse cancer care due to "God's will" concept; therefore, patients are less demanding all possible treatments.
- Limited available cancer treatment in terms of radiotherapy and chemotherapy: While there is debate in developed countries about the use of newly advanced technology radiotherapy (e.g., proton beam, IGRT, IMRT, etc.), there are debates in some countries of the ME about who gets treated and who does not. Radiotherapy is cost-effective and 40% of cancer cure could be achieved by radiotherapy that should be available in every ME country. Analgesic opioids consumption is also the lowest in ME. Many physicians face patients' treatment rejection for fear of addiction.

One of the strategic actions for cancer prevention and control in the Eastern Mediterranean region was to improve cancer management and support PC and pain relief through the following action plans:

- Strengthen cancer diagnosis and treatment programs through all levels of care.
- Promote and implement interventions in childhood cancers at different levels of the health system.
- Strengthen the development of human resources in cancer management.
- Develop or strengthen PC services, including promotion of community nursing and home care.
- Ensure accessibility and affordability of PC medicines.
- Support integration of cancer management and PC in primary health care.

It is crucial to put resources difference apart from in ME countries and to develop a third part collaboration to integrate PC like Middle East Cancer Consortium (MECC). It is a nongovernmental unique valuable organization that collaborates with regional ministers of health and international health care organizations. Members of it include Egypt, Jordan, Palestine, Israel, Cyprus, Turkey, and the USA. Countries such as Pakistan, United Arab Emirates, Lebanon, Morocco, Iraq, Sudan, Qatar, and Oman are actively participating in MECC's training programs. MECC seeks to over bridge the barriers of palliative care by offering training for oncologists, developing palliative care programs, and improving communication between health care givers and patients. Many countries are in the capacity building stage to improve PC. Turkey initiated a model to other countries through a novel program whereby population-based and home-based palliative care teams were developed throughout the country, including peripheral areas where appropriate care was not available. Bahrain started PC unit in 2009 that serves patients through follow-up, pain clinics, and a hotline for home care patients. Jordanian Ministry of health is now integrating PC in inpatient and outpatient services with around 400 new patients per year and care for about 60–80 patients a month (30–40 patients through home care). However, attention should be paid to postgraduate education and training programs for health professionals. Oman is still in the earliest stage of PC services. The increasing incidence of cancer in Oman necessitates training of oncologists especially for opioid administration, and awareness programs for patients with cancer to help with the opioidphobia. Egypt with >90 million residents in 2015 and with cancer prevalence of 215,000 cases in 2012 has no oral morphine which is problematic in patients with end-stage cancer. Slow-release morphine tablets (30 mg) were previously prescribed and manufactured under licence by a single supplier in the United Kingdom of Great Britain and Northern Ireland. Nowadays, it is not available in Egypt. Immediate action should be taken to avoid such shortage and support cancer pain relief. PC is not also well established in other countries as in Iraq and Syria relative to advances in Saudi Arabia where specialized PC centers are available and embrace intensive management unit, consultation service, outpatient clinics, and home health care programs. The Saudi PC team consists of physicians, nurses, social workers, and other supportive care providers (dieticians, physical therapist, a unit translator, home health care nurses, health educator, and pharmacists). Dedicated PC centers in UAE and Qatar were well established and efforts are made to best integrate home health care services.

See also: Cancer Disparities. Cancer in Sub-Saharan Africa. Cancer in Populations in Transition.

Further Reading

El-Akel, W., El-Sayed, M.H., Kassas, M.E., et al., 2017. National treatment programme of hepatitis C in Egypt: Hepatitis C virus model of care. Journal of Viral Hepatitis. https://doi.org/10.1111/jvh.12668.

Fadhil, I., Lyons, G., Payne, S., 2017. Barriers to, and opportunities for, palliative care development in the eastern Mediterranean region. Lancet Oncology 18 (3), e176–e184. https://doi.org/10.1016/s1470-2045(17)30101-8.

Forman D, Bray F, Brewster DH, et al. 2013. Cancer Incidence in Five Continents (CI5). vol. 10. Retrieved from http://publications.iarc.fr/Databases/Iarc-Cancerbases/Cancer-Incidence-In-Five-Continents-Ci5-Volumes-I-To-X-2014.

Giordano, L., Bisanti, L., Salamina, G., et al., 2016. The EUROMED CANCER network: State-of-art of cancer screening programmes in non-EU Mediterranean countries. European Journal of Public Health 26 (1), 83–89. https://doi.org/10.1093/eurpub/ckv107.

Hacikamiloglu, E., Utku, E.S., Cukurova, Z., et al., 2016. Community palliative care in Turkey: A collaborative promoter to a new concept in the Middle East. Journal of Public Health Management and Practice 22 (1), 81–88. https://doi.org/10.1097/phh.0000000000000252.

Kim, J.J., Sharma, M., O'Shea, M., et al., 2013. Model-based impact and cost-effectiveness of cervical cancer prevention in the extended Middle East and North Africa (EMENA) (provisional abstract). Vaccine 31 (Suppl. 6), G65–G77. http://onlinelibrary.wiley.com/o/cochrane/cleed/articles/NHSEED-22013054067/frame.html.

Lehman, E.M., Wilson, M.L., 2009. Epidemiology of hepatitis viruses among hepatocellular carcinoma cases and healthy people in Egypt: A systematic review and meta-analysis. International Journal of Cancer 124 (3), 690–697. https://doi.org/10.1002/ijc.23937.

Lemoine, M., Eholié, S., Lacombe, K., 2015. Reducing the neglected burden of viral hepatitis in Africa: Strategies for a global approach. Journal of Hepatology 62 (2), 469–476.

Lemoine, M., Thursz, M.R., 2017. Battlefield against hepatitis B infection and HCC in Africa. Journal of Hepatology 66 (3), 645–654. https://doi.org/10.1016/j.jhep.2016.10.013.

Omar, S., Alieldin, N.H., Khatib, O.M., 2007. Cancer magnitude, challenges and control in the eastern Mediterranean region. Eastern Mediterranean Health Journal 13 (6), 1486–1496.

Sancho-Garnier, H., Khazraji, Y.C., Cherif, M.H., et al., 2013. Overview of cervical cancer screening practices in the extended Middle East and North Africa countries. Vaccine 31 (Suppl. 6), G51–57. https://doi.org/10.1016/j.vaccine.2012.06.046.

Sharma, M., Seoud, M., Kim, J.J., 2017. Cost-effectiveness of increasing cervical cancer screening coverage in the Middle East: An example from Lebanon. Vaccine 35 (4), 564–569. https://doi.org/10.1016/j.vaccine.2016.12.015.

Silbermann, M., Daher, M., Fahmi-Abdalla, R., et al., 2015. The Middle East cancer consortium promotes palliative care. Lancet 385 (9978), 1620–1621. https://doi.org/10.1016/s0140-6736(15)60791-7.

Silbermann, M., Epner, D.E., Charalambous, H., et al., 2013. Promoting new approaches for cancer care in the Middle East. Annals of Oncology 24 (Suppl. 7), vii5–vii10. https://doi.org/10.1093/annonc/mdt267.

Strategy for cancer prevention and control in the Eastern Mediterranean Region 2009–2013. Retrieved from. http://www.emro.who.int/health-topics/cancer/index.html (30 May 2017)

Relevant Websites

http://globocan.iarc.fr/—WHO. International Agency for Research on Cancer. GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012.
http://www.emro.who.int—World Health Organization. Regional Office for the Eastern Mediterranean.

Cancer Risk Reduction Through Lifestyle Changes☆

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Glossary

Epidemiology The science concerned with the study of factors determining and influencing the frequency and distribution of disease and other health-related events and their causes in a defined human population.

Micronutrient A vitamin or mineral that the body must obtain from outside sources. Micronutrients are essential to the body in small amounts because they are either components of enzymes (the minerals) or act as coenzymes in managing chemical reactions.

Nutrigenetics The science to identify how genetic variation affects response to nutrients.

Nutrigenomics The science of the effects of foods and food constituents on gene expression.

Oncogene Mutated and/or overexpressed version of a normal gene that in a dominant fashion can release the cell from normal restraints on growth and thus, alone or in concert with other changes, convert a cell into a tumor cell.

Pharmacotherapy The treatment of diseases or conditions by medicines.

Polymorphism The occurrence of different forms (alleles) of a gene (typically $>1\%$) in individual organisms or in organisms of the same species, independent of sexual variations.

Primary prevention The identification, control, and avoidance of environmental factors related to cancer development.

Randomized, controlled trials A clinical trial that uses a control group of people given an inactive substance (placebo) and an intervention group given the substance or action under study.

Sunscreen A substance applied to the skin to protect it from the effects of the sun's rays; sunscreens act by either absorbing ultraviolet (UV) radiation or reflecting incident light. Their effectiveness is rated by their sun protection factor (SPF); for example, a sunscreen with an SPF of 15 allows only 1/15 of the incident UV radiation or light to reach the skin.

Introduction

Cancer remains a leading cause of mortality. It is estimated that by 2020 there will be 16 million new cancer cases, and 10 million related deaths, whereas there may be >20 million new cases of cancer in 2030. Lifestyle behaviors are modifiable key factors for cancer prevention and cancer survivorship. It is now acknowledged that lifestyle habits including tobacco use, poor diet, physical inactivity, alcohol abuse and overexposure to sunlight are associated with 50% to 70% of all cancers, meaning that 281,000 to 395,000 cancer deaths each year could be prevented through changes in health behaviors in population level.

Effective interventions have been suggested in clinical trials for modifying lifestyle habits and specifically so for reducing tobacco use, increasing physical activity, reducing sun exposure, and reducing alcohol excess intake. Nonetheless millions of adults are engaged in risky behaviors: approximately 23% smoke, $<25\%$ follow dietary recommendations (e.g., about fruits and vegetables) and about 40% are physically inactive.

The previous figures may indicate a failure in transferring evidence-based findings to the general public. Therefore, disseminating the scientific knowledge about the importance of behavioral changes in cancer prevention and survivorship, as well as, delivering, through health care systems, effective interventions towards healthier lifestyle choices, are major, urgently needed tasks for public health policy makers worldwide.

Diet and Cancer

The association of diet with health outcomes, including cancer is of special interest due to the essential role of diet in human being. On the other hand, diet is a modifiable risk factor which can be directed towards healthier choices, in individual and population level through clinical advice and public health policies. Many studies focused on single foods, food groups or nutrients in association to cancer incidence/mortality worldwide. In addition, studies have also focused on the association of *dietary patterns* with cancer incidence and survival. Notably, the importance of the growing body of research regarding the benefits of healthy eating in cancer prevention/mortality is reflected in the recent reports of the World Cancer Research Fund (WCRF)/American Institute

☆*Change History:* November 2017. C. Bamia updated the text and updated Table 1 with two additional smoking cessation methods.
This article is an update of Peter Greenwald, Darrell E. Anderson, and Sharon S. McDonald, Cancer Risk Reduction (Diet/Smoking Cessation/Lifestyle Changes), in Encyclopedia of Cancer (Second Edition), edited by Joseph R. Bertino, Academic Press, 2002, Pages 311–317.

for Cancer Reduction (AICR) and in certain dietary guidelines (e.g., US Department of Health and Human Services and US Department of Agriculture, 2015).

Individual Foods, Food Groups and Nutrients

The strength of the overall evidence for specific dietary constituents in relation to cancer incidence depends on the number and quality of published studies, as well as, on the consistency of the reported findings. The level of evidence can be ranked from "low/unlike" to "convincing".

In the 2017 major review of WCRF/AICR on the role of diet, somatometry and physical activity in cancer risk and mortality, the global research based on studies published up to 2017, was evaluated. The evidence for *increased* cancer risk was rated as "convincing" for the following associations: aflatoxins and liver cancer; processed meat and colorectal cancer; beta-carotene, in the form of high dose supplements, and lung cancer; alcohol intake and cancers of: (a) mouth, pharynx and larynx, (b) esophagus (squamous cell carcinoma), (c) liver, (d) colorectum and (e) postmenopausal breast. In addition, the evidence for increased cancer risk was evaluated as "probable" for the following associations: red meat and colorectum cancer; processed meat, foods preserved by salt and alcohol and stomach cancer; alcohol and premenopausal breast cancer.

On the other hand, the evidence concerning foods and nutrients associated with *decreased* risk of cancer has been evaluated at the most as "probable' (but not convincing). Such associations have been highlighted for: colorectum cancer and consumption of wholegrains, foods rich in dietary fiber, dairy products and calcium intake, either from diet or from through supplements (200–1000 mg/day); lung cancer and fruit intake; mouth, pharynx and larynx and fruit, as well as, starchy vegetables intake; liver and endometrium cancers with increased coffee consumption, and; kidney cancer with alcohol intake.

Associations that have been investigated, but were judged as "unlikely" were those for beta carotene intake either through diet or as supplement intakes in relation to prostate and skin cancer risk.

Dietary Patterns

Dietary patterns (DPs) are an alternative approach to assess diet in a holistic way, that is, as a combination of, rather than as isolated, dietary components. A DP can be hypothesis-driven (a priori DP), expressing adherence to a distinct diet for example, the Mediterranean Diet (MD), or, level of compliance with formal dietary guidelines such as the WCRF/AICR guidelines for cancer prevention. DPs can be also empirically derived (a posteriori DPs) through the application of mathematical/statistical methods which identify suitable combinations of a set of dietary variables.

A-priori DPs

The Mediterranean Diet Score (MDS), which quantifies the traditional diet of people living in olive-growing areas around the Mediterranean basin is characterized by high intake of vegetables, fruits, fish (high in n-3 fatty acids), legumes, cereals and olive oil (high in monounsaturated fatty acids), low intake of dairy and meat, and moderate alcohol intake, and has been repeatedly investigated with respect to cancer incidence/mortality. Other frequently-used indices in studies of cancer outcomes include: (a) the Healthy Eating Index (HEI) based on the 1992 USDA Food Guide Pyramid and the 1995 Dietary Guidelines for Americans, (b) the Dietary Approaches to Stop Hypertension (DASH) diet index, (c) the Diet Diversity scores, and (d) WCRF/AICR index, as well as variations of the above.

A-posteriori DPs

A-posteriori DPs may include both, "healthy" and "unhealthy" dietary choices and are labeled in various ways, the most frequent ones being: "prudent", "vegetable/fruit", "traditional", "western-type", "animal", "fat and salty", "refined", "alcohol-based", "drinking", based on the foods mainly characterizing these patterns and their suspected relation with specific cancer sites. A-posteriori DPs that share common labels (e.g., "healthy") may, however, include different foods/food groups.

Dietary patterns and cancer incidence/mortality

Many systematic reviews and meta-analyses for adult populations have been undertaken on the indicated a priori and a posteriori DPs in relation to cancer risk/mortality overall, as well as, by cancer site. A 2016 comprehensive review examined 64 studies of prospective design, including many large multiregion cohorts reporting on a priori dietary patterns. Meta-analyses have been also undertaken for quantifying the overall evidence regarding the association of MDS, as well as, HEI, AHEI, and DASH diet with cancer incidence or mortality. A posteriori patterns have been also studied in systematic reviews in relation to colorectal, breast, gastric, esophageal and lung cancer.

Accumulated evidence from the aforementioned research indicates that there may exist a priori and a posteriori DPs that are associated with reduced cancer risk, and specifically so for colon cancer, breast cancer (especially for the postmenopausal breast cancer) and gastric cancer. From the a priori DPs, collective evidence seems to consistently indicate inverse associations of overall cancer risk with MDS, HEI, AHEI and DASH dietary indices and variations of those. The same holds for a posteriori DPs that have been considered/labeled as "healthy". Evidence is less conclusive for DPs that have been found to be associated with increased cancer risk, mainly the a posteriori "unhealthy" DPs.

Interactions likely occur among many dietary constituents. However, neither these interactions nor their influence on cancer risk are well understood. Thus, at present it is difficult to tease out the specific effects of individual dietary components from diet and cancer research data.

Randomized, Controlled Trials

Most of the evidence relating diet with cancer risk/mortality relies on observational studies. On the other hand randomized controlled trials (RCTs) offer one of the best means for testing diet and cancer hypotheses developed from the insights provided by epidemiologic and experimental studies. Specific dietary constituents have been investigated in RCTs such as vitamins A, C, D and E, folic acid, calcium, and selenium, beta-carotene etc. Interventions in these trials consisted of high-dose daily supplements of the aforementioned vitamins compared to placebo or to no special dietary advice in the group of controls. From these, vitamin D, calcium and selenium have shown promise in reducing overall/site-specific cancer risk.

A few RCTs have been also undertaken with respect to dietary patterns such as low-fat diets for the prevention of breast cancer and MD for the prevention of cancer in general, but due to limited number of studies results are still inconclusive.

Gene–Nutrient Interactions

Recently, research on the relationship between diet and cancer has also focused in gene–nutrition interactions, since genetic factors play a key role in the development of cancer *and* are affected by nutrition. Exposure to the same quantitative level of dietary factor(s)/carcinogens can increase cancer risk in one individual but not in another, depending on specific susceptibilities to gene–nutrient interactions. Therefore, studying the gene–nutrition interactions may help to explain inter-individual differences in response to the same nutritional intakes and, therefore, may shed light to the underlying mechanisms linking diet to the risk of developing cancer. *Nutrigenetics* and *nutrigenomics* are the related research areas and are defined according to Wikipedia as: "the science to identify how genetic variation affects response to nutrients (nutrigenetics)", and "the science of the effects of foods and food constituents on gene expression (nutrigenomics)", respectively.

Genes are involved in carcinogenesis through metabolic activation/detoxification, DNA repair, chromosome stability, activity of oncogenes or tumor suppressors, cell cycle control, signal transduction, hormonal pathways, vitamin metabolism pathways, immune function, and receptor or neurotransmitter action. To date many animal and observational studies have evaluated a number of hypotheses regarding specific diet–gene interactions with respect to cancer risk and prognosis, focusing on cancer sites for which certain diet-related genes, as well as, nutrients/foods have been identified as possible risk factors. Perhaps, some results on colorectal cancer risk may hold promise, for example, the interplay between high consumption of well-done meat (as a source of exposure to heterocyclic amines (HAAs)), and NAT2 and CYP1A2 phenotypes; the association of folic acid and vitamin 12 with DNA methylation and DNA synthesis processes; the association of fruit and vegetable intake with *K-ras* mutations. Results, however, from such studies are still in very early stage.

Understanding gene–nutrient interactions and individual differences in genetic susceptibilities will enhance, in the long run, the development of personalized nutrition strategies for cancer prevention. Moreover it will help researchers to design future dietary intervention strategies to reduce cancer risk. Focusing on polymorphisms in intervention studies will allow investigators to develop study designs, stratify participants, and analyze results based on genetic differences within the study population. For example, polymorphisms in genes affecting the use of vitamin D seem to confer different levels of risk for prostate cancer. This kind of information is important for researchers in designing trials to investigate the role of vitamin D, or its analogs, in prostate cancer risk.

Notwithstanding the promising results of nutrigenetics and nutrigenomics, it should be acknowledged that, at this point in time, any evidence coming from this area is still at premature stage. The challenge for the future is to use any knowledge gained about how genetic polymorphisms influence cancer risk through specific dietary constituents or patterns in order to allow for tailor-made dietary recommendations based on genetic testing or gene expression, as well as, metabolic/nutritional status biomarkers. Under this concept of individualized dietary recommendations, diagnosis, prevention and treatment of cancer could be optimized.

Dietary Guidelines

As research into the role of diet in cancer risk continues, it is important that clinicians and the public be advised about the importance of modifying diets to reduce cancer risk. Since the mid-1970s, various scientific organizations around the world, including the WCRF/AICR, the American Cancer Society (ACS), and the US National Cancer Institute (NCI), have developed dietary guidelines to promote cancer risk reduction as a population strategy. As an example, the WCRF dietary recommendations for the prevention of cancer advise to: (1) avoid high-calorie foods (e.g., processed foods high in fat or sugar intake) and sugary drinks, (2) eat more of wholegrains, vegetables, fruit and pulses (e.g., legumes and beans), (3) limit red meat intake to no more than 500 g/week (cooked weight) and avoid totally, if possible, processed meat (e.g., ham and bacon), (4) avoid alcohol intake or limit alcoholic drinks to the level suggested by national guidelines, (5) avoid eating foods preserved in salt and limit salt intake to less than 6 g/day, (6) avoid mouldy grains and cereals because they may be contaminated with aflatoxins, and, (7) do not rely on supplements but follow a healthy diet in order to protect against cancer.

Dietary guidelines are not followed, however, by the general public. For example the average American consumes too much fat (too much saturated fat, too little monounsaturated fat), too little fiber, and too few vegetables, fruits, and whole grains. This pattern is seen in many developed countries worldwide. It is therefore urgently needed that physicians and nutritionists try to better educate people about components of healthful and cancer-preventing diets and small changes in eating patterns that can lower cancer risk and improve cancer outcomes—this is particularly important nowadays that information on "good" or "bad" diets has become readily available but with questionable scientific gravity and validity.

Smoking Cessation

Smoking and Cancer Risk

In the United States, health conditions related to smoking are responsible for 480,000 deaths each year, 12 years in advance of what would be normally expected (given sex and age distributions). These figures translate to an aggregated annual loss of >5 million life-years. Moreover, about 32% of cancer deaths and 20% of all premature deaths in the United States are attributable to smoking.

Tobacco use is a major avoidable contributor to the cancer burden, accounting for one third of all cancer deaths and 87% of lung cancer deaths each year. Cigarette smoking is a risk factor contributing highly to the amount of cancers not only of the lung, but also of trachea, bronchus, larynx, pharynx, oral cavity, nasal cavity, and esophagus, and is also related to cancers of the pancreas, kidney, bladder, stomach, and cervix. At least 20 carcinogens (e.g., the polycyclic aromatic hydrocarbons (PAHs), including the carcinogen benzo[*a*]pyrene (BaP)) and the nicotine-derived tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)) that are components of tobacco smoke cause lung tumors in either animals or humans. Smoking also affects nonsmokers. Secondhand tobacco smoke as a result of household or occupational exposure, is responsible for inhalation and metabolization of components of the smoke and, thus, has been associated with increased incidence of lung cancer.

In 2014, estimated rates of current smokers in the US adults were 18.8% and 14.8% among men and women, respectively. The prevalence of smoking is inversely associated with education level and with years of education. In the last 30 years, the prevalence of current smoking decreased overall and especially so among men. This decline in smoking prevalence since 1980s was followed by a decline in mortality rates from lung cancer in men.

Smoking cessation has a dramatic impact in reducing the risk of cancer among former smokers even if these quit smoking at older ages. Smokers who stop smoking before the age of 50, succeed in halving mortality risk within 15 years after quitting as compared with people who continue to smoke. In specific, lung cancer risk after 10 years of abstinence is 30% to 50% lower than that of people who continue smoking, whereas the risk of oral and esophageal cancer is 50% lower in former (as compared to current) smokers after 5 years of smoking cessation.

From the above figures it is evident that educational strategies to prevent the start of smoking and promotion of effective approaches to permanently stop tobacco use by smokers are very important elements in reducing smoking-related cancer risk. Policy strategies encouraging reduction of tobacco use at the community level in various countries have been credited with notable reductions in smoking prevalence. These strategies include, pursuing smoke-free laws, media campaigns, increasing the minimum legal age of access to tobacco products, increasing the cost and/or apply excise taxes to tobacco, and offering quitting programs to people through primary health and other health care organizations.

About 70% of smokers claim to be interested in quitting smoking. When smokers, however, try to quit on their own, only about 7% succeed. Long-term success rates can be increased to 15%–30% if smoking cessation interventions are used. These include pharmacotherapies, as well as, behavioral therapies such as individual or group counseling, intensive physician advice, or combinations of the above.

Pharmacotherapies for Smoking Cessation

Pharmacotherapies are effective interventions for smoking cessation. These can be divided to nicotine replacement therapies (NRTs) and non-nicotine medications (**Table 1**). Nicotine, present in all tobacco products, is an addictive substance. For most users, tobacco use results in drug dependence, although in different levels, depending on patterns and quantity of daily smoking. Evidence demonstrating that pharmacotherapies can help people addicted to nicotine to quit smoking is strong and consistent, and NRTs such as nicotine gum, patch, spray, lozenge, and inhaler have been approved for smoking cessation by the US Food and Drug Administration. A 2004 meta-analysis of 110 randomized trials concluded that NRT treatments, alone or in combination, improved significantly cessation rates over placebos after 6 months of use.

There are also non-nicotine pharmacotherapies that have been efficacious for smoking cessation, by lessening nicotine craving and withdrawal symptoms, namely bupropion, an antidepressant, and varenicline, a selective alpha-4-beta-2 nicotinic receptor. Based on 31 randomized trials comparing bupropion to placebo, bupropion was associated with a statistically significant increase in the likelihood of quitting smoking after 6 months of follow-up. Varenicline also improves cessation rates over placebo as shown

Table 1 Pharmacotherapies for smoking cessation

Pharmacotherapy	*Availability*	*Side effects*
Nicotine gum	Over-the-counter only	Mouth soreness, indigestion, stomatitis, sore throat
Nicotine patch	Over-the-counter and prescription	Skin reaction at patch site, insomnia
Nicotine Lozenges	Prescription only	Local irritation (warmth and tingling)
Nicotine nasal spray	Prescription only	Nasal/throat irritation, sneezing, coughing
Nicotine inhaler	Prescription only	Mouth/throat irritation, coughing
Bupropion	Prescription only	Insomnia, dry mouth, dizziness, rhinitis
Varenicline	Prescription only	Nausea, insomnia

in a meta-analysis of nine trials including 7267 participants (4744 under varenicline) where the pooled risk ratio (RR) for continuous abstinence at ≥ 6 months for varenicline versus placebo was 2.33. Moreover in this meta-analysis varenicline was shown to be superior to bupropion or NRTs after 1 year of follow-up.

Both bupropion and varenicline have certain adverse effects. In 2009 and after post marketing surveillance the risk of serious neuropsychiatric symptoms associated with both products were added to the Boxed Warnings Symptoms—these include: "changes in behavior, hostility, agitation, depressed mood, suicidal thoughts and behavior, and attempted suicide."

Combinations of pharmacotherapy

Using more than one NRTs seems to increase smoking cessation rates as compared to using a single type NRT. Some studies have shown that NRT in combination with bupropion may be more efficacious than bupropion alone.

Behavioral Interventions

Many behavioral interventions are available to encourage smoking cessation in adults. These interventions can be delivered in the primary or in the community care settings and include in-person behavioral support and counseling, telephone counseling, and self-help materials. It has been shown that behavioral interventions may increase smoking abstinence from a baseline 5%–11% in control groups to 7%–13% in intervention groups.

Effective individual or group counseling is essential to help a smoker, who is willing to quit, achieving long-term success in smoking cessation and can be delivered by various types of primary care providers, such as physicians, nurses, psychologists, social workers, and specialized counselors. Counseling aims to helping smokers to organize a specific plan on how to quit, acknowledge situations that increase their risk of smoking and, accordingly, develop resistance skills. Effective counseling, therefore, offers basic information about smoking/quitting, describes coping strategies and identifies sources of social support that can be useful (e.g., community resources).

Physician-advice of either minimal (<20 min in 1 visit) or intensive (≥ 20 min plus >1 follow-up visit) type is also effective for successful smoking cessation. Relevant studies seem to suggest a dose–response relationship of the intensity of counseling and smoking cessation rates. Accordingly, the Public Health Service guidelines of the United States, state that "smokers should receive at least 4 in-person counseling sessions".

Telephone counseling provided by trained counselors or health care providers can be also effective. Self-help materials targeting to the needs of the individual smoker rather than simply presenting the adverse health consequences of smoking can be also helpful for smoking abstinence. Recently, computer- or mobile phone-based programs have been also proposed as behavioral interventions for smoking cessation.

Combinations of behavioral and pharmacotherapy interventions

Interventions that combine behavioral and pharmacotherapy strategies seem to increase cessation rates from about 8% to 14% as compared to these interventions given separately. These combinations frequently consist of NRTs and a variety of behavioral approaches usually delivered in >1 sessions from specialized staff.

Tobacco Cessation Guidelines

In 2008 the US Public Health Service, published the updated 2008 guidelines, "Treating Tobacco Use and Dependence" which are available online. In brief, the broad elements of these guidelines include: the need from primary care providers of: recording the smoking status of every patient at every visit, enquiring about his/her willingness to quit (if smoker) and informing those who wish to quit about effective interventions, brief or intense. The guidelines also emphasize on the importance of: (a) encouragement and assistance by the clinicians to those who are trying to quit smoking, (b) close monitoring of the process of smoking cessation in patients' every visit and, (c) training patients under smoking cessation programs in order to confront with situations that may provoke their smoking-abstinence program.

Lifestyle Changes

Physical Activity

Physical activity (PA) is any form of movement using skeletal muscles. Physical activity increases energy expenditure and is a factor of energy balance.

Different activities can be classified according to when (time window) and how (in terms of intensity) these are realized. According to "when", activities are usually grouped as occupational, household, transport, and recreational. Based on "how", PAs can be vigorous, moderate, light, or sedentary. People can be moderately or vigorously physically active at work (manual/labor) or can be occupied in jobs that request light or sedentary PA. In transport (walking, riding, cycling, driving or using public transportation) people can be lightly or moderately physically active. Exercise and other forms of physical training but also activities done during household are types of recreational physical activity—these may be of light, moderate, or vigorous intensity depending on the

nature/intensity of activities, hobbies, and pursuits. Total PA levels for a given timeframe (e.g., day, month, year, etc.) are evaluated as the combination of frequency, intensity, and duration of all activities that take place at the specific time frame.

In the 2017 WCRF/AICR report the only factor for which the evidence regarding its inverse association with cancer risk was judged as "convincing" was PA (moderate and vigorous) in relation to colon cancer. According to this report, PA also "probably" decreases the risk of post- and pre-menopausal breast, as well as, of endometrial cancers. The evidence suggesting that PA protects against cancers of the lung and pancreas was evaluated as "limited." The report also notes that "… all types and degrees of physical activity are or may be protective, excluding extreme levels of activity: the evidence for any specific type or degree of physical activity is limited."

These results are consistent with the message that, the more physically active people are the better. Regular PA may be associated with reduced cancer risk through various mechanisms, including altering hormone levels and increasing immune system activity, energy expenditure, and antioxidant activity. Given that PA is a way to preserve energy balance and retain normal body weight it seems that PA protects against cancers the risk of which is increased by overweight, weight gain, and obesity, such as cancer of the colon, rectum, prostate, endometrium, breast, and kidney.

Physical activity guidelines

Most people in urbanized and industrialized settings worldwide lead sedentary ways of life. The amount of PA needed to maintain a healthy weight, lose weight, and promote good health, including reducing cancer risk, as recommended by various organizations in the United States, including the NCI, ACS, and WCRF/AICR is, at least 30 min of moderate physical activity on most days of the week either continuously, or, in time intervals. This level of activity might include walking briskly (3–4 miles/hour) for about 2 miles, gardening and yard work, jogging, or swimming. In the recommendations of WCRF/AICR it is also emphasized that one: (a) should limit sedentary habits such as watching television and, (b) as fitness improves, he/she should aim for 60 min or more of moderate, or for 30 min or more of vigorous, physical activity every day.

Sun Exposure

Cancer of the skin is the most frequent type of cancer. Basal cell and squamous cell carcinomas, both highly curable, account for the majority of skin cancers whereas malignant melanoma, the most dangerous form of skin cancer is less frequent. The incidence of skin cancer is increasing over the last 30 years, partly due to an increase in awareness about and detection of this cancer. It is estimated that non melanoma skin cancers (NMSCs) affected 3,000,000 subjects living in the United States in 2012. For melanoma skin cancer the number of people living in the United States who will be diagnosed with melanoma in 2017, is predicted to be about 87,000 whereas the associated deaths are expected to be about 10,000.

People with red or blond hair and fair skin that freckles, tans poorly or burns easily are at especially high risk for skin cancer. Increased (in intensity and/or duration) exposure to sunlight, the main source of ultraviolet (UV) radiation, is implicated as a causative factor in the development of skin cancer in observational studies.

Abundant evidence has established that skin cancer risk can be reduced by increasing awareness in people about skin type prone to skin cancer and of skin damage (burn and solar keratoses) due to sun exposure, as well as, by limiting exposure to sunlight and, thus, to UV radiation. For melanoma cancer there is some evidence that patterns of sun exposure may also be important. In 2009 a pooled analysis of 15 case–control studies (5700 melanoma cases and 7216 controls) investigated patterns of sun exposure, sunburn and solar keratoses (only three studies) with melanoma cancer risk. This study concluded that melanoma cancer risk at different body sites is associated with different amounts and patterns of sun exposure. Recreational sun exposure and sunburn were consistently associated with melanoma cancer at all latitudes, whereas occupational and total sun exposure were associated with melanoma risk, predominately at low latitudes.

Suggested effective protective behaviors include avoiding the sun during midday (especially between 10 AM and 2 PM), wearing protective clothing and broad-brimmed hats, wearing sunglasses, avoiding tanning beds and sun lamps (these are also sources of UV radiation), and using sunscreen that has a sun protection factor (SPF) of 15 or higher, even on hazy or cloudy days—however the respective evidence of these strategies acting as interventions for reducing NMSCs is not yet conclusive.

The relationship between sunscreen use and melanoma cancer risk, as practiced in the general population, is, also, not absolutely clear. Many epidemiologic studies investigating this association have found either reduced risk or no clear association. There are also findings from some studies suggesting that sunscreen use is associated with an increased risk for melanoma. This may be attributed to the fact that people who use sunscreen primarily to avoid sunburn during intentional sun exposure, might increase their sun exposure time when using sunscreen, thus increasing their exposure to UV radiation and risk for melanoma. A meta-analysis of 18 studies that evaluated the association between sunscreen application and melanoma risk highlighted different degrees of quality among the included studies and estimated an overall weak inverse association.

At present, accumulated evidence on sun exposure and skin cancer warrants using sunscreen as part of an overall sun protection strategy, during both intentional and unintentional exposures (e.g., in gardening or hiking).

See also: Cervical Cancer: Screening, Vaccination, and Preventive Strategies. Dietary Factors and Cancer. Diet and Cancer. Environmental and Occupational Exposures.

Further Reading

American Cancer Society, 2017. Cancer facts & figures 2017. American Cancer Society, Atlanta. https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2017/cancer-facts-and-figures-2017.pdf.

Braicu, C., Mehterov, N., Vladimirov, B., et al., 2017. Nutrigenomics in cancer: Revisiting the effects of natural compound. Seminars in Cancer Biology 46, 84–106.

Byers, T., 1999. What can randomized controlled trials tell us about nutrition and cancer prevention? CA: a Cancer Journal for Clinicians 49, 353–361.

Fenech, M., El-Sohemy, A., Cahill, L., et al., 2011. Nutrigenetics and nutrigenomics: Viewpoints on the current status and applications in nutrition research and practice. Journal of Nutrigenetics and Nutrigenomics 4, 69–89.

Hu, F.B., 2002. Dietary pattern analysis: A new direction in nutritional epidemiology. Current Opinion in Lipidology 13, 3–9.

Jamal, A., Agaku, I.T., O'Connor, E., King, B.A., Kenemer, J.B., Neff, L., 2014. Current cigarette smoking among adults–United States, 2005–2013. MMWR. Morbidity and Mortality Weekly Report 63, 1108–1112.

Klein, W.M.P., 2014. Behavioral research in cancer prevention and control: A look to the future. American Journal of Preventive Medicine 46, 303–311.

Lancaster, T., Stead, L., 2004. Physician advice for smoking cessation. The Cochrane Database of Systematic Reviews 4, CD000165.

Lemmens, V., Oenema, A., Knut, I.K., Brug, J., 2008. Effectiveness of smoking cessation interventions among adults: A systematic review of reviews. European Journal of Cancer Prevention 17, 535–544.

Potter, J., Brown, L., Williams, R., Byles, J., Collins, C., 2016. Diet quality and cancer outcomes in adults: A systematic review of epidemiological studies. International Journal of Molecular Sciences 17, 1052.

Siu, A., US Preventive Services Task Force, 2015. Behavioral and pharmacotherapy interventions for tobacco smoking cessation in adults, including pregnant women: U.S. Preventive Services Task Force Recommendation Statement. Annals of Internal Medicine 163, 622–634.

Stead, L.F., Perera, R., Bullen, C., Mant, D., Lancaster, T., 2004. Nicotine replacement therapy for smoking cessation. Cochrane Database of Systematic Reviews 3, CD000146.

Saraiya, M., Glanz, K., Briss, P., Nichols, P., White, C., Das, D., 2003. Preventing skin cancer: Findings of the task force on community preventive services on reducing exposure to ultraviolet light. MMWR—Recommendations and Reports 52 (RR-15), 1–12.

Task Force on Community Preventive Services, 2002. Recommendations to increase physical activity in communities. American Journal of Preventive Medicine 22, 67–72.

World Cancer Research Fund and American Institute for Cancer Research, 2007. Food, nutrition, physical activity and the prevention of cancer: A global perspective. AICR, Washington, DC.

World Cancer Research Fund and American Institute for Cancer Research, 2017. Cancer prevention and survival. Continuous Update Project. AICR, Washington, DC.

Cancer Survival and Survivorship

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Glossary

Actuarial life-table method The survival experience of a group is summarized by cumulative survival probabilities that are calculated only at the end of each equally spaced follow-up interval.
Cancer fatality rate The proportion of individuals who experienced death within the population of individuals diagnosed with cancer, over the course of the disease.
Cancer mortality rate The number of deaths due to a cancer within a population during a period of time (1 year or more). It is usually expressed per 100,000 person-years. Mortality rate = (Cancer deaths/population) × 100,000 person-years.
Cancer survival The state of continued life in spite of a diagnosis of malignant cellular growth.
Cancer survivorship Examines the state of the cancer survival experience of an individual or population, considering the complex reality of a present or long-term cancer diagnosis that can substantially influence survival. This includes physical symptoms and conditions, psychosocial concerns, health-related quality of life, health behaviors, tools and platforms for research, special populations, economic impact, and quality of care.
Censoring A condition in which the survival experience of the study subject has not experienced the event of interest because (1) he/she is lost to follow up or drop out of the study, (2) if the study ends before the event can occur, or (3) have an event other than the outcome of interest. A censored subject is counted as alive or disease-free for the time he or she was enrolled in the study.
Corrected survival rate When reliable data on cause of death are available, a correction is made for deaths due to causes other than the disease under study by censoring causes of death other than the cause of interest. Also sometimes called net survival rate, net survival, or cause-specific survival.
Follow-up In a survival analysis and to calculate survival rates, enrolled subjects must be followed at intervals to assess vital status from the time elapsed between beginning and end of observation period.
Hazard function $\lambda(t)$ The probability that the event of interest did not occur before the study observation period, t.
Independent-censoring assumption The assumption that the event of interest in survival analyses is unrelated to whether or not a subject is censored.
Kaplan–Meier method Estimates the proportion of people living after each increment of time during the study observation period. Cumulative survival probabilities are calculated when an event occurs or when a subject is censored.
Lead-time bias A systematic error of apparently increased survival that occurs when disease is detected at an early stage. Lead-time bias is evident when disease detection does not alter the time from disease conception until the event of interest, but only appears so because the observation period after diagnosis ended before the event could occur. Also called information bias.
Log-rank test A univariate statistical analysis comparing the survival distributions between two groups during the observation period, based on the null hypothesis that there is no difference in survival. Also called the Mantel–Cox test.
Observed survival rates The proportion of individuals who did not experience death over the population diagnosed with cancer during an observed study period (e.g., 5 years). Also called the overall survival rate.
Relative survival rate The ratio of the observed survival rate of cancer patients to the expected or background survival rate in a comparable set of cancer-free individuals. Often used for populations when cause-of-death data is not available.
Survival function $S(t)$ The probability that the event S did not occur before the study observation period, t.
Time-to-event The time from entry into a study until the subject has a particular outcome. The "event" can be death, recurrence, symptom reduction, or hospital discharge.

Introduction

Cancer survival is an all-inclusive term that refers to the state of continued life in spite of a diagnosis of malignant cellular growth. It is measured by survival statistics that display the proportion of patients alive over a specific observation period. In addition to life or death, survival statistics can examine outcomes that influence survival, such as cancer progression and recurrence within the observed time period. Cancer survivorship broadens the cancer survival definition, taking into consideration the complex reality of a present or long-term cancer diagnosis that can substantially influence survival. This includes physical signs and symptoms,

Table 1 Positive influences that increase cancer survival rate for common cancers by cancer type

	Early diagnosis/screening	*Treatment*
Lung	(−)[a]	−
Prostate	(−)[b]	+
Breast	+	+
Colorectal	+	+
Stomach	(−)[c]	−
Cervix	+	+
Liver	−	−
Acute lymphoblastic leukemia in children	−	+

[a]Screening is recommended in the United States only for current heavy smokers or those who have quit in the past 15 years and are between 55 and 80 years old, but has not been implemented in most other countries yet. Clinical trials evaluating the benefits and risks of lung cancer screening are ongoing.
[b]Prostate-specific antigen level screening is recommended for some populations, but not widely recommended to all male population with average risk.
[c]In countries with very low incidence, gastric cancer screening is not recommended. Gastric cancer screening programs exist nationwide for Japan and Korea and some regions of China.

psychosocial concerns, health-related quality of life, health behaviors, tools and platforms for research, special populations, economic impact, and quality of care.

Cancer survival is influenced by two main determinants that are consistent across populations: early stage at diagnosis or screening and improved cancer management strategies (Table 1). These factors vary by cancer type and thus play a key role in observed differences in cancer survival rates. Acute lymphoblastic leukemia and testicular cancer, which have effective cancer treatment strategies, have 5-year survival rates greater than 85%, while pancreatic cancer, which has no effective treatment, has a 5-year survival rate of 1%. Access and utilization of oncological health care is another factor influencing each of the two survival determinants. These differ between populations based on a breadth of sociopolitical and economic factors and are reflected in survival rates across countries (Table 2).

Survival and survivorship data are routinely collected and analyzed by cancer registries to monitor and assess cancer control programs in all patients registered in a population-based cancer registry. Survival data may also be collected in observational and randomized-control studies to compare groups of patient and effects of treatment and/or management strategies. The appropriate analysis and interpretation of survival data requires an understanding of the correct application of quantitative tools, metrics, assumptions, and limitations imposed by data source and availability.

Cancer survival rates often use a 5-year survival rate, although 1-year and 10-year rates are also used. Survival rates of interest are cancer type, stage, and treatment groups, for a multitude of survival outcomes, including death, recurrence, and/or new symptoms. Although certain cancers can recur beyond the 5-year period, chance of a later recurrence for most cancers is very small.

It is worth noting that survival rates and morality rates are not direct opposites. Survival rates differ from mortality rates in that they are highly dependent on when cancer is diagnosed or treated. While mortality rates of cancer within a certain time interval are absolute measures of cancer deaths regardless of when the cancer was diagnosed, cancer survival rates provide an outlook of the possibility of living past a fixed time period using the time of diagnosis or treatment as the starting point. The practical goal for analyzing survival data is to provide an estimated cancer prognosis for a patient based on the survival rates of a large number of patients diagnosed or treated for that cancer. However, as a prognostic benchmark, survival probability may be different for each individual patient and strongly depends on stage at medical diagnosis, his or her comorbidities, past medical history, and tumor characteristics.

Table 2 Five-year net survival, age-standardized estimates, %, adults (aged 15–99 years), 2005–2009

	United Kingdom	*United States*	*Norway*	*Germany*	*Italy*	*Slovenia*	*Ecuador*	*Brazil*	*China*	*India*	*Japan*	*Tunisia*[a]	*Mauritius*[b]
Lung	9.6	18.7	15	16.2	14.7	11.4	28.7	18	17.5	9.6	30.1	10.3	37.2
Prostate	83.2	97.2	86.3	91.2	89.7	78.1	92.4	96.1	62.9	58.1	86.8	100	77.3
Breast	81.1	88.6	85.9	85.3	86.2	80.2	83.2	87.4	80.9	60.4	84.7	68.4	87.4
Colon	53.8	64.7	61.8	64.6	63.2	56	68.2	58.2	54.6	37.3	64.4	67.6	55.5
Stomach	18.5	29.1	24.1	31.6	32.4	26.7	31.9	24.9	31.3	18.7	54	49	40.7
Cervical	60.2	62.8	71.4	64.9	68.3	68.9	61.7	61.1	59.9	45.8	66.3	42.4	86.7
Liver	15.2	9.3	9.5	14.4	17.9	5.2	17.7	11.6	12.5	4.3	27	–	52.6
Childhood leukemia	89.1	87.7	89.7	91.8	87.7	75.7	62.6	65.8	61.1	64.7	81.1	50.1	–

[a]Survival estimates considered less reliable.
[b]Survival estimates are considered less reliable and are based on few cases.
Data from Allemani, C., Weir, H. K., Carreira, H., et al. (2014). Global surveillance of cancer survival 1995–2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet*. https://doi.org/10.1016/S0140-6736(14)62038-9.

Concepts of Survival Data

A database suited for survival studies must have three key features: a defined event of interest, a specific start and end of each individual subject's observation period, and a set observation time-scale, such as years or months. Randomized-control trials may use date of randomization, date of entry into the cohort, or date of diagnosis as start points. The dataset may also contain variables that could be evaluated for their relationships with survival. These variables can include sex, cancer stage and type, carcinogenic exposures, or treatment modalities.

Survival statistics use time-to-event data. This is the time (t) from study entry until an individual is observed to have a particular outcome. Both the time enrolled in the study and the event of interest are recorded for each individual. A binary outcome of interest is typically used: no occurrence of event (0) or occurrence of event (1). Occurrence or nonoccurrence of the event is restricted to the observed study period only.

If an individual leaves a study before the event of interest, the outcome is censored. Individuals are censored if they are lost to follow-up, drop out from study for any reason, or if the study is terminated before the outcome of interest occurs. The dataset would contain a record that the individual survived at least to time t. A study proposing to assess the probability that the event did not occur before t is interested in the survival function $S(t)$. The proportion of persons not observed to experience the event by the time the study ends is reported as the survival experience for that group.

Vital status of study subjects can be determined through two methods of follow-up: active and passive. Active follow-up collects vital status information by repeated residential visits, surveys, physician notes, death registry offices, or other social or community gatherings. Passive follow-up is more common in regions with established cancer registries and is accomplished by linking a subject's personal identifiers to an institutionally recorded vital status.

Although survival data often use life or death as the events of interest, other outcomes may be of clinical interest. Examples of other outcomes include developing a complication of disease, treatment of a side effect, resolution of symptoms, progression, and recurrence.

Methods for Survival Analyses: Descriptive Measures

The first step in survival analysis is to estimate the overall survival experience of the entire population or cohort. Unlike incidence or mortality statistics where the total population is included in the ratio denominator, only cancer-diagnosed patients are included in survival fractions. The observed survival rate or overall survival rate is the proportion of individuals alive after receiving a cancer diagnosis or treatment. It includes all deaths for these patients, regardless of whether the cause was from the cancer itself, a cardiovascular disease, injury, or other cause. When reliable information on cause of death is available, a corrected, net, or cause-specific survival rate can be calculated. Deaths occurring only due to the disease of interest are counted as an observation of the event, while deaths due to other causes are treated as a nonevent. Treating other causes is controversial, particularly for studies examining the relationship between exposures and cancer. The cancer may still have occurred had an individual not experienced the competing cause of death. In these circumstances, competing risks can be calculated (see section "**Competing Risks**").

When cause of death data is unavailable or unreliable, a relative survival rate can account for survival from noncancer causes. Relative survival is a ratio of the observed survival rate of cancer patients to the expected or background survival rate in a comparable set of cancer-free individuals. An example of background survival rate can be cancer-free patients or overall survival of cancer patients when race, sex, and age, race, sex, or year information is missing. In practice, overall, corrected, and relative survival rates are very similar. Because they are calculated differently, relative and corrected survival estimates cannot be compared.

Disease-free survival rate is another specific type of survival rate. This is the proportion of subjects with cancer who achieve complete remission. Progression-free survival rate is the proportion of subjects who still have cancer, but the cancer has not progressed over a specific time period. To be free of progression may have an impact on further therapy and is correlated to tumor-specific survival.

When survival rates are compared between populations of varying age distributions, such as different countries, an age-adjusted survival rate is necessary to account the different age structures of different patient populations. Age-adjusted survival rates are calculated by multiplying specific survival rate for each age group with the corresponding proportion of persons in that age group in the population.

Survival probabilities can be summarized in a tabular and graphical form in a survival curve. These cumulative survival probabilities can be calculated by the actuarial life-table method or the Kaplan–Meier method. In the actuarial life-table method, cumulative survival probabilities are calculated only at the end of each equally spaced follow-up interval. If the percent of the patients surviving until the end of the first, second, and third intervals are 90%, 83%, and 67%, the cumulative survival percentage (or the chance of surviving until the beginning of the fourth interval) is 50% ($0.9 \times 0.83 \times 0.67 = 0.50$) (**Table 3**). Conversely, the Kaplan–Meier method calculates survival probabilities at the time that the death takes place, resulting in a stepped figure (**Fig. 1**). Both curves allow the estimation of the time beyond which 50% of study subjects are expected to survive (median survival time), comparison of survival of different groups in the study over time.

Table 3 Calculating cumulative survival probabilities, actuarial life-table

Time interval (months)	*Number at risk during interval*	*Number of deaths during interval*	*Probability of death during interval*	*Probability of surviving during interval*	*Cumulative probability of surviving at the beginning of the time interval*
0–4	100	10	0.10	0.90	1.00
5–9	90	15	0.17	0.83	0.90
10–14	75	25	0.33	0.67	0.75
15–19	50	25	0.50	0.50	0.50
20–24	25	3	0.12	0.88	0.25

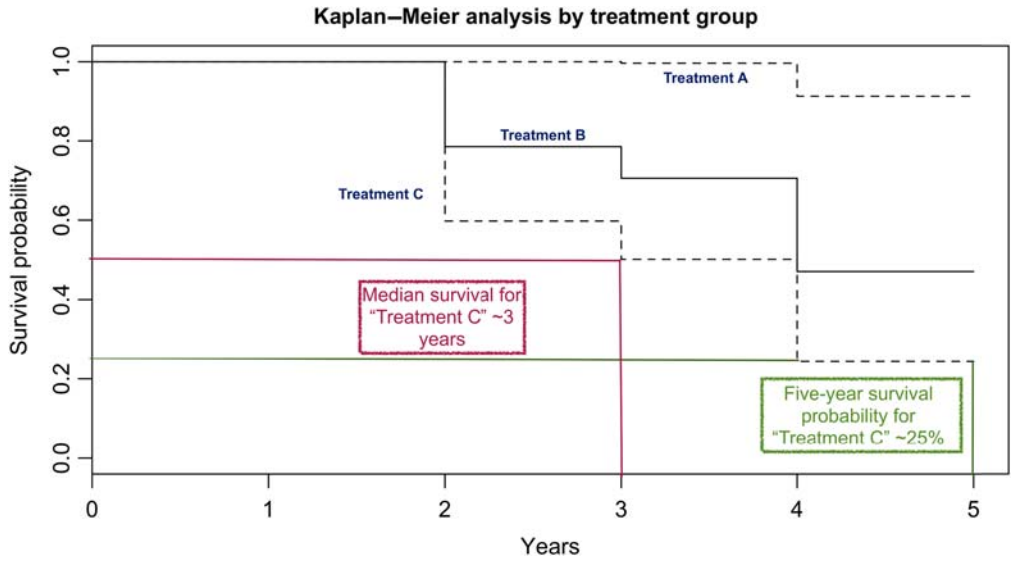

Fig. 1 Five-year survival of cancer patients by treatment modalities A, B, and C using the Kaplan–Meier method.

Care must be taken when interpreting cumulative survival probabilities and two main underlying assumptions must be accounted for. Firstly, at any point during the study course, subjects who are censored at one time have the same survival probability as those who continue to be followed. Events such as undergoing new treatment or achieving complete remission within time t may otherwise alter the survival rate. Secondly, in studies with long enrollment periods, survival probabilities must be the same for subjects recruited early and late in the study. Some individuals recruited earlier in a study before effective treatment availability may be more susceptible to a cause of death that differs from subjects recruited later and the interpretation of the survival rate may be erroneous.

Comparison of cumulative survival rates is calculated by a log-rank test, comparing the survival distributions, based on the null hypothesis that there is no difference in survival. This calculation has the form of a chi-square probability distribution, equally weighing the differences of observed survival rate compared to the expected survival rate for each group at each event time. The number of degrees of freedom is the number of groups to be compared, -1. If the difference between the observed and expected survival distribution has $<5\%$ probability to be due to chance, then the null hypothesis is rejected and the alternative hypothesis of a significant difference in survival is expected. The log-rank test is only a test of significance and provides no estimate of the magnitude of difference between the groups studied. For comparative methods with ability to account for multiple variables influencing survival rates, multivariate models are used.

Methods for Survival Analyses: Basic Statistical Assessments for Comparisons Between Groups

To evaluative the survival–predictor relationship or quantitatively compare groups, multivariate regression models such as the Cox model or proportional hazard regression analysis and Poisson regression are applied. These models both assume that the time-estimated hazard ratios (Cox) or incidence rate ratios (Poisson) are proportional to the length of time t and that the impact of each predictor in the model is constant in the relationship to the outcome for time t. Like any survival analysis, censoring must be independent or noninformative. That is, the censoring of subjects must not be related to the probability of an event occurring.

Table 4 A comparison of the Cox model versus Poisson regression for survival analysis

	The Cox model or proportional hazards regression	*Poisson regression*
Distribution of response event	Semiparametric; does not estimate baseline hazard but instead uses arbitrary baseline hazard	Parametric; baseline hazard is estimated
Response variable in equation	Hazard function gamma(t); reported as hazard ratio (HR)	Incidence rate: natural log counts of event with a model offset for the amount of risk each individual had until the event (person-years)
Dataset preconditions	Nonaggregate or individual subject data are needed	Can be used for both aggregate and nonaggregate data
Survival distribution	Does not make assumptions about survival distribution	The incidence must fit a Poisson distribution (the mean and variance are equal)
Use in longitudinal studies	Allows to account for repeated observations for each record	Allows to account for repeated observations for each record, but must be compressed prior to analysis
Proportional hazard assumption	Proportional to the predictor over time t and constant	Proportional to the predictor over time t and also constant
Limitations	Cannot use if assumption of proportionality is violated, though stratification for some covariates can be done	Difficult to set up dataset. Data on individual subjects must be organized into event-time tables that are stratified on time and other covariates
Advantages	No assumption is made about the shape of the baseline hazard function	If Poisson distribution assumption is violated, alternative methods are available, including quasi-likelihood and binomial regression

The Cox model and Poisson regression differ in assumptions of survival distributions. For the event rate of interest, the Cox model assumes a semiparametric distribution whereas the Poisson model assumes a parametric distribution. Differences between the two model types are displayed in Table 4.

The Cox Model

A Cox model is a statistical technique that can be used for survival-time (time-to-event) outcomes on one or more predictors. The response variable is the hazard function $\lambda(t)$, which assesses the probability that the event of interest (in this case, death) occurred before t. The equation models this hazard as an exponential function (exp) of an arbitrary baseline hazard (λ_0) when all covariates are null, and β is the regression coefficient of the covariate, x.

$$\lambda(t) = \lambda_0(t)\exp(\beta_1 x_1 + \cdots \beta_k x_k)$$

The Cox proportional hazards model makes two assumptions: (1) survival curves for different strata must have hazard functions that are proportional over the time t and (2) the relationship between the log hazard and each covariate is linear, which can be verified with residual plots. Examples of covariates can be categorical such as race or treatment group, or continuous such as biomarker concentrations.

Competing Risks

So far, we have assumed that only a single event of interest occurs in a subject. In practice, one cause of death may alter the survival observed for another cause of death. An increase in cardiovascular deaths in a susceptible population may lead to an apparent increased survival for cancer deaths. The basic Cox model does not differentiate between subjects lost to the competing death and subjects who were alive at the end of his or her observation period. This leads to an erroneous estimation of the cancer-specific survival rate and, in comparisons between predictors or characteristics, an erroneous interpretation of the effect of these variables on survival. A second example occurs when the suspected predictor is linked to multiple causes of death. Smoking, for instance, is linked to multiple cancers of interest, but the event of interest is prostate cancer death, which is not as lethal as lung cancer. Lung cancer would be a competing cause of death. Another phenomenon can occur in clinical trials. A particular cancer management strategy may result in two outcomes: death or recurrence. Recurrence is related to death, but is a competing event that alters survival risk and would result in earlier death. Each of these scenarios violates the assumption that the risk of death is unrelated to censoring (independent-censoring assumption).

There are a few strategies for handling competing risks that quantify more accurate hazard probabilities. Adjusting models for competing risks is useful in circumstances where it is highly suspected that the competing event alters the risk of the event of interest. For example, the patient could have died from the cause of interest given the predictor, but the competing cause of death obscured the true risk. Unlike censoring, which obstructs you from viewing the event probability, a competing event prevented the event of interest from occurring altogether. The most frequently used methodologies for adjusting analyses to take competing risks into account is the Fine and Gray method. The Fine and Gray method is a modification of the Cox model that postulates a model for the sub-hazard function of a failure event of primary interest. Instead of a binary outcome with one possible event of interest, the Fine and Gray method is able to account for additional competing events.

Poisson Regression

Poisson regression uses the natural log of event counts and an exposure or offset parameter (person-years) to measure the relationship between predictors and the event of interest. Instead of comparing hazard rates, as the Cox model, the Poisson model compares incidence rates for predictors:

$$\text{In } (\lambda) = \beta_0 + \beta_1 x_1 \ldots \beta_k x_k$$

In this case, In is the natural logarithm of the incidence rate, λ, β represents the coefficient of each predictor, x, and β_0 is the baseline rate or intercept. The event rates over time t follow a Poisson distribution where Ln(λ) has mean equal to its variance.

Both Poisson regression and the Cox model can be used to analyze survival data. Both equations enable an analysis aimed at understanding the relationship between predictors to survivorship outcomes. However, Poisson regression is useful for aggregate data in which only cancer rates for the co-variates of interest, rather than each individual, are known. This may be the case for public use or registry-based datasets. Although the response variables differ (hazard ratios vs. incidence rate ratios), both methods provide an estimated relative risk (Table 4).

Cancer Survivorship at a Glance

The number of cancer survivors has increased substantially over the past four decades. Many patients diagnosed with most cancers are now expected to survive. In countries with a high gross-domestic-product per capita, this survivor proportion is well over half of those diagnosed with cancer. In high-income countries, the increase has been by 40%–70% over the past four decades. Although cancer survival is increasing, aging populations in both high- and low-income countries suggest that the projected number of people living with or beyond a cancer diagnosis will grow. With more cancer survivors and advances in cancer diagnosis and treatment, greater emphasis is being placed on improving survival estimations, identifying key survival determinants, and utilizing these summary measures to improve cancer preventive planning and investment.

Cancer survivorship studies merge clinical and public health efforts to improve patient cancer outcomes. Survival statistics' accuracy and completeness requires systematic collaboration between clinicians and cancer registries. General practitioners, health institutions, hospitals, and death registries notify cancer registries using a standardized coding system. Cancer registries systematically collect data for the purposes of calculating cancer statistics and conducting cancer research. It must therefore be stated that the currently available survival statistics are less reliable for many low-income countries with fewer resources and infrastructure available than for high-income countries.

Cancer survivorship is being recognized as a multifaceted field that is influenced not only by key differences in cancer survival, such as by cancer type and age at diagnosis, but also by several non-prediagnostic factors such as economics, culture, and society. These community-related factors include an individual's socioeconomic status, healthcare-seeking behavior and consistency with follow-up, health care service efficiency, and personal treatment choices. In considering these many cancer survivorship variables, it is important to observe them within the context of stage at diagnosis and treatment access/availability.

Cancer stage makes a significant impact on survival—those with higher clinical stage cancers are likely to have a lower survival rate than those with low-stage cancers. Cancers can be detected at a lower stage when the length of survival would be longer than the 5-year follow-up period. This cancer can be said to have a lead-time bias. Lead-time or information bias can lead to the erroneous interpretation that earlier cancer screening prolongs life, when in actuality, the cancer was only detected at an earlier starting point in time. In other words, the patients would have died at the same time whether they had screening or not, but would have known about the disease earlier. It is important to note that while 5-year survival is useful in monitoring progress for early detection and treatment of cancer, it does not represent the total proportion of people who are cured because cancer death can occur beyond 5 years after diagnosis. Lead-time bias is often observed for cancers in which a diagnostic method is discovered, but no known effective treatment exists regardless of stage at diagnosis.

Factors that affect survival are unique to each type of cancer. For cancers without early detection or effective treatment, such as liver, lung, or pancreatic cancer, survival rates vary little across countries. In contrast, the largest survival differences between countries are observed for cancers with a better prognosis through screening and access to treatment: female breast, colorectal cancer, cervical cancer, and childhood leukemia (treatment only).

Within countries, it has been widely recognized that survival differences are distributed differently across socioeconomic status groups and race. While survival has mostly improved over time, differences in survival by race that existed in 1975 are still evident in contemporary times. Indeed, survival rates for the most common cancers have improved among both US blacks and whites due to early detection and treatment, but US blacks still have an approximately 10% lower 10-year survival rate (Fig. 2).

Breast Cancer

Among women worldwide, breast cancer survivors make up a third of those living with a cancer diagnosis in the past 5 years. When breast cancer is detected at an early stage, treatment is more effective and a cure is more likely. The 5-year survival rate for breast cancer in the United States in 2005–2009 was 88.6%, compared with 60.4% in India and 68.4% in Tunisia (Table 2). Within India, 5-year survival for early-stage (I or II) breast cancer ranged from 41% to 78% by region, indicating wide variation in health services

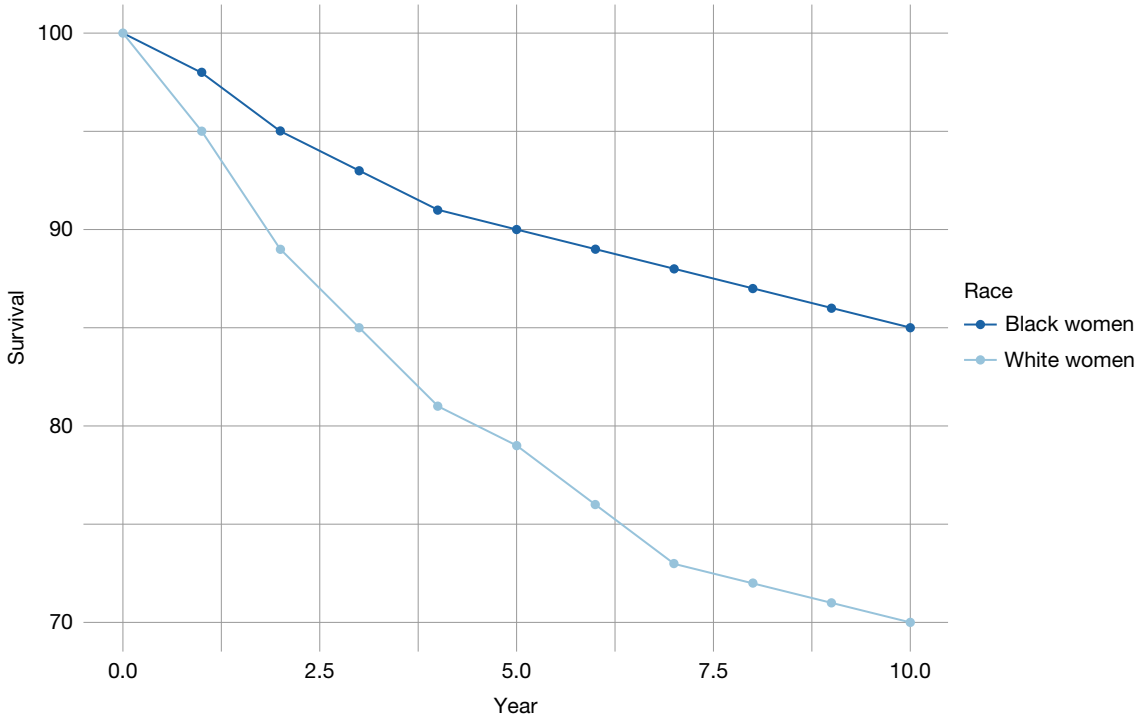

Fig. 2 Five-year relative survival by race for female breast cancer, United States, 1988–2013. Data from Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence – SEER 9 Regs Research Data, Nov 2016 Sub (1973–2014) <Katrina/Rita Population Adjustment>—Linked to County Attributes—Total U.S., 1969–2015 Counties, National Cancer Institute, DCCPS, Surveillance Research Program, released April 2017, based on the November 2016 submission.

delivery. In high-income countries, over 75% of breast cancers are diagnosed at an early stage, which is reflected in the high 5-year overall survival rate (80%+). By contrast, in several low-to-middle income countries, the majority of women are diagnosed with late-stage disease (clinical stage III or IV). Reasons for late-stage diagnoses include lack of awareness as well as limited access to adequate detection and diagnostic services. In some cultures, breast cancer or having any cancer at all, is associated with a social stigma and reduces a woman's social status or the potential for her childrens' marriage within her community. Cancer fatalism, a belief that cancer is preordained in one's destiny and cannot be altered, can also hinder cancer screening programs and may delay or prevent cancer treatment. Additionally, women living in deprived or remote areas are less likely to receive breast-conserving surgery, and would have lower 5-year survival than women in more affluent areas.

Cervical Cancer

When detected at an early stage, invasive cervical cancer is one of the most successfully treated cancers. The 5-year net survival rate ranges from 42.4% in Tunisia to 77% in South Korea and 71% in Norway, although it is between 60% and 70% in most countries. The Papanicolaou (Pap) test screens for precancerous cervical cells that can be treated to prevent cervical cancer, but this test requires a clinician to swab for a cell sample directly from the cervix and a trained cytologist, cytotechnician, or pathologist to examine these cells under a microscope. As the main cause of cervical cancer is a persistent infection with high-risk strains of human papilloma virus (HPV), HPV tests are being currently introduced as a mean of primary or secondary screening in several countries. This test has a higher sensitivity but lower specificity than the Pap test and using HPV in screening will be particularly useful in the future, when most women will hopefully become vaccinated for HPV. In many cultures, social barriers to screening exist, such as the reluctance to have a gynecological exam by a male doctor. Additionally, many remote areas or low-resource countries do not have the technology, expertise, or resources to support an effective cervical cancer screening program.

Colorectal Cancer

Survival rates for colorectal cancer vary worldwide. In high-income countries, colon and rectum cancer 5-year net survival is about 50%–65%, while in Asia a wider range exists. Five-year colon and rectal cancer survival rates of more than 65% have been reported in Israel and South Korea, while they range from about 20% to 55% in other countries in Asia. As colorectal cancer is not one of the highest causes of cancer deaths in many Asian countries (with cervical cancer and cancer of the stomach being more common), there is less public awareness about screening in many areas. Survival is much higher when colorectal cancer is detected at an early stage.

Colorectal cancer is amenable to screening, and there are several screening modalities available such as fecal immunochemical test, fecal occult blood test, sigmoidoscopy, and colonoscopy. However, these screening modalities have been implemented almost exclusively in high-income countries. In some countries with routine screening practices for middle-age adults, colorectal cancers are diagnosed at an earlier and more treatable stage, and survival rates are consequently higher as observed in the United States and Germany, with 5-year survival rates of 64.7% and 64.6%, respectively.

Liver Cancer

Liver cancer is one of the most fatal cancer types, with 5-year survival rates ranging between 10% and 20% across all countries. A cancer fatality rate is the proportion of individuals who experienced death within the population of individuals diagnosed with cancer, over the course of the disease. Liver cancer risk factors vary substantially by country. It main risk factors are alcohol abuse, liver cirrhosis, exposure to food contaminants such as aflatoxins, and—importantly—different types of hepatitis infections. Hepatitis infections, mainly hepatitis B and C, are the largest cause of liver cancer in many parts of the world. There is an effective vaccine for hepatitis B to be given to newborns/infants, and persons at risk, and hepatitis C can be effectively cured.

Liver cancer incidence rate is increasing worldwide, making preventive studies and randomized clinical trials a high priority for patients' prognostic improvement.

Lung Cancer

Although there have been improvements in surgical techniques and combined therapies over the past several decades, lung cancer is one of the most lethal cancers and the most difficult to treat. For the general population, no screening is available and it is often not diagnosed until late stages. Most lung cancers are also difficult to remove surgically even for localized cases, although survival for this small proportion of patients is higher. For populations with less access to trained surgeons and tertiary health care centers, the 5-year survival rate is only slightly lower (around 10%) than high-resource countries (up to 30% or slightly more). Clinical trials are currently underway to determine the risks and benefits of lung cancer screening modalities. Currently, the United States is the only country with lung cancer screening for certain populations. The US Preventive Task Force recommends annual screening for lung cancer with low-dose computed tomography in adults aged 55–80 years who have a 30 pack-year smoking history and currently smoke or have quit within the past 15 years. Once a person has not smoked for 15 years, he develops a health problem that limits life expectancy, or cannot have curative lung surgery, screening should be discontinued. Screening eligibility constitutes a fraction of all lung cancer patients and it should be noted that the US population 5-year survival rate for all lung cancers is 18.7%, which is not substantially higher than other countries.

Stomach Cancer

In addition to differences in screening availability and treatment, international differences in stomach cancer survival rates are also affected by differences in screening policy and patient awareness, as well as access to treatment. For example, Japan has a high stomach cancer survival rate due to nationwide gastric screening; the 5-year net survival is of 54% and most cases are diagnosed at an early stage. In China, another country with high stomach cancer incidence rates but without nationwide screening, the average 5-year survival rate is 31.3%. In the United States, where only about 26% of cases are diagnosed at an early stage, the 5-year survival rate is 29%. Generally, the observed differences have been attributed to lack of awareness of signs and symptoms and greater delay in seeking care among particular groups.

Prostate Cancer

Among men worldwide, over a quarter of all cancer survivors have had a diagnosis of prostate cancer. Since the widespread use of prostate-specific antigen testing in the late 1980s to early 1990s, the dramatic survival improvement in high-income countries has been attributed to lead-time bias. Many asymptomatic cancers would never have become clinically evident and for elderly men, the risks of invasive surgery outweigh the benefit. The 5-year net survival rate for patients diagnosed with prostate cancer is more than 90% in some countries (Israel, Canada, United States, Brazil, Ecuador, Germany, and many Western European countries). Survival rates are lower in Asia, where prostate cancer is less common or where public health programs or access to early diagnosis and treatment are unavailable.

Outlook in Cancer Survivorship

Cancer has become a significant health burden internationally with survivorship not always being attainable for a variety of socioeconomic, cultural, and environmental reasons. There are currently more survivors in high-income countries than in low- and middle-income countries (**Table 5**). Given the economic and political transitions, diagnostic, treatment, and care may not be accessible and/or available and result in different survival rates. Efforts are underway by the SURVCAN project to estimate, compare, and identify survival in low-to-middle income countries. This new survival database established at the International Agency for Research

Table 5 Prevalence of cancer survivors (number of survivors diagnosed with cancer within the past 5 years per 100,000 adults) (15+ years), 2012

Country	*Prevalence per 100,000 persons*
United Kingdom	1594
United States	1892.1
Norway	2020.1
Germany	1964.6
Italy	1933.8
Slovenia	1648.8
Ecuador	534.3
Brazil	720.2
China	456
India	202.9
Japan	1830.7
Tunisia[a]	310.1
Mauritius[a]	530.2

[a]Survival estimate considered less reliable.

Data from Ferlay, J., Soerjomataram, I., Ervik, M., et al. GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC cancer base no. 11. Available from: http://globocan.iarc.fr (accessed July 16, 2014).

on Cancer allows a comparison for cancer survival across cancer types and by country, incorporating survival analyses methodology and recommendations for improving survival statistics in lower-resources areas.

Patients face medical and nonmedical challenges in cancer survivorship. Results of cancer survivorship statistics reflect the impact of health-seeking behaviors, social inequities, natural histories, and health care service efficiencies in responding to diagnosis, treatment, and follow-up.

Sharing expertise between clinical and population-based research will simultaneously enhance the inference potential of data collected in both types of resources and also provide a cost-effective solution to targeting key issues in oncological research.

Increasing access to and improving screening program quality in high-risk groups, improving access to cancer treatment and management programs, and creating public health programs tailored toward health care barriers faced by each community are necessary to improve cancer survivorship.

Prospective Vision

Accurate estimation and interpretation of survival statistics is necessary for understanding patient prognosis. Currently, survival statistics are mostly unreliable or unavailable for many low-resource countries. Methodologies and public health efforts aimed at building institutions and programs are currently underway. As with any clinical diagnosis, cancer patients are not all the same and have different survivorship struggles. Cancer survivorship is a new and burgeoning field that seeks to understand the barriers to cancer diagnosis and treatment faced by individuals and their communities. Several indicators are being explored by the collaborative efforts of epidemiologists and clinicians to make public health programs relevant to patients and their community, and to improve cancer survivorship.

See also: Aging and Cancer. Chemoprevention Trials. Symptom Control.

Further Reading

Allemani, C., Weir, H.K., Carreira, H., et al., 2015journal. Global surveillance of cancer survival 1995–2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). Lancet 385, 977–1010.

American Cancer Society, 2016book. Cancer treatment & survivorship facts & figures 2016–2017. American Cancer Society, Atlanta. Available from: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/cancer-treatment-and-survivorship-facts-and-figures/cancer-treatment-and-survivorship-facts-and-figures-2016-2017.pdf.

Braaten, T., Weiderpass, E., Lund, E., 2009journal. Socioeconomic differences in cancer survival: the Norwegian Women and Cancer Study. BMC Public Health 9, 178.

Brenner, H., Hakulinen, T., 2002other. Advanced detection of time trends in long-term cancer patient survival: experience from 50 years of cancer registration in Finland. American Journal of Epidemiology 156 (6), 566–577.

Breslow, N.E., Day, N.E., 1987book. Fitting models to grouped data. In: Statistical methods in cancer research. Volume II – the design and analysis of cohort studies. World Health Organization, Lyon. IARC Scientific Publications No. 82. Available from: http://www.iarc.fr/en/publications/pdfs-online/stat/sp82/.

Ferlay, J., Soerjomataram, I., Ervik, M., et al.. GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC cancer base no. 11. Available from: http://globocan.iarc.fr. (Accessed 16 July 2014).

Feuerstein, M. (Ed.), 2007. Handbook of cancer survivorship. Springer, New York.
Hashim, D., Boffetta, P., La Vecchia, C., Rota, M., Bertuccio, P., Malvezzi, M., Negri, E., 2016journal. The global decrease in cancer mortality: trends and disparities. Annals of Oncology 27 (5), 926–933. https://doi.org/10.1093/annonc/mdw027.
Hosmer, D., Lemeshow, S., May, S., 2008other. Applied survival analysis. Wiley series in probability and statistics, 2nd edn. Wiley, Hoboken.
Marubini, E., Valsecchi, M.G., 2004book. Analysing survival data from clinical trials and observational studies. Wiley, New York.
Sankaranarayanan, R., Swaminathan, R., Lucas, E., 2011other. Cancer survival in Africa, Asia, the Caribbean and Central America. IARC Scientific Publications No. 162. International Agency for Research on Cancer, Lyon. Available from: http://survcan.iarc.fr/indexsurvcan1.php. (Accessed 16 July 2017).
Selvin S (2008). Survival analysis for epidemiologic and medical research. Cambridge: Cambridge University Press.
Therneau, T.M., Grambsch, P.M., 2000book. Modeling survival data: extending the Cox model. Springer-Verlag, New York.

Relevant Websites

http://eco.iarc.fr/eureg—European Cancer Observatory project at the International Agency for Research on Cancer.
https://seer.cancer.gov/publications/survival-1975-2010/—Cancer Survival From a Policy and Clinical Perspective: US Surveillance, Epidemiology, and End Results (SEER) Program, 1975–2010.
http://survcan.iarc.fr/—Cancer survival in Africa, Asia and Latin America (SURVCAN).
https://surveillance.cancer.gov/survival/index.html—Measures of cancer survival including cancer survival downloadable statistical software.

Cancer Vaccines: Dendritic Cell-Based Vaccines and Related Approaches☆

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Glossary

C-type lectin receptors Receptors that bind to carbohydrates in a calcium-dependent manner. They are able to recognize microbial antigens and can be expressed on dendritic cells and monocytes/macrophages.

Dendritic cells Professional antigen-presenting cells located in the skin, mucosa, lymphoid tissues, and circulating in blood. Dendritic cells form a cellular network of cells able to capture, process antigens and present them on the cell surface to activate T cells. They link the innate and the adaptive immune systems.

Immune checkpoint blockers Immunotherapeutic drugs (usually antibodies) that help release the brakes cancer cells put on the immune system to prevent their destruction. They have revealed efficient, particularly in patients with metastatic melanoma. They target for example CTLA-4, PD-1, or PD-L1 coinhibitory molecules.

Neoantigens Antigens specifically expressed by tumor cells and not by normal cells, generated from somatically mutated genes. They are ideal target for personalized cancer vaccine design.

Therapeutic cancer vaccine Vaccines developed to treat an existing cancer by stimulating patients' natural immune response against its own cancer. They are designed to activate cytotoxic T cells.

Toll-like receptors Receptors expressed on sentinel cells such as macrophages and dendritic cells, that recognize structurally conserved molecules derived from pathogens. TLR play a key role in the initiating immune responses.

Tumor-associated antigens Antigens differentially expressed by tumor cells compared to normal cells, corresponding to overexpressed proteins, tissue-specific molecules, cancer-testis or oncofetal antigens.

Whole exome sequencing Exome sequencing allows to identify mutations found in the coding region of genes which affect protein sequences.

Vaccination was first developed to prevent infectious diseases, and in this specific context, its efficacy relies on the induction of neutralizing antibodies targeting viral or bacterial pathogens. In the past decades, vaccines have also been developed to prevent virus-induced cancers such as uterine cancers driven by papillomaviruses, or hepato-carcinomas associated with hepatitis B virus. In these cases, blocking cell infection prevents cancer development. Therapeutic vaccination is different, as it aims at curing patients with cancer or chronic viral infection by boosting immune responses targeting diseased cells, whether malignant or infected, for elimination. Cytotoxic T lymphocytes (CTL) are the main immune effectors involved in these responses, hence the need to develop new vaccinations strategies that cannot simply reproduce disease prevention schemes that aim at triggering B cell responses. The scope of therapeutic vaccination covers priming of naïve T cells to reactivation of existing but anergic or dormant antitumor memory T cells.

Cellular cancer therapeutic vaccines can be based on killed cancer cells that carry a specific cancer-associated antigen(s) or on immune cells that are modified to present such an antigen(s) on their surface. Other types of cancer vaccines use viruses, yeast, or bacteria as vehicles to deliver both antigens and immunogenic signals. Alternatively, molecules of DNA or RNA coding for tumor-associated antigens (TAA) can be injected either alone (naked nucleic acid) or packaged into a vector.

Among immune cells, dendritic cells (DC) are considered "professional" antigen presenting cells (APC), due to their unique capacity to prime naive T cell responses. They function as sentinels, searching for and integrating so-called "danger" signals, and upon infection, they drive the most suitable immune response (Steinman and Banchereau, 2007). For this reason, dendritic cells are central for the antitumor vaccines approaches we describe here (Saxena and Bhardwaj, 2018; Bol et al., 2016; Palucka and Banchereau, 2013a): the injection of DC loaded with tumor antigen (Ag), and the injection of medicinal products targeting DC in vivo. Priming of both CD4+ helper and CD8+ cytotoxic antigen-specific T cells by DC is fundamental for therapeutic vaccine efficacy. These lymphocytes can recognize Ag expressed by tumor cells, either derived from mutation containing proteins (neoantigens), or overexpressed compared to healthy cells, or aberrantly expressed by tumor cells (spatially or temporally) (Coulie et al., 2014). Tumor-specific neoantigens are often considered as ideal tumor-rejection antigens, enabling the development of personalized vaccines.

The Ag recognized by T lymphocytes are peptides, arising from processed endogenous proteins, and presented in association with major histocompatibility (MHC) complex molecules (human leukocyte antigen (HLA) system in human). Antitumor T

☆*Change History*: March 2018. Laurence Chaperot, Olivier Manches, and Caroline Aspord rewrote the entire chapter.
This article is an update of James W. Young, Jean-Baptiste Latouche and Michel Sadelain, Cancer Vaccines: Gene Therapy and Dendritic Cell-Based Vaccines in Encyclopedia of Cancer (Second Edition) edited by Joseph R. Bertino, Academic Press, 2002, Pages 319–325.

lymphocytes activated upon vaccination will be efficient if they succeed in (i) migrating toward the tumor and their metastases, (ii) escaping the immune-subversion mechanisms found inside tumors (e.g., regulatory T cells, immunosuppressive soluble or membrane-associated factors), and (iii) lysing malignant cells. In order to exert long-lasting effects and prevent relapses, these T lymphocytes must retain their capacity to proliferate and persist as memory cells (Palucka and Banchereau, 2013a).

Dendritic Cells: Professional Antigen Presenting Cells

Dendritic cells represent critical regulators of the host immune system, involved in immune surveillance and bridging innate and adaptive immunity. They are native adjuvants, dictating the specificity and type of immune responses, depending on the microorganism or diseased cells they encounter. Multiple dendritic cell subsets have been described; they are produced in the bone marrow and derive from common myeloid progenitors (Gardner and Ruffell, 2016), and are found in almost all tissues, including blood, lymph nodes, spleen, skin or mucosae, where they act as sentinels and first activators for immune responses. Dendritic cells localized in tissues can take up Ag through specialized receptors, (by phagocytosis, endocytosis, or pinocytosis), detect associated danger signals, and migrate to secondary lymphoid organs. They integrate the environmental signals they receive, mature accordingly, process and present the Ag they have captured, in association with major histocompatibility complex class II and class I molecules, to finally activate both CD4+ and CD8+ T lymphocytes (Reis e Sousa, 2006). The diversity of dendritic cells in mice and humans is still under investigation, and the differential role that these subsets play in antitumor responses remains a matter of debate. DC subsets have been classified according to ontogeny, functionality, location or by transcriptome analyses. They are characterized by the expression of different surface molecules allowing the recognition of conserved pathogenic structures, their activation, migration, and interaction with various immune cells. Differential cytokine and chemokine secretion by different DC subsets accounts for the diverse mobilization, activation, and polarization of adaptive immune responses. A better understanding of DC heterogeneity is essential to improve the development DC-based antitumor immunotherapies.

Human Dendritic Cell Subsets

In human blood, three major DC subsets have been identified: plasmacytoid DC (pDC), CD1c+and CD141+ DC, that lack lineage markers (CD3, CD19, CD14, CD56, and GlycophorinA) and express high levels of MHC class II molecules (Dzionek et al., 2000). PDC express BDCA-2(CD303) and BDCA-4 (CD304), and are known to produce high levels of type I interferon (IFN) upon viral infection (Cella et al., 1999). Conventional or classical myeloid DC (cDC) produce interleukin (IL)-12 and are comprised of two subsets: BDCA-1 (CD1c)+ DC that may be particularly efficient for priming CD4+, and BDCA-3 (CD141) high DC, a minor population of DC in blood that are specialized in cross-presentation of exogenous antigen, allowing priming of CD8+ T cells against pathogens that do not infect DC. The diversity of DC subsets is still under investigation, and recently, single-cell RNA-seq and CyTOF uncovered an underestimated phenotypic heterogeneity of cDC, identifying a new AXL+SIGLEC6+ circulating cDC subset named AS DC (Villani et al., 2017; Alcantara-Hernandez et al., 2017) (**Table 1**). In the skin, Langerhans cells (LC) are localized in the epidermis, while the dermis contains mostly DC expressing CD1a or CD14 (thought to be monocyte-derived cells), but also CD141^{hi} DC (Gardner and Ruffell, 2016) and CD16+ SLAN DC (Gulley et al., 2016). Upon inflammation, the skin can also present with increased numbers of inflammatory DC (CD11c+, CD1c+, HLA-DR+) and pDC (Nestle et al., 2009). In the intestinal mucosae, three subsets of classical DC are found, characterized by their differential expression of Sirpα and CD103 (Watchmaker et al., 2014), whereas in the lung CD1c+ and CD141+ cDC have been described (Yu et al., 2013).

Table 1 Circulating DC subsets in human

	cDC CD1c+	*cDC CD141+*	*DC CD16+*	*AS DC*	*PDC*
Phenotype	CD45+, lin (CD3, CD19, CD56, CD14, GlycophorinA)-, HLA-DR+				
	CD11c+, CD123-			CD11c+/−, CD123+/−	CD11c-, CD123+
	CD1c+	BDCA-3+, CLEC9A+	CD16+	AXL+, SIGLEC6+	BDCA-4+
TLR expression	TLR1, **2**, 3, **4**, **5**, 6, 8, 10	TLR1, 2, **3**, 6, 8, 10	TLR1, **2**, **4**, **5**, 6, 8, 10	?	TLR1, 6, **7**, **9**, 10
C-type lectin expression	Dectin1, DEC-205, DCIR			?	Dectin1, DEC-205, DCIR
	DC-SIGN, CD206, CLEC10a	Dectin2, DC-SIGN, langerin, CLEC9A	Dectin2	?	BDCA-2, ILT7
Cytokine production	IL12p70, IL23	IL12p70, IFN-λ	IL-1β, TNF-α	IL12p70	Type I IFN

Expression of surface markers, Toll-like receptors, C-type lectin, and cytokine production by human DC populations.
−, negative; +, positive; bold numbers, main TLR expressed; ?, for controversial data, or not yet defined expression.

Antigen Capture by Dendritic Cells

In peripheral tissues, skin and mucosae where they are located, DC function as sentinels sampling self and foreign antigens for presentation. PDC and cDC express complementary sets of receptors allowing the recognition of different kinds of pathogens. Antigen (Ag) capture by cDC involves C-type lectin receptors (Caminschi et al., 2008; Sancho and Reis e Sousa, 2012): mannose receptor (MR, CD206), DC-SIGN (CD209), langerin (CD207), dectin-1 and 2, DEC-205 (CD205), LOX-1, DCIR, CLEC-9a, as well as receptors for immunoglobulin Fc regions (CD32 and CD64). PDC express CD32, allowing endocytosis of antibody-opsonized Ag; they may also capture Ag using BDCA-2, and DCIR. Following their uptake by DC, Ag are first directed to endosomes and *endo*-lysosomes, and processed via the exogenous pathway for presentation on MHC class II molecules to CD4 + T lymphocytes. Ag-derived peptides can also be loaded on MHC class I molecules via two cross-presentation pathways: the cytosolic and the vacuolar pathways (Blum et al., 2013). Cross-presentation, that is, the presentation of peptides derived from exogenous Ag by MHC class I molecules (Joffre et al., 2012), is crucial for initiation of CD8 + T cell responses, and is predominantly performed by DC, acting as bona fide professional APC.

Dendritic Cells Maturation and Migration

Ag capture mediated by CLR must be associated with the recognition of a danger signal for DC to trigger a protective immune response. Pattern recognition receptors (PRR) are environmental sensors that include the surface or endosomal toll-like receptors (TLR), and intracellular NOD-like (NLR) and helicases (RIG-like) (Krishnaswamy et al., 2013; van Kooyk and Geijtenbeek, 2003; Geijtenbeek and Gringhuis, 2009; Goutagny et al., 2012). These receptors recognize highly conserved structures expressed by pathogens: the so-called pathogen-associated molecular patterns (PAMP). The expression of TLR is somehow difficult to evaluate, since specific antibodies are not available for all TLR, and most analyses were made by RT-qPCR or functional assays with TLR-ligands. While CD1c+ cDC mainly express TLR 2, 4, 5, allowing recognition of peptidoglycan, LPS, flagellin, or zymozan, CD141+ cDC express high levels of TLR3, and pDC express TLR7 and 9, specific for viral single-stranded RNA (ssRNA) and hypomethylated CpG motifs of bacterial double-stranded DNA (dsDNA) (Hemont et al., 2013; Leal Rojas et al., 2017). Triggering of PRR modulates DC phenotype, DC maturation (upregulation of MHC class II molecules, expression of costimulatory molecules, for example, CD40, CD80, CD86, 4-1BBL, OX-40L) and cell shape modifications (dendritic morphology is acquired). DC migration to secondary lymphoid organs is driven by chemokine receptors such as CCR7 (receptor for the lymph node homing chemokines CCL19 and CCL21), where their cytokine secretion allows efficient T cell priming, activation, and polarization (Reis e Sousa, 2006). Mature DC phenotype can vary according to the nature of PAMP detected, and is further influenced by microenvironment clues, tissue integrity or local inflammation. Of note, DC are able to detect immunogenic signals produced by other cells, such as cell and tissue damage, and recognize "eat-me signals" expressed by necrotic and apoptotic cells (phosphatidyl-serine, calreticulin); they can also be activated by soluble factors released by dying-cells (ATP, HMGB-1) (Hemont et al., 2013).

DC are also found infiltrating tumors, and correlations have been described between tumor prognosis and the presence of mature or immature cDC, or the presence of pDC (Vacchelli et al., 2013). However, the tumor-microenvironment may contain many immunosuppressive factors (e.g., IL-10, TSLP, TGFβ, IDO), secreted by tumor cells, stromal cells, myeloid-derived suppressor cells (MDSC), or regulatory T cells that can locally suppress DC functions, blocking their recruitment, Ag presentation capacity, their activation or their maturation (Hargadon, 2013; Palucka and Banchereau, 2012; Faget et al., 2012; Le Mercier et al., 2013; Sisirak et al., 2012; Aspord et al., 2013). Understanding the complex interactions between dendritic cells and the tumor microenvironment is crucial to decipher how tumors subvert DC functions, in order to restore these functions (Palucka and Banchereau, 2012).

T Cell Responses Driven by Dendritic Cells

In secondary lymphoid organs, mature DC loaded with Ag present antigenic peptides and initiate CD4 + and CD8 + responses, and their functional plasticity is responsible for triggering different types of response according to the context of Ag encounter. In addition, the functional specialization of different DC subsets may help drive various classes of T cell responses. PDC are specialized in initiating antiviral immune responses and through their capacity to secrete huge amounts of type I interferon (Liu, 2005), they are involved in innate and adaptive immune responses (Colonna et al., 2004; Lande and Gilliet, 2010; Reizis et al., 2011). In vitro, pDC have been shown to capture and cross-present tumor or viral antigens (Lui et al., 2009), and stimulate specific adaptive T cell responses. In vivo, they are crucial for the development of cytotoxic effector cells in the context of cancer or viral infections (Villadangos and Young, 2008). Because they secrete cytokines such as IFNα and express costimulatory molecules (ICOSL, GITRL, OX40L, or CD70…), they can interact with innate and adaptive immune cells, modulate natural killer (NK) and iNKT cell responses, and activate B lymphocytes (Swiecki and Colonna, 2015). PDC can also prime Th1 or Th2 responses following contact with virus or IL-3 (Ito et al., 2004). Functional specialization of cDC has also been described, and different subsets can produce IL-12, IL-15, type I or III interferon, as well as regulatory cytokines, depending on the context of their activation. Among cDC, CD14 + DC preferentially activate follicular helper T cells, facilitating/enhancing humoral responses; CD1c+ DC may polarize T cells toward Th1 or Th17 pathway through their IL-12p70 and IL-23 secretion, CD141+ cDC seem as efficient as CD1c+ cDC to prime Th1 polarization, however, they can also induce Th2 cells through their expression of OX-40L (Leal Rojas et al., 2017; Durand and

Segura, 2015; Yu et al., 2014). Ag-specific T cells recognizing their cognate Ag presented by DC differentiate and proliferate (Kapsenberg, 2003). DC-derived IFNα, IL-12, or IL-15 skew differentiation of CD4+ T lymphocytes toward the Th1 phenotype, that favors differentiation of CD8+ CTL. Conversely, if DC express IDO (indoleamine 2,3-dioxygenase), secrete IL-10, TGFβ, or express coinhibitory molecules such as CTLA-4 or PD-L1, they can drive the differentiation of CD4+ regulatory T cells, with tolerogenic functions (Clark and Kupper, 2005). Of note, immature DC are intrinsically tolerogenic, and can induce specific T cell anergy. Altogether, the quality of the generated T cell response depends on the functional status of DC and the DC subtype, and DC plasticity is a key element for orchestrating this diversity of immune responses.

Naïve T lymphocytes migrate to secondary lymphoid organs where they scan MHC-bound peptidic antigens on DC through their T cell receptor (TCR). Upon specific antigen encounter, T lymphocytes are activated, proliferate and differentiate, and then leave the lymph node to reach the tissues where the Ag is expressed. This specific migration is governed by expression of a set of chemokine receptors and adhesion molecules at the surface of T cells (Sheridan and Lefrancois, 2011). T lymphocytes expressing CLA, CCR4, and CCR10 migrate to the skin, if they express CD49a they will preferentially home to the lung, whereas CCR9 and α4β7 integrin expressing T lymphocytes migrate to the intestine. Thus, effector T cell function clearly depends on their capacity to migrate to the site where Ag are located, and to exert their function despite an immunosuppressive environment, to effectively counteract immune evasion (Hargadon, 2013; Gajewski et al., 2013).

Dendritic Cell-Based Therapeutic Vaccination

The goal of cancer vaccines is to induce efficient CTL responses directed toward tumor-associated antigens. Several approaches have been developed, based on the injection of the defined antigens, in the form of whole proteins or antigenic peptides, or by the delivery of genes encoding these peptides or proteins, associated or not with adjuvants or costimulatory signals. Since dendritic cells are very efficient APC, many cancer vaccine approaches use these cells, either by injecting them loaded with the Ag, or by using vectors to address the Ag to specific DC subsets in vivo. Several parameters must be taken into account to optimize the design of therapeutic cancer vaccines based on DC or on other approaches of Ag delivery.

Diversity of Dendritic Cells

As described above, DC belong to a complex multicellular system whose role in initiating potent immune responses is crucial for antitumor immunotherapy. Different strategies taking advantage of this potency are used at the forefront of cancer immunotherapy. However, due to the heterogeneity and plasticity of DC subtypes, several parameters have to be taken into account and tested individually to devise an optimal DC-based immunogen.

Previous studies have shown how difficult it is to identify the best DC subset and DC differentiation protocol to be used for maximal clinical efficacy. Blood DC represent a tiny percentages of mononuclear cells (less than 1% for CD1c+ DC and even less for CD141+ DC), so DC purified from blood have been used only sporadically in immunization protocols (see for example the clinical trial NCT01690377 in advanced melanoma that relies on injection of purified CD1c+ DC). To obtain sufficient numbers of DC, and for logistic and economic purposes, monocyte-derived DC have been the predominant source for vaccination in human clinical trials.

Indeed, monocyte-derived DC (moDC) can be obtained in large numbers by isolation of monocytes, followed by incubation with GM-CSF and IL-4, yielding pure moDC populations in a relatively easy and scalable way. However, moDC may not be endowed with the same stimulation, migration, and functional capabilities as endogenous DC (transcriptomic analysis suggest a phenotype close to inflammatory DC) (Segura et al., 2013), although they are very potent at MHC-II presentation and CD4+ T cell activation. DC generated from monocytes by culture in GM-CSF and IFNα may be more efficient for cross-presentation to CD8+ T cells (Lapenta et al., 2006). CD34+ hematopoietic progenitor cells (HPCs) can be used as precursors for DC differentiation. HPC treated with c-kit ligand and tumor necrosis factor-α (TNF-α) yield subsets of myeloid DC in different stages of differentiation, including Langerhans cells (Caux et al., 1996). Alternatives to in vitro-derived DC include cell lines with similar functionalities as primary DC, such as the myeloid MUTZ-3 cell line derived from leukemic myeloid cells; however these cells have not yet been used in clinical trials. As described further below, a cell line was derived from the leukemic cells of a patient with plasmacytoid DC leukemia, which shares many phenotypic and functional features with primary pDC; this cell line (GENius-Vac) was recently used in a phase I clinical trial in melanoma.

Provenge®/Sipuleucel, developed by Dendreon Corporation, which was FDA approved in 2010, represents a unique case, as the injected cells are not strictly DC per se. Indeed, patients' monocytes are incubated for a short period of time (4 days) with a fusion protein of GM-CSF and prostatic acid phosphatase (PAP). No differentiation factor, such as IL-4 or IL-15, is added, and no maturation is induced. The actual mechanisms of action of the vaccine are not known, but injected cells may complete their differentiation in vivo to stimulate CD4+ and CD8+ T cells.

It is to be noted that injected DC may not directly prime specific T cells. Thus, it has been demonstrated in mouse models that antigen-loaded DC do not prime T cell responses without the help from endogenous DC (Yewdall et al., 2010). Antigen transfer between DC populations in the lymph node, through transfer of MHC-peptide (cross-dressing), exosome secretion, or capture of apoptotic cell, likely plays a role in CD4+ T cells activation and cross-presentation to CD8+ T cells. Studies of T cell activation in viral infection have identified choreographed interactions between antigen-carrying migratory DC and

lymph node resident XCR1+ DC that are necessary to cross-present viral antigen to CD8+ CTL (Hor et al., 2015; Eickhoff et al., 2015).

Nature and Formulation of Tumor Antigens

A critical aspect of vaccination is the nature of the targeted antigens, as well as the form with which they are delivered to DC. Most clinical trials have used DC pulsed by MHC class I restricted peptides to specifically induce CD8+ T cells. In order to optimize the uptake and presentation of antigens, in vitro-derived DC can be loaded with short (9-mer) or long peptides, whole proteins, tumor antigen coding RNA, DNA, or tumor lysate. In other approaches, the Ag (peptides, proteins, RNA, or DNA) can also be directly injected in diverse formulations, associating vectors and adjuvants for optimal targeting to tissue dendritic cells. Peptide or protein-based vaccines can be associated with heat shock proteins, or encapsulated into liposomes (Hu et al., 2018). DNA-vaccines seem safe and promising. Autologous cells at the injection site, upon electroporation or other delivery processes, will up take the nucleic acids and synthesize the antigens, allowing development of immune responses directed toward these antigens. Different mechanisms of action are proposed for DNA vaccines, depending on the route of injection. If the vaccine is injected intranodally, or in the skin, Ag are directly produced by local dendritic cells, whereas if the DNA is injected intramuscularly, Ag are secreted by muscular cells, and must be taken-up by dendritic cells to trigger immune responses. The current strategies to improve the efficacy of DNA vaccine have been described recently (Tiptiri-Kourpeti et al., 2016): they include optimization of delivery systems, association with adjuvants, and introduction of sequences coding for costimulatory signals, cytokines, chemokines, or danger signals to improve vaccine immunogenicity.

Whatever the form chosen for the vaccine candidate antigens, it is crucial to succeed in targeting them to dendritic cells, in order to ensure optimal T cell priming. Targeting multiple defined or undefined (lysate) antigens is likely to be advantageous, by limiting the risk of tumor escape through antigen loss. In addition, recent studies have shown that tumors can take advantage of MHC locus inactivation to prevent presentation of too diverse a repertoire of peptides, such that tumors with loss of heterozygosity in HLA class I, resulting in HLA-homozygosity, is a prevalent immune escape mechanism in lung cancer (McGranahan et al., 2017). Maximal heterozygosity at HLA class I loci (A, B, and C) and particular HLA class I supertypes are associated with increased overall survival after immune checkpoint blockade (Chowell et al., 2018). Long antigenic sequences (DNA, RNA encoded or long peptides) can potentially enable both CD4+ and CD8+ T cell activation. Concomitant activation of helper CD4+ T cell responses and CD8+ cytotoxic T cells has been associated with clinical benefit, as demonstrated with DC pulsed by MHC-I and MHC-II restricted peptides (Aarntzen et al., 2013). Antigen choice is crucial, as highlighted by the fact that not all tumor-associated antigenic sequences are immunogenic, and that not all immunogenic sequences can induce T cell-mediated tumor rejection, which defines a limited subset of tumor rejection antigens. The size and functional status of the specific T cell repertoire has to be considered. As an example, differentiation antigens, such as Melan-A or tyrosinase in melanoma, are immunogenic and a number of DC vaccine trials have shown potent CD8+ T cell amplifications against these antigens. However, the functionality and avidity of specific T cell may be suboptimal to mediate tumor rejection, due to the potential peripheral tolerance to these self-antigens. Ideal tumor antigens are either selectively expressed by the tumor, but not by healthy somatic tissues, or are specifically and uniquely expressed by the tumor. The first category is exemplified by the so-called cancer-testis (CT) antigens (e.g., NY-ESO-1, MAGE antigens), whose expression is restricted to germline and placental tissue, and absent in somatic tissues. Tumors of diverse origins often express CT antigens due to epigenetic dysregulation. CT antigens are being used in a large number of clinical trials and have been shown to be highly immunogenic, despite relatively limited clinical responses up to now. In the latest years, progress in sequencing technologies has opened the door to the identification of the full spectrum of somatic mutations in one particular tumor. In conjunction with the improvements in MHC binding prediction algorithms, it is now possible to identify and select tumor neoantigens (derived from somatic mutations) expressed by the tumor in a highly specific fashion (Kroemer and Zitvogel, 2012). As an example, a large scale high-throughput analysis was performed by the Xpresident platform, combining sequencing of HLA-bound peptides, RNA sequencing and whole exome DNA sequencing of renal cell carcinoma and healthy autologous tissue, to identify a combination of 10 peptides specifically expressed by the tumor (TUMAP). These peptides are now being used for vaccination in conjunction with GM-CSF injection (Walter et al., 2012). An extensive catalog of somatic mutations in 7042 cancers was published, identifying 4,938,362 mutations and 20 distinct mutational signatures, of which some are shared among different cancers, and others are unique (Alexandrov et al., 2013). Such neoantigens are now being targeted in a large number of clinical trials (Hu et al., 2018). Preliminary results seem to indicate that both the quantity and the quality of neoantigens expressed by a tumor are important in determining the efficacy of vaccination. Thus, tumor with the highest somatic mutation and neoantigen burden (e.g., lung cancer, melanoma), patients with microsatellite instability, tend to respond better to immune checkpoint inhibitors (Rizvi et al., 2015; Van Allen et al., 2015). Most mutations are in nonessential genes (passenger mutations) and vastly outnumber mutations in driver genes. Recent studies have uncovered the role of presumably immunodominant neoantigenic sequences in determining clinical outcome in long-term cancer survivors (Luksza et al., 2017). Both the probability of binding to MHC molecules and homology with microbial peptide sequences signal a high probability of recognition by specific CD8+ T cells, and tumor expression of these high quality neoantigens predicts a better clinical outcome. Characterization of the specificity of tumor infiltrating lymphocytes (TILs) will further validate these observations and enhance predictions of immunogenic tumor antigens (Linnemann et al., 2013). Finally, DC vaccination can potentially be enhanced by the use of epigenetic drugs that can augment the expression of tumor antigens and MHC molecules, in addition to their direct antioncogenic and pro-apoptotic effect (Heninger et al., 2015).

Dendritic Cell Maturation and Injection Route

We mentioned the importance of DC maturation stage, which determines the quality and magnitude of induced T cell responses. The generation of local inflammation increasing the immunogenicity of a vaccine can be obtained by using adjuvants such as Montanide water-in-oil emulsions (Clancy-Thompson et al., 2013; Valmori et al., 2007). By using TLR or NLR agonists (mimicking microbial stimulation) as adjuvants, one can specifically activate one or the other DC subset, endowing them with the requisite immunostimulatory properties. Thus, imiquimod, a TLR7 agonist, can activate pDC to produce type I IFN, whereas poly-IC can activate cDC through TLR3 to produce IL-12. Poly-I:C stimulation seems to be optimal for CD8 T cell priming by moDC, whereas triggering other TLR, such as TLR4, may induce suppressive IL-10 secretion that could impede on IL-12 secretion and Th1 priming (Bogunovic et al., 2011). CD40 agonists can also be used for maturing and "licensing" DC, partially alleviating the need for CD4+ T cell help. GM-CSF has been incorporated in several cancer vaccines to enhance their immunostimulatory capacities, for example in the GVAX strategy based on transduction of autologous or allogeneic whole tumor cells (Burkhardt et al., 2013; Lipson et al., 2015). Transduction of DC with constructs knocking down negative regulators of DC activation, such as SOCS1 (Evel-Kabler et al., 2006), can help potentiate cytokine secretion and prolong antigen presentation by injected DC to enhance antitumor immune responses (Shen et al., 2004).

Many questions remain as to what is the best route for therapeutic vaccine injection. Regarding DC-based vaccines, subcutaneous injection is the most frequently used, while intranodal injection has also been tested to bypass DC migration to secondary organs. DNA vaccine can be administered by intradermal, intranodal, or intramuscular routes. Other approaches include device-mediated (gene gun) DNA delivery to keratinocytes or Langerhans cells. Electroporation by local application of electrical pulses causes transient cell membrane permeabilization, allowing DNA entry into cell cytoplasm.

It is crucial to garner knowledge on DC circulation upon immunization, as this will likely impact on activated T cell trafficking, or on the differentiation of central and effector memory T cells. Thus, immunization against cutaneous and pulmonary tumors probably requires different routes of injection for optimal antitumor T cell activation and homing (Sandoval et al., 2013). Preconditioning of draining lymph nodes in animal models has shown that it can highly increase DC migration and vaccine efficacy (MartIn-Fontecha et al., 2003). Some strategies currently being tested in clinical trials involve direct intratumor injection of TLR agonists, with or without concomitant T cell costimulatory activation. They aim at modifying the tumor microenvironment, while recruiting DC and T cells, inducing DC migration to draining lymph nodes. Remarkably, in situ injection of CpG oligonucleotides (TLR9 agonists) in conjunction with OX40L agonistic antibodies in various murine tumors triggers systemic cytotoxic immune responses, eradicating metastasizing tumor cells and preventing tumor growth upon rechallenge (Sagiv-Barfi et al., 2018). Despite these advances, it is not clear yet which DC activation protocol and vaccine injection route will induce adequate T cell stimulation, in view of the frequent immune dysfunction observed in various malignancies.

Dendritic Cells in Cancer Immunotherapy Clinical Trials

Increased knowledge of DC biology and optimized protocols for DC generation have translated into the design of immunotherapeutic strategies that are now being tested in multiple clinical trials. Safety and clinical evaluations are most of the time complemented with immunomonitoring protocols that quantify the induction of specific helper and CTL responses against tumor antigens.

Ongoing DC-Based Vaccines

A pilot clinical trial in lymphoma utilizing DC pulsed by tumor-specific idiotype was performed more than two decades ago. However, as of today, only two commercial DC vaccines are approved as drug products. The most prominent is Provenge®/Sipuleucel for the treatment of advanced prostate cancer. CreaVaxRCC® developed by the company CreaGene was approved in 2007 in Korea for the treatment of metastatic renal carcinoma. The National Institute of Health website (clinicaltrials.gov) represents an invaluable database cataloguing worldwide information about clinical trials. An analysis of the number of ongoing and completed DC vaccine trials highlights the positive dynamics of therapeutic DC vaccines for the treatment of cancer. Thus, a keyword search for "dendritic cell" and "vaccine" identifies 400 clinical studies. Sixty-seven of them are actively recruiting at the time of this writing. A large number of these studies are in phase 1 (257) and phase 2 (221), whereas only 10 protocols are currently tested in phase 3 trials, reflecting the challenges facing many early clinical trials and the difficulty in demonstrating clinical benefit. The ongoing trial NCT00045968 in glioblastoma multiforme is one of the few phase 3 DC-based studies. Developed by Northwest Biotherapeutics, DCVax®-L consists in autologous DC pulsed with an autologous tumor lysate. Patients are injected with DCVax®-L over a period of 120 weeks, while the placebo group receives mononuclear cells. Phase 1/2 evaluation of DCVax®-L demonstrated an increase in median overall survival (>4 years in 33% of patients and more than 6 years in 27%, compared to 14.6 months with conventional treatments) warranting further evaluation in a phase 3 trial.

Many approaches are being tested to optimize immunization by DC. The vast majority (99%) of these studies focus on immunization by cDC, whether directly purified from blood, or differentiated from monocytes or CD34+ HSC. However, two teams have also exploited the antigen presenting capabilities of pDC to immunize patients with metastatic melanoma. In the first trial (NCT0190377) (Tel et al., 2013), patients received intranodal injection of purified and tumor antigen-loaded autologous pDC.

In the second one (NCT01863108), the semi-allogeneic (HLA-A2+) pDC cell line GENius-Vac was used as vaccination platform upon loading pDC with melanoma-associated HLA-A2-restricted peptides. This approach, relying on the use of a well-characterized cell line, allows large cell numbers of a homogeneous cell preparation to be injected, at reduced costs. Similarly, in clinical trial NCT01373515 HLA-A2+ patients with acute myeloid leukemia were injected with a semi-allogeneic (HLA-A2+) acute myeloid leukemia cell line that differentiates into functional mature dendritic cells upon anthracycline treatment. Rather than relying on injection of whole dendritic cells, an original approach exploits the immunogenic properties of DC-derived nanovesicles termed "exosomes" that can mediate MHC-dependent immune responses. A phase II trial of vaccination with tumor antigen-loaded dendritic cell-derived exosomes was performed in nonsmall cell lung cancer (NCT01159288).

Source of Antigen

It is still unclear which antigenic targets will prove to be the most beneficial for long term clinical efficacy. Defined shared antigens, such as cancer-testis antigens or carcinoembryonic antigens are either loaded as peptides onto in vitro-derived DC, or targeted to DC through endocytic receptors. An example of this latter strategy is the intranodal injection of a DEC-205- NY-ESO-1 fusion protein with or without Sirolimus (rapamycin) administration in astrocytoma (NCT01522820). Targeting of antigens to DEC-205 on DC has been shown to potentiate cross-presentation to CD8+ T cells (Bonifaz et al., 2002). As mentioned above, it is becoming widely appreciated that including multiple antigens in a vaccine could prevent tumor immune escape by outgrowth of antigen-loss variants. This may be one of the reason for the efficacy of the DCVax®-L trial, where undefined but numerous antigens are loaded onto DC in the form of a cellular lysate. Several clinical trials are ongoing with DNA vaccines (Tiptiri-Kourpeti et al., 2016), mainly in melanoma, prostate or HPV-associated cancers, targeting various kinds of tumor-associated Ag (over-expressed, CT, or onco-viral). Recently, a clinical trial based on a DNA vaccine coding for two epitopes from gp100 melanoma-derived Ag inserted in a human IgG1 antibody sequence has been shown to be safe and stimulated T cell responses in melanoma patients (Patel et al., 2018).

The new sequencing technologies (whole exome sequencing, RNAseq analysis) provide the opportunity to identify and target many tumor-specific neoantigens at the same time. So far, several phase I trials evaluating vaccination with personalized neoantigens are ongoing or have been completed in melanoma. The NCT00683670 study used DC loaded with short HLA-A2-restricted neoantigen peptides (Carreno et al., 2015). The NeoVax trial (NCT01970358) involved subcutaneous administration of up to 20 long neopeptides, admixed with poly-IC-LC (carboxymethylcellulose, polyinosinic-polycytidylic acid, and poly-L-lysine) (Ott et al., 2017). In the IVAC MUTANOME trial (NCT02035956) several doses of RNA encoding shared tumor antigen (tyrosinase and/or NY-ESO-1) and neoantigens were injected into inguinal lymph nodes (Sahin et al., 2017). The latter two studies rely on neoantigen uptake and presentation by tissue- or lymph-node resident DC to activate antitumor T cells. Although performed on a small number of patients, impressive immune responses against neoantigens were observed in all three studies. Some patients experienced vaccine-related objective clinical responses in the NeoVax and IVAC MUTANOME trials, warranting further evaluation in large scale studies. Neoantigens can also be used in DNA vaccine approaches, as in the ongoing clinical trial NCT03122106, in pancreatic cancer patients. This neoantigen DNA vaccine, administered with an electroporation device, incorporates neoantigens and personalized mesothelin polyepitopes, with the aim to activate both CD4+ and CD8+ T lymphocytes.

Reversing Immunosuppression

Due to the systemic and local immune dysregulation observed in the tumor microenvironment, and the sensitivity of DC to immunosuppressive cytokines and ligands, it is likely that the best immunization regimens will require coadministration of DC vaccines with other therapies. Current combination trials testing DC vaccines have been extensively described (Saxena and Bhardwaj, 2018), and we will briefly mention here a few important themes. One goal is to improve antigen delivery to DC in an immunogenic environment, which is being tested in different ways. DC vaccination combined with chemotherapy inducing "immunogenic cell death" (doxorubicine and cyclophosphamide) (e.g., NCT00082641), or with concomitant injection of antitumor T cells (unmodified autologous T cells (e.g., NCT00338377), antigen-specific TCR transduced T cells (e.g., NCT00910650, NCT01697527)), will test this concept in different malignancies. Another aim is to bypass immunosuppressive pathways, whether metabolic (e.g., IDO), induced by tumor-secreted mediators (e.g., prostaglandins) or through surface receptors (such as the PD-1/PD-L1 interaction). Thus, the breast cancer clinical trial NCT01042535 combines p53-targeted DC immunization with administration of the IDO inhibitor 1-methyl-D-tryptophan, whereas in trial NCT01560923 patients with advanced prostate cancer receive Sipuleucel with the IDO inhibitor indoximod. Immune checkpoint inhibitors (anti-PD-1 antibodies nivolumab, pembrolizumab, and anti-PD-L1 atezolizumab) are used to reawaken and prevent deactivation of T cell immune responses. Building on the results of the first DCVax-L trial, a phase 2 trial is being initiated, that combines vaccination with tumor lysate-loaded DC with nivolumab injection (NCT03014804). Finally, a few studies test sequential immunotherapy with multiple immunomodulators, in a multipronged strategy to enhance vaccine-induced immune responses. In trial NCT02677155, patients with lymphoma receive sequential intratumor injections of low-dose rituximab (anti-CD20) combined with local radiotherapy, and autologous DC with subcutaneous GM-CSF injection, with the aim of enhancing tumor antigen delivery and uptake by DC. Patients also receive intravenous anti-PD-1 antibody (Pembrolizumab) to overcome tumor tolerance. This strategy reflects the growing trend toward combination of standalone promising approaches, such as DC vaccination, immunogenic conditioning by cytotoxic therapy, neoantigen targeting, and immune checkpoint inhibitors blockade, which when used together will hopefully translate into greater clinical benefits for cancer patients.

Defining Responders and Nonresponders Patients

Understanding the effects of DC vaccines on the immune system is crucial to better characterize the pathways required to induce protective antitumor responses. The status of patient immune system affects the capacity to trigger antitumor T cell responses, modulates responses to immunotherapies, and also affects clinical responses. It is crucial not only to define the factors required for the activation of effector T cells, but also to define the interactions between DC and innate immune cells. The modulation of suppressive cells in peripheral blood but also at the tumor site have to be better characterized to potentiate vaccine efficacy and design. Identification of response biomarkers differentiating early responder and nonresponder patients will guide future developments. System-wide immunologic approaches, combining transcriptome profiling with multiparametric analysis by flow cytometry and proteomic analysis, may lead to the identification of new biomarkers of efficacy (Pulendran et al., 2010). Isolation of molecular and cellular signatures predicting the immunogenicity and efficacy of immunotherapies, and their correlation with induction of protective immune responses, will help optimize future vaccines.

Clinical Evaluation

In addition, RECIST (response evaluation criteria in solid tumors) criteria of clinical evaluation (Eisenhauer et al., 2009) may not be appropriate to evaluate the clinical efficacy of immunomodulatory therapies. Indeed, in early time points after the beginning of immunotherapeutic treatment, a temporary tumor "growth" can be observed preceding a durable regression, the former reflecting inflammatory processes associated with active immune responses and tumor infiltration. Clinical evaluation based on irRC (immune-related response criteria) (Wolchok et al., 2009) will be more suitable to define the real clinical impact of immunotherapies.

Future Directions With Cancer Vaccines

Therapeutic DC vaccines in clinic trials have often been disappointing, despite exciting results obtained in animals and preclinical trials. However with the development of new generation products such as combination with novel adjuvants or other immunotherapies, the use of new DC subsets (pDC) and clinical evaluation based on immunological criteria (Wolchok et al., 2009), these approaches should evolve toward potent anticancer therapies. Clinical trials performed in the last 20 years proved that DC-based vaccines are well tolerated, highly immunogenic, and trigger tumor-specific and functional T-cell responses in many patients. Despite limited objective clinical responses, these responses are often durable, correlate with the induction of antitumor immune responses, and have been associated with objective tumor regressions in some instances, demonstrating the capacity of the immune system to control tumor development. New clinical strategies are needed to improve the potential of DC vaccines to trigger tumor regression. One major problem of DC-based trials is that patients are treated in advanced stages of cancer, when the immune system is subverted by the unfavorable tumor microenvironment. Critical points to overcome for a better efficacy are (1) the absence of costimulatory signals within tumor microenvironment, (2) the presence of inadequate signals that lead to DC subversion and prevent triggering of protective antitumor immune responses, and (3) a better understanding of the mechanisms directing the response to immunotherapies. Advances in the knowledge of DC biology and in the understanding of interactions between tumor cells and the immune system have led to the emergence of new strategies that exploit the properties of various DC subsets and neutralize the immunosuppressive tumor microenvironment. Whereas past developments often required individual DC production, the trend is now to design universal products such as vaccines targeting DC in vivo, for broader use with simplified, standardized, and less expensive products. However, there is a parallel trend toward the development of personalized approaches using neoantigens. All in all, these strategies offer new therapeutic perspectives in the treatment of cancer and will hopefully translate into better clinical success (**Fig. 1**).

Exploitation of Various DC Subsets

As mentioned earlier, DC used in immunotherapy are generated from monocytes, differentiated in vitro from CD34+ HSC, or directly purified from circulating immune cells of patients. The discovery of new DC subsets with unique properties allows their use as vectors for cancer immunotherapy (Ueno et al., 2011; Palucka and Banchereau, 2013b). Distinct DC subsets trigger various T cell responses, and the quality of CTL induced by DC is crucial for antitumor efficacy. Various cytokine cocktails drive differentiation of DC with specific properties in vitro. Thus, moDC generated in the presence of GM-CSF and IL-15 induce CTL exhibiting higher antitumor activity compared to CTL induced by moDC generated in presence of GM-CSF and IL-4 (Dubsky et al., 2007), which may transfer into higher clinical efficacy. HPC can also be used to generate multiple DC subsets in sufficient numbers for immunotherapy. Differentiation of the cross-presenting CLEC9A+ DC or Langerhans cells from CD34+ HPCs brings the opportunity to use specialized DC subsets for vaccination, and is currently being tested in clinical trials. Alternative strategies consist in directly purifying DC subsets from circulating immune cells of the patients. The use of primary circulating CD1c+ DC activated and loaded with tumor-derived peptides led to the induction of strong antitumor T cell responses associated with favorable progression free survival in stage IV melanoma patients (Schreibelt et al., 2016). pDC are also crucial actors of antitumor immune responses, and represent promising vectors for immunotherapy (Tel et al., 2012). In melanoma patients, injection of purified circulating pDC that

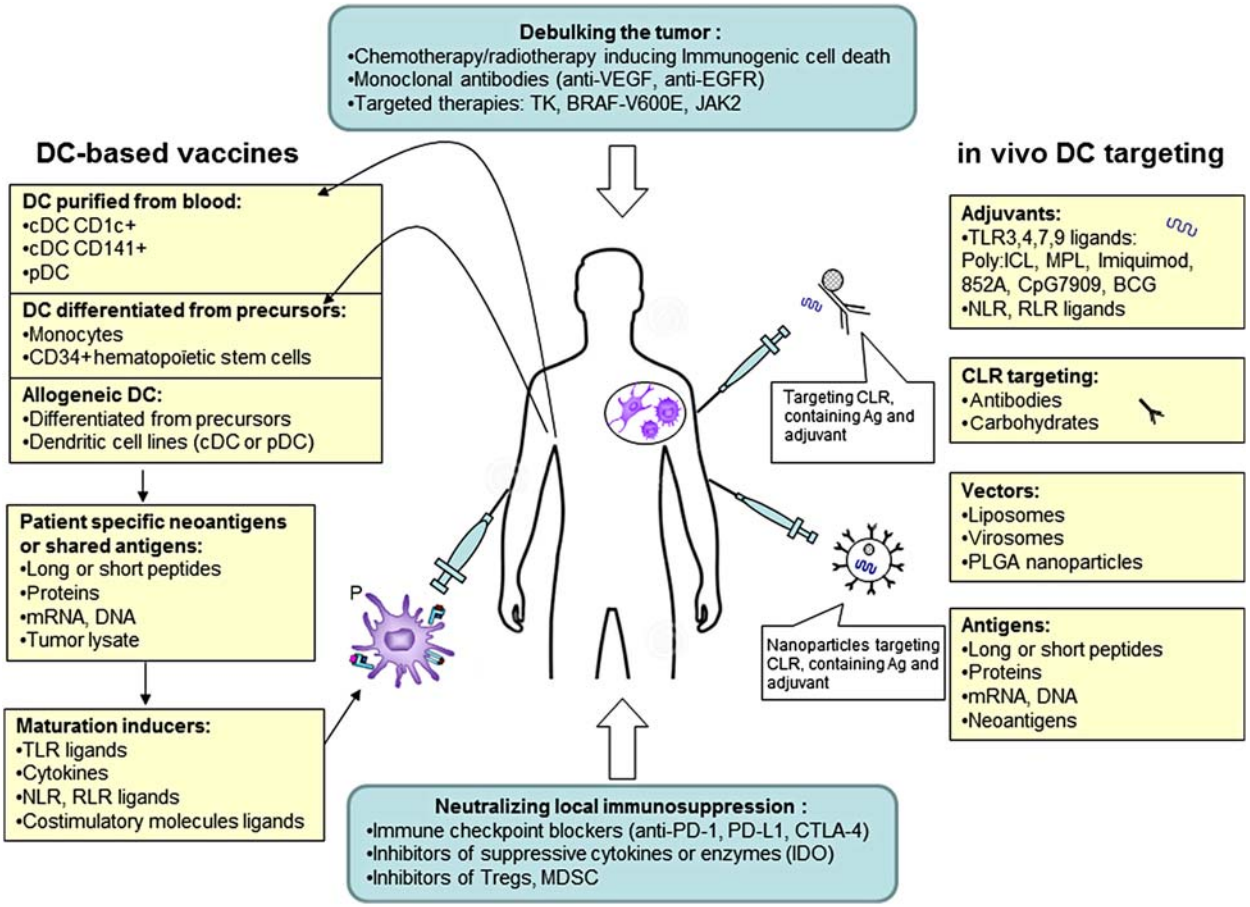

Fig. 1 Optimizing DC-based therapy. The current approaches are based on injection of autologous dendritic cells generated from monocytes or hematopoietic stem cells, or purified from patient's blood, or of allogeneic DC lines. These DC are loaded in vitro with tumor Ag (neoantigens or shared Ag), activated with different adjuvants, and injected to the patients. DC in vivo targeting approaches are emerging; they aim at specifically targeting DC in vivo using pattern recognition receptors (PRR) or C-type lectin (CLR) ligands. CLR can be targeted through antibodies or glycans conjugated to an antigen, while TLR triggering activates a signaling cascade resulting in DC activation. Combination strategies may be the key for DC vaccine success, with the use of drugs neutralizing local immunosuppression, or debulking the tumor.

have been activated and pulsed with peptides derived from tumor antigens triggered significant CD4+ and CD8+ T cell responses, and was associated with promising clinical responses (Tel et al., 2013). However, large scale therapeutic use of autologous primary DC is difficult due to the low number of circulating DC, their potential functional alteration induced by the tumor, and the individual preparation required for each patient. These obstacles can be overcome by using semi-allogeneic DC cell lines. The MUTZ3 cell line derived from human leukemic myeloid cells harbor cDC phenotype and function (Santegoets et al., 2008). We developed an alternative strategy based on the exploitation of a human pDC line HLA-A*0201+ derived from leukemic pDC. This pDC line shares phenotypic and functional properties with primary pDC. The GENiusVac strategy (Aspord et al., 2011a) is based on the use of the irradiated pDC line loaded with HLA-A*0201+-restricted peptides derived from tumor or viral antigens to trigger antigen-specific cytotoxic CD8 T cells in allogeneic but HLA-A*0201+ matched settings. We demonstrated the strong potency of this strategy to induce multispecific and highly functional antitumor and antiviral T cell responses in vitro, its therapeutic efficacy in vivo in humanized mice, and its clinical relevance ex vivo with patients' T cells (Aspord et al., 2010, 2011b, 2012). The GENiusVac strategy is currently tested in a phase I trial in metastatic melanoma. Such "one to many" strategy offers many advantages compared to existing immunotherapies, including its standardizable design, broad potential use for numerous tumoral and viral pathologies. Partial HLA matching between the vaccine and the patients further exploits the allogeneic effect to potentiate the immunogenicity of tumor or viral antigens (Fabre, 2001). Such semi-allogeneic settings have been explored in strategies using hybrid vaccines between DC and allogeneic tumor cells (Kugler et al., 2000), in another setting, allogeneic skin graft has been shown to trigger antitumor response in patients with cancer prostate (Muir et al., 2006).

Exploitation of Neoantigens

As described above, neoantigens are generated by tumor-related (driver) or spontaneous (bystander) mutations that can be presented as highly specific tumor antigens. It has been shown that the mutational load and microsatellite instability in solid tumors

correlates with the response to immune checkpoint blockers (Rizvi et al., 2015; Van Allen et al., 2015). The use of DC loaded with patient-specific peptides derived from mutated neoantigens is a powerful alternative, currently under investigation, that may trigger epitope spreading, diversification of the TCR repertoire and elicit specific CD8 T cells in cancer patients (Carreno et al., 2015).

In Vivo DC Targeting

DC scan the environment through PRR and take up antigens recognized by their CLR. Various DC subsets differentially express PRR, and CLR allowing their specific targeting to endow them with desired functionalities. Thus, emerging strategies aim at specifically targeting DC in vivo using PRR or CLR ligands. CLR can be targeted through an antibody or a glycan conjugated to an antigen that will be internalized, allowing the cross-presentation of peptides derived from the antigen. TLR triggering activates a signaling cascade resulting in DC activation: upregulation of costimulatory molecules expression and cytokine secretion, which favor innate and adaptive immune responses. Such in vivo DC targeting offers the advantage to develop universal off-the-shelf products, which is highly convenient for future transfer into the clinic.

Targeting DC through CLR

A large number of CLR can be exploited to target different DC subsets (Ueno et al., 2011). Current approaches targeting CLR with antibodies show promising preclinical results: targeting of DEC-205, DC-SIGN or MR expressed by cDC, langerin expressed by LC, CLEC9A expressed by BDCA-3+ DC, or dectin-1 and DCIR expressed by pDC (Palucka and Banchereau, 2013a; Ueno et al., 2011) allows the capture and cross-presentation of Ab-associated antigens, leading to induction of specific T cell responses. The use of DC-targeting antibodies combined to antigens together with adjuvants leads to efficient presentation of antigens by DC in vivo and to induction of specific CD4 and CD8 T cell responses (Tacken and Figdor, 2011). Many preclinical studies have demonstrated the efficacy of directly targeting DC in vivo to trigger specific immune responses (Caminschi et al., 2008; Tacken et al., 2005) and to control tumor growth (Sancho et al., 2008; He et al., 2007). CLR can also be targeted by carbohydrates ligands (Lepenies et al., 2013). Engineered glycans that have been optimized for increased binding affinity to CLR show promise as a means to enhance tumor antigen uptake and presentation; preclinical studies combining gp100 to glycans targeting DC-SIGN have demonstrated the feasibility and efficacy of these approaches. The location of targeted DC is important to determine the tropism of primed T cells, as DC from one tissue can induce T cells with migratory properties specific for that tissue. In addition, DC stimulated with different adjuvants can induce T cells with various migratory properties, so that the choice of adjuvant is critical to induce the desired type of immunity at the right location. Proof-of-concept clinical trials are on-going to determine the safety of such strategies. Targeting antigens to DC-specific receptors in vivo bypasses the need for ex vivo DC production, a long and critical step which can potentially alter DC functionality. It further allows antigen uptake and stimulation of specific DC subsets, even rare population with interesting properties. This strategy represents an alternative to strategies based on DC injections which are often costly, restricted to a limited number of patients, and limited to particular DC subsets (the ones that can be purified or produced in sufficient numbers).

Activating DC through TLR

As described in detail above, TLR recognize conserved pathogenic motifs present on virus and bacteria, and TLR ligands (TLR-L) represent potent immunomodulatory molecules that can trigger DC maturation and modulate their function. Several TLR-L are under preclinical investigation due to their adjuvant properties for antitumor vaccines (Krieg, 2007) and tested in many clinical trials in cancer. By activating DC (upregulation of MHC and costimulatory molecules, secretion of inflammatory cytokines such as IL-12 and IFNs), they provide the missing signal in DC/T interactions within the tumor microenvironment, allowing induction of Th1-oriented adaptive immunity. TLR agonists already approved by the FDA (BCG, MPL, and imiquimod) together with others (TLR-4 agonist LPS, TLR-3 agonist poly-IC, TLR-7/8 agonist resiquimod) undergo promising clinical developments (Vacchelli et al., 2012). Synthetic TLR agonists efficiently trigger antitumor immunity in vivo in melanoma. In particular, agonists engaging TLR7 and TLR9, expressed by pDC, allow the induction of antitumor immune responses by stimulating pDC function. Imiquimod (TLR7 agonist) is approved for basal carcinoma, and systemic immune activation was observed in melanoma patients in response to TLR7-L (Dummer et al., 2008) or TLR9-L (Molenkamp et al., 2008) administration. The administration of CpG- oligodeoxynucleotides potentiates patients' response to vaccination (Speiser et al., 2005) and stimulates pDC and NK cell functions (Pashenkov et al., 2006). Recently mRNA transfection-based delivery of a costimulatory molecule cocktail (CD40L, CD70, and constitutively active TLR4) called the TriMix-DC emerged as a potent DC maturation strategy leading to strong T cell responses (Van Lint et al., 2014). Vaccination with autologous TriMix-DC transfected with tumor associated antigens can trigger durable tumor responses in melanoma patients (Van Nuffel et al., 2012).

Combination of CLR and TLR

A recent development combining DC targeting through CLR and DC activation via TLR is based on biodegradable nanoparticles constituted of PLGA (poly lactic–*co*-glycolic acid), covered with anti-DC-SIGN antibodies and containing tumor antigens and adjuvants (TLR3-L, TLR7/8-L) (Tacken et al., 2011). Another strategy is based on liposomes covered with anti-DEC-205 antibodies and containing tumor antigens and adjuvants (van Broekhoven et al., 2004). Such a combination of DC targeting via CLR, antigen delivery and direct in vivo activation of DC through TLR, seems promising (Zitvogel and Palucka, 2011) due to its simple and scalable design.

Toward Combinations of DC-based Immunotherapies With Other Antitumor Therapies

With immune checkpoint blockers and inactivation of immunosuppressive cells

The discrepancy between the potent immune responses and the disappointing clinical responses observed in clinical trials using DC vaccines is partly due to the presence of regulatory T cells and an immunosuppressive tumor microenvironment (Palucka et al., 2008). Inhibition of immunosuppressive mechanisms is required to improve the clinical efficacy of immunotherapies, especially the ones based on DC vaccines or DC targeting in vivo. Antitumor effector T cells are inhibited by engagement of inhibitory molecules (CTLA-4, PD1) and the often massive infiltration by immunosuppressive cells (regulatory T cells, MDSC) at the tumor site. Immunomodulatory therapies such as anti-CTLA4 and anti-PD1/anti-PD-L1 blockade have achieved prolongation of patient overall survival for 30%–40% of patients (Ott et al., 2013), demonstrating that inhibition of immunosuppressive mechanisms can lead to tumor control. The higher efficacy of combined DC vaccine with immune checkpoint blockers compared to monotherapy is supported by many preclinical studies (Vasaturo et al., 2013), and by a clinical trial in melanoma patients in which durable tumor regressions were observed following DC vaccine combined with anti-CTLA4 treatment (Ribas et al., 2009). Enhancement of tumor-specific immunity with DC vaccines combined to reversal of T cell exhaustion by immune checkpoint blockers is an attractive strategy. In addition, inhibition of MDSC can be achieved by COX-2 and arginase inhibitors (Wesolowski et al., 2013). Regulatory T cells and MDSC can also be targeted by inhibitors of the IDO pathway (Prendergast et al., 2014), a novel class of immunomodulators that may help reverse the dysfunction of antitumor effector cells. Combination of DC vaccines with IDO inhibitors are currently under clinical investigation. The combination of optimized DC vaccines with inhibition of immunosuppressive and immunoregulatory mechanisms is likely to trigger strong antitumor immune responses, priming potent effectors T cells while protecting them from exhaustion.

With chemotherapies

Besides lowering tumor burden, many studies demonstrated that some classical antitumor chemotherapies can affect many aspects of antitumor immune responses and display immune-potentiating effects. DC may contribute to the efficacy of conventional antitumor chemotherapies that induce immunogenic cell death of tumor cells, defined as apoptotic processes favoring, rather than suppressing, immune responses. The positive immunologic consequences of certain chemotherapies are well characterized (Zitvogel et al., 2008, 2011). Immunogenic signals from tumor cells undergoing apoptosis can be detected by DC and potentiate DC maturation and cross-presentation of tumor antigens. In particular, chemotherapeutic drugs such as anthracyclin or oxaliplatin can induce an immunogenic tumor cell death of cells characterized by surface exposure of calreticulin which enhances phagocytosis of tumor cells by DC. Similarly, secretion of HMGB1 (a TLR4-L) by tumor cells inhibits phagosome–lysosome fusion in APC, preventing rapid tumor antigen degradation and enabling MHC-I restricted presentation and subsequent priming of T cells (Kroemer et al., 2013). The synergy of chemotherapies with DC vaccines has been demonstrated in colorectal cancer patients treated with DC vaccine combined to oxaliplatin (Lesterhuis et al., 2010).

With monoclonal antibodies and targeted therapies

Many preclinical studies have demonstrated the beneficial immunomodulatory effects of antibodies and targeted drugs, and the potential synergy between immunotherapy and targeted therapy (Marabelle and Caux, 2012). The efficacy of therapeutic monoclonal antibodies may also involve DC (Galluzzi et al., 2012). Indeed, some antibodies such as cetuximab, anti-EGFR, not only attenuate oncogenic signals, but also induce antibody-driven effector mechanism (antibody-dependent cell-mediated cytotoxicity, phagocytosis, complement-dependent cytotoxicity) leading to tumor cell lysis or engulfment, and help elicit specific CD8 T cells by potentiating antigenic presentation by DC and their activation (Banerjee et al., 2008; Correale et al., 2012). Bevacizumab, an anti-VEGF antibody, inhibits angiogenesis and also induces DC differentiation and tumor infiltration by CTL (Osada et al., 2008). Inhibition of angiogenic factors such as VEGF restores DC function within the tumor microenvironment and synergizes with DC-based vaccines (Tartour et al., 2011; Gabrilovich et al., 1999). Recent preclinical studies suggest that targeted therapies such as dasatinib or sunitinib (tyrosine kinase inhibitors) used in c-KIT mutated tumors, especially melanoma, gastrointestinal cancer (GIST) and mastocytomas (Yang et al., 2012), may decrease MDSC and immunosuppressive cytokines within the tumor microenvironment (Ko et al., 2009; Ozao-Choy et al., 2009) and potentiate DC vaccines in patients (Amin et al., 2015). JAK2 inhibitors not only interfere with tumor cell survival, but also potentiate DC functions by blocking STAT3 signaling, an inhibitory pathway leading to the induction of suppressive T cells (Lee et al., 2011). Indeed, in a preclinical model, the combination of anti-JAK2 targeted therapies with a DC vaccine enhanced DC maturation, induction of T cell responses, and suppressed tumor growth (Nefedova et al., 2005). Inhibition of mutated BRAF by vemurafenib blocks IL-10 and VEGF secretion by tumor cells, consequently improving DC functionality within the tumor microenvironment (Sumimoto et al., 2006). Thus, targeted therapies can directly antagonize the immunosuppressive microenvironment and restore DC functions.

Overall, antitumor treatments or targeted therapies can be beneficial or deleterious for the immune system, and this needs to be taken into account for the design of optimal combinations to potentiate DC function and promote the emergence of a robust immunity. The synergistic effect of chemotherapies or targeted therapies with immunotherapies, may help prevent escapes due to mono-therapies while acting simultaneously at different points of control of tumor progression. Such combination strategies (Vanneman and Dranoff, 2012) may achieve a better clinical success. However, optimization of duration, sequence and delivery dose of the diverse therapies is crucial for the success of these combinatorial approaches and to avoid increased toxicity and side

effects. Numerous ongoing phase I/II clinical trials are testing combinations of targeted therapies or immunomodulatory therapies with DC vaccines.

Conclusion

DC are crucial actors of the immune system, orchestrating a large panel of responses that can result in potent antitumor immune responses. However tumor cells hijack the immune system, often compromising DC function. Recent clinical trials have demonstrated that DC vaccines could trigger antitumor responses associated with a clinical benefit in subgroups of patients. DC-based cancer immunotherapy has evolved a lot, starting from initial ex vivo DC vaccines to a panel of new therapeutic options. Better knowledge of DC subsets, their biology and unique properties, together with a better understanding of the type of response triggered by specific DC subsets or engaged target receptors, open the door to the development of rationally optimized vaccines. In addition, characterization of tumor immune escape mechanisms and of DC subversion by tumors will help restoring DC functionality within the tumor microenvironment. Understanding the immunological consequences of conventional antitumor treatments will help potentiate immunotherapies. New approaches in preclinical development are promising, taking advantage of DC diversity and plasticity, of neoantigens identification, and exploitation of the synergies between immunotherapies and conventional therapies. Future developments will likely trend toward optimized personalized combined strategies depending on the properties of the immune system and characteristics of the patient's tumor, to interfere with the cancer–immunity cycle (Chen and Mellman, 2013). Combination approaches are under development to optimize tumor control, involving both DC vaccines, small molecules inhibiting signal transduction pathways, and antibodies blocking immune checkpoints. The combined expertise of immunologists and oncologists is crucial to develop innovative and efficient antitumor treatments. The current challenge of antitumor immunotherapy is to reverse the balance from tolerance to immunity within a suppressive tumor microenvironment, but also to overcome many regulatory and financial issues associated with the transfer of new strategies in human clinic.

References

Aarntzen, E.H., et al., 2013. Targeting CD4(+) T-helper cells improves the induction of antitumor responses in dendritic cell-based vaccination. Cancer Research 73 (1), 19–29.

Alcantara-Hernandez, M., et al., 2017. High-dimensional phenotypic mapping of human dendritic cells reveals interindividual variation and tissue specialization. Immunity 47 (6), 1037–1050 e6.

Alexandrov, L.B., et al., 2013. Signatures of mutational processes in human cancer. Nature 500 (7463), 415–421.

Amin, A., et al., 2015. Survival with AGS-003, an autologous dendritic cell-based immunotherapy, in combination with sunitinib in unfavorable risk patients with advanced renal cell carcinoma (RCC): Phase 2 study results. Journal for Immunotherapy of Cancer 3, 14.

Aspord, C., et al., 2010. A novel cancer vaccine strategy based on HLA-A*0201 matched allogeneic plasmacytoid dendritic cells. PLoS One 5 (5), e10458.

Aspord, C., et al., 2011a. GENiusVac, a novel antitumor vaccine strategy based on allogeneic plasmacytoid dendritic cells. La Revue de Médecine Interne 32 (5), 329–332.

Aspord, C., et al., 2011b. Induction of antiviral cytotoxic T cells by plasmacytoid dendritic cells for adoptive immunotherapy of posttransplant diseases. American Journal of Transplantation 11 (12), 2613–2626.

Aspord, C., et al., 2012. HLA-A(*)0201(+) plasmacytoid dendritic cells provide a cell-based immunotherapy for melanoma patients. The Journal of Investigative Dermatology 132 (10), 2395–2406.

Aspord, C., Leccia, M.T., Charles, J., Plumas, J., 2013. Plasmacytoid dendritic cells support melanoma progression by promoting Th2 and regulatory immunity through OX40L and ICOSL. Cancer Immunology Research 1 (6), 402–415.

Banerjee, D., et al., 2008. Enhanced T-cell responses to glioma cells coated with the anti-EGF receptor antibody and targeted to activating FcgammaRs on human dendritic cells. Journal of Immunotherapy 31 (2), 113–120.

Blum, J.S., Wearsch, P.A., Cresswell, P., 2013. Pathways of antigen processing. Annual Review of Immunology 31, 443–473.

Bogunovic, D., et al., 2011. TLR4 engagement during TLR3-induced proinflammatory signaling in dendritic cells promotes IL-10-mediated suppression of antitumor immunity. Cancer Research 71 (16), 5467–5476.

Bol, K.F., et al., 2016. Dendritic cell-based immunotherapy: State of the art and beyond. Clinical Cancer Research 22 (8), 1897–1906.

Bonifaz, L., et al., 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. The Journal of Experimental Medicine 196 (12), 1627–1638.

Burkhardt, U.E., et al., 2013. Autologous CLL cell vaccination early after transplant induces leukemia-specific T cells. The Journal of Clinical Investigation 123 (9), 3756–3765.

Caminschi, I., et al., 2008. The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. Blood 112 (8), 3264–3273.

Carreno, B.M., et al., 2015. Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science 348 (6236), 803–808.

Caux, C., et al., 1996. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. The Journal of Experimental Medicine 184 (2), 695–706.

Cella, M., et al., 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nature Medicine 5 (8), 919–923.

Chen, D.S., Mellman, I., 2013. Oncology meets immunology: The cancer-immunity cycle. Immunity 39 (1), 1–10.

Chowell, D., et al., 2018. Patient HLA class I genotype influences cancer response to checkpoint blockade immunotherapy. Science 359 (6375), 582–587.

Clancy-Thompson, E., et al., 2013. Peptide vaccination in Montanide adjuvant induces and GM-CSF increases CXCR3 and cutaneous lymphocyte antigen expression by tumor antigen-specific CD8 T cells. Cancer Immunology Research 1 (5), 332–339.

Clark, R., Kupper, T., 2005. Old meets new: The interaction between innate and adaptive immunity. The Journal of Investigative Dermatology 125 (4), 629–637.

Colonna, M., Trinchieri, G., Liu, Y.J., 2004. Plasmacytoid dendritic cells in immunity. Nature Immunology 5 (12), 1219–1226.

Correale, P., et al., 2012. Cetuximab +/− chemotherapy enhances dendritic cell-mediated phagocytosis of colon cancer cells and ignites a highly efficient colon cancer antigen-specific cytotoxic T-cell response in vitro. International Journal of Cancer 130 (7), 1577–1589.

Coulie, P.G., et al., 2014. Tumour antigens recognized by T lymphocytes: At the core of cancer immunotherapy. Nature Reviews. Cancer 14 (2), 135–146.

Dubsky, P., et al., 2007. IL-15-induced human DC efficiently prime melanoma-specific naive CD8+ T cells to differentiate into CTL. European Journal of Immunology 37 (6), 1678–1690.
Dummer, R., et al., 2008. An exploratory study of systemic administration of the toll-like receptor-7 agonist 852A in patients with refractory metastatic melanoma. Clinical Cancer Research 14 (3), 856–864.
Durand, M., Segura, E., 2015. The known unknowns of the human dendritic cell network. Frontiers in Immunology 6, 129.
Dzionek, A., et al., 2000. BDCA-2, BDCA-3, and BDCA-4: Three markers for distinct subsets of dendritic cells in human peripheral blood. Journal of Immunology 165 (11), 6037–6046.
Eickhoff, S., et al., 2015. Robust anti-viral immunity requires multiple distinct T cell-dendritic cell interactions. Cell 162 (6), 1322–1337.
Eisenhauer, E.A., et al., 2009. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). European Journal of Cancer 45 (2), 228–247.
Evel-Kabler, K., et al., 2006. SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. The Journal of Clinical Investigation 116 (1), 90–100.
Fabre, J.W., 2001. The allogeneic response and tumor immunity. Nature Medicine 7 (6), 649–652.
Faget, J., et al., 2012. ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells. Cancer Research 72 (23), 6130–6141.
Gabrilovich, D.I., et al., 1999. Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. Clinical Cancer Research 5 (10), 2963–2970.
Gajewski, T.F., Schreiber, H., Fu, Y.X., 2013. Innate and adaptive immune cells in the tumor microenvironment. Nature Immunology 14 (10), 1014–1022.
Galluzzi, L., et al., 2012. The secret ally: Immunostimulation by anticancer drugs. Nature Reviews. Drug Discovery 11 (3), 215–233.
Gardner, A., Ruffell, B., 2016. Dendritic cells and cancer immunity. Trends in Immunology 37 (12), 855–865.
Geijtenbeek, T.B., Gringhuis, S.I., 2009. Signalling through C-type lectin receptors: Shaping immune responses. Nature Reviews. Immunology 9 (7), 465–479.
Goutagny, N., et al., 2012. Targeting pattern recognition receptors in cancer immunotherapy. Targeted Oncology 7 (1), 29–54.
Gulley, J.L., et al., 2016. Perspectives on sipuleucel-T: Its role in the prostate cancer treatment paradigm. OncoImmunology 5 (4), e1107698.
Hargadon, K.M., 2013. Tumor-altered dendritic cell function: Implications for anti-tumor immunity. Frontiers in Immunology 4, 192.
He, L.Z., et al., 2007. Antigenic targeting of the human mannose receptor induces tumor immunity. Journal of Immunology 178 (10), 6259–6267.
Hemont, C., et al., 2013. Human blood mDC subsets exhibit distinct TLR repertoire and responsiveness. Journal of Leukocyte Biology 93 (4), 599–609.
Heninger, E., Krueger, T.E., Lang, J.M., 2015. Augmenting antitumor immune responses with epigenetic modifying agents. Frontiers in Immunology 6, 29.
Hor, J.L., et al., 2015. Spatiotemporally distinct interactions with dendritic cell subsets facilitates CD4+ and CD8+ T cell activation to localized viral infection. Immunity 43 (3), 554–565.
Hu, Z., Ott, P.A., Wu, C.J., 2018. Towards personalized, tumour-specific, therapeutic vaccines for cancer. Nature Reviews. Immunology 18 (3), 168–182.
Ito, T., et al., 2004. Plasmacytoid dendritic cells regulate Th cell responses through OX40 ligand and type I IFNs. Journal of Immunology 172 (7), 4253–4259.
Joffre, O.P., et al., 2012. Cross-presentation by dendritic cells. Nature Reviews. Immunology 12 (8), 557–569.
Kapsenberg, M.L., 2003. Dendritic-cell control of pathogen-driven T-cell polarization. Nature Reviews. Immunology 3 (12), 984–993.
Ko, J.S., et al., 2009. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. Clinical Cancer Research 15 (6), 2148–2157.
Krieg, A.M., 2007. Development of TLR9 agonists for cancer therapy. The Journal of Clinical Investigation 117 (5), 1184–1194.
Krishnaswamy, J.K., Chu, T., Eisenbarth, S.C., 2013. Beyond pattern recognition: NOD-like receptors in dendritic cells. Trends in Immunology 34 (5), 224–233.
Kroemer, G., Zitvogel, L., 2012. Can the exome and the immunome converge on the design of efficient cancer vaccines? OncoImmunology 1 (5), 579–580.
Kroemer, G., et al., 2013. Immunogenic cell death in cancer therapy. Annual Review of Immunology 31, 51–72.
Kugler, A., et al., 2000. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nature Medicine 6 (3), 332–336.
Lande, R., Gilliet, M., 2010. Plasmacytoid dendritic cells: Key players in the initiation and regulation of immune responses. Annals of the New York Academy of Sciences 1183, 89–103.
Lapenta, C., et al., 2006. IFN-alpha-conditioned dendritic cells are highly efficient in inducing cross-priming CD8(+) T cells against exogenous viral antigens. European Journal of Immunology 36 (8), 2046–2060.
Le Mercier, I., et al., 2013. Tumor promotion by intratumoral plasmacytoid dendritic cells is reversed by TLR7 ligand treatment. Cancer Research 73 (15), 4629–4640.
Leal Rojas, I.M., et al., 2017. Human blood CD1c(+) dendritic cells promote Th1 and Th17 effector function in memory CD4(+) T cells. Frontiers in Immunology 8, 971.
Lee, H., et al., 2011. STAT3: A target to enhance antitumor immune response. Current Topics in Microbiology and Immunology 344, 41–59.
Lepenies, B., Lee, J., Sonkaria, S., 2013. Targeting C-type lectin receptors with multivalent carbohydrate ligands. Advanced Drug Delivery Reviews 65 (9), 1271–1281.
Lesterhuis, W.J., et al., 2010. A pilot study on the immunogenicity of dendritic cell vaccination during adjuvant oxaliplatin/capecitabine chemotherapy in colon cancer patients. British Journal of Cancer 103 (9), 1415–1421.
Linnemann, C., et al., 2013. High-throughput identification of antigen-specific TCRs by TCR gene capture. Nature Medicine 19 (11), 1534–1541.
Lipson, E.J., et al., 2015. Safety and immunologic correlates of melanoma GVAX, a GM-CSF secreting allogeneic melanoma cell vaccine administered in the adjuvant setting. Journal of Translational Medicine 13, 214.
Liu, Y.J., 2005. IPC: Professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annual Review of Immunology 23, 275–306.
Lui, G., et al., 2009. Plasmacytoid dendritic cells capture and cross-present viral antigens from influenza-virus exposed cells. PLoS One 4 (9), e7111.
Luksza, M., et al., 2017. A neoantigen fitness model predicts tumour response to checkpoint blockade immunotherapy. Nature 551 (7681), 517–520.
Marabelle, A., Caux, C., 2012. Immunobiology. Combined targeted and immunotherapy: The future of personalized medicine. Blood 120 (23), 4454–4455.
Martln-Fontecha, A., et al., 2003. Regulation of dendritic cell migration to the draining lymph node: Impact on T lymphocyte traffic and priming. The Journal of Experimental Medicine 198 (4), 615–621.
McGranahan, N., et al., 2017. Allele-specific HLA loss and immune escape in lung cancer evolution. Cell 171 (6), 1259–1271 e11.
Molenkamp, B.G., et al., 2008. Local administration of PF-3512676 CpG-B instigates tumor-specific CD8+ T-cell reactivity in melanoma patients. Clinical Cancer Research 14 (14), 4532–4542.
Muir, G., et al., 2006. Preliminary evidence that the allogeneic response might trigger antitumour immunity in patients with advanced prostate cancer. BJU International 98 (5), 989–995.
Nefedova, Y., et al., 2005. Regulation of dendritic cell differentiation and antitumor immune response in cancer by pharmacologic-selective inhibition of the janus-activated kinase 2/signal transducers and activators of transcription 3 pathway. Cancer Research 65 (20), 9525–9535.
Nestle, F.O., et al., 2009. Skin immune sentinels in health and disease. Nature Reviews. Immunology 9 (10), 679–691.
Osada, T., et al., 2008. The effect of anti-VEGF therapy on immature myeloid cell and dendritic cells in cancer patients. Cancer Immunology, Immunotherapy 57 (8), 1115–1124.
Ott, P.A., Hodi, F.S., Robert, C., 2013. CTLA-4 and PD-1/PD-L1 blockade: New immunotherapeutic modalities with durable clinical benefit in melanoma patients. Clinical Cancer Research 19 (19), 5300–5309.
Ott, P.A., et al., 2017. An immunogenic personal neoantigen vaccine for patients with melanoma. Nature 547 (7662), 217–221.
Ozao-Choy, J., et al., 2009. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. Cancer Research 69 (6), 2514–2522.
Palucka, K., Banchereau, J., 2012. Cancer immunotherapy via dendritic cells. Nature Reviews. Cancer 12 (4), 265–277.
Palucka, K., Banchereau, J., 2013a. Dendritic-cell-based therapeutic cancer vaccines. Immunity 39 (1), 38–48.

Palucka, K., Banchereau, J., 2013b. Human dendritic cell subsets in vaccination. Current Opinion in Immunology 25 (3), 396–402.
Palucka, A.K., et al., 2008. Dendritic cells: A critical player in cancer therapy? Journal of Immunotherapy 31 (9), 793–805.
Pashenkov, M., et al., 2006. Phase II trial of a toll-like receptor 9-activating oligonucleotide in patients with metastatic melanoma. Journal of Clinical Oncology 24 (36), 5716–5724.
Patel, P.M., et al., 2018. Targeting gp100 and TRP-2 with a DNA vaccine: Incorporating T cell epitopes with a human IgG1 antibody induces potent T cell responses that are associated with favourable clinical outcome in a phase I/II trial. OncoImmunology 7, e1433516.
Prendergast, G.C., et al., 2014. Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer. Cancer Immunology, Immunotherapy 63 (7), 721–735.
Pulendran, B., Li, S., Nakaya, H.I., 2010. Systems vaccinology. Immunity 33 (4), 516–529.
Reis e Sousa, C., 2006. Dendritic cells in a mature age. Nature Reviews. Immunology 6 (6), 476–483.
Reizis, B., et al., 2011. Plasmacytoid dendritic cells: Recent progress and open questions. Annual Review of Immunology 29, 163–183.
Ribas, A., et al., 2009. Dendritic cell vaccination combined with CTLA4 blockade in patients with metastatic melanoma. Clinical Cancer Research 15 (19), 6267–6276.
Rizvi, N.A., et al., 2015. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 348 (6230), 124–128.
Sagiv-Barfi, I., et al., 2018. Eradication of spontaneous malignancy by local immunotherapy. Science Translational Medicine 10 (426), eaan4488.
Sahin, U., et al., 2017. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. Nature 547 (7662), 222–226.
Sancho, D., Reis e Sousa, C., 2012. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. Annual Review of Immunology 30, 491–529.
Sancho, D., et al., 2008. Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. The Journal of Clinical Investigation 118 (6), 2098–2110.
Sandoval, F., et al., 2013. Mucosal imprinting of vaccine-induced CD8(+) T cells is crucial to inhibit the growth of mucosal tumors. Science Translational Medicine 5 (172), 172ra20.
Santegoets, S.J., et al., 2008. Human dendritic cell line models for DC differentiation and clinical DC vaccination studies. Journal of Leukocyte Biology 84 (6), 1364–1373.
Saxena, M., Bhardwaj, N., 2018. Re-emergence of dendritic cell vaccines for Cancer treatment. Trends Cancer 4 (2), 119–137.
Schreibelt, G., et al., 2016. Effective clinical responses in metastatic melanoma patients after vaccination with primary myeloid dendritic cells. Clinical Cancer Research 22 (9), 2155–2166.
Segura, E., et al., 2013. Human inflammatory dendritic cells induce Th17 cell differentiation. Immunity 38 (2), 336–348.
Shen, L., et al., 2004. Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. Nature Biotechnology 22 (12), 1546–1553.
Sheridan, B.S., Lefrancois, L., 2011. Regional and mucosal memory T cells. Nature Immunology 12 (6), 485–491.
Sisirak, V., et al., 2012. Impaired IFN-alpha production by plasmacytoid dendritic cells favors regulatory T-cell expansion that may contribute to breast cancer progression. Cancer Research 72 (20), 5188–5197.
Speiser, D.E., et al., 2005. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. The Journal of Clinical Investigation 115 (3), 739–746.
Steinman, R.M., Banchereau, J., 2007. Taking dendritic cells into medicine. Nature 449 (7161), 419–426.
Sumimoto, H., et al., 2006. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. The Journal of Experimental Medicine 203 (7), 1651–1656.
Swiecki, M., Colonna, M., 2015. The multifaceted biology of plasmacytoid dendritic cells. Nature Reviews. Immunology 15 (8), 471–485.
Tacken, P.J., Figdor, C.G., 2011. Targeted antigen delivery and activation of dendritic cells in vivo: Steps towards cost effective vaccines. Seminars in Immunology 23 (1), 12–20.
Tacken, P.J., et al., 2005. Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. Blood 106 (4), 1278–1285.
Tacken, P.J., et al., 2011. Targeted delivery of TLR ligands to human and mouse dendritic cells strongly enhances adjuvanticity. Blood 118 (26), 6836–6844.
Tartour, E., et al., 2011. Angiogenesis and immunity: A bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with immunotherapy. Cancer Metastasis Reviews 30 (1), 83–95.
Tel, J., et al., 2012. Harnessing human plasmacytoid dendritic cells as professional APCs. Cancer Immunology, Immunotherapy 61 (8), 1279–1288.
Tel, J., et al., 2013. Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. Cancer Research 73 (3), 1063–1075.
Tiptiri-Kourpeti, A., et al., 2016. DNA vaccines to attack cancer: Strategies for improving immunogenicity and efficacy. Pharmacology & Therapeutics 165, 32–49.
Ueno, H., et al., 2011. Targeting human dendritic cell subsets for improved vaccines. Seminars in Immunology 23 (1), 21–27.
Vacchelli, E., et al., 2012. Trial watch: FDA-approved toll-like receptor agonists for cancer therapy. OncoImmunology 1 (6), 894–907.
Vacchelli, E., et al., 2013. Trial watch: Dendritic cell-based interventions for cancer therapy. OncoImmunology 2 (10), e25771.
Valmori, D., et al., 2007. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. Proceedings of the National Academy of Sciences of the United States of America 104 (21), 8947–8952.
Van Allen, E.M., et al., 2015. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science 350 (6257), 207–211.
van Broekhoven, C.L., et al., 2004. Targeting dendritic cells with antigen-containing liposomes: A highly effective procedure for induction of antitumor immunity and for tumor immunotherapy. Cancer Research 64 (12), 4357–4365.
van Kooyk, Y., Geijtenbeek, T.B., 2003. DC-SIGN: Escape mechanism for pathogens. Nature Reviews. Immunology 3 (9), 697–709.
Van Lint, S., et al., 2014. Optimized dendritic cell-based immunotherapy for melanoma: The TriMix-formula. Cancer Immunology, Immunotherapy 63 (9), 959–967.
Van Nuffel, A.M., et al., 2012. Intravenous and intradermal TriMix-dendritic cell therapy results in a broad T-cell response and durable tumor response in a chemorefractory stage IV-M1c melanoma patient. Cancer Immunology, Immunotherapy 61 (7), 1033–1043.
Vanneman, M., Dranoff, G., 2012. Combining immunotherapy and targeted therapies in cancer treatment. Nature Reviews. Cancer 12 (4), 237–251.
Vasaturo, A., et al., 2013. Clinical implications of co-inhibitory molecule expression in the tumor microenvironment for DC vaccination: A game of stop and go. Frontiers in Immunology 4, 417.
Villadangos, J.A., Young, L., 2008. Antigen-presentation properties of plasmacytoid dendritic cells. Immunity 29 (3), 352–361.
Villani, A.C., et al., 2017. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. Science 356 (6335), eaah4573.
Walter, S., et al., 2012. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nature Medicine 18 (8), 1254–1261.
Watchmaker, P.B., et al., 2014. Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. Nature Immunology 15 (1), 98–108.
Wesolowski, R., Markowitz, J., Carson 3rd, W.E., 2013. Myeloid derived suppressor cells—A new therapeutic target in the treatment of cancer. Journal for Immunotherapy of Cancer 1, 10.
Wolchok, J.D., et al., 2009. Guidelines for the evaluation of immune therapy activity in solid tumors: Immune-related response criteria. Clinical Cancer Research 15 (23), 7412–7420.
Yang, Y., et al., 2012. Antitumor T-cell responses contribute to the effects of dasatinib on c-KIT mutant murine mastocytoma and are potentiated by anti-OX40. Blood 120 (23), 4533–4543.
Yewdall, A.W., et al., 2010. CD8+ T cell priming by dendritic cell vaccines requires antigen transfer to endogenous antigen presenting cells. PLoS One 5 (6), e11144.
Yu, C.I., et al., 2013. Human CD1c+ dendritic cells drive the differentiation of CD103+ CD8+ mucosal effector T cells via the cytokine TGF-beta. Immunity 38 (4), 818–830.
Yu, C.I., et al., 2014. Human CD141+ dendritic cells induce CD4+ T cells to produce type 2 cytokines. Journal of Immunology 193 (9), 4335–4343.

Zitvogel, L., Palucka, K., 2011. Antibody co-targeting of DCs. Blood 118 (26), 6726–6727.
Zitvogel, L., et al., 2008. Immunological aspects of cancer chemotherapy. Nature Reviews. Immunology 8 (1), 59–73.
Zitvogel, L., Kepp, O., Kroemer, G., 2011. Immune parameters affecting the efficacy of chemotherapeutic regimens. Nature Reviews. Clinical Oncology 8 (3), 151–160.

Further Reading

Alcantara-Hernandez, M., et al., 2017. High-dimensional phenotypic mapping of human dendritic cells reveals interindividual variation and tissue specialization. Immunity 47 (6), 1037–1050 e6.
Bol, K.F., et al., 2016. Dendritic cell-based immunotherapy: State of the art and beyond. Clinical Cancer Research 22 (8), 1897–1906.
Chen, D.S., Mellman, I., 2013. Oncology meets immunology: The cancer–immunity cycle. Immunity 39 (1), 1–10.
Hu, Z., Ott, P.A., Wu, C.J., 2018. Towards personalized, tumor-specific, therapeutic vaccines for cancer. Nature Reviews. Immunology 18 (3), 168–182.
Saxena, M., Bhardwaj, N., 2018. *Re*-emergence of dendritic cell vaccines for cancer treatment. Trends Cancer 4 (2), 119–137.
Tiptiri-Kourpeti, A., et al., 2016. DNA vaccines to attack cancer: Strategies for improving immunogenicity and efficacy. Pharmacology & Therapeutics 165, 32–49.
Veglia, F., Gabrilovich, D.I., 2017. Dendritic cells in cancer: The role revisited. Current Opinion in Immunology 45, 43–51.

Relevant Websites

https://cancerresearch.org/scientists/meetings-and-resources/peptide-database—Cancer Research Institute. Peptide Database.
https://clinicaltrials.gov/—US National Library of Medicine. Clinical Trials.

Cancer-Related Inflammation in Tumor Progression

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Glossary

Tumor microenvironment The ecological niche of cancer including inflammatory cells, immune cells and stroma.

Nomenclature

TME Tumor microenvironment
TAM Tumor-associated macrophages
IL Interleukin

The perception that cancer and inflammation are linked processes goes back to the 19th century. This perception has not attracted the limelight for a long time. Different lines of work have led to a renaissance of the inflammation-cancer connection, leading to a generally accepted paradigm. Inflammatory cells and mediators are present in the microenvironment of most neoplastic tissues, including those not causally related to an obvious inflammatory process (e.g., breast cancer). Essential characteristics of cancer-related inflammation include the presence of leukocytes, prominently tumor-associated macrophages (TAM); the presence of inflammatory cytokines (e.g., tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IL-23, IL-17, chemokines such as CCL2); prominent tissue remodeling and angiogenesis.

In the last 20 years we have moved from a cancer cell centric view of the essence of cancer to one that includes the tumor microenvironment (TME) and immunity. A better understanding of innate and adaptive immunity in cancer has paved the way to development of immunotherapy approaches in its broad sense (Fig. 1), from checkpoint blockade inhibitors to inhibition of inflammatory cells and cytokines.

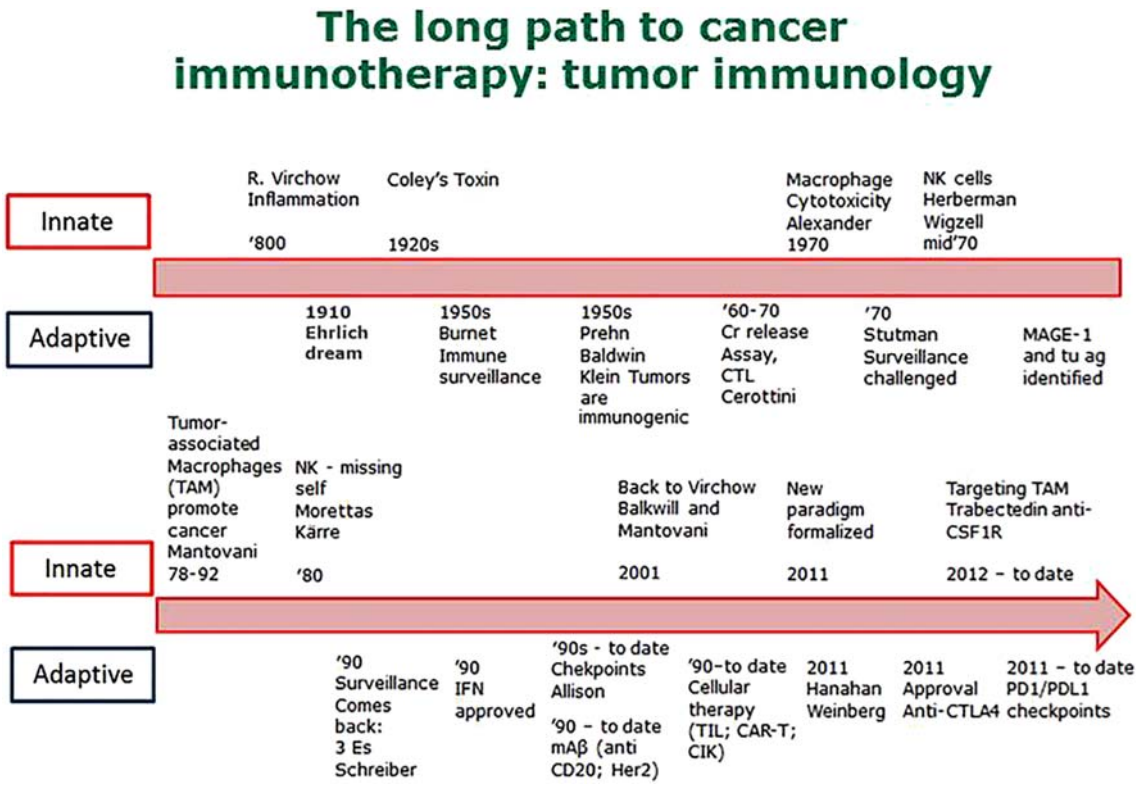

Fig. 1 A historical perspective of the development of tumor immunology and immunotherapy.

The TME includes components of inflammation and adaptive immune responses. Pathways connecting inflammation, carcinogenesis and progression are complex (Fig. 2). Under selected conditions, smoldering local od systemic inflammation increases the cancer risk. We have referred to this pathway as "extrinsic." The extrinsic pathway connection between local inflammation and cancer is illustrated by ulcerative colitis increasing the risk of developing colorectal cancer. At a systemic level, obesity causes a subclinical inflammation state and is associated to an increased risk to develop cancer, in this same extrinsic pathway perspective.

Tumors epidemiologically unrelated to overt inflammatory conditions have inflammatory cells and mediators in their TME. For instance macrophages are a component of the TME in breast cancer and promote growth and metastasis. Moreover, in triple negative breast cancer tumor-associated macrophage (TAM) infiltration is associated with poor prognosis. Activation of the kinase MER TK which drives epithelial to mesenchymal transition is an important determinant of TAM recruitment under these conditions.

Key orchestrators at the intersection of the intrinsic and extrinsic pathway include transcription factors (e.g., NFkB; STAT3), cytokines (e.g., IL-1; TNF) and chemokines. Thus, cancer-related inflammation (CRI) is an essential component of the tumor microenvironment and is now a recognized essential characteristic of cancer. TAM are now being considered as prognostic markers and therapeutic targets. Targeting TAM is a key component in the antitumor activity of Trabectedin, an EMA and FDA approved antitumor agent, thus providing proof of principle for TAM directed strategies. Based on preclinical work. Based on preckinical work, antibodies or simple molecules targeting the CSF1 pathway are undergoing clinical assessment per se or in concert with checkpoint blockade inhibitors.

A variety of inflammatory cells and mediators are present in TME (Fig. 2). Macrophages have served as a paradigm of cancer-related inflammation. Other inflammatory cells include neutrophils, eosinophils and basophils. Components of the humoral arm of innate immunity are present in the TME and have recently emerged as important drivers of tumor progression. Evidence now suggests that complement can be a component of tumor promoting inflammation. In the same perspective of complement mediated tumor promotion, the humoral pattern recognition gene PTX3 has been shown to be an extrinsic oncosuppressor gene. PTX3 interacts with factor H which regulates complement-driven inflammation in preclinical models and selected human tumors. Complement drives tumor promoting inflammation by recruiting macrophages which are the key cells in tumor promotion. Thus complement can have a yin yang role in cancer, on the one hand mediating tumor cell killing of recognition by effector cells after monoclonal antibody therapy. On the other hand, complement can be a driver of tumor promoting inflammation.

The TME is remarkably different in tumors originating in different organs and among tumors which originate in the same organ or tissue. Within the same histological type, tumors are characterized by widely different TME. For instance, in colorectal cancer four TME phenotypes have been identified based on profiling.

Assessment of components of the TME has prognostic significance. T cell infiltration ("Immunoscore") is an independent prognostic indicator of favorable outcome. Conversely, TAM infiltration is generally associated with bad prognosis. A notable exception is colorectal cancer. Recent evidence indicate that TAM infiltration predicts response to 5FU chemotherapy.

Dissection of the role and diversity of cancer-related inflammation may pave the way to improving on current immunotherapy strategies and may impact on diagnosis and prevention. The prototypic inflammatory cytokine IL-1 was shown early on to promote tumor progression. These early observations have now been translated. In the CANTOS study with over 10,000 patients treated with

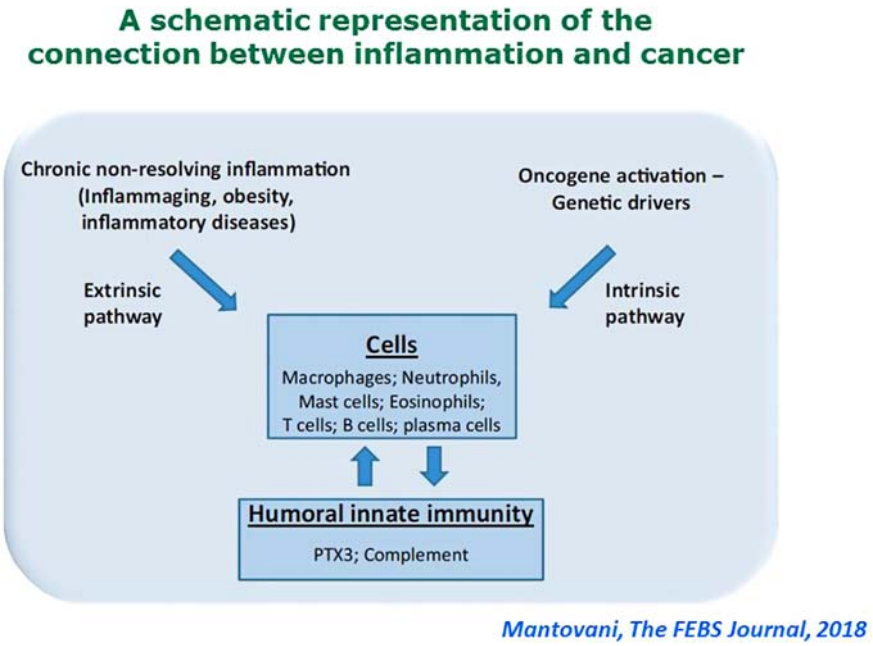

Fig. 2 A schematic representation of the connection between inflammation and cancer. Reproduced with permission from Mantovani A. (2018). *FEBS J.* **285**(*4*):638–640.

anti-IL-1 (canakinumab) with high C reactive Protein. Over 50% protection against incidence and mortality from lung cancer were observed.

Immunity is an essential component of the tumor microenvironment (TME) and a key determinant of metastasis. Inflammatory cells, tumor-associated macrophages (TAM) in particular, pave the way to tissue invasion and intravasation and provide a nurturing microenvironment for metastasis, serving as a component of the cancer cell niche at distant sites. NK cells are innate lymphoid cells (ILCs) which have long been considered to play a role in resistance against hematogenous dissemination of cancer cells, in particular to the lungs. Finally, tumor progression and escape are associated with activation of pathways of suppression of innate and adaptive anti-tumor responses which include, among others, immunosuppressive myeloid cells, activation of checkpoint blockade, induction and recruitment of T regulatory cells.

Invasion and metastasis are key to malignancy. There is also evidence for a role for neutrophils and their major growth factor, granulocyte colony stimulating factor (G-CSF), in cancer progression to metastasis. The interplay between tumor-associated macrophages and neutrophils with the extracellular matrix shapes the TME and promotes tumor growth and dissemination.

Quantification of the immune and inflammatory landscape of TME has provided novel prognostic indicators of cancer progression as shown by quantification of tumor infiltrating T cells and TAM. Genomic technologies have added a new dimension to the characterization of the TME and to classification of cancers. Finally, dissection of the mode of action of conventional cytoreductive strategies, the impact of checkpoint blockade inhibitors and adoptive cell therapy of hematological malignancies have proven the principle that the immune system can be harnessed to cope with advanced disseminated neoplastic diseases.

Full exploitation of the diagnostic and therapeutic potential of innate and adaptive immunity will require an integrated in depth analysis of its components in primary versus spreading, metastatic tumors, dissection of the diversity of metastatic niches, identification and development of new molecular and cellular tools. Given the diversity of the involved innate and adaptive players and of the environmental clues involved, only an integrated effort with expertise from different fields can lead to the identification of new biomarkers and therapeutic strategies for metastatic disease.

See also: *Helicobacter pylori*-Mediated Carcinogenesis.

Further Reading

Balkwill, F., Mantovani, A., 2001. Inflammation and cancer: Back to Virchow? Lancet 357, 539–545.

Bonavita, E., Gentile, S., Rubino, M., Maina, V., Papait, R., Kunderfranco, P., Greco, C., Feruglio, F., Molgora, M., Laface, I., Tartari, S., Doni, A., Pasqualini, F., Barbati, E., Basso, G., Galdiero, M.R., Nebuloni, M., Roncalli, M., Colombo, P., Laghi, L., Lambris, J.D., Jaillon, S., Garlanda, C., Mantovani, A., 2015. PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer. Cell 160, 700–714.

Bonelli, S., Geeraerts, X., Bolli, E., Keirsse, J., Kiss, M., Pombo Antunes, A.R., Van Damme, H., De Vlaminck, K., Movahedi, K., Laoui, D., Raes, G., Van Ginderachter, J.A., 2018. Beyond the M-CSF receptor—Novel therapeutic targets in tumor-associated macrophages. The FEBS Journal 285 (4), 777–787.

Bottai, G., Raschioni, C., Székely, B., Di Tommaso, L., Szász, A.M., Losurdo, A., Győrffy, B., Ács, B., Torrisi, R., Karachaliou, N., Tőkés, T., Caruso, M., Kulka, J., Roncalli, M., Santoro, A., Mantovani, A., Rosell, R., Reis-Filho, J.S., Santarpia, L., 2016. AXL-associated tumor inflammation as a poor prognostic signature in chemotherapy-treated triple-negative breast cancer patients. NPJ Breast Cancer 2, 16033.

Coussens, L.M., Zitvogel, L., Palucka, A.K., 2013. Neutralizing tumor-promoting chronic inflammation: A magic bullet? Science 339, 286–291.

Fridman, W.H., Zitvogel, L., Sautes-Fridman, C., Kroemer, G., 2017. The immune contexture in cancer prognosis and treatment. Nature Reviews. Clinical Oncology 14, 717–734.

Gonzalez, H., Robles, I., Werb, Z., 2018. Innate and acquired immune surveillance in the postdissemination phase of metastasis. The FEBS Journal 285 (4), 654–664.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: The next generation. Cell 144, 646–674.

Jeannin, P., Paolini, L., Adam, C., Delneste, Y., 2018. The roles of CSFs on the functional polarization of tumor-associated macrophages. The FEBS Journal 285 (4), 680–699.

Ma, C., Zhang, Q., Greten, T.F., 2018. Nonalcoholic fatty liver disease promotes hepatocellular carcinoma through direct and indirect effects on hepatocytes. The FEBS Journal 285 (4), 752–762.

Majety, M., Runza, V., Lehmann, C., Hoves, S., Ries, C.H., 2018. A drug development perspective on targeting tumor-associated myeloid cells. The FEBS Journal 285 (4), 763–776. https://doi.org/10.1111/febs.14277.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. Nature 454, 436–444.

Mantovani, A., Marchesi, F., Malesci, A., Laghi, L., Allavena, P., 2017. Tumor-associated macrophages as treatment targets in oncology. Nature Reviews. Clinical Oncology 14, 399–416.

Mazzone, M., Menga, A., Castegna, A., 2018. Metabolism and TAM functions—It takes two to tango. The FEBS Journal 285 (4), 700–716.

Mouchemore, K.A., Anderson, R.L., Hamilton, J.A., 2018. Neutrophils, G-CSF and their contribution to breast cancer metastasis. The FEBS Journal 285 (4), 665–679.

Murray, P.J., 2018. Nonresolving macrophage-mediated inflammation in malignancy. The FEBS Journal 285 (4), 641–653.

Pollard, J.W., 2008. Macrophages define the invasive microenvironment in breast cancer. Journal of Leukocyte Biology 84, 623–630.

Porta, C., Sica, A., Riboldi, E., 2018. Tumor-associated myeloid cells: New understandings on their metabolic regulation and their influence in cancer immunotherapy. The FEBS Journal 285 (4), 717–733.

Reis, E.S., Mastellos, D.C., Ricklin, D., Mantovani, A., Lambris, J.D., 2018. Complement in cancer: Untangling an intricate relationship. Nature Reviews. Immunology 18 (1), 5–18.

Ridker, P. M., MacFadyen, J. G., Thuren, T., Everett, B. M., Libby, P. & Glynn, R. J. (2017) Effect of interleukin-1beta inhibition with canakinumab on incident lung cancer in patients with atherosclerosis: Exploratory results from a randomized, double-blind, placebo-controlled trial, Lancet, 390(10105):1833–1842.

Varol, C., Sagi, I., 2018. Phagocyte—Extracellular matrix crosstalk empowers tumor development and dissemination. The FEBS Journal 285 (4), 734–751.

Cancers as Ecosystems: From Cells to Population

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Much of our research focuses on the causes and outcomes of cancer. Epidemiology studies the genetic and nongenetic risk factors (genes and environmental factors (MacMahon and Pugh, 1970)) and places these in the context of the assumed underlying disease process. Historically epidemiology has defined non-genetic factors as environmental, thought this embraces a broad range of lifestyle, occupational, and reproductive factors as well as medications. Using a reductionist approach, we work to separate the effects of each factor from the others, focusing on measurement and the role of exposures in etiology and modifying survivorship. However, humans live in communities and broader societies, and often the interplay between cells, human bodies, individuals, and society are ignored (Colditz and Sutcliffe, 2016). This is particularly important for considerations of the underlying causes of variation in cancer risk, and the role of lifestyle in survivorship (Colditz, 2001). Early studies of causes of cancer focused heavily on variation in incidence between countries and within countries according to occupation (Ziegler et al., 1993; MacMahon, 1965).

Let me use the example of breast cancer. We have identified a range of reproductive and other lifestyle factors that modify risk (Hankinson et al., 2004). Each may be studied as an independent risk factor or combined into a model that summarizes risk making assumptions of the underlying disease process (Rosner et al., 1994, 2013; Rosner and Colditz, 1996), which Pike called breast tissue age (Pike et al., 1983). The individual risk factors such as first pregnancy may be studied in humans in relation to changes in circulating hormone levels (Hankinson et al., 1995), and intermediate markers such as breast mammographic density (McCormack et al., 2010). Pregnancy may also be studied in animal models to refine understanding of the cellular changes associated with a first full-term pregnancy (Medina, 2013). Other lifestyle causes of breast cancer include alcohol (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010), obesity (Lauby-Secretan et al., 2016), physical activity (International Agency for Research on Cancer, 2002), and use of contraceptives and menopausal hormone therapy (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007). These have been studied for action at the cellular level and then with more recent and larger epidemiologic studies they are related to risk of subtypes of breast cancer defined by receptor status (Colditz et al., 2004). For weight, the field has evolved to address timing of adiposity and weight gain (Rosner et al., 2015, 2017).

In parallel to these studies of environmental causes of cancer, the details of genetic predisposition have been refined from a family history of breast cancer diagnosis (1800s) to genetic changes that carry varying levels of risk (Colditz et al., 1996; Miki et al., 1994; Couch et al., 2014). Molecular mechanism for genetic predisposition are described in other articles.

After diagnosis of breast cancer, lifestyle is again a focus, as components of lifestyle may modify survival outcomes (adiposity increases mortality (Chan et al., 2014), physical activity may reduce mortality (Holmes et al., 2005)). Furthermore, socially isolated women have a greater reduction in their physical function after cancer diagnosis than those who are more socially integrated (Michael et al., 2002). Similar associations are studied for outcomes after colorectal cancer (Van Blarigan et al., 2018; Chan et al., 2008) and prostate cancer (Khan et al., 2018; Imm et al., 2017), to name a few.

These risk factors do not occur in isolation. Rather they are the proximate measures that are easily accessible for epidemiologists to study. Many of these factors studied in isolation have been reviewed by the International Agency for Research on Cancer (IARC) and classified as carcinogens (IARC, 2011). However, many of these exposures and the attained level of risk is often driven by broader social forces. Take parity as an example. Before commercial contraception was readily available women had an average of 6–8 pregnancies. This level continued in rural China and other low-income countries until very recently (Lewington et al., 2014). Meanwhile, with industrialization and access to contraception parity has fallen. In addition, with increasing education and career options, women have deferred childbearing to later in life. Mean age at first pregnancy is now 30 or more in most OECD countries (OECD, 2012). Thus, the risk factor age at first birth is not isolated from the broader social changes of education, income, and opportunity (Bumpass et al., 1978). Changes in sun exposure over decades reflect changing social norms regarding sunbathing, swim suits, development of sunscreens, and now commercial tanning industries (Wehner et al., 2014). Likewise, consumption of sugar sweetened beverages has changed dramatically over decades with increasing serving size reflecting marketing and commercial targets over public health concerns.

Age at menarche is another long-studied risk factor, marking the beginning of menstrual cycling and monthly breast proliferation and involution (Pike et al., 1993). The drivers of age at menarche have changed dramatically—age at menarche has fallen from 18 to 12 or less in a century (Lewington et al., 2014), and with it more adiposity in childhood (Dietz and Gortmaker, 2001), greater attained height (Sung et al., 2009), and fewer infections have covaried. In Korea, for example, mean age at menarche decreased from 16.90 ± 1.25 years for women born between 1920 and 1925 to 13.79 ± 1.37 years for those born between 1980 and 1985, indicating a downward trend of 0.68 years per decade (95% CI, 0.64–0.71) in age at menarche (Cho et al., 2010).

Some have worked to understand the relations between these societal changes and the change in age at menarche (Berkey et al., 2000). Nutrition is one clear driver of both adiposity (excess energy intake for energy expenditure) and sources (animal vs. vegetable) modifying age at menarche (Berkey et al., 2000). Higher animal protein intake is related to greater height and also earlier menarche. So, while the cellular changes in the breast may be the driver of breast cancer risk; societal changes in access to food, healthy living conditions, and so forth modify the expression of the underlying biologic process.

To refine understanding of pathways and modifiers of predisposition, studies have more recently included blood markers (hormone levels, nutrients, inflammatory markers) and related them to cancer risk (Colditz and Hankinson, 2005). Often this is in the context of understanding or refining understanding of exposures and cancers (McMichael, 1994). While these markers may serve as more proximate measures of exposure, to date they do not address the cellular environment in all levels of richness and are not specific to individual organ tissues.

Moving From Causes to Prevention

The causal role of HPV in the etiology of cervical cancer demonstrates the refinement of understanding of causes of cancer over time, and the broader social context that can modify the effectiveness of prevention even when effective strategies are scientifically justified. The evolution of epidemiologic understanding from number of sexual partners as a risk factor for cervical cancer (hypothesized to reflect transmission of on infectious agent) (Pereyra, 1961; Schiffman et al., 1993; Beral, 1974) to defining the etiologic agent (Franco et al., 1999), and then developing an effective vaccine for cervical and anal cancer (Giuliano et al., 2011), has variable effectiveness as a prevention strategy at the population level (Walling et al., 2016). This is not due to varying biologic effectiveness at the cellular level, but rather due to differences in society approaches to implementing comprehensive vaccination programs (Walling et al., 2016). In the span of 20 years or less global evidence moved such that Australia has among the most effective programs (vaccine purchased by the Federal government and administered by school nurses) (Gertig et al., 2013) while the US on the other hand has hap hazard prescribing by primary care providers (USA) (Rahman et al., 2015). Australia has documented the dramatic decrease in premalignant cervical lesions on biopsy, and modified its surveillance program to every 5 year vaginal testing for HPV (Brotherton et al., 2016). The US still has a vaccination rate of only 43% of adolescents up to date on all recommended doses of HPV (https://www.cdc.gov/hpv/hcp/vacc-coverage.html).

Synthesis

From these perspectives and others, the social ecologic model for prevention has been proposed as an approach to place the cellular level biologic processes of carcinogenesis in the broader context of society (Swinburn et al., 2011). For obesity, Story described how access to food and nutrients, as well as safe places to play and exercise modify energy balance; the built environment also supports or inhibits physical activity, active commuting, and so forth (Story et al., 2008; Shelton et al., 2011). A similar model applies globally (Swinburn et al., 2011). Food and agriculture policies modify access to foods/nutrients and the composition of diet. Marketing, home and workplace environments, also contribute to the enticement for food and drinks, such as sugar sweetened beverages, alcohol, etc.; and modify how we spend our time—watching television—or in occupations that do not burn energy the way our forebears did in manual labor. In this spirit Warnecke and others have defined how the interplay of processes from the cellular level through individual and community level measure and broader social policies interact to drive cancer risk and outcomes (Warnecke et al., 2008). Moving forward to reduce disparities in cancer risk across populations and within populations greater focus from cell to society will be needed to maximize our return on investment in cancer research (Emmons and Colditz, 2017).

Conflict of Interest

Authors declare no potential conflict of interest.

See also: Cancer Risk Reduction Through Lifestyle Changes. Prevention and Control: Nutrition, Obesity, and Metabolism.

References

Beral, V., 1974. Cancer of the cervix: A sexually transmitted infection? Lancet 1 (7865), 1037–1040.
Berkey, C.S., et al., 2000. Relation of childhood diet and body size to menarche and adolescent growth in girls. American Journal of Epidemiology 152 (5), 446–452.
Brotherton, J.M., et al., 2016. HPV vaccine impact in Australian women: Ready for an HPV-based screening program. The Medical Journal of Australia 204 (5), 184.
Bumpass, L., Rindfuss, R., Janosik, R., 1978. Age and marital status at first birth and the pace of subsequent fertility. Demography 15, 75–86.
Chan, J.A., et al., 2008. Association of family history with cancer recurrence and survival among patients with stage III colon cancer. JAMA 299 (21), 2515–2523.
Chan, D.S., et al., 2014. Body mass index and survival in women with breast cancer-systematic literature review and meta-analysis of 82 follow-up studies. Annals of Oncology 25 (10), 1901–1914.
Cho, G.J., et al., 2010. Age at menarche in a korean population: Secular trends and influencing factors. European Journal of Pediatrics 169 (1), 89–94.
Colditz, G.A., 2001. Cancer culture: Epidemics, human behavior, and the dubious search for new risk factors. American Journal of Public Health 91 (3), 357–359.
Colditz, G.A., Hankinson, S.E., 2005. The Nurses' health study: Lifestyle and health among women. Nature Reviews. Cancer 5 (5), 388–396.
Colditz, G.A., Sutcliffe, S., 2016. The preventability of cancer: Stacking the deck. JAMA Oncology 2 (9), 1131–1133.
Colditz, G.A., Rosner, B.A., Speizer, F.E., 1996. Risk factors for breast cancer according to family history of breast cancer. For the Nurses' Health Study Research Group. Journal of the National Cancer Institute 88 (6), 365–371.

Colditz, G.A., et al., 2004. Risk factors for breast cancer according to estrogen and progesterone receptor status. Journal of the National Cancer Institute 96 (3), 218–228.
Couch, F.J., Nathanson, K.L., Offit, K., 2014. Two decades after BRCA: Setting paradigms in personalized cancer care and prevention. Science 343 (6178), 1466–1470.
Dietz, W.H., Gortmaker, S.L., 2001. Preventing obesity in children and adolescents. Annual Review of Public Health 22, 337–353.
Emmons, K.M., Colditz, G.A., 2017. Realizing the potential of Cancer prevention—The role of implementation science. The New England Journal of Medicine 376 (10), 986–990.
Franco, E., Rohan, T., Villa, L., 1999. Epidemiologic evidence and human papillomavirus infection as a necessary cause of cervical cancer. Journal of the National Cancer Institute 91, 506–511.
Gertig, D.M., et al., 2013. Impact of a population-based HPV vaccination program on cervical abnormalities: A data linkage study. BMC Medicine 11, 227.
Giuliano, A.R., et al., 2011. Efficacy of quadrivalent HPV vaccine against HPV infection and disease in males. The New England Journal of Medicine 364 (5), 401–411.
Hankinson, S.E., et al., 1995. Reproductive factors and family history of breast cancer in relation to plasma estrogen and prolactin levels in postmenopausal women in the Nurses' health study (United States). Cancer Causes & Control 6 (3), 217–224.
Hankinson, S.E., Colditz, G.A., Willett, W.C., 2004. Towards an integrated model for breast cancer etiology: The lifelong interplay of genes, lifestyle, and hormones. Breast Cancer Research 6 (5), 213–218.
Holmes, M.D., et al., 2005. Physical activity and survival after breast cancer diagnosis. JAMA 293 (20), 2479–2486.
IARC, 2011. Monographs on the evaluation of carcinogenic risks to humans. In: A review of carcinogen—Part B: Biological agents, vol. 100. International Agency for Research on Cancer, Lyon.
IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007. I.A.f.R.o.C. World Health Organization Combined estrogen–progestogen contraceptives and combined estrogen–progestogen menopausal therapy IARC monographs on the evaluation of carcinogenic risks to humans. International Agency for Research on Cancer; Distributed by WHO Press, Lyon, France; Geneva, p. 528. ix.
IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010 (2007, Lyon France). Alcohol consumption and ethyl carbamate. IARC monographs on the evaluation of carcinogenic risks to humans. International Agency for Research on Cancer; Distributed by WHO Press, Lyon, France.
Imm, K.R., et al., 2017. African American prostate cancer survivorship: Exploring the role of social support in quality of life after radical prostatectomy. Journal of Psychosocial Oncology 35 (4), 409–423.
International Agency for Research on Cancer, 2002. Weight control and physical activity. In: IARC Handbook on cancer prevention, vol. 6. International agency for research on Cancer, Lyon, p. 315.
Khan, S., et al., 2018. The association of weight change in young adulthood and smoking status with risk of prostate cancer recurrence. International Journal of Cancer 142 (10), 2011–2018.
Lauby-Secretan, B., et al., Body fatness and Cancer—Viewpoint of the IARC working group. The New England Journal of Medicine, 2016. 375 (8): p. 794–8.
Lewington, S., et al., 2014. Temporal trends of main reproductive characteristics in ten urban and rural regions of China: The China Kadoorie biobank study of 300 000 women. International Journal of Epidemiology 43 (4), 1252–1262.
MacMahon, B., 1965. Epidemiologic methods in cancer research. The Yale Journal of Biology and Medicine 37 (6), 508–522.
MacMahon, B., Pugh, T.F., 1970. Epidemiology: Principles and methods, 1st edn. Little, Brown and Company, Boston, MA, p. 376.
McCormack, V.A., Perry, N.M., Vinnicombe, S.J., Dos Santos Silva, I., 2010. Changes and tracking of mammographic density in relation to Pike's model of breast tissue aging: a UK longitudinal study. International Journal of Cancer 127 (2), 452–461.
McMichael, A., 1994. Invited commentary: Molecular epidemiology. New pathway or new travelling companion. American Journal of Epidemiology 140, 1–11.
Medina, D., 2013. Pregnancy protection of breast cancer: New insights reveal unanswered questions. Breast Cancer Research 15 (3), 103.
Michael, Y.L., et al., 2002. Social networks and health-related quality of life in breast cancer survivors: A prospective study. Journal of Psychosomatic Research 52 (5), 285–293.
Miki, Y., et al., 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266 (5182), 66–71.
OECD, 2012. SF-2.3 Mean age of mothers at first childbirth. In: OECD Family database. OECD Social Policy Division, Paris, France.
Pereyra, A., 1961. The relationship of sexual activity to cervical cancer. Obstetrics and Gynecology 17, 154–159.
Pike, M.C., et al., 1983. "Hormonal" risk factors, "breast tissue age" and the age-incidence of breast cancer. Nature 303, 767–770.
Pike, M.C., et al., 1993. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiologic Reviews 15, 48–65.
Rahman, M., Islam, M., Berenson, A.B., 2015. Differences in HPV immunization levels among young adults in various regions of the United States. Journal of Community Health 40 (3), 404–408.
Rosner, B., Colditz, G.A., 1996. Nurses' health study: Log-incidence mathematical model of breast cancer incidence. Journal of the National Cancer Institute 88 (6), 359–364.
Rosner, B., Colditz, G.A., Willett, W.C., 1994. Reproductive risk factors in a prospective study of breast cancer: The Nurses' health study. American Journal of Epidemiology 139 (8), 819–835.
Rosner, B.A., et al., 2013. Validation of Rosner-Colditz breast cancer incidence model using an independent data set, the California teachers study. Breast Cancer Research and Treatment 142 (1), 187–202.
Rosner, B., et al., 2015. Short-term weight gain and breast cancer risk by hormone receptor classification among pre- and postmenopausal women. Breast Cancer Research and Treatment 150 (3), 643–653.
Rosner, B., et al., 2017. Weight and weight changes in early adulthood and later breast cancer risk. International Journal of Cancer 140 (9), 2003–2014.
Schiffman, M.H., et al., 1993. Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. Journal of the National Cancer Institute 85, 958–964.
Shelton, R.C., et al., 2011. The association between social factors and physical activity among low-income adults living in public housing. American Journal of Public Health 101 (11), 2102–2110.
Story, M., et al., 2008. Creating healthy food and eating environments: Policy and environmental approaches. Annual Review of Public Health 29, 253–272.
Sung, J., et al., 2009. Height and site-specific cancer risk: A cohort study of a korean adult population. American Journal of Epidemiology 170 (1), 53–64.
Swinburn, B.A., et al., 2011. The global obesity pandemic: Shaped by global drivers and local environments. Lancet 378 (9793), 804–814.
Van Blarigan, E.L., et al., 2018. Marine omega-3 polyunsaturated fatty acid and fish intake after colon cancer diagnosis and survival: CALGB 89803 (alliance). Cancer Epidemiology, Biomarkers & Prevention 27 (4), 438–445.
Walling, E.B., et al., Interventions to improve HPV vaccine uptake: A systematic review. Pediatrics, 2016, 138(1). pii: e20153863.
Warnecke, R.B., et al., 2008. Approaching health disparities from a population perspective: The National Institutes of Health centers for population health and health disparities. American Journal of Public Health 98 (9), 1608–1615.
Wehner, M.R., et al., 2014. International prevalence of indoor tanning: A systematic review and meta-analysis. JAMA Dermatology 150 (4), 390–400.
Ziegler, R.G., et al., 1993. Migration patterns and breast cancer risk in Asian-American women. Journal of the National Cancer Institute 85, 1819–1827.

Further Reading

1. Emmons, K., 2000. Behavioral and social science contributions to the health of adults in the United States. In: Smedley, B., Simes, S. (Eds.), Promoting health: Intervention strategies from social and behavioral research. National Academy Press, Washington DC, pp. 254–321.

2. Shelton, R.C., et al., 2011. The association between social factors and physical activity among low-income adults living in public housing. American Journal of Public Health 101 (11), 2102–2110.
3. Atwood, K., Colditz, G.A., Kawachi, I., 1997. From public health science to prevention policy: Placing science in its social and political contexts. American Journal of Public Health 87 (10), 1603–1606.
4. Colditz, G.A., Wei, E.K., 2012. Preventability of cancer: The relative contributions of biologic and social and physical environmental determinants of cancer mortality. Annual Review of Public Health 33, 137–156.

Carcinogen—DNA Adducts☆

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Glossary

Carcinogen-DNA adduct The addition product between two molecules, in this case a carcinogen and DNA, involving one or more covalent bonds.
Cytochrome P450 A member of a superfamily of closely related heme-containing oxygenases that oxidize a wide variety of substrates.
Exposure biomarker A xenobiotic, its metabolite, or the product of an interaction between a xenobiotic and some target molecule such as DNA or proteins that are reflective of an individual's exposure.
Metabolic activation The metabolism of a compound lacking the ability to react covalently with DNA (or proteins) to a product(s) that does.
Xenobiotic Describing or relating to a foreign compound.

Introduction

Some years ago Christopher Wild, director of the International Agency for Research on Cancer (IARC), introduced the "exposome" as a complementary concept to the "genome." The exposome reflects all of the encountered exposures of an individual over the course of her/his lifetime. According to epidemiological studies, approximately 90% of cancer deaths can be attributed to the presence of certain environmental factors, and are not related to the genetic makeup of an individual. So far IARC has classified around 120 compounds as carcinogenic to humans (Group 1 carcinogens). The list of carcinogens contains organic chemicals, inorganic material, biological agents, mixtures, and radiation. The underlying pathways leading to cancer for most of those compounds are not yet fully understood.

Chemical carcinogens can exert their effect either through genotoxic (involving DNA damage) or nongenotoxic mechanisms. Some highly reactive genotoxic carcinogens are capable of directly interacting with DNA (e.g., alkylating agents) but most chemical carcinogens (e.g., polycyclic aromatic hydrocarbons [PAHs], aromatic amines, heterocyclic aromatic amines, nitrosamines) are not chemically reactive as such and require metabolic activation to reactive intermediates capable of binding to DNA (i.e., nucleobases), forming covalent adducts. Xenobiotics that need metabolic activation to exert their genotoxic/carcinogenic properties are termed pro-carcinogens or indirect-acting carcinogens.

A wide variety of enzymes are involved in xenobiotic metabolism and these xenobiotic-metabolizing enzymes can be divided into two groups. Phase I (functionalisation) reactions include oxidation, reduction and hydroxylation catalyzed by enzymes such as cytochrome P450s (CYPs), cyclooxygenases (COXs), and aldo-keto reductases (AKRs) with the aim to make hydrophobic xenobiotics more hydrophilic and thus excretable. Phase II (conjugation) reactions further add polar moieties, such as glucuronic, acetic or sulfuric acids or glutathione thereby increasing polarity, which further assist in excretion. Examples of phase II enzymes include uridine diphosphate glucuronosyltransferases, *N*,*O*-acetyltransferases, sulfotransferases and glutathione *S*-transferases. However, phase I and II enzymes can arguably be in either category.

As an example Fig. 1 shows the bioactivation and DNA adduct formation of benzo[*a*]pyrene (BaP), a PAH that has been classified as human carcinogen (Group 1) by IARC. The major pathway in humans involves the oxidation of BaP by CYP1A1 or CYP1B1 to an epoxide (i.e., BaP-7,8-oxide) that is then converted to BaP-7,8-dihydrodiol by microsomal epoxide hydrolase (mEH). BaP-7,8-dihydrodiol can be further activated by CYPs (i.e., CYP1A1 or CYP1B1) or COX to BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) which is the ultimate reactive species capable of reacting with DNA to form covalent adducts preferentially at the exocyclic amino group at N^2-guanine (i.e., 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP [dG-N^2-BPDE]) (see Table 1). Other activation pathways for BaP lead to radical-DNA interactions and DNA depurination but their role in BaP carcinogenesis is under discussion.

Some representative carcinogens, their environmental sources, their reactive metabolites and their most abundant DNA adduct are shown in Table 1.

☆*Change History:* November 2017. Annette M. Krais, Rajinder Singh, and Volker M. Arlt wrote a new article. Only couple of sentences of the old chapter have been incorporated into the present version of chapter.
This article is an update of Alan M. Jeffrey, M.I. Straub, Carcinogen–DNA Adducts, in Encyclopedia of Cancer (Second Edition), edited by Joseph R. Bertino, Academic Press, 2002, Pages 345–357.

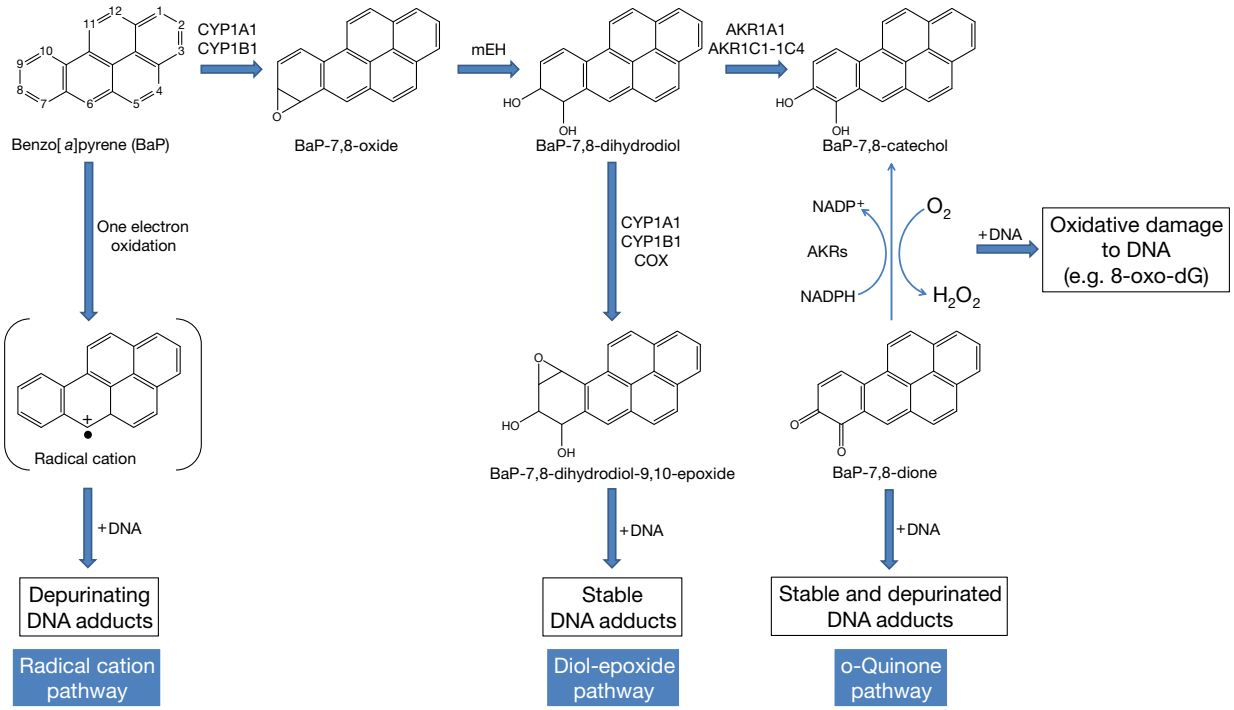


Fig. 1 Pathways of BaP bioactivation leading to DNA adduct formation. *CYP*, cytochrome P450; *mEH*, microsomal epoxide hydrolase; *COX*, cyclooxygenase; and *ARK*, aldo–keto reductase. See text for details.

DNA adducts can also originate from endogenous processes, including normal metabolism, oxidative stress, lipid peroxidation and chronic inflammation (Fig. 2). The most abundant lesion linked to oxidative damage to DNA is 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-guanine [8-oxo-dG]) which is formed by free radical attack on DNA (i.e., addition of •OH to the C8 position of guanine). As shown in Fig. 1 BaP quinone redox cycling can generate reactive oxygen species which leads to the formation of 8-oxo-dG in DNA (see Fig. 2). Thus some genotoxic carcinogens that do not appear to directly modify DNA can instead damage it through inducing reactive oxygen species leading to oxidative base modifications.

The DNA adductome is defined as the sum of all DNA adduct types and levels present in cellular DNA and can be considered reflective of the internal exposome. These DNA adducts, if not repaired before DNA replication, can lead to DNA mutations or chromosomal damage, thus possibly resulting in carcinogenesis. Therefore as adducts can lead to mutations, and mutations in critical genes are a characteristic feature of tumors, DNA adduct formation is considered to be the first critical step (i.e., initiation) in chemical carcinogenesis (Fig. 3).

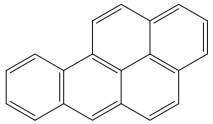
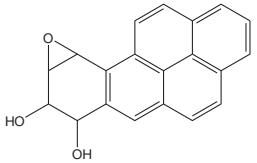
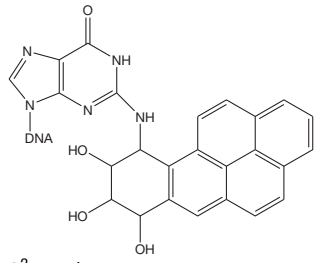
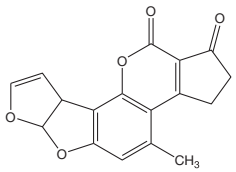
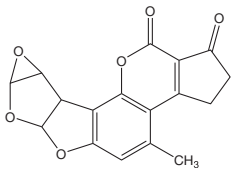
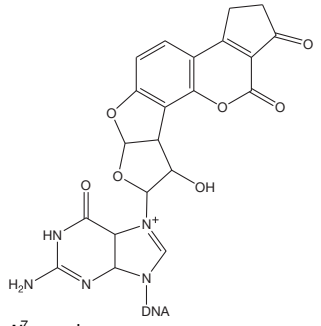
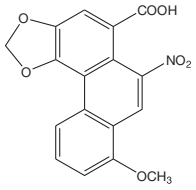
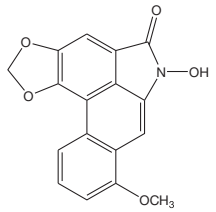
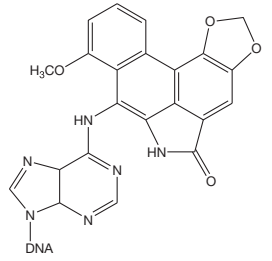
With few exceptions, however, carcinogens form DNA adducts in many tissues (in both target and nontarget tissues), leading to the view that adducts are necessary, but not sufficient, for carcinogenesis. Thus, although there are linear relationships between dose, adduct levels in target tissues and tumor incidence, it may be difficult to identify which tissues are targets for carcinogenicity by an agent purely from a consideration of the levels and persistence of DNA damage. Nevertheless, the identification and structural characterization of DNA adducts in human tissues can provide important clues on the etiology of human cancer. DNA adducts have the potential to be used as biomarkers of exposure (to carcinogens) and markers of cancer risk (from exposure to carcinogenic agents).

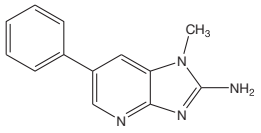
Application of DNA Adduct Analysis

A major goal of adduct analysis is to improve our ability to extrapolate data on carcinogen exposure to human risk. It is reassuring to find that good correlations have been obtained between carcinogenicity of genotoxic compounds and their ability to bind to DNA, especially when closely related series of compounds are investigated. Sensitive DNA adduct detection methods make it possible to monitor carcinogen-DNA adduct formation in humans. Thus, DNA adduct formation in an exposed cell or tissue can be used: (*i*) to determine the mutagenic or carcinogenic potential of an agent; (*ii*) for monitoring human exposure to environmental carcinogens to study the etiology of cancer; (*iii*) for mechanistic investigations into carcinogen activation, tumor initiation or DNA repair; (*iv*) monitoring the environment for the presence of genotoxins (e.g., by detecting DNA adducts in aquatic species); and (*v*) assessing a patient's response to cytotoxic cancer drugs.

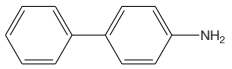
Since humans are exposed not only to one but to a complex mixture of carcinogens, direct proofs of an association of chemical exposure to the development of a specific cancer type are scarce. The plant carcinogen aristolochic acid and the mycotoxin aflatoxin B_1 are two rare examples where distinct environmental exposures are linked to tumor development in humans. For both agents DNA adduct formation could be linked to cancer incidence, by combining molecular epidemiological data on chemical exposures,

Table 1 Examples of environmental carcinogens and drugs that form DNA adducts

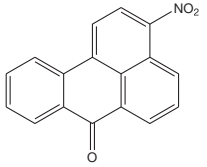
Carcinogen	*Major active metabolite*	*Major DNA adduct/site of modification*	*Environmental source*
 Benzo[*a*]pyrene (BaP)	 BaP-7,8-dihydrodiol-9,10-epoxide (BPDE)	 N^2-guanine	Tobacco smoking combustion processes diet
 Aflatoxin B_1(AFB_1)	 AFB_1-8,9-epoxide	 N^7-guanine	Mycotoxin
 Aristolochic acid I	 *N*-Hydroxy-aristolactam I	 N^6-adenine	*Aristolochia* species



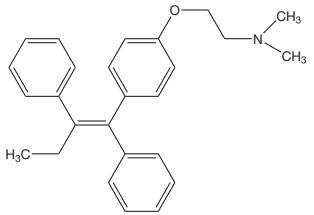
2-Amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP)



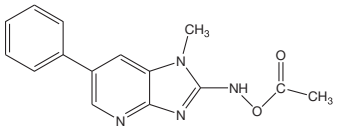
4-Aminobiphenyl



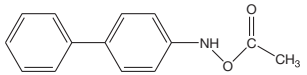
3-Nitrobenzanthrone



Tamoxifen



N-Acetoxy-PhIP



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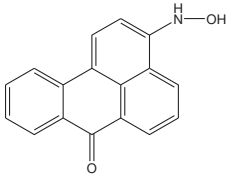

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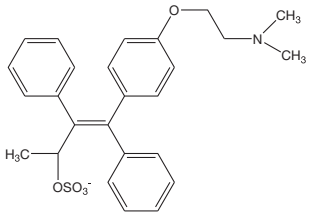

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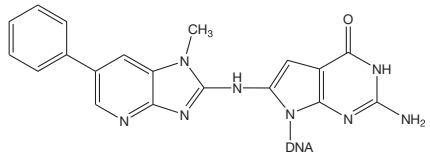
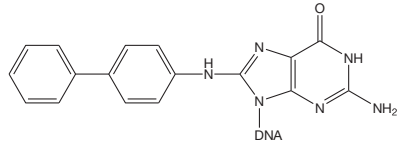


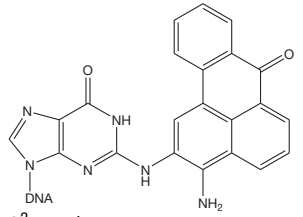
C8-guanine

Food processing



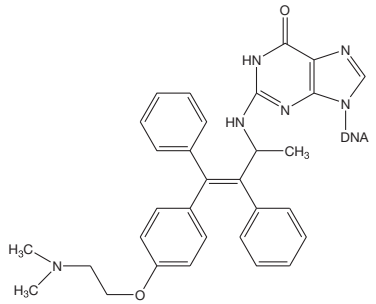
C8-guanine

Tobacco smoking, Azo dyes



N^2-guanine

Diesel exhaust air pollution



N^2-guanine

Anticancer drug

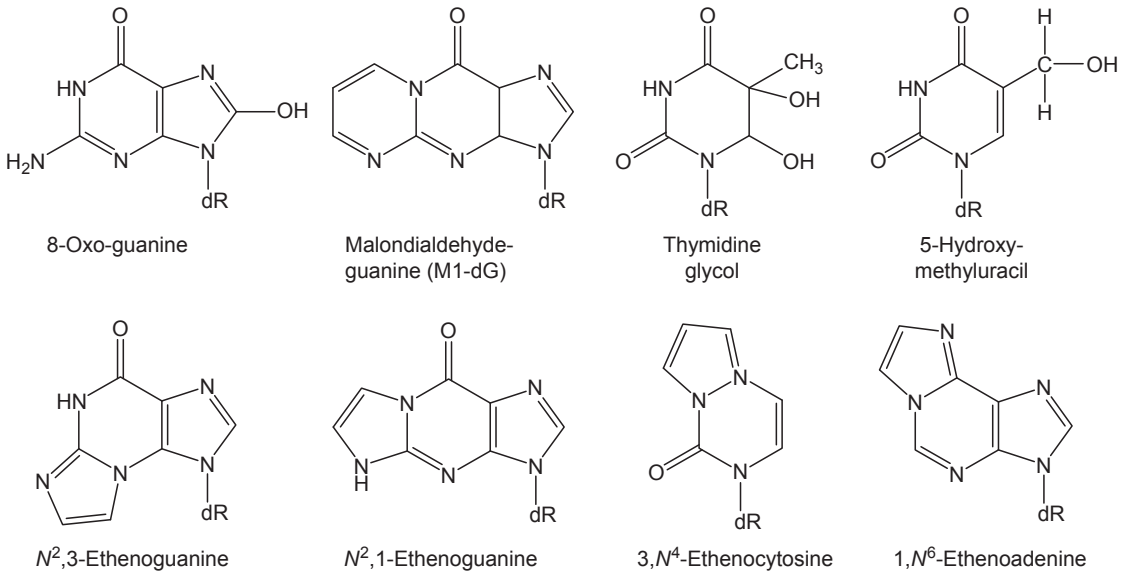


Fig. 2 Structures of endogenous DNA adducts arising from oxidative stress, lipid peroxidation, and chronic inflammation.

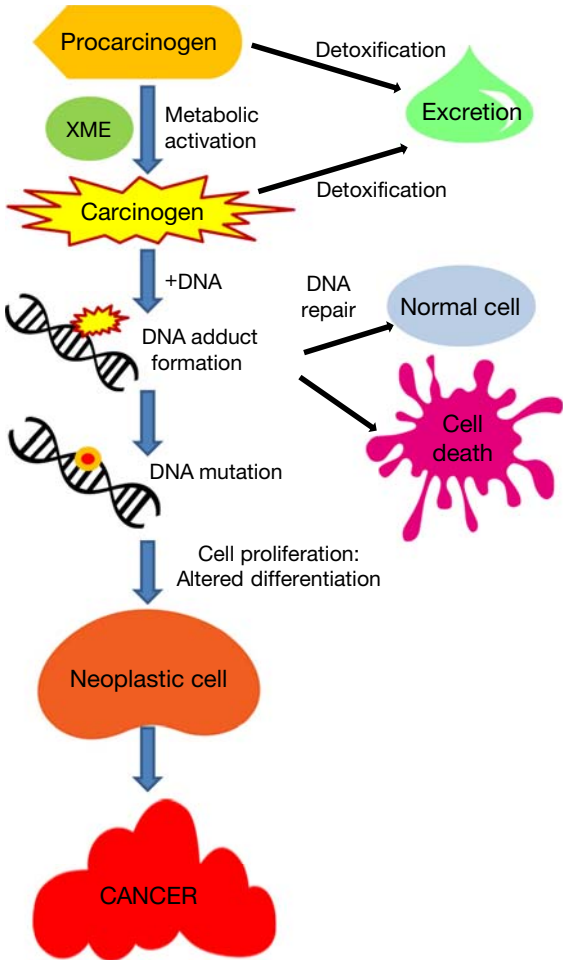


Fig. 3 The role of DNA adducts in the initiation process of carcinogenesis. See text for details.

chemical-specific DNA adducts in target tissues and mutation spectra in tumor-related genes. Thus aristolochic acid and aflatoxin B_1 have both been classified as Group 1 human carcinogen by IARC. Aflatoxin B_1 is a potent liver carcinogen that can be found as a contaminant in various crops and dietary exposure to aflatoxin B_1 has been associated with hepatocellular carcinoma in certain areas of Asia and Africa. Those observations have been strengthened by the detection of N^7-guanine adducts of aflatoxin B_1 (see **Table 1**) in the urine of exposed individuals. These adducts lead to characteristic G:C → T:A transversion mutations selectively in codon 249 of the *TP53* tumor suppressor gene in aflatoxin B_1-induced liver cancers. Aristolochic acid, the plant extract derived from *Aristolochia* species, causes upper urothelial tract cancer in patients suffering from aristolochic acid nephropathy (AAN) and Balkan endemic nephropathy (BEN). Exposure to aristolochic acid has been demonstrated in many parts of the world and linked to medicinal herbal remedies or dietary intake. The 7-(deoxyadenosin-N^6-yl)aristolactam I adduct (see **Table 1**) is the most abundantly found in urothelial tissue of AAN/BEN patients. It has even been detected in individuals over 20 years after exposure to aristolochic acid had ceased. The abundance and exceptionally long-term persistence of 7-(deoxyadenosin-N^6-yl)aristolactam I in DNA can therefore be exploited as specific biomarker of AA exposure in human biomonitoring. This adduct also leads to otherwise rare A:T → T:A transversion mutations, which are frequently detected in *TP53* in urothelial tumors of AAN/BEN patients. This characteristic mutation pattern is also found by whole-genome sequencing of AA-exposed tumors.

It is clear from numerous studies that DNA adducts are intrinsically present in DNA. These "background" levels of adducts or DNA damage have been detected in individuals for whom no specific exposure to the agent of interest was known. Background DNA damage can have multiple origins including diet, air pollution or smoking and thus need to be taken into account when selecting control groups of individuals for comparison with a putative "exposed" population in molecular epidemiology studies. This will ensure that any difference in adduct levels between the study groups are due to the exposure of interest and not just linked to differences in other lifestyle factors. Besides "normal" environmental exposures, endogenous sources also contribute to background levels of DNA damage.

DNA adducts have been detected in the range of 1 adduct per 10^7–10^{11} normal nucleotides in human tissues. The remainder of the chapter considers methods for carcinogen-DNA adduct detection in humans and how identified adducts in human tissues can be used to study cancer etiology.

Methods for Adduct Detection

A variety of sensitive methods have been developed for the detection and characterization of carcinogen-DNA adducts (**Table 2**). In order to be applicable for human biomonitoring the assays used must be sufficiently sensitive to detect low levels of DNA adducts, require only small amounts of DNA, provide results quantitatively related to the exposure, be applicable to unknown DNA adducts that may be formed from complex mixtures and be able to resolve, quantitate, and identify DNA adducts.

The main methods that have been used to detect DNA adducts include ^{32}P-postlabeling, immunoassay, gas chromatography mass spectrometry (GC-MS), liquid chromatography (LC) coupled with electrochemical, fluorescence or mass spectrometry (MS) and accelerator mass spectrometry (AMS). Each of these methods adopts very different approaches for the detection of carcinogen-DNA adducts, and differs in their detection limits and requirements for quantities of DNA for analysis (**Table 2**). They all are sufficiently sensitive to be employed in human biomonitoring but they all have their strengths and weaknesses, so the method of choice has to be selected on a case-by-case basis.

Radioactive Labels

Most early work on adducts required the use of radiolabeled compounds (labeled either with ^{3}H or ^{14}C). The DNA binding is measured by the detection of radioactivity in DNA isolated from exposed animal tissue or cells in culture achieving sensitivities of detection of 1 adduct in 10^8 nucleotides; ^{14}C-labeling is less sensitive than ^{3}H-labeling. The advantage of ^{3}H is that it is relatively easily introduced into the carcinogen and can be obtained at higher specific activities than ^{14}C because of the former's shorter half-life. However, ^{3}H is also very susceptible to loss during metabolism or by exchange reactions. This can result in the formation of tritiated water which in turn may become incorporated into nonexchangeable positions in newly synthesized DNA. Although

Table 2 DNA adduct detection methods applicable to human biomonitoring and their limits of detection

Method	*Variations*	*Amount of DNA required*	*Approximate detection limits*
^{32}P-postlabeling	Nuclease P_1 digestion, butanol extraction, TLC, HPLC, PAGE	1–10 μg	1 adduct per 10^9–10^{10} nucleotides
Mass spectrometry		Up to 100 μg	1 adduct per 10^9 nucleotides
Immunoassay	ELISA, DELFIA, CIA, IHC	20 μg	1.5 adduct per 10^9 nucleotides
Fluorescence	HPLC fluorescence, SFS	100–1000 μg	1 adduct per 10^9 nucleotides
Accelerator mass spectrometry		Up to 100 μg	1 adduct per 10^8–10^{12} nucleotides

TLC, thin-layer chromatography; *HPLC*, high-performance liquid chromatography; *PAGE*, polyacrylamide gel electrophoresis; *ELISA*, enzyme-linked Immunosorbent assay; *DELFIA*, dissociation-enhanced lanthanide fluoroimmunoassay; *CIA*, chemoluminescence immunoassay; *IHC*, immunohisto-chemistry; *SFS*, synchronous fluorescence spectroscopy.

now at very low specific activity, this ^{3}H can contribute significantly to the overall level of apparent binding. The major limitation of this approach to prelabel the carcinogens is the requirement of highly radioactive test compounds which cannot be used in human biomonitoring studies.

The ^{32}P-postlabeling assay also uses radiation as a measure to detect and quantify carcinogen-DNA adducts, the underlying principle is however that adducts are labeled after their formation. ^{32}P can be obtained at about 300 times higher specific activity than ^{3}H and can be counted with higher efficiency due to the higher energy of the emitted β particle allowing the method to be intrinsically much more sensitive. The detection limit is approximately 1 adduct per 10^{9}–10^{10} nucleotides (see **Table 2**) using only a few micrograms of DNA which makes the assay widely applicable in human biomonitoring. As shown in **Fig. 4**, the method consists of four principle steps: (*i*) modified DNA is digested enzymatically to deoxyribonucleoside-3′-monophosphates using micrococcal

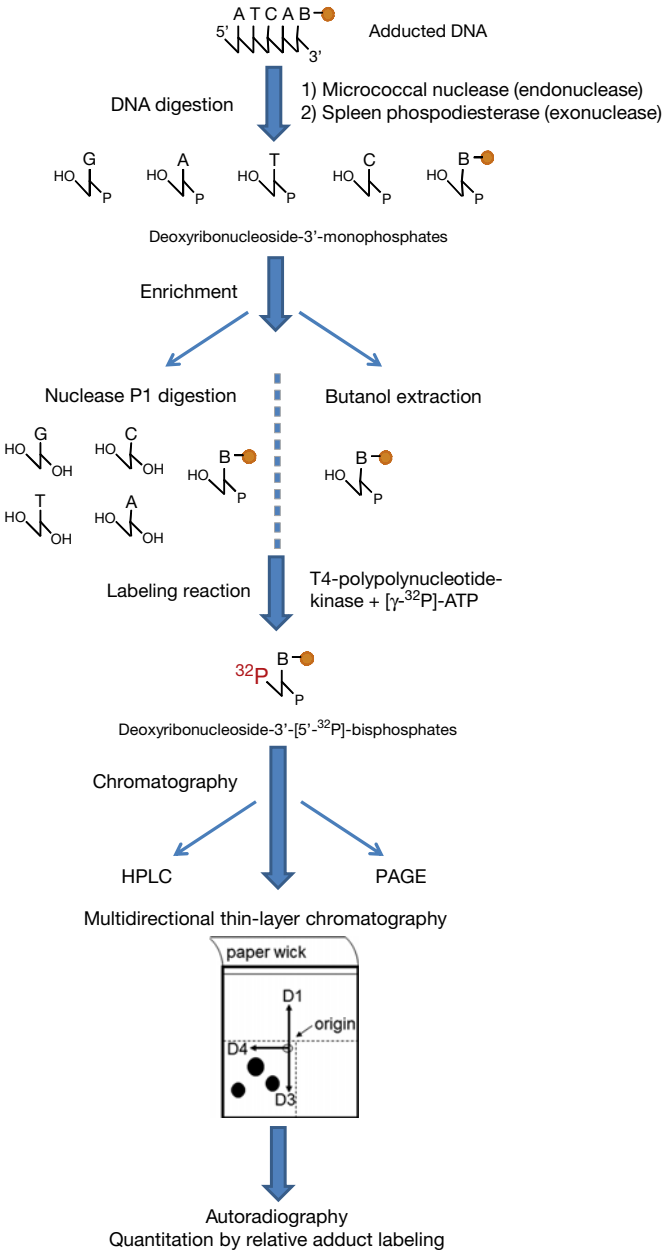


Fig. 4 DNA adduct analysis using ^{32}P-postlabeling. DNA (B indicates the modified base) is digested with micrococcal nuclease and spleen phosphodiesterase and subsequently phosphorylated by radioactive ^{32}P using T4-polynucleotide kinase. The enrichment procedures include nuclease P_1 digestion, which hydrolyzes most of the unmodified nucleotides to nucleosides, which are not kinase substrates. Many bulky DNA adducts, not linked to C8 purine position, are resistant to this enzyme. Butanol enrichment mainly extracts hydrophobic adducts. Radioactive-labeled adducts are separated by chromatography, mainly by using multidirectional thin-layer chromatography (TLC), but also high-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE) are occasionally used. Adducts separated by TLC are subsequently visualized by autoradiography and quantitation is achieved by calculating relative adduct labeling.

nuclease (endonuclease) and spleen phosphodiesterase (exonuclease); (*ii*) the adduct fraction is enriched using nuclease P1 digestion or butanol extraction; (*iii*) the DNA adduct is 5′-labeled by transfer of ^{32}P-orthophosphate from [γ-^{32}P]-ATP using T4-polynucleotide kinase resulting in deoxyribonucleoside-3′,[5′-^{32}P]-bisphosphates; and (*iv*) the chromatographic or electrophoretic separation of ^{32}P-labeled adducts and quantitation by the measurement of their radioactive decay.

As shown in Fig. 4, two principle enrichment procedures that follow DNA digestion have been developed. Digestion of deoxyribonucleosides-3′-monophosphates (modified or unmodified) with nuclease P_1 prior to labeling, resulting in normal nucleotides but not many adducts, being converted to nucleosides which are no substrate for T4-polynucleotide kinase. Enrichment using nuclease P_1 digestions works well for PAH-like adducts but is less suited for many aromatic amine adducts. Often adducts formed at C8-guanine appear not to be resistant to nuclease P1 digestion. The other enrichment procedure uses extraction of the aromatic/hydrophobic adducts into butanol to separate them from the unmodified normal nucleotides (which remain in the water phase) for selective ^{32}P-postlabeling.

Chromatographic resolution of ^{32}P-labeled adducts is mainly achieved by thin-layer chromatography (TLC) or alternatively by high-performance liquid chromatography (HPLC) (Fig. 4). Electrophoretic separation using polyacrylamide gel electrophoresis (PAGE) are also be utilized but is less frequently used. The major difficulty is the separation of the overwhelming excess of radioactivity associated with residual ATP, the normal nucleotides which also become phosphorylated, and any radiochemical decomposition products that might have been formed. Clearly, depending on the level of modification of the DNA by the carcinogen, which may be in the range of 1 adduct per 10^6–10^{10} nucleotides, only about that fraction of the disintegrations will be that of interest. Subsequently an elaborate separation technique was developed to remove any excess of radioactivity associated with residual ATP. This is illustrated here for the TLC version of the assay (see Fig. 4). The enriched fraction of adducts which have been labeled with ^{32}P is placed in the center of a polyethylenimine cellulose TLC plate and the plate is washed first in one direction (D1) using a phosphate buffer, cutting away the edge of the plate with an attached filter paper which contains the bulk of the unwanted radioactivity moved. Multidirectional resolution in two directions of chromatography (i.e., D3 and D4), using solvent systems optimized for the adduct(s) of interest, allow for the separation of the carcinogen-modified bases. Chromatography in D2 is now often omitted. Subsequently, DNA adducts are visualized by placing the TLC plates in cassettes using autoradiography films or plates are scanned using radiation imaging systems (e.g., Instant imager or phosphoimager).

^{32}P-postlabeling can be applied to detect a variety of bulky carcinogen-DNA adducts as illustrated in Table 1. Prior structural characterization of adducts is not required, although some assumption about the likely chromatographic properties may be necessary. Thus the method can also be applied to the detection of DNA adducts formed by complex mixtures such as those present for example, in tobacco smoke. Total smoking-related adducts can be measured as a diffuse radioactive zone on the TLC plate that can be considered representative of PAH-DNA and other aromatic/hydrophobic adducts thereby providing a summary measure of a complex mixture of adducts present in the postlabeling chromatograms. Although the method does not provide direct structural information of adducts, identification of adducts can be achieved by co-chromatography with characterized synthetic standards, usually using a combined approach employing TLC and HPLC for confirmation.

Mass Spectrometry

Mass spectrometry analysis is currently recognized as the "gold standard" for DNA adduct detection. MS analysis enables accurate quantitation and provides structural information about the DNA adduct, with only micrograms amounts of DNA or small volumes (from μL to mL) for adducts excreted in urine required for analysis. Detection is possible for DNA adducts at levels of up to one DNA modification per 10^9 deoxynucleosides (see Table 2), and sensitivity is still improving due to technical advancements in the MS instrumentation with regard to the efficiency of ionization, ion transmission and detection. Samples can be analyzed by using chromatographic separation coupled with various MS detection methods, for example, GC-MS, LC-MS/MS and AMS (see Section "Accelerator Mass Spectrometry"). Two different types of approaches can be used when studying genotoxin exposure using MS-based methods, targeted DNA adduct determination and screening of multiple DNA adducts (either targeted or untargeted); the latter adductomics approach is described in the "Adductomics" section. The targeted approach implies the identification and quantification of a limited number of DNA modifications. Using targeted DNA adduct detection, all known exposure types or exposure to specific chemicals can be analyzed in order to characterize the exposure. This implies that the MS system specifically scans for the presence of certain DNA adducts of interest, while the analysis of all other adducts that may be present in the sample is omitted.

GC-MS can be used for the analysis of several DNA adducts, but samples require chemical derivatization prior to analysis to increase the volatility of the DNA adducts under investigation. It provides excellent separation and resolution due to the employment of capillary column chromatography as well as structural information about the adduct. Furthermore, GC-MS can use different MS modes for carcinogen-DNA adduct detection such as electronic impact (EI) and negative ion chemical ionization (NICI) mode. GC-EI-MS has been used to analyze polar deoxynucleosides and aglycone adducts as well as BaP-tetrols (i.e., *r*-7,*t*-8,*t*-9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydro-BaP) released by acid hydrolysis of DNA, as a marker for BPDE-DNA adduct formation. The main application of GC-EI-MS has been the detection of oxidized DNA bases, such as those resulting from the reaction of reactive oxygen species with DNA (e.g., 8-oxo-dG). The chemical derivatization of the DNA hydrolysate at high temperature has to be carefully monitored as artifacts of oxidized bases can easily be generated. Compared to the positive-ion EI mode, NICI possesses higher selectivity and superior sensitivity toward compounds with high electron affinities. GC-NICI-MS has been applied for DNA adducts caused by lipid peroxidation by-products, and tobacco smoke. However, GC-MS is less commonly used for carcinogen-DNA adduct

detection compared to LC-MS, due to labor-intensive sample preparation such as the extensive derivatization steps required to increase the volatility of the adduct and the potential to generate artifact adducts.

LC-MS/MS is the primary method for the detection and quantitation of DNA adducts in DNA from biological matrixes. LC is more suited than GC for the analysis of DNA adducts since it allows the analysis of polar and nonvolatile molecules. Most analyses are performed using electrospray ionization (ESI) which is a soft ionization technique. LC-ESI-MS allows for the detection of adducted nucleobases, adducted deoxynucleosides, deoxyribose-phosphate adducts as well as modified oligonucleotides. LC-MS/MS typically employs two quadrupole mass analysers in series separated from each other by a collision cell, which yields fragment ions following collision-induced dissociation (CID), and normally analyses are performed in selected reaction monitoring (SRM) mode. Several studies have used LC-MS/MS for the detection of modified bases in human urine including 8-oxo-dG, etheno-DNA adducts (see **Fig. 2**), N^7-ethylguanine, and N^7-guanine adducts of aflatoxin B_1 (see **Table 1**). Examples for the application of LC-MS/MS in human tissues include 1,N^2-propanodeoxyguanosine adducts derived from acetaldehyde and crotonaldehyde and acetaldehyde-derived adducts (as a possible marker of ethanol genotoxicity), 4-aminobiphenyl, aristolochic acid and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) adducts (see **Table 1**).

Accelerator Mass Spectrometry

AMS is an analytical technique that can quantify long-lived isotopes with attomole (amol) (10^{-18}) sensitivity in isotope-labeled drugs and toxicants. AMS counts atoms of a rare isotope of interest and reports the ratio of the counted isotope to that of the total number of atoms of the element. Radiocarbon ^{14}C ($t_{1/2} = 5730$ years) and tritium ^{3}H ($t_{1/2} = 12.3$ years) are commonly used to label the compound or drug of interest for DNA adduct analyses. The excellent sensitivity of AMS enables analysis of DNA adduct formation for human biomonitoring. Human studies using AMS include adduct formation of the food carcinogen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MelQx) and tamoxifen, a compound used in the treatment of breast cancer. AMS has also been used to determine DNA adduct formation in rodents following the exposure of ^{14}C-labeled benzene, ochratoxin A, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Despite the excellent sensitivity of AMS, sample preparation is labor-intensive and the instrumentation requires high levels of maintenance. Additionally, AMS does not provide any structural information about the DNA adduct, but it can be coupled to HPLC and identification can be achieved by using adduct standards. For example, HPLC separation followed by AMS analysis was employed for determining the distribution of [^{14}C]oxaliplatin-induced DNA adducts in drug-sensitive testicular and drug-resistant breast cancer cells at a clinically relevant drug concentration.

Immunological Methods

These approaches rely on the preparation of antisera which recognize specifically the carcinogen-DNA adduct. To date around 20 different carcinogen-DNA adducts can be detected by immunological methods, limited by the need to raise antibodies against synthetically prepared and characterized adducts in order to detect them in human tissues. These antisera include those raised against alkylation adducts such as O^6-methyl- and O^6-ethylguanine, N^7-methylguanine, and N^6-methyladenine, or adducts formed by oxidative stress such 8-oxo-dG (see **Fig. 2**). Other antibodies that detect bulky DNA adducts include those derived from BaP, 4-aminobiphenyl and aflatoxin B_1. Many variations exist on the method by which the antisera may be used. Most of the immunological studies have been based on enzyme-linked immunosorbent assay (ELISA) but modified assays such as the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) and chemiluminescence immunoassay (CIA) have been employed in molecular epidemiology because they have sensitivities approaching those of ^{32}P-postlabeling.

The underlying principle for the ELISA is that DNA to be tested is coated onto the surface of a suitable plate. Antiserum, specific for the adduct of interest, is added and after incubation any noncomplexed antibody is removed. A secondary antibody linked to a suitable marker enzyme, for example, alkaline phosphatase, is then added. This secondary antibody will bind to the plate only if there was an interaction between the adducted DNA and the primary antibody. After washing the plate, *p*-nitrophenylphospate is added as the enzyme substrate. As hydrolysis proceeds, which will be proportional to the extent of modification originally present in the DNA, the color formed from the released nitrophenyl anion can be measured. A standard curve is generated by serial dilution of either modified denatured DNA or monoadduct and mixing with the adduct-specific primary antibody. Samples with unknown levels of DNA adducts are tested in a similar way. The sensitivity of the ELISA can be further increased by using competitive versions of the assay, using substrates which yield fluorescent products. In the DELFIA assay the alkaline phosphatase conjugate is replaced by a biotin-europium-labeled streptavidin signal amplification system which releases europium into the solution forming a highly fluorescent lanthanide chelate complex. The assay principle of CIA is similar to that of competitive ELISA but uses chemiluminescene instead of a colored end product.

Immunohistochemistry can also be used to detect DNA adducts in cells and tissue sections. Although the method is less sensitive than the immunological assays described above, it can provide valuable information on the localization of carcinogen-DNA adduct formation within a tissue or cell population. Assessment uses subjective estimation of staining intensity and/or number of positively stained cells with an arbitrary scale and thus need to be regarded as semiquantitative in its assessment of adduct levels. As with all immunohistochemical methods appropriate control experiments need to be conducted to validate the specificity of cell staining.

Slot- or dot-blot immunological methods also provide a means to detect DNA adducts using antisera. The principle of the slot/dot-blot methodology is that DNA is immobilized on nitrocellulose and then incubated with primary adduct-specific antibodies. Again enzyme-labeled secondary antibodies are subsequently used to measure the degree of binding using colored or

chemiluminescent end products. Immuno-dot/slot-blot methods have been developed to detect DNA adducts derived from PAHs, alkylating agents or malondialdehyde (see Fig. 2).

Fluorescence Techniques

DNA adducts derived from PAHs (e.g., BaP) or aflatoxin B_1 that contain fluorescent chromophores can be detected by means of their fluorescent emissions. This also includes some methylated DNA adducts. Usually HPLC separation coupled with fluorescence detection is used for the determination of carcinogen-DNA adduct formation. Combining the fluorescence characteristics (specific excitation and emission wavelengths) with HPLC separation makes it even possible to detect stereoisomers. The advantage of fluorescence analysis is that it requires no prior knowledge or preparation of the DNA adduct, only the knowledge that it is adequately fluorescent. However this is also a major limitation as only few carcinogen-DNA adducts are intrinsically fluorescent. HPLC with fluorescence detection has been applied in molecular epidemiological studies to determine DNA adducts but necessitated using fairly large quantities of DNA to achieve the required sensitivity.

Other applications include using the fluorescence generated of BaP-tetrols, the hydrolysis products of BPDE-DNA adducts, as measures of BaP-derived DNA adduct formation by HPLC. Synchronous fluorescence spectrometry can also be used to detect BPDE-DNA adducts (sensitivity of $\sim$1 adduct/10^7 nucleotides). Samples are scanned synchronously with a fixed difference between the excitation and emission wavelengths (e.g., 34 nm for BPDE-DNA adducts) where the peak formed correlates linearly with the level of adducts. Fluorescent labeling of nonfluorescent DNA adducts is another approach to exploit fluorescence spectroscopy. Capillary electrophoresis and laser-induced fluorescence has been used to measure DNA adducts formed by BaP and aristolochic acid (sensitivity of $\sim$1.5 adducts/10^7 nucleotides).

Electrochemical Detection

Electrochemically active DNA adducts which readily undergo reduction or oxidation reactions can be determined by HPLC coupled with electrochemical detection including 8-oxo-dG and N^7-alkylguanines.

Adductomics

The DNA adductome is part of the exposome. It is defined as the sum of all DNA adduct types and levels present in a DNA sample reflecting both known and unknown exogenous and endogenous exposures. Untargeted analysis refers to the total amount of all DNA adducts present, and in case of sufficient sensitivity, full scan MS data can be searched for the presence of known or unknown DNA adducts, in parallel or retrospectively (see also Section "Mass Spectrometry"). This approach however requires specialized untargeted screening technologies and methodologies as well as extensive data processing using specialized software. Despite its expense and complexity, screening studies have been suggested to be the future of cancer epidemiology. Recent improvements in MS scanning acquisition capabilities and instrument sensitivity potentially allow the use of MS in high-throughput applications in DNA adductomic approaches.

DNA adductomics analyses usually takes advantage of the common structural feature of adducted deoxyribonucleosides which involves the cleavage of the labile glycosidic bond between the deoxyribose moiety (dR) and the adducted nucleobase. Hence, different approaches for the detection of these DNA adducts can be used by screening of the neutral loss of the dR moiety (116 *u*) and detecting the adducted base (Fig. 5A), which is the most common approach utilized or by analyzing the formation of the nucleobase-ion from the aglycone nuceloblase adduct (Fig. 5B).

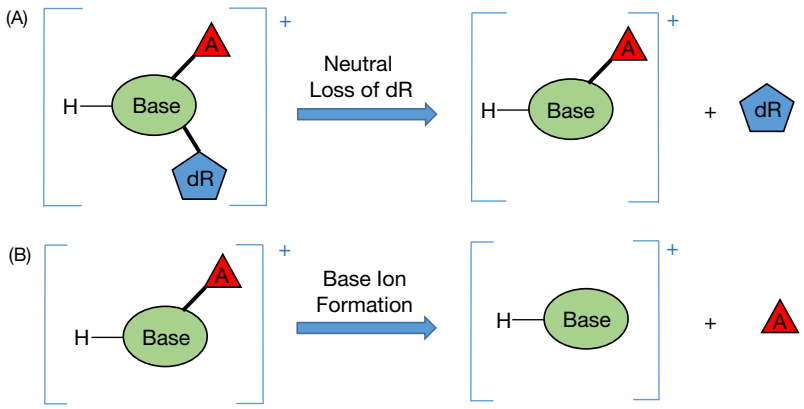


Fig. 5 The most common fragmentation pathways for adducted deoxynucleosides are (A) the neutral loss of the 2′-deoxyribose moiety and (B) formation of the nucleobase ion. Base, nucleobase; A, adduct; and dR, 2′-deoxyribose.

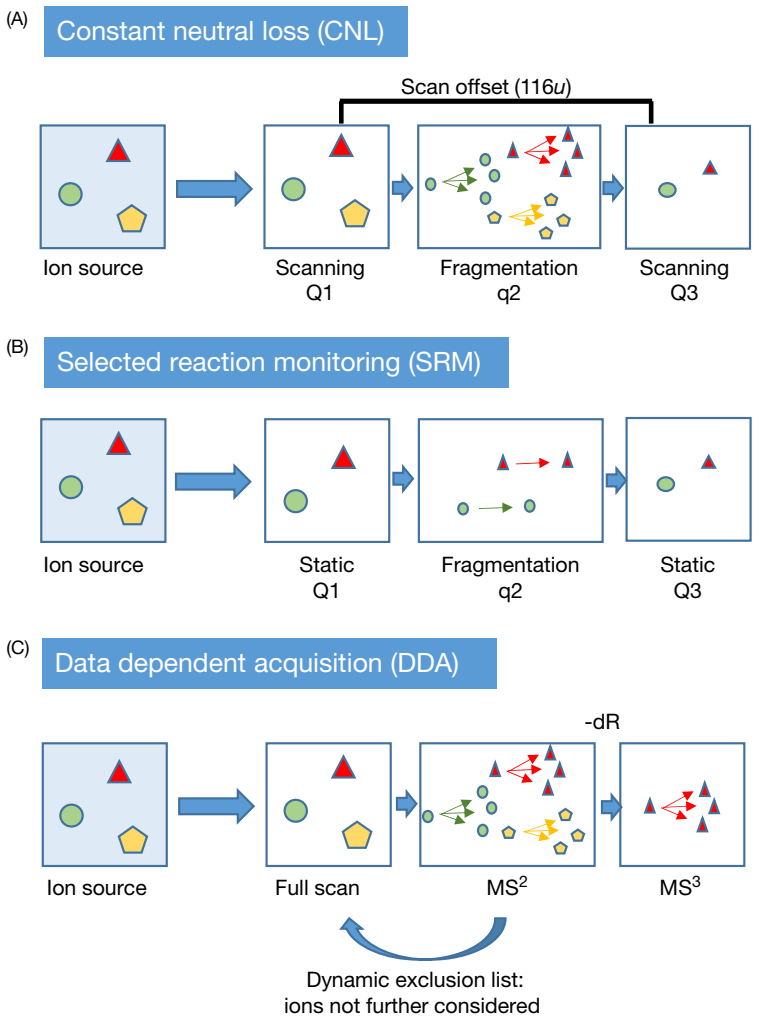


Fig. 6 Schematic illustrating the variety of data acquisition modes in the context for DNA adduct screening. The acquisition of DNA adductomics data can be performed in (A) CNL mode (only ions that fragment with the neutral loss of 116 *u* will be recorded as a signal), (B) in SRM mode (both Q1 and Q2 are operated in static mode and only ions of the user-specified *m/z* that produce specific fragments in q2 are detected), or (C) in DDA mode (the ions not showing the neutral loss of 116 *u* [*green* and *yellow*] are put into an exclusion list so that they are no longer eligible for fragmentation).

Several different modes of detection can be applied for DNA adductomic analyses, SRM, constant neutral loss (CNL), or data dependent acquisition (DDA) (Fig. 6). SRM methods offer the greatest sensitivity and are used primarily for targeted detection and quantitative analysis of DNA adducts, while the other two modes provide structural information and are useful for identification and characterization of novel DNA modifications.

Constant neutral loss (CNL) scanning using a triple quadrupole mass spectrometer has been a common mode of detection for DNA adductomic analysis. This scan mode monitors the neutral loss of 2′-deoxyribose (from positively ionized 2′-deoxynucleoside adducts). As shown in Fig. 6A, quadrupole 1 (Q1) and Q3 are run in scan mode with an offset of mass difference 116 *u*, while ions are fragmented in the collision cell (q2). Only ions that fragment with the specific neutral loss of 116 *u* will be recorded as a signal. Alternatively, instead of using both quadrupoles in scan mode, a number of contiguous SRM transitions can be monitored all involving the neutral loss of 116 *u*. As shown in Fig. 6B, Q1 precursor ions are selected that undergo fragmentation (q2), followed by product ion selection Q3, thus allowing for selective detection of adducted 2′-deoxynucleosides using the $[M+H]^+$ to $[M+H-116]^+$ transition. The potential of using MS for the screening of DNA adducts by monitoring a large range of such SRM transitions led to the concept of producing an adductome map of all DNA adducts in a sample. This approach was first applied for the mapping of the DNA adductome by Kanaly and colleagues that used a series of SRM transitions totalling 374 for monitoring the neutral loss of 2′-deoxyribose. This LC-MS/MS approach for monitoring multiple SRMs has been used to analyze tissue from human lung for multiple DNA adducts derived from lipid peroxidation, furfuryl alcohol, and methyleugenol. Similar methods have been used for the simultaneous characterization of exocyclic DNA adducts derived from exogenous industrial chemicals and endogenous by-products of lipid peroxidation in human DNA. An alternative LC-MS/MS adductomic approach can be used for the detection of N^7-alkylguanine guanine adducts which are released from the DNA by thermal depurination. The transition of the adducted

nucleobase $[M+H]^+$ precursor ion to the protonated guanine (m/z 152) product ion can be used as a generic method for monitoring N^7-alkylguanines.

An advancement for the detection and identification of unknown DNA adducts is the development of high resolution mass spectrometry (HRMS) and MS^n detection. HRMS can provide accurate mass information enabling the determination of minute differences in mass between two compounds that would appear to be identical on a standard low resolution triple quadrupole instrument. By providing accurate mass measurements, HRMS provides data for the determination of elemental composition allowing identification of the DNA adduct. New approaches for DNA adductomics research may be achieved by using quadrupole-TOF (time of flight) instruments which provide accurate mass data. TOF-MS instruments can be used for the DNA adduct screening and characterization using DDA. DDA uses repeated acquisition of spectra from full scans as well as multistage scans (MS^n). This scanning mode is performed in real-time and it thus requires preprogrammed decision making by the MS software during consecutive scans. For example, masses of ions selected for fragmentation can be placed into a dynamic exclusion list and these masses are no longer eligible for fragmentation in subsequent full scan spectra (Fig. 6C). Alternatively, an inclusion list containing masses of interest can be used to trigger analysis of targeted analytes. Furthermore, a reject mass list can be programmed containing a list of interfering background ions that will be excluded from analysis. DDA can also be used with ion trap instrumentation whereby ion trap MS analyzers allow the acquisition of multistage scan events (MS^n) that can provide additional structural information about the DNA adduct. A linear ion trap LC-DDA-CNL-MS^3 approach was developed, where the detection of a DNA adduct ion (listed in a targeted mass-list) in a limited m/z scan range leads to MS^2 acquisition. Then the detection of the $[M+H-116]^+$ ion among the top 10 of the most abundant MS^2 ions triggers MS^3 fragmentation. This approach has been used to study the formation of DNA adducts of derived from aromatic amines, heterocyclic aromatic amines, PAHs, and aldehydes.

Other types of mass analysers such as the Orbitrap also provide very accurate mass detection information due to a high resolving power and mass accuracy. The Orbitrap mass analyzer is often coupled to a quadrupole or an ion trap and is well suited for small molecule analysis and untargeted adductomics applications and a high resolution LC-DDA-CNL-MS^3 method was developed, using a linear ion trap-Orbitrap instrument. The instrumentation can also be applied to data-independent acquisitions (DIAs). In DIA, the MS data is collected systematically and independent of precursor ion information. The aim of DIA is to comprehensively obtain fragmentation data on all analytes that are present thereby allowing the data to be searched for the presence of known or unknown DNA adducts.

Matrix-assisted laser desorption ionization (MALDI) is like ESI a soft ionization technique, but it provides far fewer multiply charged ions in the gas phase compared to ESI. This potentially simplifies data interpretation as well as analysis of complex mixtures. It has been a useful tool for the analysis modified oligonucleotides as well as determining their sequences. MALDI-TOF MS has been applied to characterize oligonucleotides carrying a DNA photoproduct as well as 5-hydroxymethylcytosine, 5-formylcytosine and 6-oxothymine. Nontargeted analysis of modified nucleotides in DNA (as well as RNA) has been performed, involving derivatization of the deoxynucleotides with isotopologue benzoylhistamines followed by MALDI-TOF or MALDI-TOF/TOF analysis.

Triple quadrupole LC-MS/MS instruments operated in SRM mode are still the most sensitive mass spectrometers for detecting low levels of DNA adducts. New technological improvements enable very fast scanning times and rapid switching between SRM transitions without loss of sensitivity. Modern instruments operated in SRM mode can increase throughput with the capability of simultaneously acquiring hundreds of SRM transition channels per second while maintaining accuracy and precision, including multiple transitions per analyte. Though HRMS systems are excellent tools for identifying unknown DNA adducts, sensitivity may not be sufficient to detect background levels of DNA adducts that are present in human and animal DNA. However the sensitivity of these instruments can be improved by coupling with nano-LC and a nanospray ionization source.

Summary

For many carcinogens the mechanism of tumor initiation is causally associated with carcinogen-DNA adduct formation. DNA adducts, if not repaired prior to DNA replication, can lead to mutation in critical genes of carcinogenesis (e.g., proto-oncogenes, tumor suppressor genes) which can drive malignant transformation of the cell and tumor development. DNA adduct formation is considered a critical step in the initiation of carcinogenesis and in many molecular epidemiology studies it has been shown that DNA adducts are valid biomarkers of exposure and cancer risk. A variety of sensitive methods have been developed for the detection and characterization of carcinogen-DNA adducts which are suitable for their applicability in human biomonitoring in order to study cancer etiology. The measurement of carcinogen-DNA adducts can also employed to evaluate mechanism(s) of carcinogenesis including host factors mediating the carcinogenic response/potency. All methods available for DNA adduct detection have their strengths and weaknesses, so the method of choice has to be decided on a case-by-case basis considering the study design and research question.

Prospective Vision

Looking into the future, the field of DNA adductomics is still under development with many challenges still remaining however the hybrid HRMS/MS^n methodology represents an important advance in the investigation of DNA adduct structures in complex

mixtures. This approach could be an extremely powerful tool for DNA adductome mapping. If sensitivity is sufficient, this could enable the discovery of yet unknown DNA adduct biomarkers. MS-based DNA adductomics is particularly suited for research on the exposure of the human body to both known and unknown hazardous agents (either *exo*- or endogenous) and any subsequently formed DNA adducts. However, limited availability of DNA adduct standards (in particular stable isotope labeled standards) is currently limiting full characterization and correct identification of unknown DNA adducts with MS. To improve DNA adductomics, a database could be set up with information on chemical structure and characteristics of DNA adducts, stability, prevalence, origin, and route of exposure. A multidisciplinary approach is needed, involving epidemiologists, clinicians, pathologists, analysts and bioinformaticians, to further unravel the DNA adductome.

See also: Aflatoxins. Cancer Risk Reduction Through Lifestyle Changes. Cell Responses to DNA Damage. Genetic Instability. Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2. Molecular Epidemiology and Cancer Risk. Radiation Therapy-Induced Metastasis and Secondary Malignancy. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics.

Further Reading

Arlt, V.M., Meinl, W., Florian, S., Nagy, E., Barta, F., Thomann, M., Mrizova, I., Krais, A.M., Liu, M., Richards, M., Mirza, A., Kopka, K., Phillips, D.H., Glatt, H., Stiborova, M., Schmeiser, H.H., 2017. Impact of genetic modulation of SULT1A enzymes on DNA adduct formation by aristolochic acids and 3-nitrobenzanthrone. Archives of Toxicology 91, 1957–1975.

Arlt, V.M., Poirier, M.C., Sykes, S.E., John, K., Moserova, M., Stiborova, M., Wolf, C.R., Henderson, C.J., Phillips, D.H., 2012. Exposure to benzo[*a*]pyrene of hepatic cytochrome P450 reductase null (HRN) and P450 reductase conditional null (RCN) mice: Detection of benzo[*a*]pyrene diol epoxide-DNA adducts by immunohistochemistry and ^{32}P-postlabelling. Toxicology Letters 213, 160–166.

Arlt, V.M., Singh, R., Stiborova, M., Gamboa da Costa, G., Frei, E., Evans, J.D., Farmer, P.B., Wolf, C.R., Henderson, C.J., Phillips, D.H., 2011. Effect of hepatic cytochrome P450 oxidoreductase deficiency on 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-DNA adduct formation in reductase inducible Cre (RIC) mice. Drug Metabolism & Disposition 39, 2169–2173.

Arlt, V.M., Stiborova, M., Henderson, C.J., Thiemann, M., Frei, E., Singh, R., da Costa, G.G., Schmitz, O.J., Farmer, P.B., Wolf, C.R., Phillips, D.H., 2008. Metabolic activation of benzo[*a*]pyrene in vitro by hepatic cytochrome P450 contrasts with detoxification in vivo: Experiments with hepatic cytochrome P450 reductase null mice. Carcinogenesis 29, 656–665.

Arlt, V.M., Schmeiser, H.H., Osborne, M.R., Kawanishi, M., Kanno, T., Yagi, T., Phillips, D.H., Takamura-Enya, T., 2006. Identification of three major DNA adducts formed by the carcinogenic air pollutant 3-nitrobenzanthrone in rat lung at the C8 and N^2 position of guanine and at the N^6 position of adenine. International Journal of Cancer 118, 2139–2146.

Arlt, V.M., Ferluga, D., Stiborova, M., Pfohl-Leszkowicz, A., Vukelic, M., Ceovic, S., Schmeiser, H.H., Cosyns, J.P., 2002. Is aristolochic acid a risk factor for Balkan endemic nephropathy-associated urothelial cancer? International Journal of Cancer 101, 500–502.

Balbo, S., Turesky, R.J., Villalta, P.W., 2014. DNA adductomics. Chemical Research in Toxicology (3), 356–366.

Balbo, S., Hecht, S.S., Upadhyaya, P., Villalta, P.W., 2014. Application of a high resolution mass spectrometry-based DNA adductomics approach for identification of DNA adducts in complex mixtures. Analytical Chemistry 86, 1744–1752.

Bessette, E.E., Goodenough, A.K., Langouet, S., Yasa, I., Kozekov, I.D., Spivack, S.D., Turesky, R.J., 2009. Screening for DNA adducts by data-dependent constant neutral loss-triple stage mass spectrometry with a linear quadrupole ion trap mass spectrometer. Analytical Chemistry 81, 809–819.

Brown, K., Tompkins, E.M., White, I.N., 2006. Applications of accelerator mass spectrometry for pharmacological and toxicological research. Mass Spectromtry Reviews 25, 127–145.

Divi, R.L., Beland, F.A., Fu, P.P., Von Tungeln, L.S., Schoket, B., Camara, J.E., Ghei, M., Rothman, N., Sinha, R., Poirier, M.C., 2002. Highly sensitive chemiluminescence immunoassay for benzo[*a*]pyrene-DNA adducts: Validation by comparison with other methods, and use in human biomonitoring. Carcinogenesis 23, 2043–2049.

ESCODD (European Standards Committee on Oxidative DNA Damage), 2002ESCODD(EuropeanStandardsCommitteeonOxidativeDNADamag. Comparative analysis of baseline 8-oxo-7,8-dihydroguanine in mammalian cell DNA, by different methods in different laboratories: An approach to consensus. Carcinogenesis 23, 2129–2133.

Farmer, P.B., Singh, R., 2008. Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: The increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens. Mutation Research 659, 68–76.

Biomarkers of carcinogen exposure and early effects. In: Farmer, P.B., Emeny, J.M. (Eds.), 2006. Environmental cancer risk, nutrition and Indvidual susceptibility (ECNIS), a network of excellence within the European Union Sixth Framework Programme, priority 5: Food quality and safety. The Nofer Institute of Occupational Medicine, Lodz.

Gamboa da Costa, G., Singh, R., Arlt, V.M., Mirza, A., Richards, M., Takamura-Enya, T., Schmeiser, H.H., Farmer, P.B., Phillips, D.H., 2010. Quantification of 3-nitrobenzanthrone-DNA adducts using online column-switching HPLC-electrospray tandem mass spectrometry. Chemical Research in Toxicology 22, 1860–1868.

Gavina, J.M., Yao, C., Feng, Y.L., 2014. Recent developments in DNA adduct analysis by mass spectrometry: A tool for exposure biomonitoring and identification of hazard for environmental pollutants. Talanta 130, 475–494.

Hemeryck, L.Y., Moore, S.A., Vanhaecke, L., 2016. Mass spectrometric mapping of the DNA adductome as a means to study genotoxin exposure, metabolism, and effect. Analytical Chemistry 88, 7436–7446.

Kanaly, R.A., Matsui, S., Hanaoka, T., Matsuda, T., 2007. Application of the adductome approach to assess intertissue DNA damage variations in human lung and esophagus. Mutation Research 625, 83–93.

Kanaly, R.A., Hanaoka, T., Sugimura, H., Toda, H., Matsui, S., Matsuda, T., 2006. Development of the adductome approach to detect DNA damage in humans. Antioxidant Redox Signaling 8, 993–1001.

Klaene, J.J., Sharma, V.K., Glick, J., Vouros, P., 2013. The analysis of DNA adducts: The transition from ^{32}P-postlabeling to mass spectrometry. Cancer Letters 334, 10–19.

Krais, A.M., Speksnijder, E.N., Melis, J.P., Singh, R., Caldwell, A., Gamboa da Costa, G., Luijten, M., Phillips, D.H., Arlt, V.M., 2016. Metabolic activation of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine and DNA adduct formation depends on p53: Studies in Trp53(+/+),Trp53(+/−) and Trp53(−/−) mice. International Journal of Cancer 138, 976–982.

Krais, A.M., Speksnijder, E.N., Melis, J.P., Indra, R., Moserova, M., Godschalk, R.W., van Schooten, F.J., Seidel, A., Kopka, K., Schmeiser, H.H., Stiborova, M., Phillips, D.H., Luijten, M., Arlt, V.M., 2016. The impact of p53 on DNA damage and metabolic activation of the environmental carcinogen benzo[*a*]pyrene: Effects in Trp53(+/+), Trp53(+/−) and Trp53(−/−) mice. Archives of Toxicology 90, 839–851.

Liu, S., Wang, Y., 2015. Mass spectrometry for the assessment of the occurrence and biological consequences of DNA adducts. Chemical Society Reviews 44, 7829–7854.

Monien, B.H., 2014. Mass spectrometric DNA adduct quantification by multiple reaction monitoring and its future use for the molecular epidemiology of cancer. Advances in Experimental Medicine and Biology 806, 383–397.

Nortier, J.L., Martinez, M.C., Schmeiser, H.H., Arlt, V.M., Bieler, C.A., Petein, M., Depierreux, M.F., Pauw, L., Abramowicz, D., Vereerstraeten, P., Vanherweghem, J.L., 2000. Urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fangchi). New England Journal of Medicine 342, 1686–1692.
Phillips, D.H., 2013. On the origins and development of the ^{32}P-postlabelling assay for carcinogen DNA adduct. Cancer Letters 334, 5–9.
Phillips, D.H., Arlt, V.M., 2009. Genotoxicity: Damage to DNA and its consequences. Experientia Supplementum 99, 87–110.
Phillips, D.H., 2008. Biomarker of exposure: Adducts. In: Wild, C., Vineis, P., Garte, S. (Eds.), Molecular epidemiology of chronic disease. John Wiley & Sons, Chichester, pp. 111–125.
Phillips, D.H., Arlt, V.M., 2007. The ^{32}P-postlabeling assay for DNA adducts. Nature Protocols 2, 2772–2781.
Phillips, D.H., 2007. The formation of DNA adducts. In: Alison, M.R. (Ed.), The cancer handbook, 2nd edn. John Wiley & Sons, Chichester, pp. 338–350.
Poirier, M.C., 2016. Linking DNA adduct formation and human cancer risk in chemical carcinogenesis. Environmental and Molecular Mutagenesis 57, 499–507.
Santella, R.M., 1999. Immunological methods for detection of carcinogen-DNA damage in humans. Cancer epidemiology biomarkers and. Prevention 8, 733–739.
Singh, R., Teichert, F., Seidel, A., Roach, J., Cordell, R., Cheng, M.K., Frank, H., Steward, W.P., Manson, M.M., Farmer, P.B., 2010. Development of a targeted adductomic method for the determination of polycyclic aromatic hydrocarbon DNA adducts using online column switching liquid chromatography/tandem mass spectrometry. Rapid Communication Mass Spectrometry 24, 2329–2340.
Singh, R., Farmer, P.B., 2006. Liquid chromatography-electrospray ionization-mass spectrometry: The future of DNA adduct detection. Carcinogenesis 27, 178–196.
Schmeiser, H.H., Nortier, J.L., Singh, R., Gamboa da Costa, G., Sennesael, J., Cassuto-Viguier, E., Ambrosetti, D., Rorive, S., Pozdzik, A., Phillips, D.H., Stiborova, M., Arlt, V.M., 2014. Exceptionally long-term persistence of DNA adducts formed by carcinogenic aristolochic acid I in renal tissue from patients with aristolochic acid nephropathy. International Journal of Cancer 135, 502–507.
Stiborova, M., Arlt, V.M., Schmeiser, H.H., 2017. DNA adducts formed by aristolochic acid are unique biomarkers of exposure and explain the initiation phase of upper urothelial cancer. International Journal of Molecular Sciences 18, 2144.
Tretyakova, N., Villalta, P.W., Kotapati, S., 2013. Mass spectrometry of structurally modified DNA. Chemical Reviews 113, 2395–2436.
Villalta, P.W., Balbo, S., 2017. The future of DNA adductomic analysis. International Journal of Molecular Sciences 18, 1870.
Wild, C.P., 2012. The exposome: From concept to utility. International Journal of Epidemiology 41, 24–32.
Wogan, G.N., Kensler, T.W., Groopman, J.D., 2012. Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review. Food Additives & Contaminants. Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment 29, 249–257.

Carcinogenesis: Role of Reactive Oxygen and Nitrogen Species☆

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Glossary

Complete carcinogen A carcinogen that causes cell initiation and promotes cell transformation and growth to benign tumors, as well as mediates progression to malignancy.

Growth factors Low molecular weight substances produced by cells that induce growth of the same (autocrine response) or other (paracrine response) cells.

Inflammation A very complex process that is initiated by a release of cytokines and chemokines leading to the infiltration of phagocytic cells. Upon stimulation, phagocytes generate copious amounts of reactive oxygen and nitrogen species, proteases, as well as other enzymes and proteins. It can be manifested by fever, edema, hyperplasia, phagocytic infiltration, and oxidative stress.

Oxidative stress Excessive production of and/or impaired removal of oxidants with a concomitant decrease in reducing capacity of cells.

Oxidatively generated DNA damage Direct oxidation is characterized by a decrease in electron density. Such a modification can occur by oxidation of a double bond in a normal DNA base, of a methyl group or by ionization. Indirect oxidatively produced modifications occur when another molecule or macromolecule is oxidized and the product or by-product of that oxidation reacts with DNA.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) These are important players in normal physiology and signal transduction. They also participate in a plethora of damaging reactions, which lead to the dysregulation of normal cell controls, thus contributing to various pathologies, including cancer.

Transcription factors Factors needed for the transcription of genes into mRNA by binding to the specific recognition sequences in the promoter or enhancer regions of DNA.

Tumor promoters Agents that support the development of initiated cells into transformed cells and tumors.

Tumor suppressor genes Genes that prevent cell transformation and growth of tumors. Mutation of such genes usually abrogates their normal functioning and facilitates tumor development.

Nomenclature

•NO Nitric oxide
•NO_2 Nitrogen dioxide
•OH Hydroxyl radical
1,N^2-εGua 1,N^2-ethenoguanine
$^1O_2$ Singlet oxygen
4-HNE 4-Hydroxy-2-nonenal
5-CaCyt 5-Carboxycytosine
5-ClCyt 5-Chlorocytosine
5-ClGua 5-Chloroguanine
5-FoCyta 5-Formyluracil
5-FoUra 5-Formylcytosine
5-HmCyt 5-Hydroxymethylcytosine
5-HmUra 5-Hydroxymethyluracil
5-OHCyt 5-Hydroxycytosine
8-ClAde 8-Chloroadenine
8-oxoAde 8-Oxo-7,8-dihydroadenine
8-oxodG 8-Oxo-7,8-dihydro-2′-deoxyguanosine
8-oxoGua 8-Oxo-7,8-dihydroguanine
AID Activation-induced deaminase

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APE1 Apurinic/apyrimidinic endonuclease 1
APOBEC Apolipoprotein B mRNA editing enzyme catalytic polypeptide 1
BER Base excision repair
$CO_3^{\bullet -}$ Carbonate radical anion
Cu^+ Cuprous ion
DNMTs DNA methyltransferases
DSB Double strand break
DSBR Double strand break repair
Endo III Endonuclease III
FapyAde 4,6-Diamino-5-formamidopyrimdine
FapyGua 2,6-Diamino-4-hydroxy-5-formamidopyrimidine
Fe^{2+} Ferrous ion
Fpg Formamidopyrimidine DNA *N*-glycosylase
GC–MS Gas chromatography coupled to mass spectrometry
H_2O_2 Hydrogen peroxide
HOCl/OCl^- Hypochlorous acid/hypochlorite
hOGG1 Human 8-oxoguanine glycosylase 1
HPLC-ECD High performance liquid chromatography coupled to electrochemical detection
HPLC-ESI-MS/MS High performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry
HPLC-MS High performance liquid chromatography coupled to single mass spectrometer
HR Homologous recombination
IDH Isocitrate dehydrogenase
ImidCyt 1-Carbamoyl-4,5-dihydroxy-2-oxoimidazolidine
iNOS Inducible nitric oxide synthase
LM-PCR Ligation-mediated polymerase chain reaction
M1Gua Pyrimidopurinone 3-pyrimidopurin-10 (3H) one
MBD4 Methyl CpG binding protein 4
MDA Malondialdehyde
MMR Mismatch repair
MUT1 Human mutT protein
MUTHY mutY DNA glycosylase
N^2,3-εGua N^2,3-ethenoguanine
NEIL1–3 Endonuclease 8-like proteins
NER Nucleotide excision repair
NHEJ Nonhomologous end joining
NOS Nitrogen reactive species
NTH1 Endonuclease III-like protein 1
$O_2^{\bullet -}$ Superoxide anion radicals
$OONO^-$ Peroxynitrite
Oz 2,2,4-Triamino-5(2*H*)-oxazolone
ROS Reactive oxygen species
ROO• Peroxyl radical
SMUG1 Single strand specific mono-functional uracil-DNA glycosylase 1
SSB Single strand break
TDG Thymine DNA glycosylase
TET1–3 Ten-eleven translocation family of enzymes
ThyGly 5,6-Dihydroxy-5,6-dihydrothymine
ThyHyd 5-Hydroxy-5-methylhydantoin
UNG Uracil-DNA *N*-glycosylase
UraHyd 5-Hydroxyhydantoin
UV Ultraviolet
εA 1,N^6-ethenoadenine
εCyt 3,N^4-ethenocytosine

Introduction

Carcinogenesis is a complex multistage process often taking decades until malignancy appears. Conventionally, the carcinogenic process has been divided into three main stages: initiation, promotion, and progression. Initiation requires an irreversible genetic damage causing mutations in transcribed genes. Promotion consists of a potentially reversible oxidant-mediated conversion step followed by a clonal expansion of the initiated cells into benign tumors, which can progress to malignancy when they acquire many additional genetic changes. Those genetic changes include modifications of DNA bases, insertions and deletions, genetic instability consisting of loss of heterozygosity, chromosomal translocations, and sister chromatid exchanges, activation of oncogenes, and suppression of tumor suppressor genes. Although cell initiation is a frequent occurrence, tumor promotion and progression usually require a long time because of all of the genetic changes that have to accumulate within the same few cells.

It has been known for many years that antioxidants inhibit formation of tumors, even though they might not decrease DNA adducts, thought to be the initiating lesions. Hence, antioxidants are likely to interfere with the oxidant formation during promotion and/or progression stages of tumor development. Since then, numerous publications have shown that various types of reactive oxygen species (ROS) are generated during all stages of carcinogenesis, but especially during that long time required to take an initiated cell to a fully disseminated cancer. This long period between the initiation stage and cancer development provides a large window of opportunity to interfere with and suppress the carcinogenic process. This can be accomplished more readily when processes of tumor promotion/progression and factors responsible for them are known.

The importance of oxidative stress to human cancer is underscored by the existence of cancer-prone syndromes characterized by the production of high levels of oxidants, loss of adequate defense, or deficiencies in DNA repair processes. Human congenital syndromes related to oxidative stress include Fanconi's anemia, Bloom's syndrome, ataxia telangiectasia, Wilson's disease, and hemochromatosis, among others. The last two conditions point to the contribution of an excess of bioavailable transition metal ions, such as copper and iron, leading to an overload of oxygen radicals in the liver and to the progression and outcome of those diseases. Hussain and colleagues clearly showed that livers of patients with Wilson's disease and hemochromatosis contain increased levels of inducible nitric oxide synthase (iNOS), as well as mutations in the p53 tumor suppressor gene, especially G:C to T:A transversions at codon 249. This finding is particularly important because hydrogen peroxide (H_2O_2)/iron treatment, as well as lipid peroxide-derived mutagenic reactive aldehydes, such as 4-hydroxy-2-nonenal (HNE), cause the same type of mutations at codon 249. Etheno-base adducts are present in liver DNA from patients having these two diseases. Interestingly, HNE causes the formation of etheno derivatives in DNA and induces mutations at the 249 codon of the *p53* gene in HNE-treated lymphoblastic cells. Notably, not only congenital syndromes exhibit mutations in the p53 gene, but also noncancerous colon tissues from ulcerative colitis patients, a colorectal cancer-prone chronic inflammatory disease. Frequencies of p53 mutations at codons 247 and 248 are strongly correlated with the progression of the disease being appreciably higher in inflamed than in noninflamed tissues. These studies strengthen the idea that chronic inflammation contributes to cancer, and the results are consistent with the hypothesis that p53 mutations at 247–249 codons are due to chronic inflammation-associated oxidative stress.

Oxidants are continuously formed and scavenged during various normal cellular processes including mitochondrial respiration. ROS are required for the normal functioning of an organism and for the protection from invading bacteria. They are produced or utilized during mitochondrial respiration, metabolism of fats and xenobiotics, melanogenesis, and other peroxide reactions. Many types of cellular defenses keep these oxidants under control, but when they fail, extensive repair systems remove the damage attempting to restore DNA integrity. ROS produced in small amounts can serve as second messengers in signal transduction. However, when ROS are formed at a time when they are not needed and/or in amounts exceeding antioxidant defenses and DNA repair capacity, then ROS can contribute to various diseases, with cancer being prominent among them. Tumor promoters and complete carcinogens induce chronic inflammation, ROS production, and oxidatively generated DNA damage. Some of the oxidized DNA base derivatives are mutagenic, cytotoxic, and cross-linking agents. They can also cause a change in DNA methylation that leads to changes in gene expression. This process might perhaps account for the ROS-mediated enhanced levels of various growth and transcription factors, and genes involved in antioxidant defenses.

Oxidants: Formation and Reactivity

The nucleus is subjected to continuous exposure to ROS that are generated during mitochondrial respiration as the result of incomplete one-electron reduction. In addition, the generation of ROS and also of reactive nitrogen species (RNS), likely involved in one-electron oxidation of guanine is enhanced during inflammation processes as further discussed below. Both types of oxidation reactions are also associated with exposure of exogenous physical agents including solar UV and ionizing radiation.

Reactive Oxygen and Nitrogen Species

The three main reactive species involved in oxidative reactions included ROS, RNS and halogenating agents. Superoxide anion radical ($O_2^{\bullet -}$) is the predominant initial ROS that is generated during respiration and numerous various biochemical and chemical reactions. These include solar radiation activation enzymes such as of NADPH oxidase, UV and ionizing radiation and photosensitized reactions. Extensive amounts of ($O_2^{\bullet -}$) and RNS are produced during inflammation, a normal bactericidal and tumoricidal process. However, chronic inflammation contributes to the long-lasting pathologic effects of the carcinogenic course of action,

regardless of the type of cancer. ROS and RNS are generated by activated phagocytic cells (PMNs, neutrophils, granulocytes) and both circulating and resident macrophages. Target cells (i.e., epidermal keratinocytes or hepatocytes) can also form these species in response to treatment with appropriate stimuli, such as tumor promoters or allergens. Although absolute amounts of ROS and RNS are much lower than those produced by stimulated phagocytes, these oxidants can set off the synthesis and release of a cascade of various cytokines and chemotactic factors, which are instrumental to the initiation of inflammatory responses by phagocytic cells. ROS and RNS also mediate increased formation of growth and transcription factors needed for the accelerated proliferation of initiated cells.

In addition to $O_2^{\bullet -}$ four main other ROS are generated in cells, which include hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), peroxyl radicals (ROO$^{\bullet}$) and singlet oxygen ($^1O_2$). H_2O_2 is mainly produced by enzymatic dismutation of $O_2^{\bullet -}$ by superoxide dismutase. $^{\bullet}OH$ arises from either transition metal-mediated reduction of H_2O_2 according to Fenton reactions or radiolysis of water molecules through the indirect effects of ionizing radiation. Peroxyl radicals are generated by the addition of molecular oxygen to carbon-centered pyrimidine radicals. In contrast, the formation of $^1O_2$ can be explained by the involvement of endogenous type II photosensitizers that are able upon excitation by UVA radiation/visible light to transfer energy to molecular oxygen. Mammalian heme peroxidases represent other sources of oxidizing agents that are activated in inflammatory tissues. They consist of myeloperoxidases, lactoperoxidases and eosinophil peroxidases that are able to trigger the formation of hypochlorous acid/hypochlorite ($HOCl/OCl^-$).

RNS are also produced during inflammation and contribute to its pathologic effects. Nitric oxide ($^{\bullet}NO$) as a major player is generated through activation of epidermal inducible nitric oxide synthase (iNOS). The distinction between ROS and RNS is further blurred because of an avid reaction between $O_2^{\bullet -}$ and $^{\bullet}NO$ at near diffusion rate giving rise to peroxynitrite ($ONOO^-$), a potent oxidizing and nitrating agent (**Table 1**).

One-Electron Oxidants

Other main oxidative degradation pathways of DNA are initiated by the ionization of DNA bases and sugar moieties. Nucleobases are susceptible to several one-electron oxidants in the following decreasing order of reactivity guanine -> adenine > cytosine ≈ thymine. Thus, guanine preferentially undergoes one-electron oxidation by type I photosensitizers (riboflavin, menadione), bi-photonic ionization mediated by high intensity nanosecond UVC laser. In addition, guanine is oxidized by the carbonate radical anion ($CO_3^{\bullet -}$), a biologically relevant oxidizing radical that derives from the thermal decomposition of nitrosoperoxycarbonate, arising from the reaction of peroxynitrite ($OONO^-$) with CO_2 or carbonates. Another one-electron oxidant includes bromate. The highly energetic photons emitted by ionizing radiation are able to oxidize both nucleobases and the 2-deoxyribose moieties through the so-called direct effect. Lastly, purine and pyrimidine radical cations generate aminyl radicals that are strong oxidants of neighboring DNA bases in DNA.

Reactivity

Reactive oxygen species

Various cellular ROS and oxidants exhibit a wide diversity of reactivity toward nucleic acids ranging from the totally unreactive $O_2^{\bullet -}$ to the highly oxidizing $^{\bullet}OH$. H_2O_2 is another poorly reactive ROS that unlike $O_2^{\bullet -}$ is able to cross membrane and migrate within cells to reach the nucleus. In the presence of reduced transition metals such as ferrous and cuprous ions, H_2O_2 is able to generate $^{\bullet}OH$ that reacts essentially at the site where it is produced with the nearest molecule. For example, H_2O_2 will react with DNA bound Fe^{2+} and Cu^+ to generate $^{\bullet}OH$ that subsequently reacts with DNA in a site specific manner. The diffusion of $^{\bullet}OH$ in biological

Table 1 Major cellular oxidants and their reactivity toward nucleic acids

ROS or RNS	*Reactivity*
$O_2^{\bullet -}$	Undetectable toward DNA constituents
H_2O_2	Very low reactivity with adenine; involved in Fenton reaction
$^{\bullet}OH$	High reactivity with bases and the 2-deoxyribose moiety
Peroxyl radicals	Pyrimidine peroxyl radicals may either abstract hydrogen atom from vicinal thymine or add to vicinal guanine giving rise to tandem base lesions
$^1O_2$	Guanine the only reaction target via cycloaddition
One-electron oxidants	Give rise to oxidized bases and intra- and interstrand crosslinks through initial formation of base radical cation
$HOCl/OCl^-$	Chlorination of amino-substituted bases
$^{\bullet}NO$	No detectable reactivity toward nucleic acid components at the exception of deamination of nucleobases
$ONOO^-$	One-electron oxidant of guanine through the transient formation of nitrosoperoxycarbonate with subsequent release of carbonate radical anion ($CO_3^{\bullet -}$)
Lipid hydroperoxides	Reactive aldehydes generated through the thermal decomposition of lipid hydroperoxides form covalent adducts with amino-substituted bases

medium is very short (1–10 nm), and thus, difficult to scavenge with antioxidants. The reaction of •OH with nucleobases and the sugar moiety occur at close to diffusion-controlled rates with formation of modified bases and strand breaks, respectively.

Peroxyl radicals (ROO•) in general exhibit a much lower reactivity than •OH. However, when inserted into DNA, pyrimidine ROO• have been shown to efficiently react with vicinal bases, preferentially when they are located on the 5′-terminus, giving rise to tandem base modifications. Two reactions have been identified in model studies: pyrimidine ROO• is able to either abstract a hydrogen atom from the methyl group of an adjacent thymine or add to vicinal guanine at C8. $^1O_2$ is, in addition to •OH, a potent oxidizing ROS that among DNA components reacts exclusively with guanine. It may be noted that $^1O_2$ due to its highly selective reactivity shows an intracellular diffusion distance within the range of 150–220 nm that is much higher than that of •OH.

DNA base radical cations

Base radical cations of nucleobases produced by one-electron oxidation reactions are highly unstable intermediates that may undergo two main reactions. One is deprotonation that leads to the formation of carbon-centered radicals at the methyl group of thymine that is identical to the radical produced by •OH-mediated H-atom abstraction. Similarly, the competitive reaction for base radical cations involves hydration which leads to the formation of the same radicals that are generated by •OH although hydration occurs specifically at C6 of the pyrimidine ring. More generally the guanine radical cation is the predominant species in irradiated DNA partly due to redistribution on guanine bases of initially generated radical cation present on other bases. Furthermore, guanine radical cations are prone to nucleophilic addition leading to DNA–protein crosslinks and intrastrand crosslinks with opposite cytosines.

Reactive nitrogen species

•NO is a poorly reactive species with the exception however of its ability to trigger the deamination of cytosine, adenine and guanine. This explains why •NO is able to migrate within cells and efficiently react with $O_2^{\bullet -}$, another unreactive radical, giving rise to $ONOO^-$. Evidence has been provided that one of the main modes of action of $ONOO^-$, a moderately reactive compound, is to react with CO_2 or bicarbonates yielding unstable nitrosoperoxycarbonate that decomposes into carbonate radical anion ($CO_3^{\bullet -}$) and nitrogen dioxide ($^\bullet NO_2$).

Hypochlorous acid/hypochlorite

Hypochlorous/hypochlorite ($HOCl/OCl^-$) that is generated by peroxidases in inflammatory tissues is partly scavenged by amino containing compounds within membranes forming much-longer acting chloramines. However, $HOCl/OCl^-$ is able to diffuse in cells and reach the nucleus where it reacts with DNA amino-substituted nucleobases.

Reactive aldehydes as breakdown products of lipid hydroperoxides

Decomposition of lipid hydroperoxides formed by either the oxidation by •OH or $^1O_2$ gives malondialdehyde and 4-hydroxy-2-nonenal among the main reactive aldehydes. The released electrophilic species are able to migrate intracellularly from oxidized membranes to the genome where they are involved in the formation of adducts with amino-substituted nucleobases.

Oxidatively Generated Damage to DNA

Both ROS and RNS can induce a plethora of various types of modifications to DNA with the bases and the 2-deoxyribose moiety as the main targets. For this purpose numerous studies have been dedicated to the identification of the main DNA degradation products arising from exposure of isolated DNA and model compounds to •OH, one-electron oxidants, $^1O_2$, hypochlorous acid and reactive aldehydes. Thus, more than 100 different types of modifications including isomers have been identified so far in model studies and the mechanisms of their formation have been elucidated in most cases. In parallel, numerous attempts have been made to develop methods aimed at measuring oxidized bases in cellular DNA, still an analytical challenge as further discussed in the next section. It may be emphasized that in most cases, even under conditions of oxidative stress, the levels of the main oxidized bases, such as 8-oxo-7,8-dihydroguanine (8-oxoGua), is at best only a few modifications per 10^6 normal bases in nuclear DNA.

Measurement of Oxidized Bases in Cellular DNA

The accurate detection and quantitative measurements of oxidized bases in cellular DNA have been hampered by the use of inappropriate methods. This was the case of the gas-chromatography–mass spectrometry (GC–MS) method that was found to lead to artifactual overestimation of the yields of oxidized bases by at least two orders of magnitude. The main origin of unreliable GC–MS data lies on efficient spurious oxidation during the hydrolysis and derivatization step of DNA bases involving heating of the samples at 140°C prior to the chromatographic analysis. Another questionable method that unfortunately is still widely applied particularly by clinicians and biologists involves the use of poorly specific immunoassays. In this respect, it was shown that monoclonal and polyclonal antibodies directed against 8-oxoGua suffer from a lack of antigenicity, showing a cross-reactivity with guanine on the order of 10^{-4}. Other methods lead to inconsistent results including the [^{32}P]-postlabeling technique, the ligation-mediated polymerase chain reaction (LM-PCR) assay and high performance liquid chromatography analysis associated with single mass spectrometry detector (HPLC-MS). The first sensitive measurement of oxidatively generated base damage in cellular DNA concerned

8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which was achieved by HPLC coupled with electrochemical detection (ECD) operating in the oxidative mode. Versatile HPLC-electrospray-tandem mass spectrometry (EIS-MS/MS), which is more sensitive and quantitative than previous methods, allows for the unambiguous assignment of detected lesion and has become the gold standard method for measuring a wide range of oxidized nucleosides and bases. However, the possibility of adventitious oxidation of nucleobases still remains a problem if precautions are not taken to prevent this reaction from occurring during DNA extraction and the subsequent work-up prior the HPLC analysis. Thus, HPLC based analytical methods are limited to the measurement of oxidatively base damage only under conditions of severe oxidative stress. These difficulties can be partly overcome using enzymatic based assays: recognition and hydrolytic cleavage of the *N*-glycosidic modified bases is performed by DNA repair glycosylases including bacterial endonuclease III (Endo III) for oxidized pyrimidine bases and either bacterial formamidopyrimidine DNA *N*-glycosylase (Fpg) or human 8-oxoguanine DNA glycosylase (hOGG1) for modified purine bases. The resulting abasic sites are converted into single strand breaks, which are suitably measured in a highly sensitive way by either the alkaline comet assay or the alkaline elution technique. Despite a lack of specificity compared to HPLC-ESI-MS/MS measurements, the enzymatic assays are suitable tools for the detection of low variations in the levels of oxidized bases. In the subsequent sections only the oxidatively generated base lesions that were accurately measured by either HPLC based method or enzymatic assays are discussed.

Hydroxyl Radical-Mediated DNA Damage

•OH is able to react with both the bases and the sugar backbone leading to the formation of a large spectrum of modifications.

Oxidized pyrimidine bases

So far, the main •OH-mediated oxidation products that were identified in model studies in the presence of oxygen have been detected in cellular DNA. Two main classes of oxidized bases were found to be generated according to the nature of the initial •OH reaction. Thus, addition of •OH across the 5,6-double bond of pyrimidine bases gives rise to hydroxyhydroperoxides that are reduced or rearrange into more stable oxidation products. Two main stable products of thymine oxidation include 5,6-dihydroxy-5,6-dihydrothymine, also called "thymine glycol" (ThyGly) and 5-hydroxy-5-methylhydantoin (ThyHyd). In the case of cytosine, 5-hydroxycytosine (5-OHCyt), 5-hydroxyhydantoin (UraHyd) and 1-carbamoyl-4,5-dihydroxy-2-oxoimidazolidine (ImidCyt) are the main cytosine oxidation products (**Fig. 1**). The second main •OH decomposition pathway of pyrimidine bases involves initial H-atom abstraction from the methyl group of thymine and 5-methylcytosine. In both cases, the transient formation of a peroxyl radical leads to the generation of alcohol and aldehyde derivatives that were identified as 5-hydroxymetyhyluracil (5-HmUra), 5-formyluracil (5-FoUra), 5-hydroxymethylcytosine (5-HmCyt) and 5-formylcytosine (5-FoCyt), respectively.

Modified purine bases

The main degradation products of the purine bases arise from the transient formation of 8-hydroxy-7,8-dihydropurinyl radical formed by direct •OH addition at C8 of the bases or more indirectly through vicinal •OH-induced pyrimidine peroxyl radical. In a subsequent step, one-electron oxidation of 8-hydroxy-7,8-dihydroguanyl radical by O_2 gives rise to 8-oxoGua while competitive one-electron reduction of the latter radical, likely by thiol compounds, generates an open-ring 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). Similar reactions take place for adenine with the formation of 8-oxo-7,8-dihydroadenine (8-oxoAde) and 4,6-diamino-5-formamidopyrimidine (FapyAde) albeit with an about 10-fold lower efficiency than the formation of guanine products (**Fig. 2**). An additional oxidation product that arises from initial •OH at C4/C5 followed by a complex degradation pathway has been identified as 2,2,4-triamino-5(2*H*)-oxazolone (Oz). A major difference between •OH-mediated reactions and one-electron oxidation of DNA bases involves the efficient reaction of •OH with the sugar leading to DNA strand cleavage.

Strand breaks and other sugar degradation products

Evidence has been provided that •OH-mediated hydrogen atom abstraction at C3, C5 and to a lesser extent C4 of the 2-deoxyribose moieties leads to the formation of DNA single strand breaks (SSBs). As a competitive reaction to SSB generation, the C4 oxidized abasic site is also involved in the formation of interstrand crosslinks. This is rationalized by the formation of a covalent bond between the reactive aldehyde generated from the oxidized abasic site and the exocyclic amino group of cytosine opposite to the abasic site.

It may be pointed out that double strand breaks (DSBs) are not generated by one •OH attack. The formation of DSBs requires two independent radical hits on the two opposite DNA sites that are separated by less than 25 base pairs. This is typically achieved upon exposure of cellular DNA to ionizing radiations that also gives rise to non DSB oxidatively generated clustered damage.

One-Electron Oxidation of DNA

One-electron oxidation of nucleobases generates purine and pyrimidine radical cations. These radical cations are able to migrate along the DNA chains with preferential trapping by guanines bases as sinks for the positive hole that are converted into stable degradation products identified as 8-oxoGua and FapyGua. In addition, minor degradation products arise from the partial conversion of other base radical cations as detected by HPLC-ESI-MS/MS. Thus, 8-oxoAde and FapyAde are formed by oxidation and reduction, respectively, of transiently generated 8-hydroxy-7,8-dihydroadenyl radical upon hydration of the adenine radical cation. Relatively minor pyrimidine oxidation products including ThyGly, 5-HmUra, 5-FoUra and 5-OHCyt have also been detected in cellular DNA,

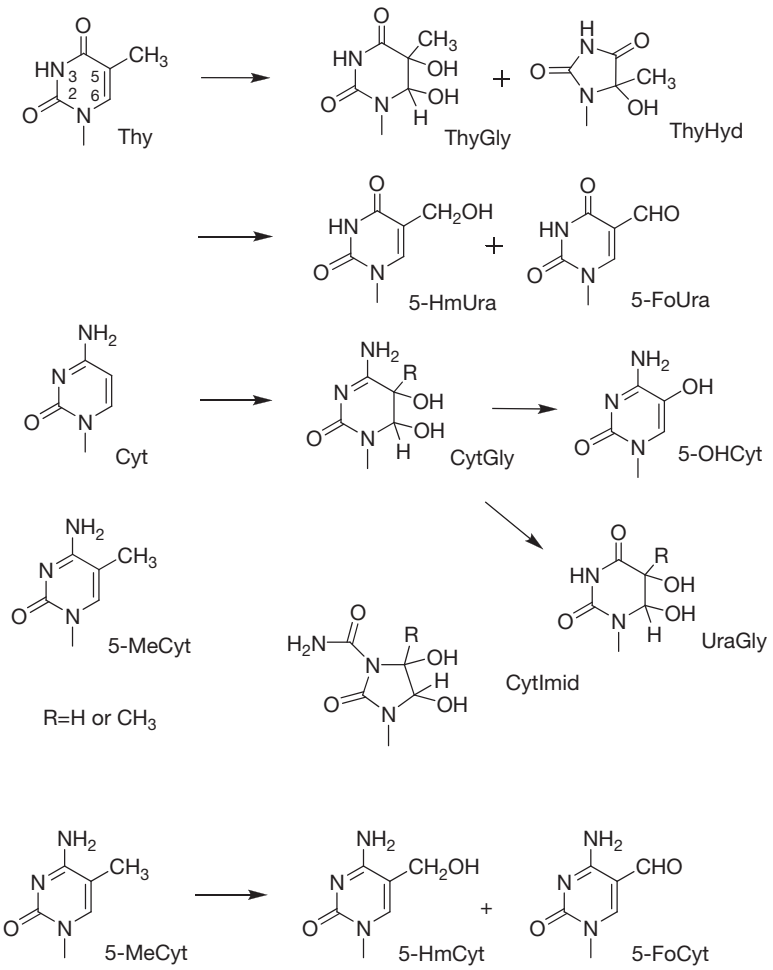

Fig. 1 Oxidation of thymine (Thy), cytosine (Cyt), and 5-methylcytosine (5-mCyt) by one-electron oxidation and •OH. One-electron oxidation and •OH give the same products except that the distribution changes, for example, one-electron oxidation gives greater methyl oxidation products (5-HmUra and 5-FoUra for Thy, and 5-HmCyt and 5-FoCyt for 5-MeCyt).

resulting from thymine and cytosine radical cations. It may also be pointed out that a guanine–thymine intrastrand cross-link is formed by nucleophilic addition of N3 of thymine to that guanine radical cation albeit the reaction is about 100-fold less efficient than the formation of 8-oxoGua.

Singlet Oxygen Oxidation of DNA

Exposure of cellular DNA to $^1O_2$ selectively reacts with guanine and leads to the predominant formation of 8-oxoGua with the exclusion of SSBs. Evidence has been provided that the UVA-induced generation of 8-oxoGua in the DNA of cells and human skin is accounted for the predominant involvement of $^1O_2$ by type II photosensitization.

Modifying Base Activity of Hypochlorous Acid/Hypochlorite

In addition to its oxidizing feature, $HOCl/OCl^-$ is able to trigger the chlorination of amino-substituted bases leading to the generation of 5-chlorocytosine (5-ClCyt), 8-chloroadenine (8-ClAde) and 8-chloroguanine (8-ClGua) as measured by HPLC-ESI-MS/MS in cellular DNA (Fig. 3).

Indirect Oxidatively Generated Damage to DNA Bases

In addition to the direct effects of oxidants on nucleobases, DNA may also be indirectly modified through interactions with electrophilic species produced by a ROS attack on other molecules or during ROS formation by such molecules. These consist essentially of etheno-base adducts formed in DNA by breakdown products of lipid peroxides. ROS including •OH and $^1O_2$ react with

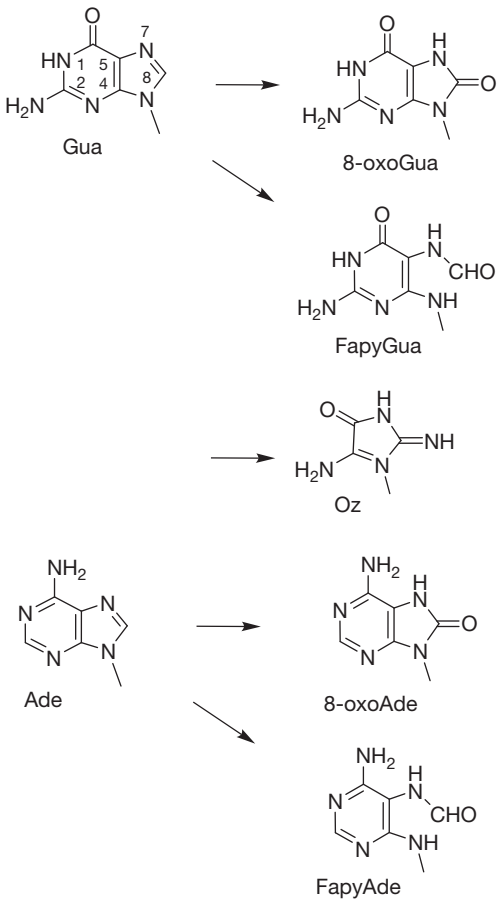

Fig. 2 Oxidation of guanine (Gua) and adenine (Ade) by •OH, one-electron oxidants and singlet oxygen ($^1O_2$). The formation of 8-oxoGua and Fapy depends on the presence of reducing agents and oxygen. The reaction of $^1O_2$ with Gua specifically gives 8-oxoGua. The yield of Ade oxidation products either by one-electron oxidation and •OH are 10-fold lower than those of Gua.

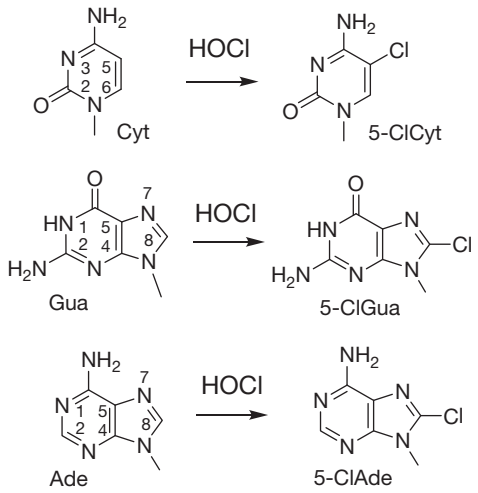

Fig. 3 Halogenation of DNA bases (Cyt, Gua and Ade).

unsaturated lipids leading to the formation of lipid peroxides, which upon decomposition release aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE). Both MDA and 4-HNE are mutagenic because they react with the exocyclic amino group of guanine, adenine and cytosine to form etheno derivatives including pyrimidopurinone 3-pyrimidopurin-10 (3H) one (M1Gua), 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εCyt), N^2,3-ethenoguanine (N^2,3-εGua), and 1,N^2-ethenoguanine (1,N^2-εGua). The aldehyde adducts transform further into products with an additional ring attached to purines and

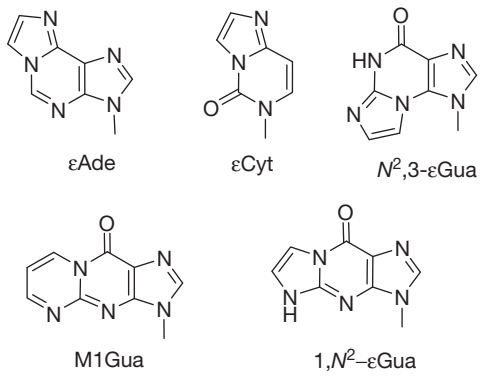

Fig. 4 Adducts of lipid peroxidation and DNA bases.

pyrimidines (Fig. 4). Etheno compounds are present in DNA isolated from various types of tumors or from nontumorous specimens of tumor-bearing animals and humans attesting to the increase of ROS-induced lipid peroxidation in cancerous cells.

DNA Repair and Mutagenesis of Oxidatively Generated Damage

There are four basic pathways of DNA repair: Base excision repair (BER); nucleotide excision repair (NER); mismatch repair (MMR); and double strand break repair (DSBR) including nonhomologous end joining (NHEJ) and homologous recombination (HR). Each of these pathways involve various proteins that recognize DNA damage, undergo enzymatic steps (DNA cleavage, polymerization, ligation), provide access, support and stability to repair complexes, engage in crosstalk between repair pathways, and elicit DNA damage response signals. In the case of ROS-induced base and sugar damage, the main repair pathway is BER although certain types of damage are repaired by NER and MMR pathways. On the other hand, the presence of excessive BER substrates and intermediates at the replication folk can also lead to double strand breaks, and thereby set DSBR repair into motion. The conventional method to study DNA repair and mutagenesis of ROS-induced damage is to insert a modification into synthetic oligonucleotides and examine the ability of purified enzymes, nuclear extracts and whole cell extracts to cleave the oligonucleotide at the site of damage by gel electrophoresis. There have been considerable methodological advances in the study of DNA repair and mutagenesis of specific lesions applying mass spectrometry strategies, using DNA repair arrays, incorporating oligonucleotides containing lesions into cellular DNA by transfection, constructing nucleosomes core particles containing lesions. The location and efficiency of repair proteins can be monitored in real time by the incorporation of fluorescent nucleotides during DNA repair synthesis or labeling repair proteins directly. A typical feature of DNA *N*-glycosylases is their broad substrate specificity toward oxidized bases in DNA and their ability to backup or be redundant in cellular and animal model systems. For example, the removal of uracil from DNA involves uracil-DNA *N*-glycosylase (UNG), single strand specific mono-functional uracil-DNA glycosylase 1 (SMUG1) as well as two mismatch specific DNA glycosylases consisting of thymine DNA glycosylase (TDG) and methyl CpG binding protein 4 (MBD4). Other DNA glycosylases in mammalian cells include endonuclease III-like protein 1 (NTH1), 8-oxoguanine DNA *N*-glycosylase (OGG1), endonuclease 8-like proteins (NEIL1–3) and apurinic/apyrimidinic endonuclease 1 (APE1), which collectively remove a broad spectrum of oxidatively induced lesions in single and double stranded DNA. To avoid mutations from 8-oxoGua formation, cells contain three other enzymes, which include hOGG1 for the removal of 8-oxoGua:C, mutY DNA glycosylase (MUTHY), which excises premutagenic 8-oxoGua:A mispairs, and human mutT protein (MUT1), which cleanses the cellular pool of 8-oxodG triphosphate. APE1 efficiently converts abasic sites to single strand breaks and also incises a number of pyrimidine modifications though a subpathway known as nucleotide incision. In contrast, the excision of purine 5′,8-cyclo-2′-deoxyribonucleosides in which the C5′ of the sugar is covalently attached to the C8 of either adenine or guanine are repaired by NER. The predominate occurrence of C to T transition mutations has largely been attributed to cytosine and 5-methylcytosine deamination or oxidation. Although thermally induced deamination is widely assumed to occur, it should be noted that ROS or RNS-induced reactions with pyrimidines lead to an enormous acceleration of the rate of deamination at the exocyclic amino group. The lifetimes of intermediate oxidation products of C and 5-mCyt, such as the corresponding 5,6-glycols and hydantoin derivatives, may explain the propensity of C to T transition mutations and the overwhelming C to T mutations at CpG dinucleotides in cancer. Once deamination occurs, the resulting modifications resemble uracil or thymine in their base pairing properties, and thus, they will mispair during replication and generate a C to T transition mutation.

Ten-Eleven Translocation Enzymatic Oxidation of 5-Methylcytosine and Epigenetics in Cancer

The methylation of DNA, mediated by DNA methyltransferases (DNMTs), converts cytosine to 5-methycytosine (5-mCyt), predominantly at CpG dinucleotides such that 60%–90% of these sites are methylated in mammals. Cytosine methylation is a well-known

epigenetic mark that generally silences the expression of genes during development and plays a regulatory role in maintaining genome integrity and the ability to modulate gene expression. In the past 8 years, a novel epigenetic mark of DNA has emerged that arises from the oxidation of 5-mCyt to 5-hydroxymethylcytosine (5-HmCyt) by ten-eleven translocation family of enzymes (TET1–3). The level of 5-HmCyt accumulates in the genome to levels as high as 20% of 5-mCyt. They are the highest in embryonic stem cells and as well in differentiated neurons that maintain a high degree of plasticity. Based on new sequencing methods that discriminate between 5-mCyt and 5-HmCyt, the distribution of 5-HmCyt in the genome is unique with respect to 5-mCyt and 5-HmCyt populated sites possess different binding partners compared to 5-mCyt. In contrast to 5-mCyt, 5-HmCyt appears to elevate gene expression with the levels of 5-HmCyt positively correlated with greater expression of hydroxymethylated genes. TET enzymes contain Fe^{2+}, require α-ketoglutarate as a cosubstrate, and have the ability to oxidize not only 5-mCyt to 5-HmCyt but also 5-HmCyt into further oxidized forms: 5-formylcytosine (5-FoCyt) and 5-carboxycytosine (5-CaCyt). The levels of 5-FoCyt and 5-CaCyt in the genome, however, are one to two orders of magnitude lower than those of 5-HmCyt. The active removal of 5-FoCyt and 5-CaCyt by BER involving TDG keeps the levels of the latter two modifications low and completes the multistep conversion of 5-mCyt to cytosine. Other enzymes from BER, which include activation-induced deaminase (AID) apolipoprotein B mRNA editing enzyme catalytic polypeptide 1 (APOBEC), SMUG, MBD2, have also been implicated in 5-mCyt demethylation. The cycle of cytosine methylation and demethylation of cytosine may explain the ability of pluripotent cells to turn genes off and on as necessary for their function and survival. The level of 5-HmCyt in the DNA of cancer cells is generally much lower than that observed in normal cells in brain, melanoma, breast and various other types of cancer. The lack of 5-HmCyt in the genome of cancer tissues has been associated with loss of TET activity. This may occur by the presence of mutations within TET genes, isocitrate dehydrogenase (IDH) genes, or other genes. Mutations in IDH switch activity from wild-type activity in which isocitrate is transformed into α-ketoglutarate to the production of 2-hydroxyglutarate, an inhibitor of TET. Interestingly, vitamin C increases the global level of 5-HmCyt in cells in culture, underlining the importance of a reducing agent to maintain the iron center of TET in a reduced state for optimal activity. Low concentrations of oxygen observed in hypoxic solid tumors may also decrease TET activity. Lastly, a reduction of 5-HmCyt in the genome may be due to passive dilution as a result of the high proliferative state of cancer cells. Despite extensive work, the role of TET and 5-HmCyt, and their interplay with DNMT enzymes and 5-mCyt, remain central questions in cancer development and growth.

Prospective Vision

There is increasing evidence today that oxidative stress, oxidatively induced DNA damage, deficiencies in DNA repair are intimately entrenched in the process of carcinogenesis and tumorigenesis. In particular, inflammation and expression of inflammatory cytokines lead to escalation of oxidative stress and the release of ROS and RNS. It is clear that mutations accumulate in cancer development and it is necessary to achieve a required number of mutations and mutant genes to achieve the transformation from normal to cancerous cells. Further structural analyses of damage has led to the characterization of many novel lesions in the past 10 years, including tandem base lesions, clustered lesions, DNA–DNA crosslinks, DNA–protein crosslinks and putative secondary purine oxidation products. The study of formation of intermediate and stable products in double stranded DNA and higher order DNA structures such as telomeres and nucleosomes has given a wealth of new information. However, the analysis in cellular DNA and the biological processing of many lesions remains incomplete. Sequencing techniques have greatly advanced our knowledge concerning the site of DNA damage and particularly toward the distribution of epigenetic marks in the genome. A major challenge today remains to home in on the location and distribution of various oxidatively generated modifications in the genome. It will be necessary to determine the effect of sequence on the formation of damage and biological processing to establish the cause of mutation signatures and align these mutations within passenger and driver genes involved in cancer. It will also be necessary to explain how epigenetic marks become profoundly altered in cancer. The development of single and multiple gene ablation and editing methods in cells and mice continues to shed much light on the biological importance of DNA damage and repair pathways in cancer.

See also: Carcinogen—DNA Adducts. Cancer-Related Inflammation in Tumor Progression. *Helicobacter pylori*-Mediated Carcinogenesis.

Further Reading

Bartsch, H., 2000. Studies on biomarkers in cancer etiology and prevention. A summary and challenge of 20 years of interdisciplinary research. Mutation Research 462, 255–279.
Bochtler, M., Kolano, A., Xu, G.L., 2017. DNA demethylation pathways: Additional players and regulators. BioEssays 39, 1–13.
Boiteux, S., Coste, F., Castaing, B., 2017. Repair of 8-oxo-7,8-dihydroguanine in prokaryotic and eukaryotic cells: Properties and biological roles of the Fpg and OGG1 DNA N-glycosylases. Free Radical Biology and Medicine 107, 179–201.
Cadet, J., Wagner, J.R., 2013. DNA base damage by reactive oxygen species, oxidizing agents and UV radiation. Cold Spring Harbor Perspectives in Biology 5, a01559.
Cadet, J., Douki, T., Ravanat, J.-L., 2015. Oxidatively generated damage to cellular DNA by UVB and UVA radiation. Photochemistry and Photobiology 91, 140–155.
Cadet, J., Davies, K.J.A., Medeiros, M.H.G., Di Mascio, P., Wagner, J.R., 2017. Formation and repair of oxidatively generated damage in cellular DNA. Free Radical Biology and Medicine 107, 13–34.
Drohat, A.C., Coey, C.T., 2016. Role of base excision "repair" enzymes in erasing epigenetic marks from DNA. Chemical Reviews 116, 12711–12729.

Knutson, C.G., Mangerich, A., Zeng, Y., Raczynski, A.R., Liberman, R.G., Kang, P., Ye, W., Prestwich, E.G., Lu, K., Wishnok, J.S., Korzenik, J.R., Wogan, G.N., Fox, J.G., Dedon, P.C., Tannenbaum, S.R., 2013. Chemical and cytokine features of innate immunity characterize serum and tissue profiles in inflammatory bowel disease. Proceedings of the National Academy of Sciences of the United States of America 110, E2332–E2341.

Kuraoka, I., Bender, C., Romieu, A., Cadet, J., Wood, R.D., Lindahl, T., 2000. Removal of oxygen free-radical-induced 5′,8-purine cyclodeoxynucleosides from DNA by the nucleotide excision-repair pathway in human cells. Proceedings of the National Academy of Sciences of the United States of America 97, 3832–3837.

Lee, A.J., Wallace, S.S., 2017. Hide and seek: How do DNA glycosylases locate oxidatively damaged DNA bases amidst a sea of undamaged bases? Free Radical Biology and Medicine 107, 170–178.

Lindahl, T., 2016. The intrinsic fragility of DNA (Nobel lecture). Angewandte Chemie International Edition 55, 8528–8534.

Lu, X., Zhao, B.S., He, C., 2015. TET family proteins: Oxidation activity, interacting molecules, and functions in diseases. Chemical Reviews 115, 2225–2239.

Marnett, L.J., 2000. Oxyradicals and DNA damage. Carcinogenesis 21, 361–370.

Menoni, H., Di Mascio, P., Cadet, J., Dimitrov, S., Angelov, D., 2017. Chromatin associated mechanisms in base excision repair—Nucleosome remodeling and DNA transcription, two key players. Free Radical Biology and Medicine 107, 159–169.

Nickoloff, J.A., Jones, D., Lee, S.-H., Williamson, E.A., Hromas, R., 2017. Drugging the cancers addicted to DNA repair. Journal of the National Cancer Institute 109 djx059.

Pfeifer, G.P., Kadam, S., Jin, S.G., 2013. 5-Hydroxymethylcytosine and its potential roles in development and cancer. Epigenetics & Chromatin 6, 10.

Cell Adhesion During Tumorigenesis and Metastasis

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Glossary

Angiogenesis Is a physiological process of blood vessel formation from the preexisting vessels. Neoangiogenesis describes the formation of blood vessels in the tumor tissue.

Epithelial-to-mesenchymal transition (EMT) Describes a process during cancer progression wherein carcinoma cells can change their epithelial phenotype into a mesenchymal phenotype. Mesenchymal cells are characterized by enhanced migratory and invasive properties required for metastasis.

Extracellular matrix (ECM) Is a collection of extracellular molecules secreted by cells that provides structural and biochemical support within a tissue. The animal ECM includes the interstitial matrix filling out the space between cells and basement membrane, which is a structured ECM found in the epithelial tissues.

Extravasation Is a process describing the migration of tumor cells or leukocytes out of the blood vessel during hematogenous metastasis or immune response.

Glycan Is carbohydrate structure composed of many monosaccharides linked via glycosidic bonds. This term is used to refer to a carbohydrate portion of a glycoconjugate, such as glycoprotein or glycolipid.

Hematogenous metastasis Is spread of cancer cells from the primary tumors through blood circulation.

Homophylic/heterophylic interactions Homophylic interactions describe cell-cell interactions through the same adhesion molecule from the adjacent cells (e.g., cadherins). Heterophylic interactions describe binding of different proteins or glycans (e.g., integrins, selectins).

Lectins Are carbohydrate-binding proteins, macromolecules that are highly specific for sugar moieties.

Myeloid cells Are leukocytes of the innate immune system. To myeloid-derived cells belongs: granulocytes, monocytes, macrophages and immature myeloid precursors that are often detected during cancer progression and associated with suppression of anti-tumor immunity.

Thromboembolism Is the development of blood clots within the vascular system and dissemination of such clots (embolism). Patients with cancer are prone to develop thromboembolic complications.

Tumor embolus Embolus of tumor cells is composed of blood constituents including platelets and leukocytes and is known to promote hematogenous metastasis.

Tumor microenvironment Is the cellular environment of a tumor, which is composed of blood vessel, immune cells, fibroblast, soluble signaling molecules and ECM. Tumor cells modulate the microenvironment by various cues that promote angiogenesis, immune suppression and thereby promote tumor growth and metastasis.

Introduction

Cell–cell adhesion determines the polarity and the physiological function of cells within tissues. On every cell, adhesion molecules facilitate interactions within the cell microenvironment that consist of other cells and the extracellular matrix. Since cell adhesion receptors are connected to signal-transduction pathways, these cell–cell and cell–matrix interactions modulate cell phenotype, survival, differentiation, and migration. As a consequence, alterations in cell adhesion directly contribute to tumorigenesis and metastasis. In a developing tumor, there is general loss of stabilizing cell adhesions between cells enabling them to gain the migratory phenotype required for invasiveness and metastasis. On contrary, the invasive capacity of metastatic tumor cells depends on efficient cell adhesion during transit in the circulation, colonization of distant organs and interactions with the extracellular matrix at secondary metastatic sites. The metastatic dissemination of tumor cells is the primary cause of death in cancer patients. Therefore, the understanding of the cell adhesion mechanisms in cancer will enable to design new targeted therapies. Finally, cell adhesion plays a major role for the immune response to cancer and could be modulated to improve cancer immunotherapy.

During tumorigenesis and metastasis several families of adhesion molecules including: cadherins, integrins, junctional-adhesion molecules, and selectins have been studied. This chapter covers the current understanding of cell adhesion mechanisms in cancer and describes the known pathophysiological functions associated with tumor progression, angiogenesis and modulation of immune responses. In solid tumors, mostly originating from epithelial tissues, the loss of cell polarity is a profound change associated with a malignant transformation resulting in a ubiquitous presence of adhesion molecules (Fig. 1). Changes in expression of cell adhesion receptors and their binding partners (ligands) define both the malignant progression and interactions within the tumor microenvironment.

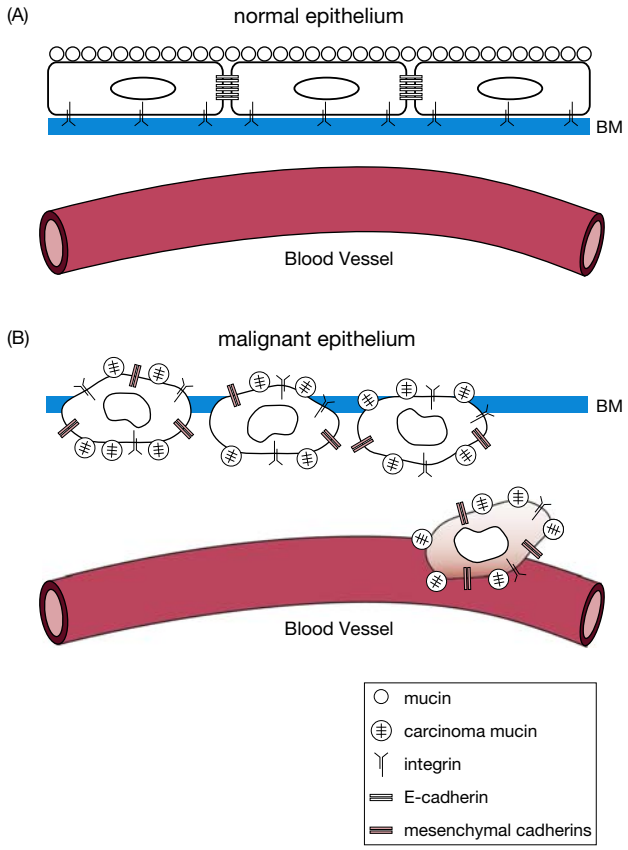

Fig. 1 *Cell adhesion changes leading to metastatic tumor cells during transformation from a normal to a malignant epithelium.* (A) *Normal epithelium* consists of polarized epithelial cells that are bound together through E-cadherin adhesions. The epithelium is organized on the basement membrane (BM) consisting of various extracellular matrix proteins (e.g., type IV collagen, glycosaminoglycans, laminin, and small adaptor proteins). On a basolateral site of the epithelium integrins facilitates the interaction with BM. On the apical site, epithelial cells produce mucus that is composed of mucins, proteins containing a large amount of O-glycans. This glycosylated mucus creates a mechanical barrier between epithelial cells and the lumen of a tissue. (B) *Malignant epithelium*. The profound change during malignancy is associated with: loss of cell polarity, loss of E-cadherin-mediated epithelial adhesions; increased production of mucus carrying altered glycan structures, degradation of BM; and increased migration and invasiveness of the malignant cells. Malignant tumor cells with altered cell adhesion preferences are able to invade into blood vessels and thereby gain access to other organs in an organism.

Cadherin-Mediated Cell Adhesion

Cadherins are cell adhesion molecules that play a major role in cell–cell interactions, tissue patterning during development and in cancer, particularly during tumor cell dissemination. E-cadherin is the most prominent cadherin expressed in epithelial cells (Fig. 1). Cadherins comprise of a large family of proteins with over 110 known members in humans with multiple functions. In a context of cancer, E-cadherin acts as a tumor suppressor. E-cadherin expression inhibits tumor progression by controlling epithelial to mesenchymal transition (EMT) that is associated with specific signaling pathways.

Cadherins are a family of transmembrane glycoproteins with at least five subfamilies. Type I cadherins, so-called classical cadherins, includes also E-cadherin. While type II cadherins are closely related to type I cadherins, there are additional subfamilies with specific structures. Type I and II cadherins, have five extracellular cadherin domains, a single transmembrane domain and a highly conserved intracellular domain that interacts with the intracellular adaptor proteins. E-cadherin mediates homophilic calcium-dependent interactions between epithelial cells that affect intracellular signaling cascades. E-cadherin-based cell adhesions regulate multiple signaling pathways that are linked to intracellular recruitment of catenins and other intracellular signaling molecules. For instance, E-cadherin alters the Wnt-signaling pathway that is important for development, tissue homeostasis and cancer progression. In mature epithelial cells, β-catenin is bound to E-cadherin in the adherent junctions. Free cellular β-catenin is sequestered in the cell through binding to adenomatous polyposis coli (APC)-Axin complex, and is immediately phosphorylated through glycogen synthase kinase 3β (GSK3β). Phosphorylated β-catenin is further ubiquitinylated and destined for degradation in the proteosomes. Upon external activation of the Wnt pathway, which blocks GSK3β activity, β-catenin is not degraded and accumulates in the cytoplasm eventually reaching the nucleus, where it induces transcription program. The nuclear signaling

pathway of β-catenin has a major role in maintaining and promoting mesenchymal and stem cell-like phenotypes that are essential in cancer progression and metastasis.

Cadherins Modulate Cancer Progression

E-cadherin is a major component of the *adherens junctions* of epithelial layers. The epithelial phenotype and the polarization of an epithelial tissue is dependent on E-cadherin expression. E-cadherin is considered as a key regulator of epithelial phenotype, since it is anchored in actin-myosin cytoskeleton and thereby contributes to stabilization of the epithelial architecture. During tumorigenesis, the loss of epithelial cell polarization (Fig. 1) is associated with reduced expression of E-cadherin coded by the *CDH1* gene. Mutation in the *CDH1* gene can lead to hereditary gastric cancer. E-cadherin loss has been found in different other cancers including hepatocellular carcinoma, squamous cell carcinoma of the skin, head and neck and esophagus, as well as in melanoma. In these cancers, E-cadherin is usually lost by epigenetic regulation via promoter methylation. A major pathway involved in the tumor suppressor function of E-cadherin is mediated by the interaction of the intracellular domain with β-catenin. E-cadherin binding to β-catenin inhibits the pro-tumorigenic β-catenin signaling. Downregulation or depletion of E-cadherin in experimental models leads to undifferentiated, highly invasive tumor cells.

E-cadherin expression is usually lost in cells that undergo EMT while the expression of other mesenchymal cadherins, including N-cadherin (CDH2), R-cadherin (CDH4) or P-cadherin (CDH3) is upregulated. These changes are known as "cadherin switch" and are regulated by several transcription factors linked to EMT such as SNAIL, SLUG, TWIST, ZEB1, and ZeB2. EMT is considered as major prerequisite for many cancers to gain an invasive phenotype and to become metastatic. The p120 catenin is an important mediator of downstream signaling linked to the cadherin switch. EMT also induces a program in epithelial cells that can generate cancer stem cells and promote thereby metastatic progression. Binding of p120 to E-cadherin suppresses tumor progression while its association with mesenchymal cadherins, such as P-cadherins, mediates interactions with Rac1 leading to promotion of tumor cell invasiveness. In addition, N-cadherin promotes anchorage-independent growth and influences growth receptor signaling and the overexpression of P-cadherin leads to an increase of MMP secretion and enhanced tumor cell invasion.

CDH2 or neuronal cadherin (N-cadherin) also has autonomous functions apart from cell adhesion. N-cadherin stimulates fibroblast growth factor receptor1 (FGFR1) and thereby promotes malignancy. Moreover, N-cadherin is also expressed on stromal cells and overexpression on tumor cells can mediate invasion and metastasis. Based on this knowledge, N-cadherin is an interesting target for cancer therapy. CDH3 or placental cadherin (P-cadherin) exercise a context-dependent role in cancer. P-cadherin upregulation during a cadherin switch has been observed in some cancers. In epithelial ovarian cancer, gonadotropin-releasing hormone receptors through interactions with p120-catenin leads to increased P-cadherin expression and tumor growth and cancer progression.

While the type I cadherins E-cadherin, N-cadherin, P-cadherin and R-cadherin (CDH4) are associated with cancer progression, type II cadherins can also influence cancer progression. In addition, vascular endothelial cadherin (VE-cadherin, CDH5) is a major cadherin in endothelial cells. VE-cadherin is important for homophilic interactions between endothelial cells and tumor cells and this interaction potentiates tumor cell intravasation and metastasis. Desmosomal cadherins, such as desmoglein and desmocollins, have tumor suppressive function through modulation of oncogenic Wnt-β-catenin signaling. Protocadherins differ from cadherins by other domains that mediate homophilic interactions and different intracelullar domains. In many cases, intracellular interaction partners are not well described, but the expression of protocadherins is often tumor suppressive.

Integrin-Mediated Adhesions

Integrins represents a family of cell adhesion molecules, which control tumor cell proliferation, migration and survival. Integrins directly binds components of extracellular matrix (ECM), and thereby provide traction necessary for cell motility and invasiveness. During cancer progression, integrins contribute to ECM remodeling by controlling the localization and activity of proteases. In addition, other integrin-expressing cells present either at the primary tumor site or at metastatic sites can contribute to the malignant process.

Integrins are heterodimeric cell surface receptors that mediate cell adhesion to ECM proteins or to other cells through binding to immunoglobulin superfamily cell adhesion molecules (IgCAMs). Two major groups of receptors belong to IgCAMs: vascular cell adhesion molecule 1 (VCAM1), and intracellular cell adhesion molecules (ICAMs). More than 24 different integrin heterodimers exist, which are composed of α- and β-subunit and bind to different counter-receptors. There are at least 18 α- and 8 β-integrins identified, that combine many unique α/β integrin receptors, with a specific preference for distinct ligands. The presence of a particular integrin on a cell surface defines the adhesion and migratory properties of the cell in dependence on the adjacent ECM (e.g., fibronectin, vitronectin, laminin, or collagen). The first binding site for integrins that has been identified is defined by a specific tripeptide RGD (arginine-glycine-aspartic acid). This motif is recognized by α5β1-integrin and αv-containing integrins. Different probes carrying the RGD epitope has been tested for integrin targeting in several pharmacological trials.

Upon adhesion to ECM, integrins cluster in the cell membrane and form structures called *focal adhesions*, which further recruit signaling and adaptor proteins. Integrins facilitate through the adaptor proteins the coupling of ECM to the cytoskeleton and allow physical attachment, which affect the cell shape and migratory properties of the cells. The integrin-mediated recruitment and

activation of kinases, such as focal adhesion kinases (FAKs), Src family kinases or GTPases of the Ras/Rho family induces signal transduction, known as "outside-in" signaling. Conversely, intracellular signals (e.g., growth factors or cytokines) can induce alterations in the integrin conformation and thereby modulate the ligand-binding affinity of integrins to ECM that is known as an "inside–out" signaling. In addition to the well-established role of integrins during migration and invasion, integrins also regulate cell proliferation, cell survival and angiogenesis, all of which contribute to cancer progression. Integrins on the cell surface constantly interrogate their microenvironment through their capacity to bind to ECM. Ligated integrins convey cell survival, while unligated integrins may trigger pro-apoptotic pathways. The enhanced cell survival triggered by ligated integrins is mediated by several mechanisms, including expression of Bcl-2 or activation of PI3K-Akt or NF-kappaB signaling. This process is further dependent on a communication between integrin-growth factor pathways. On contrary, unligated integrins may trigger activation of Caspase-8 and initiate apoptosis, which is no longer blocked by the Bcl-2. Thus, integrins can paradoxically initiate either pro-survival or pro-apoptotic pathways that is dependent on integrin engagement.

Integrins Adhesion in Cancer and During Metastasis

A wide variety of integrins on tumor cells are linked to cancer progression. Most solid tumors of the epithelial origin express integrins on a cell surface (**Fig. 1**). However, integrin expression may strongly vary between normal epithelial cells and malignant carcinoma cells. Particularly, αvβ3 and αvβ6 integrins are highly expressed in some tumors, while their presence in epithelial cells is negligible. The correlation between integrin expression and cancer patient's survival was evaluated in many cancer types as discussed elsewhere (see further reading). For example, αvβ3 integrin expression correlates with a poor prognosis in many cancers including breast, colon, prostate, pancreatic, and ovarian carcinomas.

Tumor cell seeding to a "metastatic niche" in a specific tissue is at least partially dependent on interactions between tumor cells and the available ECM. The unique binding specificities of integrins may therefore dictate the ability of tumor cells to colonize distinct tissues, and thus serve as a critical homing factor promoting metastasis. The metastatic susceptibility of certain tissue has been linked to a presence of specific ECM proteins at these sites. For example, tenascin C is a ligand for β1 and β3 integrins, and is present in the metastatic niche of the lungs, driving breast metastasis to this organ. Laminin is another ECM protein present in the lungs that is recognized by α6β4 or α6β1 integrins expressed both on tumor cells and platelets. αvβ3 of α4β1 integrins expressed on tumor cells play key roles in bone metastasis, where they enable tumor cell adhesion to ECM proteins such as osteopontin or type I collagen. The enhanced expression of α4β1 integrins on melanoma cells enables binding to VCAM1 on activated lymphatic endothelial cells thereby promoting lymph node metastasis. While the experimental evidence links integrin expression to tissue-specific metastatic seeding of tumor cells, the question how tumor cells reach ECM, which is normally not accessible from the lumen of blood vessels, remains open.

Integrins From the Tumor Microenvironment Promote Cancer Progression

Tumor microenvironment consists of a variety of host cells, where the bone marrow-derived myeloid cells represent the major population of cells virtually in any cancer type. The expression of α4β1 integrin (also known as VLA-4) on bone marrow-derived cells enables their recruitment to the tumor microenvironment. The endothelial remodeling in growing tumor induces the expression of VCAM and cellular fibronectin that facilitates integrin-based adhesion of bone marrow-derived myeloid cells. In addition, VLA-4-driven recruitment of monocytic cells to the activated endothelium, commonly observed in growing tumor and at metastatic sites, promotes angiogenesis. A positive role of platelets in tumor metastasis has been already defined. Apart from the initial P-selectin-dependent interactions, αIIbβ3 integrin on platelets was shown to facilitate tumor cell-platelet interactions. Mechanistically, ECM proteins, such as fibrinogen serve as a bridge for platelet αIIbβ3 integrin dependent adhesion and tumor αvβ3 integrin mediated interaction. This interaction contributes to arrest of tumor cell emboli leading to metastasis. Tumor-activated activated endothelial cells express αvβ3 integrin, which together with basic fibroblast growth factor (bFGF) or tumor necrosis factor α (TNF-α) stimulates angiogenesis. However, the expression of αvβ3 on activated endothelial cells is not unique to tumors, but has been detected in different pathophysiological situations. Finally, tumor-activated endothelium expresses VCAM1, ICAMs, which served as ligands for the integrin-mediated leukocyte recruitment (**Fig. 2**). The dominant members of leukocyte integrin families, facilitating leukocyte recruitment leading to their extravasation are the β2 integrins, e.g., αLβ2 = CD11a/CD18 or αMβ2 = CD11b/CD18, and β1 integrins, e.g., α4β1 = VLA-4. In addition, to integrin-mediated firm adhesion of leukocytes to VCAM1 and ICAMs, a direct interaction between αLβ2 or αMβ2 on endothelial cells directly promotes leukocyte extravasation.

Analogous to growth factor receptors, clustering of integrins in the plasma membrane directly regulate the amplification of signal transduction. In fact, there is accumulating evidence for a cooperative signaling between growth factor/cytokine receptors and integrins. This process is further regulated by other binding partners such as galectins. Galectins is a family of β-galactoside-binding lectins, which are expressed by tumor cells in soluble forms. Galectins influence tumor cell behavior through binding to carbohydrates on the cell surface receptors, such as integrins, and thereby promote their clustering. Galectin-1 expression in low metastatic lung carcinoma cells induced their migration, invasiveness and metastasis, through integrin activation. In addition, Galectin-3 induces β3 integrin-mediated tumor growth and drug resistance. Mechanistically, αvβ3 integrins on tumor cells, without binding to a ligand, recruit the oncogene KRAS to the tumor cell plasma and activate downstream signaling such as NF-kB pathway and thereby promote tumor growth. Thus, integrin αvβ3 was shown to be both necessary and sufficient for tumor initiation and resistance to receptor tyrosine kinase inhibitors in pancreatic carcinomas.

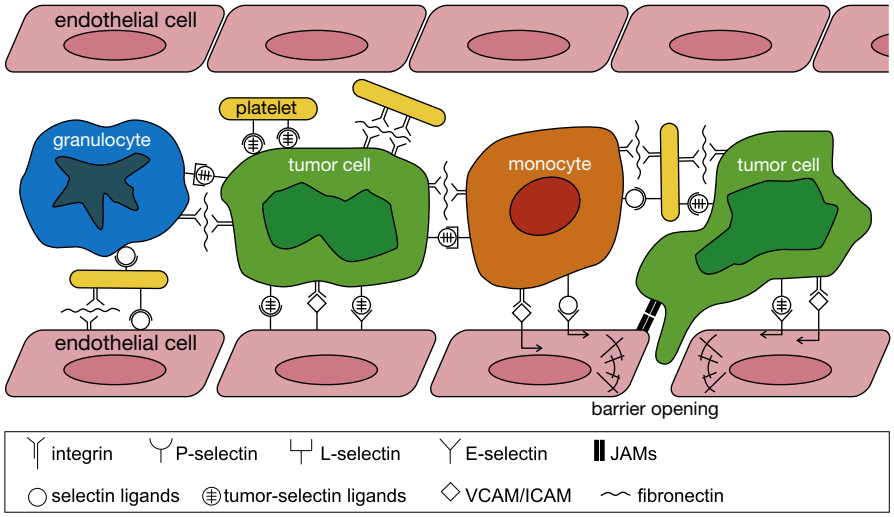

Fig. 2 *Cell adhesion facilitates tumor cell intravascular survival and tumor cell extravasation.* In the circulation, tumor cells interact with blood constituents (e.g., platelets, granulocytes, monocytes, and blood vessel wall = endothelial cells), which is primarily mediated by adhesion of selectins to altered glycan structures (carcinoma mucins) presented on the tumor cell surface. Platelet-tumor cell interactions are directly facilitated by P-selectin, and indirectly through integrin binding both from platelet and tumor cells to extracellular components (e.g., fibronectin). Platelets "coating" of tumor cells protect them from immune responses, and contribute to higher tumor cell mobility of tumor cells. Tumor cell interactions with leukocytes are both L-selectin- or integrin-mediated. Tumor cell-endothelial cells adhesion may trigger endothelial activation through *E*-selectin or immunoglobulin superfamily cell adhesion molecules (IgCAMs) such as ICAM or VCAM that are recognized by integrins from tumor cells. The resulting increase in vascular permeability is linked to specific cytoskeletal rearrangements in the endothelial cells and loosening of endothelial cell junctions. Although tumor cells may individually migrate through the endothelium, *trans*-endothelial migration of tumor cells is significantly potentiated by monocytes. In addition, interactions between tumor and endothelial cells through junction adhesion molecules (JAMs) further modulate tumor cell extravasation.

In conclusion, it is important to emphasize that individual integrins may affect very different aspects of cancer progression. While unligated αvβ3 integrin promotes tumor cell stem-like properties, its ligation to ECM drives cell invasiveness and proliferations. Thus, the role of integrins in cancer biology is dependent on particular cues present in a tumor microenvironment.

Selectin-Mediated Cell Adhesion

Selectins are vascular cell adhesion receptors that bind to glycans presented on cell surface proteins. The physiological function of selectins is to facilitate the initial tethering of leukocytes at inflammatory sites or secondary lymphoid organs before leukocyte adhesion and extravasation. Three different selectins (P-, E-, and L-selectin) can be found in mammals, which are conserved between species. Selectins are C-type lectins that bind preferentially to glycans with a terminal tetrasaccharide structure of sialyl-Lewis x (sLe^x) and sialyl-Lewis a (sLe^a) (Fig. 3), which are usually further modified with a sulfate group. The biosynthesis of sLe^x and sLe^a ligands is orchestrated by a set of glycosyltranferases, where the expression of sialyltransferases and fucosyltransferases is essential. In addition, selectins also bind to certain glycolipids (sulfatides). Heterophilic binding of selectins to glycans is usually calcium dependent. Structurally, all selectins possess an N-terminal C-type carbohydrate recognition domain (C-type lectin), an EGF domain, short consensus repeats, a transmembrane and an intracellular domain. The engagement of selectins by their ligands activates outside-in signaling through e.g., Ca^{2+} and p38 MAP kinase pathway.

P-selectin is stored in granules of platelets and endothelial cells, which bring P-selectin on the surface upon activation. *E*-selectin is present only on endothelial cells and its expression is regulated on a transcription level. L-selectin is constitutively expressed on

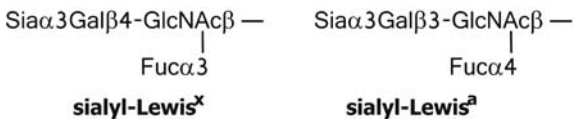

Fig. 3 *Structures of sialyl Lewis x* (sLe^x) *and sialyl Lewis a* (sLe^a). Selectins bind to glycans with a terminal tetrasaccharide structure known as sialyl Lewis $^{x/a}$. The core of the terminal tetrasaccharide is a disaccharide of galactose linked to *N*-acetylglucosamine (GlcNAc), either in α1,4 (sLe^x) or α1,3 (sLe^a) linkage. This disaccharide is further modified by α2,3sialyltransferases linking the sialic acid to the galactose. The fucosyltransferases completes the tetrasaccharide by the addition of fucose to GlcNAc either in α1,3 linkage forming sLe^x or α1,4 linkage forming sLe^a. Depending on a selectin type, this structure is further sulfated either on 6-position of the Gal or 6-position of the GlcNAc. Alternatively, the protein backbone of the selectin ligand carrier is sulfated in a close proximity to the sLe^x structure.

most subsets of leukocytes, but lost upon antigen-mediated T cell memory formation. Selectins bind to properly posttranslationally modified glycan ligands, carrying mostly sLe^x or sLe^a structures. The most studied selectin ligand is P-selectin Glycoprotein Ligand 1 (PSGL-1), where the sLe^x is further sulfated. All three selectins bind to PSGL-1 that is mostly expressed in leukocytes. In addition, E-selectin binds to a variety of glycosylated protein ligands such as E-selectin Ligand-1 (ESL-1) or CD44. L-selectin binds to a group of glycans called addressins that includes: MADCAM, PNADs like CD34, and podocalyxin. These glycans are present in high endothelial venules of lymph nodes or Peyer's patches, where they enable lymphocyte trafficking to secondary lymphoid organs. Moreover, P- and L-selectins also bind to glycosaminoglycans, like heparan sulfate and heparin, but the nature of this cell adhesion remains under investigation.

On a functional level, selectins mediate the first steps of extravasation. Endothelial cells line up the blood vessels and circulating leukocytes do not interact with the vessel wall. Inflammatory stimuli activate endothelial cells that upregulate expression of adhesion molecules including P-selectin and E-selectin. Interactions of vascular selectins with their ligands lead to the leukocyte rolling on activated endothelium, followed by integrin-mediated firm adhesion and finally leukocyte extravasation into the parenchyma, where the effector functions of leukocytes can be executed.

Selectin-mediated cell adhesion modulates two different aspects during cancer progression and metastasis: (A) tumor cell interactions with endothelial cells, platelets and leukocytes; (B) leukocyte recruitment and trafficking to developing tumors (Fig. 2).

Selectin-mediated tumor cell interactions

Malignant transformation is associated with profound changes in cell surface glycosylation of tumor cells and this leads to upregulation of ligands for selectins. These changes in glycosylation are the result of epigenetic and genetic changes of glycosyltransferases and glycosidases. While changes can lead to increased branching of *N*-glycans, the prominent glycan alterations occur on *O*-linked glycans which are present on mucin producing tumor cells. Importantly, the upregulation of sialic-acid containing glycans on tumor cells together with an increased expression of truncated glycan structures has been detected across all cancer types. The upregulation of sialic acid-containing glycans is a result of alterations in expression of sialyltransferases or sialidases. Such sialylated glycans e.g., on mucins serve as ligands for selectins and enable interactions between tumor cells and selectin-carrying cells. Thus, reciprocal interactions between platelets, leukocytes, endothelial cells and tumor cells have been associated with cancer progression. While many of these cell–cell-interactions have been demonstrated in various assays in vitro, their relevance in cancer progression has been confirmed in mouse models using selectin-deficient mice. Importantly, enhanced expression of sLe^x and sLe^a structures is frequently associated with cancer progression and cancer patient's poor prognosis in various tumors including colon, gastric, lung; renal and breast cancers; melanomas and others.

Metastasis is a multistep process which begins with dissemination of tumor cells through the blood circulation and leads to colonization of a distant organ. Selectin-mediated interactions contribute to hematogenous dissemination and organ colonization. During hematogenous metastasis platelets bind to tumor cells and this platelet-"shield" protects tumor cells from elimination by NK cells. A strong reduction of metastasis was observed in mice lacking P-selectin, indicating that P-selectin could be a target for anti-metastatic therapy. Endothelial E-selectin has been shown to promote tumor cell adhesion and thus has been implicated in metastatic dissemination. A special glycoform of CD44 in human cells, named as HCELL, has been identified as an E-selectin ligand on tumor cells. In breast cancer cells, selectin-mediated interactions with HCELL on microvascular endothelial cells promotes homing of cancer stem cells to the bone marrow. Selectin-selectin ligand interactions can influence signaling both in tumor cells and in selectin-bearing cells; thereby modulating cancer progression.

Cancer patients have a significantly increased risk to develop thromboembolic complications. This hypercoagulability first described by Trousseau is also to some part attributed to selectin-mediated interactions between cancer mucins, platelet P-selectin and L-selectin on leukocytes. Carcinoma-derived highly sialylated and glycosylated mucins enhance platelet interactions with myeloid leukocytes that promote thrombus formation. This finding might explain why heparin and heparin derivatives are potent inhibitors of thromboembolic events in cancer patients, when compared to other anticoagulants (e.g., Warfarin).

Selectin-mediated recruitment and trafficking of leukocytes in cancer

Selectin-mediated interactions promote the leukocyte recruitment to tumors and enables their interactions within a tumor microenvironment. Increased leukocyte recruitment supports cancer progression through promotion of tumor cell invasion, intravasation, dissemination, and extravasation. In particular, selectins were shown to influence the recruitment of innate immune cells during metastatic colonization. Analysis of metastatic colonization in various mouse models have shown that selectin-mediated interactions is essential for the recruitment of myeloid cells and monocytes that enhances tumor cell extravasation and facilitate organ colonization. Increased production of chemokines (e.g., CCL2, CCL5, or CXCL12) within the metastatic microenvironment further promotes recruitment of monocytes and myeloid cells, which is further supported by selectin interactions. Interestingly, both endothelial E-selectin and the leukocyte L-selectin were shown to be implicated in the process of monocyte-assisted transendothelial migration of tumor cells in lungs. These studies show that metastatic tumor cells "highjack" the physiological function of selectins and leukocyte recruitment to promote tumor growth and metastasis.

In the case of hematogenous malignancies, the tumor cells express not only selectin ligands, but also the L-selectin. The cell surface expression of L-selectin on leukemia cells can significantly influence their trafficking and distribution. For instance, in patients with chronic lymphocytic leukemia (CLL) tumor cells re-circulates to lymph nodes through L-selectin mediated rolling, which promotes cancer progression.

In conclusion, selectin-mediated interactions between tumor cells, platelets, endothelial cells and leukocytes are involved in various steps of metastasis and can facilitate the formation of a metastatic niche. Interestingly, glycosaminoglycans (GAGs) such as heparin and heparan sulfates bind to selectins and thereby inhibit interactions with tumor-associated ligands. Besides, glycomimetics of PSGL-1 can inhibit P-selectin mediated interactions and is currently tested in cancer therapy. The abundance of selectin ligands on virtually all cancer types, together with the overall access to vascular selectin during hematogenous metastasis strongly suggest that any interference in selectin-mediated cell adhesion in a temporal manner might have an antimetastatic potential also in clinical settings.

Other Cellular Adhesion During Cancer Progression

There are other cell adhesion molecules associated with cancer progression, but their function is only partially elucidated. CD44 is a cell surface adhesion molecules, which was initially shown to be the receptor for an ECM glycosaminoglycan hyaluronan. Many cancer types express high levels of CD44 that upon cleavage from the cell surface serves as a marker for cancer progression. Besides the special glycoform HCELL found in cancer that can serve as ligands for selectins (see above), tumor-specific alternatively spliced CD44 has been described. The current evidence linked altered CD44 expression to cancer stem cell phenotype, while the intracellular domain is critically involved in this process.

Junctional adhesion molecules (JAMs) are expressed on endothelial cells, where their homophilic interactions strengthen the endothelial barrier functions. During inflammation JAMs, together with endothelial ICAMs interact with αLβ2 or αMβ2 on leukocytes thereby promoting leukocyte extravasation through the retracted endothelium. Recent evidence suggests that JAM-c on melanomas actively contribute to lung metastasis though promotion of tumor cell extravasation through enhanced transendothelial migration of the tumor cells without affecting tumor cell adhesion (**Fig. 2**). Whether other tumor cells express JAMs or whether JAMs binding is relevant for metastasis to other organs remains to be defined.

Adhesion Molecules in Antitumor Immunity and Cancer-Associated Inflammation

Recent advances in our understanding how the immune system recognizes and eliminates tumor cells and how tumor cells evade this immune control has led to the introduction of successful immunotherapies into the clinical routine. In particular, targeting of inhibitory receptors on T cells including PD-1 and CTLA-4 have revolutionized the treatment of patients with advanced cancers including melanoma, nonsmall cell lung cancer, kidney cancer or bladder cancer. While antitumor immunity has gained much interest, the virtually omnipresent cancer-associated inflammation associated with recruitment and polarization of myeloid-derived cells usually supports cancer progression by the generation of an immune-suppressive microenvironment.

Dysfunction in the leukocyte recruitment with antitumor activity to tumors and increased recruitment of immune suppressive immune cells such as myeloid-derived suppressors, regulatory T cells or tumor-associated macrophages has been observed in many cancers. Cell adhesions facilitate leukocyte recruitment through selectin- and integrin-mediated interactions that influence also the adaptive immunity. Infiltration of CD8 positive T cells preferentially happens in the periphery of tumors, which is well studied in melanoma lesions. Moreover, in a murine melanoma model, endothelial cells downregulated ligands for the recruitment of cytotoxic T cells. PSGL-1 on T cells modulates T cell trafficking and the development of regulatory T cells therefore PSGL-1 represents an interesting drug target for cancer immunotherapy.

T cells including regulatory T cells (Tregs) recirculate and control immune homeostasis including peripheral tolerance. Homing of naïve T cells to secondary lymphoid organs requires L-selectin binding to addressins on high endothelial venules. Selectin receptors as well as integrins have an important function in the recruitment and homing of Tregs to sites of inflammation and to tumors. Tregs play an important role in suppressing antitumor immune responses thus targeting of cell adhesion mechanisms leading to Tregs recruitment could be an interesting way to improve antitumor immune responses.

Prospective Vision

Current evidence describes the role of cell adhesion in different processes associated with cancer progression, ranging from stemness of cancer cells; tumor growth survival, invasiveness, migration, and metastasis; to modulation of immune responses. While the biology of cell adhesion during cancer is rather complex, the function of certain integrins, cadherins and selectins has been extensively evaluated in several preclinical models. Nevertheless, no agent that specifically targets interactions of these molecules is currently used in clinical practice. Clearly, the complete identification of underlying mechanisms in the complexity of the tumor microenvironment is still ongoing and will hopefully help to develop therapeutic strategies. There are several studies testing targeted delivery of therapeutics to tumor sites through cell adhesion molecules. For example, tumor targeting by IL-2 cytokine-fusion proteins with RGD domains that bind to integrins have demonstrated promising results in mouse models together with checkpoint blockade. While timely-defined targeting of selectins resulted in reduced metastasis in many experimental models, selectins can also serve as targets for delivery of nanoparticles coated with selectin ligands. For example, P-selectin was targeted for cancer-directed drug-delivery with nanoparticles coated with a fucosylated polysaccharide. Interestingly, P-selectin expression could be initiated on endothelial cells by radiotherapy. This finding represents a very interesting concept; where drug targeting could be initiated by specific irradiation of a tumor lesion. In this context, new insights into function of cell-cell interactions, especially during

metastasis, will certainly open up new strategies to interfere in cancer progression. Interference in cell adhesion based processes during cancer lies in a combinational approach with other approaches e.g., direct tumor cell targeting or modulation of anticancer immunity.

See also: Metastatic Signatures—The Tell-Tale Signs of Metastasis.

Further Reading

Desgrosellier, J.S., Cheresh, D.A., 2010. Integrins in cancer: Biological implications and therapeutic opportunities. Nature Reviews Cancer 10, 9–22.
Hauselmann, I., Borsig, L., 2014. Altered tumor-cell glycosylation promotes metastasis. Frontiers in Oncology 4, 28.
Jeanes, A., Gottardi, C.J., Yap, A.S., 2008. Cadherins and cancer: How does cadherin dysfunction promote tumor progression? Oncogene 27, 6920–6929.
Kwan, B.H., Zhu, E.F., Tzeng, A., Sugito, H.R., Eltahir, A.A., Ma, B., Delaney, M.K., Murphy, P.A., Kauke, M.J., Angelini, A., Momin, N., Mehta, N.K., Maragh, A.M., Hynes, R.O., Dranoff, G., Cochran, J.R., Wittrup, K.D., 2017. Integrin-targeted cancer immunotherapy elicits protective adaptive immune responses. Journal of Experimental Medicine 214, 1679–1690.
Läubli, H., Borsig, L., 2010. Selectins promote tumor metastasis. Seminars in Cancer Biology 20, 169–177.
Obenauf, A.C., Massague, J., 2015. Surviving at a distance: Organ specific metastasis. Trends in Cancer 1, 76–91.
Pearce, O.M., Laubli, H., 2016. Sialic acids in cancer biology and immunity. Glycobiology 26, 111–128.
Pinho SS anf Reis CA, 2015. Glycosylation in cancer: Mechanisms and clinical implications. Nature Reviews Cancer 15, 540–555.
Schneider, J.G., Amend, S.R., Weilbaecher, K.N., 2011. Integrins and bone metastasis: Integrating tumor cell and stromal cell interactions. Bone 48, 54–65.
Shamay, Y., Elkabets, M., Li, H., Shah, J., Brook, S., Wang, F., Adler, K., Baut, E., Scaltriti, M., Jena, P.V., Gardner, E.E., Poirier, J.T., Rudin, C.M., Baselga, J., Haimovitz-Friedman, A., Heller, D.A., 2016. P-selectin is a nanotherapeutic delivery target in the tumor microenvironment. Science Translational Medicine 8, 345ra387.
van Roy, F., 2014. Beyond E-cadherin: Roles of other cadherin superfamily members in cancer. Nature Reviews Cancer 14, 121–134.
Zarbock, A., Ley, K., McEver, R.P., Hidalgo, A., 2011. Leukocyte ligands for endothelial selectins: Specialized glycoconjugates that mediate rolling and signaling under flow. Blood 118, 6743–6751.

Cell Responses to DNA Damage☆

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Glossary

AT Ataxia telangiectasia, which is an autosomal recessive genetic disease characterized by ataxia, immune deficiency, sterility, and mortality early in life, caused by loss-of-function mutation in the ATM gene

BER Base excision repair, which is mechanism that repairs damaged bases in DNA, involving excision of damaged base by N-glycosylase excising the damaged base followed by endonuclease cleavage of DNA strand at the abasic site, followed by DNA polymerase filling and ligase sealing of the strand.

BSE Bystander effect, which describes DNA damage-induced biological effects in cells that are not directly targeted by genotoxins, but are in the vicinity of directly damaged cells.

DDR DNA damage response, which describes the compilation of cellular processes activated by lesions in genomic DNA.

FA Fanconi anemia, which is an autosomal recessive genetic disease characterized by genome instability, defects in DNA repair, anemia and cancer susceptibility, caused by loss-of-function mutations in some 20 genes, collectively known as the FA genes.

FHA Fork head associated, which is an evolutionarily conserved protein domain that specifically binds to a phospho-threonine epitope.

MMR Mismatch repair, which is a mechanism that repairs mismatched base pairs and small insertions or deletions during DNA replication, involving protein complexes that recognize those lesions in newly synthesized daughter strand, followed by recruitment of nucleases to cleave the daughter stand, polymerases to fill the gap, and ligases to seal the strand.

NER Nucleotide excision repair, which is a mechanism that repairs photo-adducts of bases in DNA, involving protein complexes that recognize those lesions in actively transcribed DNA (transcription coupled repair) or genomic DNA in general, followed by endonuclease cleavage of DNA strand surrounding the photo-adducts, DNA polymerase filling and ligase sealing.

PIKK Phosphotidyl-inositol-3 (PI3K) kinase-related kinase, which is a protein kinase domain that is highly conserved in eukaryotic cells and is related to the lipid kinase PI3K. Upon DNA damage, three members of the PIKK family, namely ATM, ATR, and DNAPK, are activated by lesion recognition and sensing factors and these PIKKs function as master kinases to switch on DDR.

XP Xeroderma pigmentosa, which is an autosomal recessive genetic disease characterized by UV sensitivity, defects in NER, developmental defects, and cancer susceptibility, caused by loss-of-function mutations in a dozen or so genes, collectively known as the XP genes.

Overview

The study of DNA damage response (DDR) spans multiple disciplines and experimental approaches that defy a comprehensive review in one chapter. The goal of this chapter is therefore to provide a DDR framework that is amenable to expansion in biological directions by readers according to their interests. This DDR framework organizes factors and processes into three functional categories: (1) lesion recognition and damage sensing, (2) damage-signal transduction, and (3) downstream biological effectors (Fig. 1).

DNA lesions can be generally divided into four major groups: base modifications (alkylation, oxidation), mismatched base pairs (non-Watson Crick base pairing, small insertions, or deletions), interstrand crosslink (ICL), and strand breaks (double-stranded or single-stranded breaks). These lesions are repaired by a number of DNA repair mechanisms, for example, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), crosslink repair, nonhomologous end joining (NHEJ), and homologous recombination (HR). Each of these repair pathways is initiated by a lesion recognition factor, for example, the MSH2/MSH6 complex recognizes mismatched base pairs, Ku and MRN complexes recognize double-stranded breaks (DSB). The lesion recognition factors not only recruit DNA repair enzymes but also interact with signal transducers to activate additional DDR pathways (Fig. 1). Besides those factors that recognize specific DNA lesions, eukaryotic cells also possess an evolutionarily conserved "damage sensor," that is the 9-1-1 hetero-trimeric DNA clamp. The 9-1-1 DNA clamp is loaded unto stalled replication forks and other damage sites where structures that resemble stalled replication forks are formed.

The dual roles for lesion-recognition proteins to stimulate DNA repair and to activate damage-signal transduction ensures that DDR is terminated when lesions are repaired. With this design, the rate of DNA repair becomes an important determinant of the

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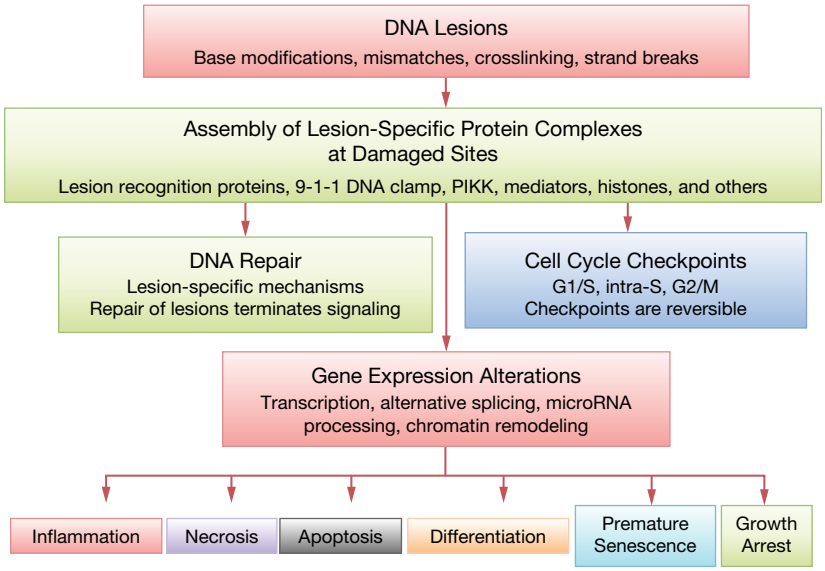

Fig. 1 A framework of DNA damage response (DDR). A variety of DNA lesions occur under physiological conditions and are further induced by exposure to genotoxins. Damage recognition by lesion-specific detectors initiates the assembly of protein complexes through regulated protein–protein interactions involving covalent modifications of preexisting proteins (see **Fig. 3** for an example of complex assembly at a double strained break site). These complexes serve two functions—to stimulate DNA repair and to propagate the DNA damage signal. The *immediate early* action (within minutes of DNA damage) is to stimulate DNA repair. In proliferating cells, another *immediate early* action is to inhibit cell cycle progression. In both proliferating and nonproliferating cells, the third *immediate* action is to alter gene expression. Because transcription of new RNA and translation of new protein require time, gene expression-dependent biological responses are *delayed* (illustrated by the longer arrow). In DDR, gene expression-dependent responses include cell cycle arrest, premature senescence, inhibition or stimulation of cellular differentiation, apoptosis, necrosis, cell–cell communication, and inflammation. Because the cell type and developmental stages control the epigenetic landscape, the *delayed* responses driven by gene expression alterations exhibit cell-context dependency and cannot be easily generalized across all cell types.

biological outcomes in DDR. Because RNA transcription and protein translation require more time (longer arrow in **Fig. 1**) than covalent modifications of preexisting factors (short arrows in **Fig. 1**), a rapid extinction of DNA lesions (efficient repair) can prevent or reduce gene expression alterations to blunt the *delayed* responses. Therefore, it is the persistence of DNA lesions that triggers the downstream effects, such as premature senescence or cell death (**Fig. 1**).

It must be emphasized that DDR exhibits not only *temporal* dependency but also cell type dependency (**Fig. 1**). In proliferating (cycling) cells, the *immediate early* effects (within minutes of lesion detection) are to stimulate DNA repair and to inhibit cell cycle progression. These rapid DDR maximizes genome protection, cell survival, and resumption of proliferation. By contrast, *delayed* effects such as premature senescence or death *eliminate* rather than *protect* the damaged cells. Therefore, the upstream factors in lesion detection and signal transduction can activate pro-survival or pro-death downstream effects. Under conditions when lesion removal (DNA repair) is highly efficient, the DDR signaling proteins contribute to cell survival. However, under conditions when DNA lesions overwhelm the repair capacity, DDR signaling proteins contribute to cell death.

Lesion Recognition and Damage Sensor

Modified Bases

Base modifications, such as guanine base alkylation (6-meG) or oxidation (8-oxoG), are recognized and repair by several different mechanisms. For example, MGMT (O^6-methylguanine DNA methyltransferase) recognizes and demethylates O^6-methylguanine. On the other hand, N-glycosylase enzymes recognize and cleave the N-glycosidic bonds to remove the modified bases in BER (base excision repair). In nucleotide excision repair (NER), which is the major repair mechanism of UV-induced photo-adducts, several XP protein complexes and the elongating RNA polymerase II serve as lesion detectors. The XP genes are those mutated in an autosomal recessive genetic disease Xeroderma pigmentosum (XP). XP patients display extreme sensitivity to UV; they also suffer from developmental defects and susceptibility to cancer.

Mismatched Base Pairs

Mistakes during DNA replication occur at an appreciable rate despite the proofreading function of DNA polymerases and must be recognized and repaired to ensure the faithful transmission of genetic information. These replication-induced mismatched bases and small deletions or insertions are repaired by the mismatch repair (MMR) mechanism. In eukaryotic cells, heterodimeric

complexes of MSH2/3 or MSH2/6 proteins detect mismatched bases and then recruit other MMR proteins (MLH1, MLH3, PMS2) to initiate repair. In human, genetic defects in MSH2 or MLH1 cause cancer susceptibility in the Lynch syndrome.

Cross-Linked DNA

Inter-strand cross-linking (ICL), where two strands of DNA are covalently linked, for example, by cisplatin, impedes transcription and DNA replication. The repair of ICL requires proteins encoded by more than 20 FA genes that are mutated in Fanconi anemia (FA), an autosomal recessive genetic disease characterized by anemia and cancer susceptibility. A complex of FA gene products recognize replication forks at ICLs to activate a complex repair mechanism that requires the coordination of nucleotide excision (NER), trans'lesion DNA synthesis, and homologous recombination (HR).

Double-Stranded Breaks (DSB)

Double-stranded breaks (DSB) occur at stalled replication forks and during the process of repairing interstrand crosslinks. DSB are also induced by genotoxins, for example, ionizing radiation or topoisomerase inhibitors. Two evolutionarily conserved protein complexes are detectors of DSB: the hetero-dimeric Ku antigen (Ku-70, Ku-80) and the hetero-trimeric MRN complex (MRE11, RAD50, NBS1). In mammalian cells, the Ku antigen recruits DNA-PK to stimulate nonhomologous end joining (NHEJ) for the non-faithful repair of DSB, whereas the MRN complex recruits ATM and many other factors to stimulate homologous recombination (HR) for the faithful repair of DSB by using an intact homolog of the broken DNA (Fig. 2).

The 9-1-1 Complex

The 9-1-1 complex is a hetero-trimeric DNA clamp consisting of three evolutionarily conserved proteins: Rad9, Rad1, and Hus1. The 9-1-1 DNA clamp is structurally similar to the homo-trimeric PCNA DNA clamp required for DNA replication and repair. The 9-1-1 complex is loaded onto double- and single-stranded hybrid DNA at stalled replication forks through the action of a damage-induced Replication Factor C (RFC) clamp loader containing the RAD17 protein. The 9-1-1 clamp is also loaded onto similar DNA structures that are generated during DNA repair. The 9-1-1 complex differs from other lesion-specific binding proteins because it is a sensor of an abnormal DNA structure rather than a modified base or a DNA end. Similar to other lesion sensors, the 9-1-1 complex serves as a scaffold to promote DNA repair and damage signal transduction (Fig. 2).

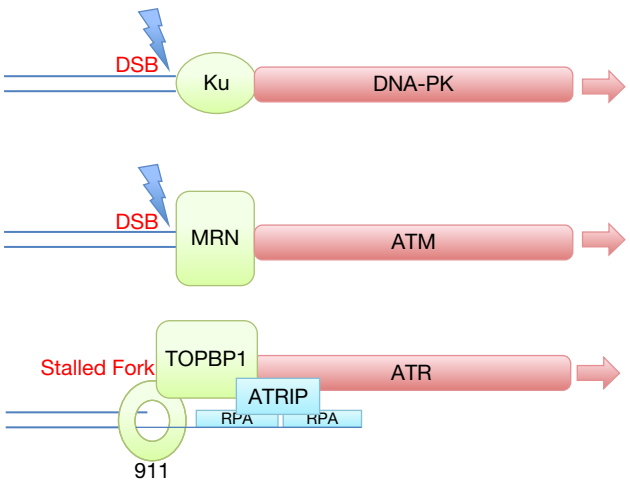

Fig. 2 Activation of PIKK by DSB or stalled replication fork. Double-stranded breaks (DSB) are recognized by the Ku-antigen (a heterodimer of Ku-70 and Ku-80) or by the MRN complex (a heterotrimer of **M**RE11, **R**AD50, and **N**BS1). Ku antigen interacts with DNA-PK (DNA-dependent protein kinase), a member of the PIKK family, which activates the nonhomologous end-joining mechanism for DSB repair. MRN interacts with ATM, another member of the PIKK family, which stimulates the phosphorylation of hundreds of substrates to regulate the homologous recombination mechanism for DSB repair, the cell cycle checkpoints and gene expression at several levels, including transcription initiation, alternative splicing and microRNA biogenesis. Stalled replication fork with double- and single-stranded hybrid DNA is sensed by the RAD17-containing RFC, which loads the 9-1-1 DNA clamp. The 9-1-1 complex and the single-stranded DNA binding protein, RPA, jointly recruit the ATR kinase associated with TOPBP1 (topoisomerase II binding protein 1) and ATRIP (ATR interacting protein). ATR, also a PIKK family of kinase, is essential to the repair and the restart of stalled replication forks, and therefore, ATR is essential to cell viability.

DNA Damage Signal Transduction

PIKK Family of Protein Kinases

In eukaryotic cells, DNA damage activates a family of evolutionarily conserved protein kinases belonging to the phosphoinositide 3-kinase-like kinase (PIKK) family. The mammalian PIKKs central to the transduction of DNA damage signal are ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3 related), and DNA-PK (DNA-dependent protein kinase) (Fig. 2). Loss of the ATM function causes Ataxia telangiectasia, an autosomal recessive genetic disease characterized by cerebellum degeneration, radiosensitivity, immune-deficiency, sterility, and cancer predisposition. Loss of DNA-PK is associated with severe combined immune deficiency (SCID) in mice.

The mammalian ATR function is required throughout S-phase and plays an essential role in DNA replication. ATR is recruited to stalled replication forks or resected DSBs by a complex array of protein–protein interactions (Fig. 2). At the stalled fork, a long stretch of single-stranded (SS) DNA is formed and this ss-DNA is recognized and bound by RPA (an evolutionarily conserved ss-DNA binding protein), which guides the loading of 9-1-1. Together, the 9-1-1 DNA clamp and RPA then facilitate the recruitment and activation of the ATR kinase in collaboration with TOPBP1 (topoisomerase II binding protein 1) and ATRIP (ATR interacting protein) (Fig. 2).

Upon activation, the PIKKs phosphorylate hundreds of cellular proteins of diverse functions to mobilize an array of biological responses to DNA damage. Among the PIKK substrates, the most highly conserved are the mediators and the checkpoint kinases.

Mediators

The mediators are scaffold proteins that play essential role in DNA damage signal transduction. The PIKKs phosphorylate the mediator proteins to regulate the assembly of protein complexes for DNA repair and signal propagation (Fig. 1). In DDR, the forkhead-associated (FHA) and the BRCA1 C-terminal (BRCT) domains serve as the phospho-epitope binding sites for the assembly of signaling and repair complexes. The FHA domain binds to phospho-threonine (pT) containing peptide motifs. The BRCT binding to phospho-peptide motifs requires two BRCT in tandem. The structural basis for phospho-epitope binding by the FHA and the BRCT domains has been elucidated.

A prototypical mediator in DNA damage signal transduction is the mammalian MDC1 (mediator of DNA damage checkpoint 1), which is a large scaffolding protein with an FHA domain at the N-terminus, two tandem BRCT domains at the C-terminus, and numerous phosphorylation sites throughout the length of the protein (Fig. 3). The phosphorylated MDC1 can recruit proteins with the FHA, the tandem-BRCT, and other phospho-binding domains. At the same time, the FHA and BRCT domains of MDC1 can interact with other PIKK-phosphorylated substrates (Fig. 3).

Checkpoint Kinases

Among the highly conserved PIKK substrates are the CHK1 and CHK2 protein kinases, which are members of the Protein Kinase superfamily, characterized by a highly conserved kinase domain that is distinct from the PIKK. CHK1 and CHK2 are phosphorylated by the PIKK, with CHK1 being primarily the substrate of ATR, and CHK2 of ATM. However, the PIKKs and the checkpoint kinases

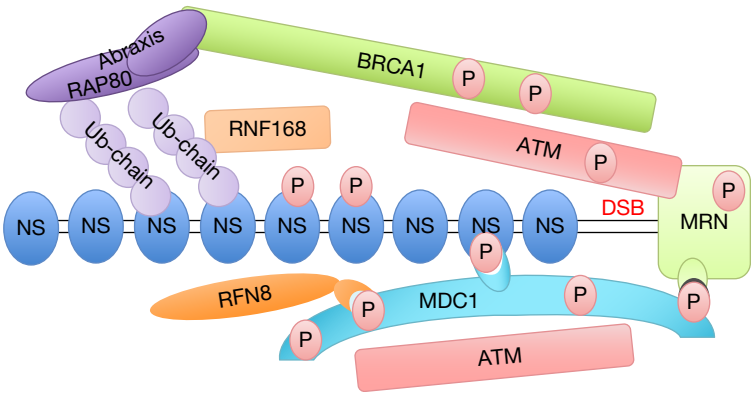

Fig. 3 Assembly of protein complexes at DSB site. This diagram illustrates an example of protein complex assembly at a double-stranded break (DSB) site. The MRN complex (MRE11, RAD50, NBS1) binds to the DSB, recruits, and activates ATM. Activated ATM phosphorylates itself (phosphorylation sites are indicated as pink balls with **P**), the MRN, the H2AX histone in the nucleosomes (NS), and the mediator MDC1. The tandem BRCT domains of MDC1 interact with the phospho-epitope in the phosphorylated H2AX (aka γH2AX). Some phospho-epitopes in MDC1 bind to the BRCT and FHA domains of NBS1 in the MRN complex. Other phospho-epitopes in MDC1 bind to RNF8, which then ubiquitinates H2A and H2AX in the nucleosomes. The poly-ubiquitin chains, further elaborated by RNF168, recruit RAP80 and Abraxis, which then recruit BRCA1 that is also phosphorylated by ATM. Note that activated ATM is distributed beyond the DSB site to phosphorylate many other substrates. The BRCA1 complex at the DSB site facilitates homologous recombination repair.

are components of a highly dynamic signaling network with nonexclusive interactions. ATM phosphorylation of CHK2 causes oligomerization of this kinase via intermolecular phospho-epitope binding to its own FHA domain and this ATM-induced CHK2 oligomerization results in CHK2 kinase activation. In addition, checkpoint kinases interact with mediators, which control the compartmentalization and stability of CHK1 and CHK2. The checkpoint kinases play essential roles in the activation of cell cycle checkpoints. They also regulate DNA repair and gene expression by phosphorylating various downstream effector proteins.

Other Kinases

Besides CHK1 and CHK2, other kinases of the Protein Kinase superfamily also participate in the transduction of DNA damage signal. These include the stress-activated protein kinases, for example, p38 and JNK, the ABL tyrosine kinase, and many others. The stress-activated protein kinases can activate the cell cycle checkpoints independent of CHK1 and CHK2. They also regulate gene expression. The ABL tyrosine kinase is localized to the cytoplasm and the nucleus. In response to DNA damage, the ABL kinase becomes accumulated in the nucleus through the action of ATM. Nuclear ABL tyrosine kinase activates pro-apoptotic gene expression by activating the p53 family of transcription factors (p53, p63, p73) and histone acetyltransferase, for example, Tip60 (KAT5).

Other Protein-Modifying Enzymes

Besides phosphorylation, DNA damage activates other protein modifying enzymes to induce a variety of protein covalent modifications, including ubiquitination, poly-ubiquitination (Fig. 3), sumoylation, neddylation, ADP-ribosylation, acetylation, methylation, and possible other less studied modifications such as nitrosylation and succinylation. For example, the mediator protein BRCA1 (together with BARD1) functions as an E3 ubiquitin ligase to ubiquitinate proteins involved in DSB repair and gene expression. Some of these protein covalent modifications occur on preexisting proteins that are assembled into complexes at the damage sites (Fig. 3). However, covalent modifications in DDR can also occur on proteins that are induced in the delayed responses to regulate biological processes other than checkpoints and repair (Fig. 1). In other words, protein covalent modifications participate in the *immediate* and the *delayed* responses to DNA damage.

Assembly of DNA Damage Signaling Complexes at DSB

Live cell imaging technology combined with laser micro-irradiation to generate DSB of defined patterns has observed a series of temporally distinct steps in the *immediate* response to DSB lesions (Fig. 3). Recruitment of the MRN complex and ATM at the DSB sites is followed by ATM acetylation, auto-phosphorylation, and the phosphorylation of H2AX (the ATM-phosphorylated H2AX is commonly known as γH2AX). Formation of γH2AX occurs initially at or near the DSB site and is then extended outward from the break site for several megabases. The mediator MDC1 is recruited to the DSB sites by binding to the phospho-epitope motif in γH2AX with its tandem BRCT domains (Fig. 3). MDC1 is then phosphorylated to generate binding sites for the FHA and BRCT domains of NBS1 in the MRN complex (Fig. 3). Other phospho-epitopes in MDC1 bind to the FHA domain of an ubiquitin ligase RNF8, which ubiquitinates H2A and H2AX at the DSB sites. The ubiquitinated histones then recruit another ubiquitin ligase RNF168 (Fig. 3). The polyubiquitin chains then recruit additional factors, such as the RAP80 protein, which contains a pair of ubiquitin interacting motifs (UIMs) that bind to polyubiquitin chains. RAP80, together with a coiled-coil scaffold protein Abraxis, in turn, create binding sites for BRCA1, which promotes homologous recombination to repair the DSB. Together, these live cell-imaging results illustrate the complexity of protein assembly at DNA lesions (Fig. 3).

Downstream Biological Effects

Cell Cycle Checkpoints

DNA damage activates three major checkpoints to halt the progression of the cell cycle (Fig. 4). The G1/S checkpoint is activated in G1 cells to inhibit S-phase entry. The intra-S checkpoint is activated in S-phase cells to prevent the not-yet-activated origins from initiating DNA replication. The G2/M checkpoint is activated in G2 cells to inhibit the entry into mitosis. The cell cycle checkpoints are triggered by protein phosphorylation that is readily reversible upon protein dephosphorylation so that cell cycle progression can resume after DNA lesions are repaired (Fig. 4).

G1/S checkpoint

A critical downstream effector in G1/S checkpoint is Cdc25A. DNA damage induces the phosphorylation, nuclear exclusion, and degradation of Cdc25A through PIKK-Mediator-CHK1/2. Inactivation of Cdc25A prevents the activation of Cdk2/Cyclin to block the initiation of DNA replication (Fig. 4). The G1/S checkpoint is an *immediate early* response to DNA damage as it is activated by protein phosphorylation within minutes of lesion detection. In mammalian cells, persistence of DNA lesions can prolong the G1/S checkpoint via the upregulation of p21/Cip1, which directly binds to and inhibits the Cdk2/Cyclin complexes. This p21Cip1-mediated G1 arrest is a *delayed* response as it takes hours for the transcription and the translation machineries to produce this protein above the levels of Cdk2/Cyclin complexes.

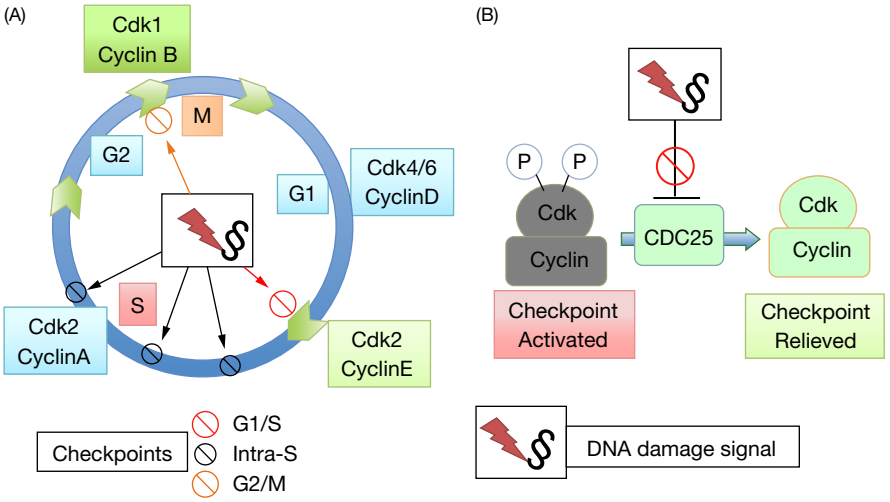

Fig. 4 DNA damage activates cell cycle checkpoints. (A) Cell cycle progression is coordinated by Cdk/Cyclin. D-type cyclins are expressed in G1 to activate Cdk4 and Cdk6. E-type cyclins are expressed in late G1 to stimulate G1/S transition by activating Cdk2. A-type cyclins are expressed throughout S and G2 to activate Cdk2. B-type cyclins are expressed in late G2 and early M to stimulate G2/M transition by activating Cdk1. DNA damage activates three checkpoints: the G1/S checkpoint (red) inhibits the entry of G1 cells into S-phase by blocking the initiation of DNA replication, the intra-S checkpoint (black) inhibits firing of replication origins that have not been activated in S-phase cells, the G2/M checkpoint (orange) inhibits the entry of G2 cells into mitosis. (B) Cell cycle checkpoint is activated when Cdk/cyclin kinase activity is inhibited. Cdc25 phosphatase, which dephosphorylates Cdk, is a critical activator of Cdk/cyclin kinase. In response to DNA damage, PIKK phosphorylates and activates the checkpoint kinases (CHK1 and CHK2) to phosphorylate, sequester, and degrade Cdc25, thus inhibiting cell cycle progression.

Intra-S checkpoint

The intra-S checkpoint is a transient inhibition of DNA replication triggered by DNA damage in S-phase cells. The eukaryotic genome is divided into tens of thousands of replication units (replicons), each with an origin where replication initiates. These replication origins are activated (fired) nonsynchronously throughout S-phase, with the timing of origin firing being dependent on the chromatin conformation and other factors. When activated, the intra-S-phase checkpoint prevents the firing of uninitiated origins (Fig. 4). The ATM kinase activates an intra-S checkpoint that requires the phosphorylation of SMCs, which are components of the sister chromatin–cohesion complex. The ATM-induced intra-S checkpoint is an *immediate early* and *short-lived* response, possibly because the resumption of DNA replication can facilitate repair.

G2/M checkpoint

The G2/M checkpoint mechanism is activated to prevent damaged cells from entering into mitosis. The Cdk1/Cyclin B kinase complex, also known as the Mitosis Promoting Factor (MPF), is the activator of mitotic entry. During late S and G2 phases of the cell cycle, Cdk1/Cyclin B is inhibited through threonine (Thr) and tyrosine (Tyr) phosphorylation of Cdk1 by the Wee1 kinase. Dephosphorylation of Cdk1 by the Cdc25 phosphatases (Cdc25A, B, and C) activates MPF to initiate mitosis (Fig. 4). DNA damage causes the inactivation of all three Cdc25 phosphatases through the actions of PIKK and checkpoint kinases to prevent MPF from being activated and thus block cells in G2. The p38 family of stress-activated protein kinases and the Polo-like kinases (Plk1 and Plk3) can also inhibit MPF to activate G2/M checkpoint.

Gene Expression

The upregulation of p21Cip1 followed by the cell cycle arrest in G1 is one of the best established examples for how gene expression controls the cellular responses to DNA damage. DNA damage alters the expression of protein-coding mRNA as well as long non-coding RNA (ncRNA) and microRNA (miR) at the levels of transcription, splicing, and miR biogenesis.

Transcription

The most well-studied transcription effector in DDR is the tumor suppressor p53, which is a classical transcription factor with a trans-activating domain (that interacts with transcription coactivators), a sequence-specific DNA binding domain, and an oligomerization domain. In response to DNA lesions, PIKK and CHK2 phosphorylate p53, MDM2 (an E3 ubiquitin ligase that targets p53 for degradation), and several other proteins to cause the stabilization and activation of p53. The activated p53 binds to enhancers containing p53-responsive elements (p53-RE) that are found in many promotes and enhancers. While p53 activation is a universal response to DNA damage across mammalian cell types, the tissue-specific responses to DNA damage are mediated by the combinatorial effects of p53 and many other tissue-specific transcription factors.

Alternative splicing

Alternative splicing of nascent RNA is a key mechanism for diversifying the coding capacity. Downstream of DNA damage signaling are effectors that regulate alternative splicing. For example, DNA damage induces covalent modifications of splicing factors to skip or to include exons, thus altering the relative levels of protein isoforms in damaged cells. Alternative splicing of DDR signal transducers themselves, such as p53 and its related p63 and p73, can have deterministic influence on the biological outcomes. However, current knowledge on DNA damage-induced alternative splicing remains insufficient to identify such deterministic effects, possibly because of the highly context-dependent nature of splicing regulation and interactions among protein isoforms.

MicroRNA biogenesis

MicroRNAs (miRs) are small (~22 nt) single-stranded noncoding RNAs that are loaded into the RNA-induced silencing complexes (RISCs) and function as guides to target specific mRNAs for translation inhibition or degradation. The DDR-activated p53 can stimulate transcription initiation from specific miR genes, with the miR-34 family being the most notable example. The biogenesis of mature miRs requires (a) transcription of primary microRNA, pri-mir, that is generally a long RNA, (b) cleavage of pri-mir by the microprocessor complex (consisting of Drosha and DGCR8) to generate a ~70 nt precursor microRNA hairpin, pre-mir, which is exported to the cytoplasm, and (c) processing of pre-mir by Dicer to form the mature miR that is loaded into the RISC complex. In response to DNA damage, the processing of pri-mir to pre-mir and from pre-mir to mir can be stimulated by ATM-dependent phosphorylation of specific RNA binding proteins. The ABL tyrosine kinase phosphorylates DGCR8 to stimulate pri-mir-34c processing to pre-mir-34c.

The biological functions of miRs are highly dependent on the cellular context, because each miR is designed to target a large battery of different mRNAs. With its interesting features of stability and versatility in target selection, DNA damage-induced microRNAs can play important roles in the execution, the maintenance, or the termination of downstream biological effects in DDR.

Chromatin Modifications

Chromatin modifications control the epigenetic landscape of a genome; thus, alterations in chromatin can have a profound and overarching influence on gene expression. Generally speaking, chromatin modifications involve regulations of: (a) the composition of histone isoforms in the nucleosomes; (b) the covalent modifications, for example, phosphorylation, acetylation, methylation, ubiquitination, sumoylation, etc., of histones; and (b) the positioning of nucleosomes. Each of these chromatin regulatory mechanisms is engaged in DDR to promote lesion recognition, repair, and signal transduction. As discussed above, PIKK-mediated phosphorylation of histone H2AX generates γH2AX (Fig. 3) that functions as a chromatin marker for the assembly of repair and signaling complexes. Phosphorylated γH2AX also recruits the INO80 complex that regulates the spacing of nucleosomes.

Cellular Differentiation

In DDR, the differentiation checkpoint describes a biological response in which DNA damage inhibits the differentiation of tissue stem cells (Fig. 5). This response has been observed in adult muscle satellite cells and embryonic muscle stem cells. This differentiation checkpoint delays terminal differentiation to allow time for DNA repair in lineage-committed muscle stem cells. With

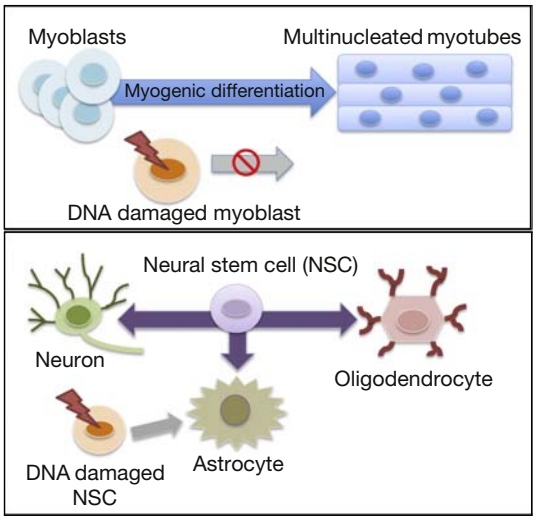

Fig. 5 Effects of DNA damage signaling on cellular differentiation. In committed muscle stem cells (myoblasts), DNA damage signal inhibits the myogenic transcription factors, in particular, MyoD, to block muscle differentiation. In multipotential neural stem cells, DNA damage signal promotes growth arrest and differentiation toward astrocytes but not other neural cell types.

multipotential neural stem cells, on the other hand, DNA damage stimulates growth arrest and accelerates differentiation toward astrocytes but not other neural cell types (Fig. 5). These findings show that DNA damage signals can regulate differentiation in stem cells and the effects are dependent on the stem cell types.

Cell Death

DNA damage activates distinct cell death mechanisms, including but not limited to apoptosis, necrosis, and mitotic death (premature senescence) (Fig. 6). Depending on the cell context, death induction can occur at widely different lesion concentrations. On one end of the spectrum is the induction of apoptosis by a single DSB. This hypersensitivity to DSB-induced apoptosis occurs in the developing central nervous system where a dose of ionizing radiation (IR) as low as 0.5 Gy is sufficient to activate ATM and p53-dependent apoptosis in neuroblasts within 6 h of irradiation. This hypersensitivity to IR-induced apoptosis is more of an exception than a rule as most mammalian cell types do not undergo apoptosis after 0.5 Gy of IR. On the other end of the spectrum is the resistance of tissue stem cells and terminally differentiated neurons or muscle cells to DNA damage-induced apoptosis. These resistances underlie the recovery of cancer patients after treatment with lesion-inducing agents, such as ionizing radiation (generates DSB) temozolomide (generates alkylated bases), cisplatin (generates ICL), and doxorubicin (generates DSB). The finding of such widely divergent sensitivity suggests that the death response in DDR is controlled not only by the concentrations and/or the persistence of DNA lesions, but it is also controlled by the proliferative status and the developmental lineages of the damaged cell (Fig. 6).

Apoptosis

The word "apoptosis" was coined to describe a specialized form of suicidal cell death that is characterized by the morphological features of chromatin condensation and degradation, as well as cell shrinkage and fragmentation. The condensed cell fragments (apoptotic bodies) express surface-exposed "eat-me" signals that stimulate their uptake by macrophages or other healthy cells. This death mechanism is employed in embryonic development to eliminate excess cells. Apoptosis also eliminates many (but not all) types of terminally differentiated cells throughout adult life.

In DDR, apoptosis induction requires alterations in gene expression (transcription initiation, alternative splicing, microRNA biogenesis). The most well established apoptotic pathway in DDR is that of p53-dependent transcription of pro-apoptotic genes, for example, PUMA (BBC3) and NOXA (PMAIP1). PUMA and NOXA are BH3-only members of the BCL2 family of apoptosis regulators. Accumulation of pro-apoptotic PUMA and NOXA proteins together with reduction in antiapoptotic BCL2, BCLxL proteins cause BAX/BAK-dependent release of cytochrome *c* from the mitochondria to activate the apoptosome and the effector caspases that bring about the morphological features of apoptosis.

It should be noted that p53 activation is an *immediate early* response in DDR, mediated by PIKK phosphorylation of MDM2 and p53. However, apoptosis execution is a *delayed* response and only occurs in some but not other cell types. Because activated p53 not only stimulates pro-apoptotic genes but also stimulates p21Cip1 to cause G1 arrest and premature senescence (Fig. 6), p53 activation is necessary but not sufficient to determine the choice of apoptosis in DDR. Generally speaking, DNA damage is more likely to induce apoptosis in highly regenerative tissue where there is continuous turnover of differentiated cells.

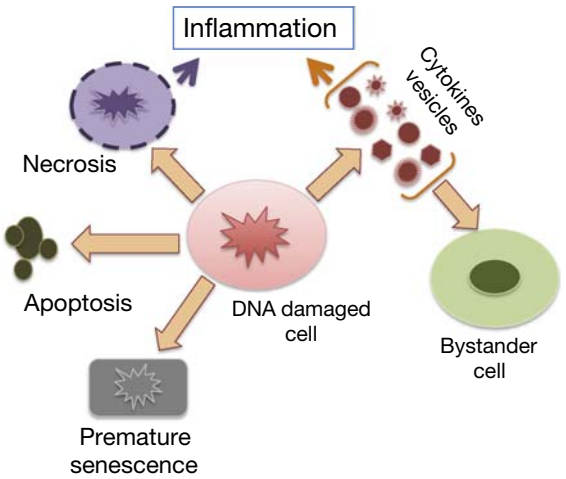

Fig. 6 Cell death choices, cell–cell communication, and the induction of inflammation in DDR. A cell suffering from DNA damage (red) can choose three types of death: namely necrosis, apoptosis, or premature senescence. The senescent cell is not physically dead but it is dead to mitosis as senescence is an irreversible cell fate. A damaged cell can communicate with neighboring bystander cell by secreting cytokines and vesicles. The DNA damage can also trigger tissue inflammatory responses that are stimulated by those factors secreted by the damaged cell or dumped by a damaged cell that underwent necrosis.

Even within a single cell type, the timing of apoptosis execution is variable, depending on the status of cell cycle. The best example to illustrate this timing variation is with neuroblasts (hypersensitive to IR-induced apoptosis) in the developing central nervous system. Experimenting with three different genetically engineered mouse strains that are knockout for ATM, p53, or with a p53 mutant lacking the ATM phosphorylation site, it can be established that IR-induced neuroblast apoptosis absolutely requires the ATM-p53 pathway. Although ATM phosphorylation of p53 is activated immediately after IR, apoptosis occurs 6 h after IR in neuroblasts that are out of S-phase at the time of irradiation. However, apoptosis only occurs 24 h after IR in neuroblasts that are in S-phase at the time of irradiation. How S-phase cells suppress apoptosis execution despite the activation of ATM and p53 is not yet understood.

Necrosis

Necrotic cell death is characterized by the loss of membrane integrity, cell bursting, and the release of cellular content. Necrosis can be accidental or regulated. The necrotic death that is actively induced by stress signals has been referred to as "*programmed necrosis*" or *necroptosis*. Upon infections or injuries, several inflammatory cytokines, for example, tumor necrosis factor-alpha (TNF), stimulate programmed necrosis of cells in the vicinity of the injuries to amplify the inflammatory signals. Either accidental or regulated, necrotic cell death contributes to the inflammatory responses triggered by infections or injuries (**Fig. 6**). In the most general and simplified terms, necrosis is activated when ATP (energy source) and/or NADH/NADPH (reducing power) become depleted and can no longer support the integrity of the plasma membrane. TNF-induced necrosis has been linked to the formation of a "mitochondrial attack complex," which may also mediate necrosis activated by calcium and reactive oxygen species.

In DDR, necrosis activation has been linked to the depletion of NAD by poly-ADP-ribose polymerase (PARP), which is a class of enzymes that convert NAD into poly-ADP-ribose chains on target proteins. PARP1 is an abundant chromatin protein that is activated by single-stranded ends in DNA and it can add poly-ADP-ribose onto a large number of proteins to stimulate DNA repair. Under conditions when excessive lesions or futile repair causes the accumulation and persistence of single-stranded ends, the continuous activation of PARP1 can lead to NAD depletion to trigger programmed necrosis. Other pathways linking DNA damage to regulated necrosis are under active investigation.

Mitotic death (premature senescence)

Mitotic death is a phrase coined by radiobiologists to describe IR-induced "death to mitosis." In the literature, this phrase is also used to describe *mitotic catastrophe* where cells die from catastrophic failure in chromosome segregation. In DDR, mitotic death and mitotic catastrophe are activated by different mechanisms. The death to mitosis is a premature senescence (**Fig. 6**), describing a prolonged or a permanent growth arrest in response to DNA damage. In DDR, premature senescence is induced in part through p53-dependent upregulation of p21Cip1, which inhibits Cdk2/Cyclin complexes to prevent phosphorylation of the RB family of pocket proteins, including RB, p107, and p130. The dephosphorylated RB-family proteins promote the assembly of repressive chromatin complexes at pro-proliferation genes to cause growth arrest. In DDR, mitotic catastrophe is induced when chromatin condensation and spindle assembly occur on incompletely replicated DNA to cause chromosome fragmentation. This event occurs as a result of failures to activate or to maintain the G2/M and/or the spindle checkpoints in cells suffering from DNA damage.

Cell–Cell Communications

The initiation of DDR occurs within the cell that suffers directly from damage to its genomic DNA. Interestingly, DDR can also be propagated to neighboring cells that are not directly targeted by genotoxins. This propagation of DDR is demonstrated by media transfer experiments, in which media conditioned by irradiated cells can activate DDR in naïve, non-irradiated, bystander cells. The radiation-induced bystander effects (BSE) result from cell–cell communications where irradiated cells produce factors to induce DDR in non-irradiated bystander cells. The directly irradiated cell can affect neighboring non-irradiated cells through gap junction communication. The directly irradiated cell can also secrete factors to communicate with other cells in its vicinity (**Fig. 6**). In whole animals, DNA damage inflicted in one part of the body can generate DDR at a distance from the site of damage. These "off-field" or "abscopal" effects may involve production of cytokines, chemokines, and vesicles by the DNA-damaged cells. For example, IR can stimulate the directly targeted cells to produce CSF1 that attracts macrophages to the tissue microenvironment where the DNA-damaged cells reside. These activated macrophages can then elaborate an inflammation reaction with long-range effects on other tissues. Because radiation and genotoxins have remained as the mainstay cancer therapeutic modalities, mechanistic understanding of the bystander and abscopal responses to DNA damage in the body is likely to provide new insights on how to mitigate the side effects and enhance the efficacy of cancer therapy.

Prospective Vision

Basic Research

DNA is the blue print for all biological processes; thus, it is not surprising to find that DNA damage activates a wide array of responses. This chapter describes a framework for DDR, from molecules to cells to organisms. The present framework, however,

is still rather primitive and based mostly on studies in model systems such as yeast, mouse, and cultured cells. While model system research is continuingly required to address many pressing questions on the molecular mechanisms of DDR, future attention should be paid to the human tissue and cell-type specificity of these processes in order for us to understand the relevance of DDR in human diseases. The DDR in different human cell types, tissues, and the whole organism needs to be investigated directly in the human systems. In particular, how cells of different developmental lineages and proliferative potential decide to live or to die, and how apoptotic versus necrotic cell death affects neighboring or distant cells, are important but as yet unsolved problems. Because DNA damaging agents are widely used to treat many different cancer types, the ongoing Cancer Genomics efforts in finding genetic and genomic determinants for tumor response to genotoxic therapies can be leveraged to expand the knowledge of DDR in the human body.

Cancer Translational Research

Knowledge gained from model organism research on DNA repair, which is the most highly conserved mechanism in DDR and is therefore readily applicable to the human system, has been translated to improve or develop cancer therapies.

The best example for how knowledge on DNA repair can impact cancer therapy is that of PARP inhibitors, which were developed to target cancer with defects in homologous recombination (HR). PARP (poly-ADP-ribose polymerase) is activated by single-stranded DNA ends to facilitate DNA repair. PARP is not found in yeast cells, and its activity is not essential to cells with competent HR repair However, HR-defective cells become dependent on PARP and are thus sensitive to the PARP inhibitors. In the clinic, PARP inhibitors have improved progression-free survival, although not overall survival, of patients with BRCA1 or BRCA2 mutant cancers. The development of PARP inhibitors to target HR-defective cancers has provided the proof of concept that DDR defects in cancer cells can be exploited to develop targeted therapies.

Cancer cells with HR defects are also hypersensitive to platinum-based drugs that generate ICLs in the genomic DNA. Because platinum resistance can be linked to recovery of HR function such as reversion of BRCA2 mutation back to wild type, deliberate disruption of HR function could in theory enhance the efficacy of platinum-based therapy. A flurry of research activities are ongoing in the public and the private sectors to find drugs that target DNA repair in cancer cells.

DNA repair defects can also impact cancer immunotherapy as exemplified by the increased response of MMR-defective cancers to immune checkpoint blockade antibodies. As discussed above, MMR corrects replication errors and MMR-defective cancer cells suffer from hypermutations. The increased mutation rate causes a higher probability in the expression of neo-antigens to trigger antitumor immunity. In patients with MMR-defective cancer, therapeutic blockade of immune checkpoints, therefore, is more likely to activate tumor-specific cytotoxic T-lymphocytes to kill tumor cells expressing neo-antigens that are generated as a result of DNA repair defects.

Given the already established therapeutic impacts from the current knowledge on DDR, it is not unreasonable to anticipate future developments of therapies to target not only repair but also the other DDR defects in cancer as we gain further understanding on DNA damage response in the human body.

Acknowledgment

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See also: Genetic Instability.

Further Reading

Friedberg, E.C., Walker, G.C., Siede, W., Wood, R.D., Schultz, R.A., Ellenberger, T., 2006. DNA repair and mutagenesis, 2nd ed. ASM Press, Washington, DC.
Article Series on "DNA Damage", Nature Reviews Molecular Cell Biology.
Blackford, A.N., Jackson, S.P., 2017. ATM, ATR and DNA-PK: The trinity at the heart of DNA damage response. Molecular Cell 66, 806–817.
Czabotar, P.E., Lessene, G., Strasser, A., Adams, J.M., 2014. Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. Nature Reviews Molecular Cell Biology 15, 49–63.
D.T. Le, J.N. Durham, et al., Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade, Science, https://doi.org/10.1126/science.aan6733.
Lord, C.J., Ashworth, A., 2017. PARP inhibitors: Synthetic lethality in the clinic. Science 355, 1152–1158.
Mukherjee, D., Coates, P.J., Lorimore, S.A., Wright, E.G., 2014. Responses to ionizing radiation mediated by inflammatory mechanisms. Journal of Pathology 232, 289–299.
Reinhardt, H.C., Yaffe, M.B., 2013. Phospho-Ser/Thr-binding domains: Navigating the cell cycle and DNA damage response. Nature Reviews. Molecular Cell Biology 14, 563–580.
Schneider, L., Pellegatta, S., Favaro, R., Pisati, F., Roncaglia, P., Testa, G., Nicolis, S.K., Finocchiaro, G., D'Adda Di Fagagna, F., 2013. DNA damage in mammalian neural stem cells leads to astrocytic differentiation mediated by BMP2 signaling through JAK-STAT. Stem Cell Reports 1, 123–138.
Seeber, A., Hauer, M., Gasser, S.M., 2013. Nucleosome remodelers in double-strand break repair. Current Opinion in Genetics and Development 23, 174–184.
Zhang, J., Walter, J.C., 2014. Mechanism and regulation of incisions during DNA interstrand cross-link repair. DNA Repair 19, 135–142.

Relevant Websites

PubMed: https://www.ncbi.nlm.nih.gov/pubmed: Search with the listed Keywords to find review and research articles on DDR.
GeneCards: http://www.genecards.org/: Search with Gene names to find basic information on genes mentioned in this article.
Google Images: https://www.google.com/: Search Google Images with "DNA damage response" to view pathway diagrams and research data relating to this topic.

Cervical Cancer: Screening, Vaccination, and Preventive Strategies

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Glossary

Ascertainment (second-level test) The test or set of tests used to ascertain the presence of a disease or clinically relevant lesion in women positive to first-level test and, in case of an algorithm including a triage test, to the triage test as well. In cervical cancer screening guidelines for high-income countries, ascertainment is always colposcopy with colposcopy-guided biopsy; see-and-treat strategies are not recommended. Treatment should follow a histological diagnosis on biopsy.

Cotesting This term defines a screening algorithm in which two primary screening tests are performed in parallel; women are managed according to the results of both tests. This strategy has been proposed for HPV and Pap test by the US guidelines. The rationale is to maximize sensitivity, although the number of false positives and the number of tests needed to screen the population do increase. It is the opposite of the triage strategy, in which two tests are performed in sequence, the second only if the first one is positive.

Detection rate The number of clinically relevant lesions found by screening divided by all the screened women.

Organized screening program An organized preventive intervention aimed at screening the population in which the target population, the testing protocols, including age to start and stop screening, interval between tests, management of abnormal tests and treatment are all clearly defined. A monitoring and quality assurance system is also in place. Programs are population-based if they actively invite the entire target population on a regular basis. The alternative model of care is opportunistic (or spontaneous) screening, where a physician takes the opportunity of any contact with each individual to recommend or prescribe the screening test.

Participation rate The proportion of women participating in the screening program out of the total target population. In the absence of an organized screening program, this indicator can be called test coverage or uptake; it is computed as the proportion of the population having had a test within the recommended interval.

Positive predictive value The probability that a woman who tests positive has a clinically relevant lesion, usually CIN2 or more severe.

Primary screening test The screening test that is performed first in the screening algorithm and the only one that is administered to the entire population. HPV DNA, Pap test and VIA are recommended as primary screening tests.

Referral rate The proportion of women that are referred to second-level test or ascertainment.

Regressive lesion In the natural history of cervical neoplasia, HPV-induced lesions often regress. Cervical intraepithelial neoplasia grade 1 (CIN1) is typically a regressive lesion; its probability of progression to a more severe lesion is so low that it is no longer considered a precancerous lesion. CIN grades 2 and 3, which are considered high-grade lesions and precancer, are highly regressive, but the balance between the risk of cancer and the very low morbidity related to the treatment favors surgical excision of these lesions. All guidelines therefore recommend it for CIN3 and most guidelines recommend it also for CIN2. In recent years, some authors have suggested that strict follow up can be appropriate for CIN2 in young women, when the probability of regression is higher.

Screening interval The period of time recommended between a negative screening test and the subsequent screening test. It defines the screening round, that is, the 3- or 5-year cycle in which a program should invite the whole target population to achieve complete invitation coverage.

Screening The systematic application of a test to a healthy population to detect a disease or its precursors early in order to begin treatment immediately, thereby improving prognosis or, in the case of targeting precursors of the disease, to avoid the onset of the disease.

Triage test In certain screening algorithms, women who test positive to the primary screening test are referred to a triage test in order to distinguish between those who need immediate ascertainment and those who can be followed up, thereby avoiding referring too many women to ascertainment test, which can be invasive and resource-consuming.

Nomenclature

CIN Cervical intraepithelial neoplasia. See regressive lesion in the glossary for a distinction between different grades of CIN.

High-risk HPV A restricted group of HPV types that infect human mucosae and that can induce cellular transformation. They are associated with some epithelial cancers in humans, and are virtually a necessary cause of cervical cancer. High-risk HPV types include the 12 types classified by the International Agency for Research on Cancer (IARC) as surely cancerogenic for humans (group 1): types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59. Some available commercial tests also include types 68 and 66, classified as probably (group 2a) and possibly (group 2b) cancerogenic, respectively, by IARC.

 https://doi.org/10.1016/B978-0-12-801238-3.65138-3

HPV Human papilloma virus. DNA capsuled virus including >200 types which infect human skin and mucosae. Some of them produce papillomas and other flat lesions, which are mostly asymptomatic.
HPV DNA test Test for the presence of HPV DNA. In screening, the test should target only high-risk types and be validated on clinical endpoints, that is, having high sensitivity and specificity for clinically relevant lesions and not for any HPV infection (Meijer et al., 2009).
Pap test Papanicolaou test is a cytological analysis of cells scraped from the cervix. Cells can be put directly on a slide, thus obtaining a smear, or suspended in a fixative medium and then transferred onto a slide in thin layer (liquid-based cytology).

Introduction

Cervical Cancer Burden of Disease: The Role of HPV and Screening

It is estimated that every year, >527,000 new cervical cancer cases and 265,672 deaths occur worldwide. The vast majority of the burden—84% of cases and 87% of deaths—occurs in less developed countries (Bruni et al., 2017). Cervical cancer affects relatively young women, with incidence peaking at between ages 40 and 55, then plateauing or decreasing slightly.

Geographic distribution is extremely heterogeneous, ranging from an age-standardized rate of >50/100,000 in some countries of East and Central Africa, to <4/100,000 in many North African and Middle Eastern countries (**Fig. 1**A). There are large differences within Europe as well: Switzerland, Finland, and some Mediterranean countries have low incidence (about 4/100,000), while many Eastern European countries have an incidence of >20/100,000 (Bruni et al., 2017; Li et al., 2011).

Both molecular biology and epidemiological studies have demonstrated that cervical cancer is causally linked to human papillomavirus (HPV) infection; persistent infection of oncogenic HPV types (hereafter named hrHPV) is a necessary cause for the onset of high-grade precancerous cervical lesions (cervical intraepithelial neoplasia, CIN grades 2 or 3) and progression to invasive cancer (zur Hausen, 1999; Walboomers et al., 1999).

Among the hrHPV types, HPV16 is responsible of >65% of cancers worldwide, followed by HPV18 (Clifford et al., 2005; Bruni et al., 2017; de Sanjose et al., 2010; Plummer et al., 2016). The distribution of HPV types does not necessarily reflect the prevalence of infections in the healthy population; in fact, as cervical cancer is a relatively rare consequence of a very common infection, the proportion of cancers due to each type is mostly related to the ability of a certain type to induce cellular transformation (de Sanjosé et al., 2007; Guan et al., 2012). Based on epidemiological studies on HPV infection, CIN and cancer incidence distribution by age and through modeling studies, it is possible to estimate that the process from infection to cancer takes decades (Schiffman and Wentzensen, 2013; Baussano et al., 2013; Bruni et al., 2017), although this time varies according to the virus and to host factors that are not yet fully understood. The natural history includes long-lasting precancerous tissue modifications that can be detected through cytological or molecular tests and treated (Schiffman et al., 2016). The combination of all these characteristics of the cervical cancer pathogenetic process makes it possible to design an effective screening strategy. Observational studies based on time trend and geographical differences, as well more analytical case–control and cohort studies have shown that screening with Pap test can effectively prevent the incidence of cervical cancer through the identification and conservative treatment of preinvasive lesions (IARC, 2005). The impact of population-based programs with high test coverage of the population can be very strong, with a >80% reduction of incidence (Anttila, 2007; Quinn et al., 1999).

The prevalence of HPV infection (**Fig. 1**B), the most important risk factor, and the diffusion of effective screening programs, which is to date the most impactful preventive measure, explain most of the differences in cervical cancer incidence (Li et al., 2011; Giorgi Rossi and Ronco, 2013). In particular, countries with low screening coverage or ineffective screening programs show high variability in cervical cancer incidence, according to the prevalence of hrHPV infection in the population in previous decades, while countries with high screening test coverage and effective programs (**Fig. 1**C), which assure correct follow up and treatment, all have incidence below 10/100,000, with very small differences even when HPV prevalence is very different (Giorgi Rossi and Ronco, 2013). This phenomenon can be observed in women immigrating to high-income countries as well: they maintain the risk of their origin country for about 10 years or more after arrival (high if coming from countries with high prevalence of HPV and low if they come from low prevalence regions); afterward, thanks to having undergone more than one screening round, their excess risk is dramatically reduced. This is true even for women from high prevalence countries (Azerkan et al., 2008; Mousavi et al., 2012; di Felice et al., 2015).

The sexual revolution at the end of 20th century caused an epidemic of HPV infection in most western countries, but the consequences of this epidemic in terms of cervical cancer incidence have been more or less completely controlled by the spread of well-organized screening programs. Several epidemiologic studies provide evidence of this phenomenon (Bray et al., 2005a, b; Peto et al., 2004).

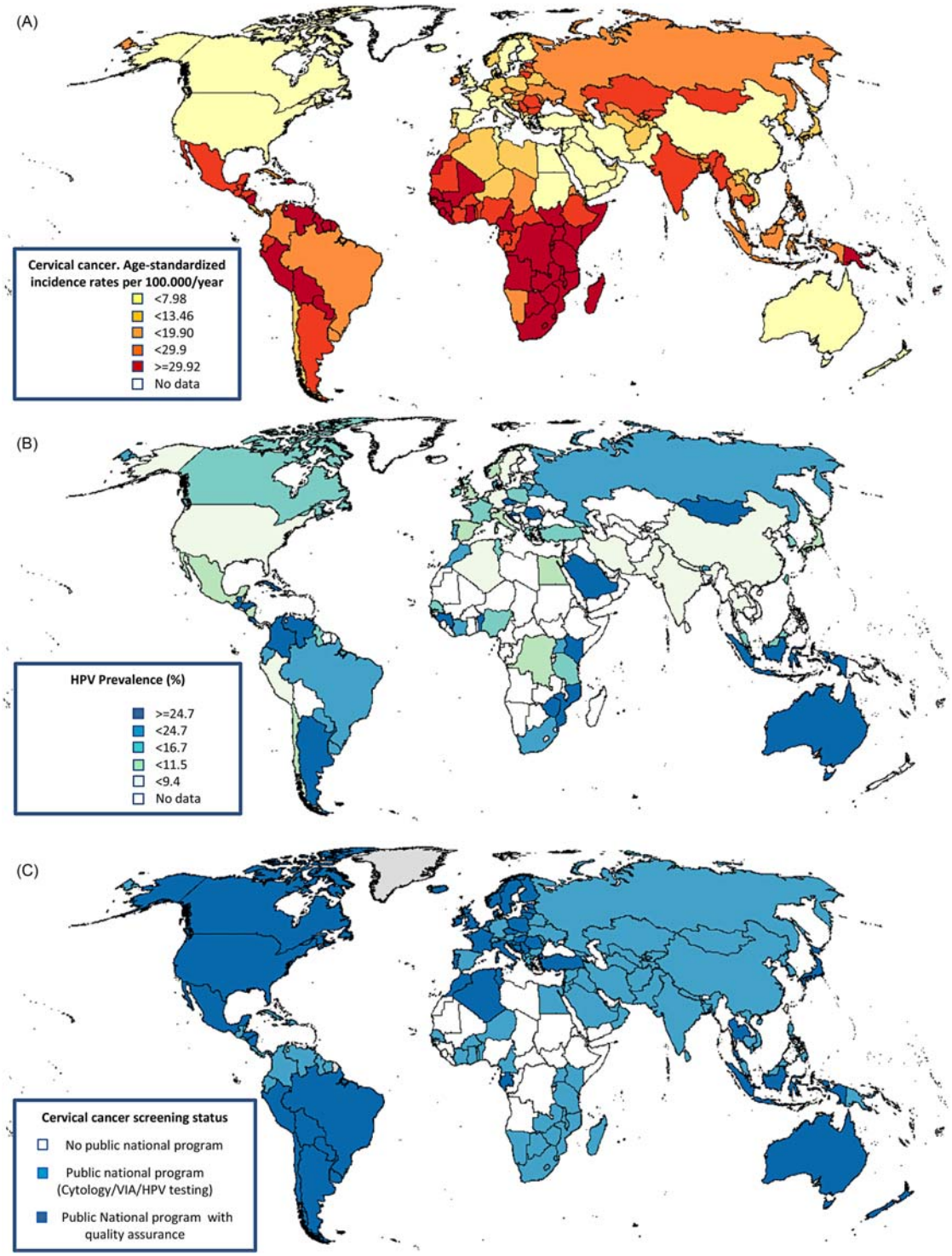

Fig. 1 Worldwide distribution of cervical cancer and its determinants. (A) Age-standardized incidence rates of cervical cancer in the world (estimates for 2012). Rates per 100,000 women per year. For Sudan, South Sudan: Estimate for Sudan and South Sudan. Data sources: Ferlay, J., Soerjomataram, I., Ervik, M., et al. (2013). *GLOBOCAN 2012 v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11*. Lyon, France: International Agency for Research on Cancer. (B). Prevalence of HPV among women with normal cervical cytology in the world. The samples for HPV testing come from cervical specimens (fresh/fixed biopsies or exfoliated cells). Systematic review updated 30 June 2015 (Bruni et al., 2017). (C) Worldwide status of cervical cancer screening programs. Availability of a cervical cancer screening program: Public national cervical cancer screening program in place (Cytology/VIA/HPV testing). Countries may have clinical guidelines or protocols, and cervical cancer screening services in the private sector but without a national public program. Publicly mandated programs have a law, official regulation, decision, directive or recommendation that provides the public mandate to implement the program with an authorized screening test, examination interval, target group and funding and copayment determined. Self-reported quality assurance: Organized programs provide for a national or regional team responsible for

Pathogenesis

The Natural History of the Disease: Preinvasive Phase

The identification of HPV infection as the necessary cause of cervical cancer has led to an enormous improvement in the knowledge of its pathogenesis. HPV infection has been identified as a carcinogen for other sites as well, but cervical cancer accounts for most of the HPV-related burden of disease and is the only cancer with virtually 100% of population attributable fraction to HPV.

More than 200 HPV genotypes have been identified and grouped into different genera. Papillomaviruses are classified into types depending on L1 sequencing; a new type is defined when its genome differs by at least 10% from that of all other classified types (Burk et al., 2013). Out of the five genera of HPVs, four (beta, gamma, mu and nu) contain only viruses that infect cutaneous epithelia. The fifth, the alpha genus, is unique in that it contains HPVs tropic to both cutaneous and mucosal epithelia. The oncogenic HPVs are a subset of the mucosotropic alpha-HPVs.

Most of our knowledge about the natural history of HPV infection comes from studies conducted on the epithelium of the uterine cervix. Twelve types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), also known as high-risk types, have been classified as certainly carcinogenic to humans according to the International Agency for Research on Cancer (WHO and IARC, 2012). Low-risk types, including HPV6 or HPV11, generally cause benign diseases such as genital warts (Egawa and Doorbar, 2017), while other types classified as probably or possibly carcinogenic are rarely found in large series of cancers, so their oncogenicity remains to be clarified (IARC, 2007).

In contrast to low-risk HPV types, hrHPV can transform infected cells, although only a minority of hrHPV-infected individuals will develop precancerous lesions or invasive carcinomas during their lives.

The papillomavirus family is a remarkably heterogeneous group of viruses that share the same genome structure and organization (Bravo and Felez-Sanchez, 2015). A circular double-stranded DNA genome of approximately 8 kb is structured into three main regions: (a) the early region (E) encodes genes that are necessary for the viral cycle and has an important role in cell transformation (E1, E2, E4, E5, E6, and E7); (b) the late region (L) encodes the L1 and L2 capsid proteins; (c) the upstream regulatory protein, referred to also as the long control region, is a noncoding region containing the replication origin and transcription factor-binding sites that contribute to regulate DNA replication by controlling the viral gene transcription.

The expression of E6 and E7, together with that of E1, E2, E4, and E5, is essential for viral genome replication and virion synthesis and release, but they also play a key role in cell transformation (Doorbar et al., 2012).

The HPV life cycle begins with the infection of the basal layer through microtraumas that compromise the epithelial barrier (Stubenrauch and Laimins, 1999). HPV genome is maintained at low copy number in the infected host basal cells. Upon differentiation of epithelial cells, the virus replicates to a high copy number and expresses the capsid genes (L1 and L2), resulting in the production of new progeny virions that are released from the epithelial surface. For persistence, HPV must infect basal cells showing stem cell-like features that are still able to proliferate (Egawa et al., 2015). This phenomenon is far less common in low-risk HPV types. The epithelial transition zones, and especially the endo-/ectocervix, are regions more susceptible to carcinogenesis by HPV type (Yang et al., 2015). High-risk types are more prone to activate cell proliferation in basal and differentiated layers, thereby promoting the transition from a productive infection to an infection that can activate several pathways essential for epithelial transformation (de Sanjose et al., 2017). One plausible explanation of the increased oncogenic capacity of the high-risk types resides in the activity of the E6 and E7 oncoproteins. Although E6 and E7 activity is present in high- and low-risk types, its role in low-risk types is limited to increasing viral fitness and viral production and is largely insufficient to trigger the development of preneoplastic lesions and cancer (Schiffman et al., 2016). Key functions of the HPV oncogenes are immune evasion, E7-mediated degradation of pRB family members, E6-mediated degradation of p53 and PDZ binding domain proteins and E6-mediated upregulation of telomerase.

The main role of early E6 and E7 proteins in the carcinogenic process is through their inhibition of p53 and pRB tumor suppressors (Psyrri and DiMaio, 2008; McLaughlin-Drubin and Munger, 2008). E6 functions also include the activation of telomerase activity and deregulation of pathways involved in immune system response, of epithelial differentiation, of cell proliferation and of survival signaling. Besides cell cycle deregulation and proliferation, E7 enhances genomic instability and promotes the accumulation of chromosomal abnormalities. The deregulation of the cell cycle, the activation of the telomerase activity and genomic instability create a favorable environment for epithelial cell transformation. HPV integration can also drive the carcinogenic process through the inactivation of the E2 expression, the main inhibitor of E6 and E7, and the disruption of host genes because of the viral

implementation and require providers to follow guidelines, rules, or standard operating procedures. They also define a quality assurance structure and mandate supervision and monitoring of the screening process. To evaluate impact, organized programs also require ascertainment of the population disease burden. Quality assurance consists of the management and coordination of the program throughout all levels of the screening process (invitation, testing, diagnosis and follow up of screen-positives) to assure that the program performs adequately and provides services that are effective and in line with program standards. The quality assurance structure is self-reported as part of the national cancer programs or plans. For some countries, when $<$50% of its regions does not have a quality assurance plan, the country is categorized as not having quality assurance (Bruni et al., 2017). From Bruni, L., Barrionuevo-Rosas, L., Albero, G. et al. (2017). *Human papillomavirus and related diseases in the world.* Summary report 27 July 2017 Barcelona: ICO Information Centre on HPV and Cancer (HPV Information Centre). (Accessed 13/11/2017).

sequence insertion (McBride and Warburton, 2017). The carcinogenic process, initiated with E6 and E7 activation, needs to be complemented by the accumulation of additional alterations in the host gene to lead to the invasive cancer phenotype.

Growing evidence suggests that epigenetic alterations directly or indirectly related with E6 and E7 activity are common events during the early phases of epithelial malignization, and have been described as potential biomarkers for cervical cancer (Mirabello et al., 2016; Clarke et al., 2012).

High-risk HPV types have developed several mechanisms to avoid host immune response, which is important for viral persistence and progression to HPV-associated neoplastic diseases. One of the first strategies to avoid detection is to maintain a very low profile (Stanley et al., 2012; Kanodia et al., 2007). The HPV cycle is exclusively intraepithelial and not lytic, thus preventing the associated proinflammatory signal. As a result, the recruitment of antigen-presenting cells such as Langerhans cells (LC) and the release of cytokines that mediate the immune response are absent or very low after HPV infection. Other mechanisms of HPV immune evasion include the regulation of interferon signaling, inhibition of LC by E6 and E7 activity, inhibition of adherence molecules such as the CDH1 and modulation of intracellular signaling pathways. At some point during most HPV infections, the cell-mediated immune system is alerted to the infection, thereby inducing regression of infected cells and lesions (Stanley, 2012). The role of the humoral immune response in natural infection is not clear; many infected individuals do not seroconvert, leaving them vulnerable to subsequent infection by the same virus. This is in contrast with the extremely high levels of humoral antibodies and the protection they provide, which results from immunization with the HPV vaccine.

In addition to the viral characteristics, environmental or exogenous factors have long been identified as modifiers of the natural history of HPV infections leading to cervical cancer. Many of the early case–control studies were conducted in populations with low screening coverage for cervical cancer. The most relevant cofactors identified at the time that increased cervical cancer risk were long-term smoking, multiparity (International Collaboration of Epidemiological Studies of Cervical Cancer, 2006) and long-term use of hormonal contraceptives (International Collaboration of Epidemiological Studies of Cervical Cancer et al., 2007), with an average increased risk in HPV-positive women of 1.5–2 times (Castellsagué et al., 2002). The fact that HPV infection has higher probability of persistence in smokers than in nonsmokers should alert women of the increased risk and reinforce the message to quit smoking. In HPV-positive long-term oral contraceptive users, a closer surveillance for cervical disease may be advisable. Coinfection with sexually transmitted infections such as Chlamydia trachomatis has been inconsistently associated with the risk of progression to cancer. HIV has always been associated with HPV as a major cofactor inducing cervical cancer. The mechanism has been largely associated to the immunosuppression conferred by active HIV infection and not by a direct effect of HIV. Many HIV-infected women are now following antiretroviral therapy (ART); it is therefore expected that good compliance with treatment will be followed by a reduction of cervical lesions and cervical cancer in this population (Kelly et al., 2015).

Stages of HPV Infection of the Uterine Cervix

Oncogenic human papillomaviruses can subsist in the epithelium in three major infection stages, referred to as latent, permissive (productive) and transforming infection (Doeberitz and Vinokurova, 2009; Doorbar, 2006; Doorbar et al., 2012). It is thought that the virus initially gains access to the epithelial basal cells via minor breaches in the epithelium (Kines et al., 2009) and that it can establish a long-term, persistent infection within these cells. Once taken up by the basal cells, the virus is transported to the nucleus, where the viral circular genome is released.

When these infected cells differentiate and move up toward the surface of the epithelium, high-level viral replication and gene expression is induced (**Fig. 2**). Virions are assembled in the superficial layers and are released from the epithelium in viral-laden squames. This strategy of infecting self-renewing cells ensures long-term viral persistence, while restricting high levels of viral proteins to more differentiated layers of the lesion is thought to help the virus escape detection by the immune system.

During the latent infection stage, the HPV genomes are believed to reside in the infected basal cells in an extra-chromosomal (episomal) physical state at low copy numbers (Kalantari et al., 2009; Maran et al., 1995). Viral activity appears to be highly restricted, potentially mediated by epigenetic silencing of the viral genome (Prigge et al., 2017). The latent stage of HPV infection generally remains clinically unapparent. However, it may account for reactivation of a clinically meaningful HPV infection later in life, for example, under circumstances of immunosuppression (Maglennon et al., 2014).

In contrast, the permissive stage of infection is characterized by high viral gene expression, replication of the virus at high copy numbers and packaging and release of newly synthesized infectious viruses at the epithelial surface (Doeberitz and Vinokurova, 2009). The expression of viral genes occurs in close relation to epithelial differentiation during the permissive infection stage. Expression of the HPV E6 and E7 oncogenes primarily occurs in the basal and suprabasal cell layers and is tightly controlled. This limited expression allows for controlled cellular proliferation, resulting in the multiplication of cells that carry viral genomes (Pyeon et al., 2009). In the more differentiated cells of the superficial cell layers, genome packaging and virus assembly occur under the increased expression of the HPV late genes L1 and L2. The infectious viruses are subsequently released from decaying, fully differentiated cells at the epithelial surface. From a histopathological point of view, the permissive infection stage is regularly accompanied by distinct morphologic hallmarks, the so-called koilocytes, which represent virus replicating cells and are characterized by nuclear hyperchromasia, perinuclear vacuolization and a thickened cytoplasmic rim. These cells are frequently found in cervical intraepithelial neoplasia grade 1 (CIN1) or low-grade squamous intraepithelial lesions of the uterine cervix (LSIL), respectively (Prigge et al., 2017).

A shift from the permissive to the transforming mode of infection may occur in a minority of HPV infections, at least through the start of a new infection from the virions released by the permissive infection. In contrast to the latent and permissive HPV infection

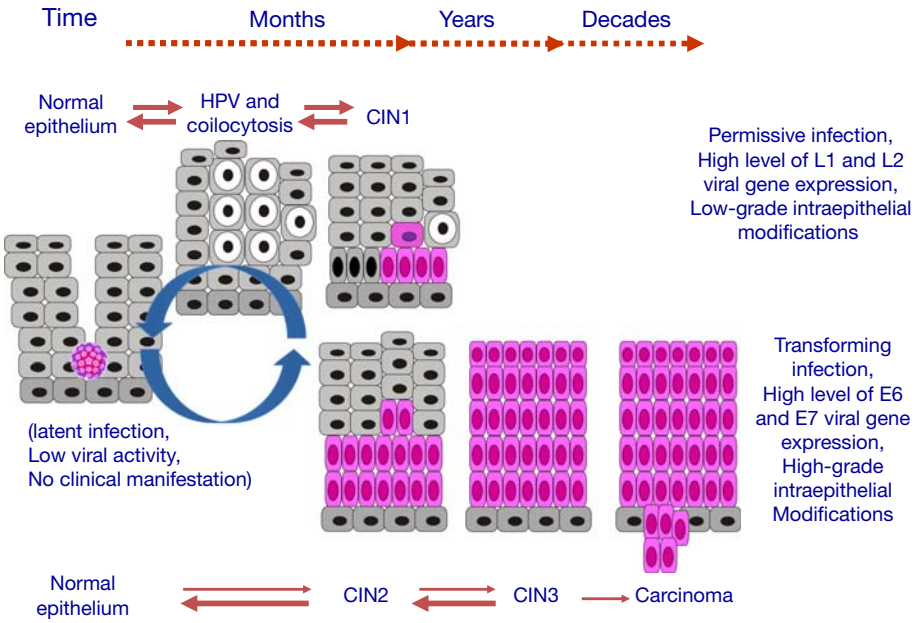

Fig. 2 Molecular and histological processes during HPV infection and neoplastic transformation in the cervix. Infection of basal cells is thought to occur through microtraumas that compromise the epithelial barrier. Infections can be latent, permissive, or transforming. During the latent infection, the HPV genomes are believed to reside in basal cells at low copy numbers; viral activity is restricted, and there are no clinical manifestations. However, latent infection may account for reactivation of a clinically meaningful HPV infection later in life, for example, under circumstances of immunosuppression. In permissive infections the virus replicates itself completely, leading to cellular death and releasing the virions from the epithelium in viral-laden squames; capside proteins encoded by the viral genes L1 and L2 are produced; the cells and the tissue morphology are that of low-grade intraepithelial neoplasia (CIN1 or LSIL); the process takes some months, and infections usually clear after 1 or 2 years. In the transforming infection the virus does not replicate itself, but tends to stimulate cell replication; the viral proteins coded by E6 and E7 genes are highly expressed; only E6 and E7 proteins of cancerogenic HPV types, that is, the high-risk types, can inhibit p53 and pRB tumor suppressors; cell and tissue morphology are that of high-grade intraepithelial lesions or cancer. The whole process from infection to invasion generally takes 10–40 years although, in a very few cases, it may only take only 2–3 years; nevertheless, regression of high-grade lesions is common. The distinction between the modes of infections occurs very early, implying that CIN1 is not considered a precancerous lesion. The presence of infecting virions in the permissive infection makes a shift from permissive to transforming infection possible, through the start of a new infection.

stages, the transforming stage is characterized by a substantial upregulation of HPV E6 and E7 oncogene expression. Clinically and histologically, the transforming HPV infection stage may present as CIN grades 2 or 3 or high-grade squamous intraepithelial lesions (HSIL, both considered as precancerous lesion) or invasive cancer. Herfs et al. suggest that the shift from a permissive to a transforming stage of infection is restricted to specific cells in the squamocolumnar junction zone that are characterized by a distinct gene expression profile (Herfs et al., 2012).

HPV Prevalence, Acquisition and Clearance in the Population

HPV prevalence shows substantial geographical, sexual behavior and age-dependent differences. The majority of anogenital HPV infections are acquired through sexual contact, and acquisition is strongly determined by the number of sexual partners and their respective sexual behavior. The probability of transmission per single episode of intercourse is high compared with other sexually transmitted infections, and it occurs with skin to skin or mucosa to mucosa contact. A new infection can be detected soon after the first sexual contact with an infected partner, and most infections will still be detectable 1 year later (Winer et al., 2008; Moscicki et al., 2010a); about 30% of young women become infected within 24 months of their sexual debut (Gravitt, 2011).

As a result of this high transmission rate, HPV infections are very common in women younger than 35 years (de Sanjosé et al., 2007), with a peak in prevalence generally around 20–25 years of age. It is estimated that the vast majority of sexually active women in western countries have at least one infection during their life. Transmission is also common among women having sex with women, suggesting a different way of transmission compared to other STDs.

An abrupt decline in the prevalence of infection follows as a result of frequent clearance and reduced exposure to new partners. This pattern is consistent in populations all over the world and confirms that sexual activity is the main mode of transmission. Instead, the prevalence of genital infections in men shows no clear age dependence (Giuliano et al., 2011a).

While persistent oncogenic HPV infections are a major risk factor for the subsequent development of HPV-induced high-grade intraepithelial neoplasia (CIN) or invasive carcinomas, clearance of HPV infections in the cervical regions is common.

Following women over time has demonstrated that up to half of HPV infections clear within 6 months and that the great majority (>90%) clear within a few years after acquisition (Rodríguez et al., 2010; Winer et al., 2011). Innate and adaptive immune responses are interplayers in the resolution of such common infections (Schiffman et al., 2016). A Th1 proinflammatory response in the genital tract has been suggested as a potential mechanism for clearance (Scott et al., 1999), but cell-mediated immune response may be the most common mechanism.

Several recent studies have observed a differential pattern of the vaginal microbiome, with more abundant *Lactobacillus* spp. among women clearing the infection compared with those with persistent infections (Shannon et al., 2017; Brotman et al., 2014; di Paola et al., 2017). Others have identified bacterial vaginosis associated with persistence of HPV infections among pregnant women (Kero et al., 2017). More data are needed to fully understand how HPV infections are modulated by the vaginal environment.

HPV antibodies acquired through natural infection are observed in a limited number of infected people. A systematic review of the acquired natural immunity against subsequent genital HPV infection identified a range of seroprevalence estimates against HPV16 of 6.2%–45.5%, with a relative modest protection for HPV16 reinfection of 0.72 (0.62–0.82); it is thus unlikely to play a major role in clearance (Beachler et al., 2016).

In some circumstances, after follow up of a positive HPV test, HPV may simulate a cleared infection and thus go undetected. It is now accepted that HPV infection may be latent and controlled by a cellular immune surveillance environment in which viruses are kept in very low copy numbers, escaping its detection. Reactivation may occur under moderate (menopausal state, aging) to relevant immunosuppressive situations (Maglennon et al., 2014), with or without hormonal influence. This reactivation could explain the second peak in HPV prevalence observed in postmenopausal women in some populations.

Only a proportion of persistent infections progress via several infection stages toward neoplasia (Moscicki et al., 2006, 2014; Nyitray et al., 2011).

Persistent carcinogenic HPV infection clearly predicts the risk of cervical cancer in women (Ronco et al., 2014; Khan et al., 2005). Although persistence does not have a standardized definition, is a very relevant concept, as many populations undergoing screening using HPV tests will rely on measures of persistent infection. Munoz et al. defined persistence as those infections that last more than the median duration, but this concept is relevant for natural history studies. Others define persistence as two consecutive positive HPV DNA tests with undetermined time interval (Marks et al., 2012; Munoz et al., 2009).

Besides age, strong risk factors for the acquisition and persistence of an HPV infection and the subsequent development of neoplasia are immunosuppression and tobacco abuse (Schiffman and Kjaer, 2003; Rositch et al., 2013).

Irrespective of the time window, major determinants of HPV persistence are HPV type and viral load at first detection. It remains unclear whether age is a key element in persistence. However, in the prospective study by Munoz et al. previously described, median duration of HPV incident infection was longer for high-risk HPV types than for low-risk types and for HPV16. Women younger than 30 years had a longer mean duration for HPV16 infections (16.6 months), while women >30 years had a significantly shorter duration (9.5 months). Others suggest that persistence increases with age (Castle et al., 2011a).

Moreover, the presence of concomitant HPV infections, a phenomenon very common in younger populations, is reported not to influence the duration of infection (Campos et al., 2011).

During the period of persistent infection, there is no apparent immune detection of the virus (Stanley, 2012). This is partly due to the viral life cycle itself, which ensures that high levels of viral activity occur only in the upper, differentiated cell layers that are not exposed to immune defenses.

Conclusions

The pathogenesis of cervical cancer historically has been interpreted as a continuum from infection to cancer in which progression to a more severe lesion was always less probable than regression until invasive cancer. The nomenclature reflects this interpretation: from normal epithelium to CIN1, CIN2 and CIN3, or from low-grade lesions to high-grade lesions. As has emerged in this section, the molecular interpretation of these morphological stages progressively suggests that in fact two different pathways can be distinguished in the course of an infection: the permissive and the transforming mode (**Fig. 2**).

Some indirect molecular and clinical evidence suggests that this distinction between the modes of infections occurs very early: the viral methylation pattern of infection without any colposcopically and histologically detected CIN that will develop into a CIN3 in the subsequent 3 years is more similar to that of CIN3 than to that of CIN1 and of many CIN2 (Ronco et al., 2017); the probability of progression to CIN2 or 3, given an hrHPV infection, is the same whether in the presence of no detectable histological abnormality or whether a CIN1 is found (Mittal et al., 2017, Giorgi Rossi et al., 2013; Castle et al., 2011b; Sørbye et al., 2011), suggesting that CIN1 is not a necessary step for progression to abortive infections, but only the manifestation of an HPV infection, and therefore it represents the same risk as any initial detection of the virus.

Cervical Cancer Prevention

The identification of HPV infection as a necessary cause of cervical cancer has opened the way to two new prevention tools: vaccines and a molecular test for screening. Although the focus of this article is the transformation of screening, it is impossible to address

this issue without a brief description of the characteristics of the available vaccine and of the main strategies adopted for HPV immunization worldwide.

While HPV testing is an improvement on an existing preventive measure, that is, screening with Pap test, vaccination is a completely new approach. Despite the fact that vaccination has the potential to decrease or to entirely eliminate the need for screening in the future, the two interventions will both be used until the near future. In fact, the vaccine is not effective in women who are infected, and mass vaccination has thus far been implemented for teenagers only (Obel et al., 2015; Dorleans et al., 2010; de Vuyst et al., 2015; GAVI Alliance Board, 2012; Bosch et al., 2016). Finally, to date, most women have been vaccinated only for HPV 16 and 18 and are thus still at risk of cervical cancer for other HPV types; this reduced but not negligible risk will require different, less intensive screening (Ronco and Giorgi Rossi, 2017; Franco et al., 2006, 2009; Cuzick et al., 2006). Screening in vaccinated women and how to implement these preventive measures more quickly is the focus of the "Prospective Vision" section of this article.

Vaccination

The existing vaccines and the proof of efficacy and safety

There are three registered vaccines against HPV: Cervarix (GlaxoSmithKline GSK, London, UK) covers HPV16 and 18; Gardasil (Merck, Kenilworth, New Jersey, USA) covers HPV 16, 18 and the low-risk types 6 and 11; Gardasil9 (Merck, Kenilworth, New Jersey, USA) covers the four types covered by Gardasil plus five other high-risk types: 31, 33, 45, 52 and 59. All the vaccines have shown almost complete protection against persistent vaccine-type HPV infections and precancerous lesions in studies recruiting 16–26-year-old women (Lehtinen et al., 2012; Joura et al., 2015). Efficacy was limited only to those women that were not infected at the time of vaccination. Studies on clinical outcomes have been conducted only in women older than 16, but the most interesting target population in order to prevent infections are preadolescent girls. Since it is impossible to observe clinically relevant outcomes in a population that is still not sexually active, studies in 9–12-year-old girls could only measure the immunogenicity of the vaccine. These so-called "bridging studies" have shown that vaccines are more immunogenic in girls than in young women (Schiller and Müller, 2015). So far, vaccines have shown long-term protection of up to at least 10 years (Elfstrom et al., 2014; Kjaer et al., 2009). More recent studies have shown protection also in women up to age 40 (Castellsagué et al., 2011; Skinner et al., 2014) and in males for precancerous anogenital lesions (Giuliano et al., 2011b). Cross protection against nonvaccine types, in particular against 31, 33 and 45 for Cervarix (Wheeler et al., 2011) and 31 for Gardasil (Brown et al., 2009), has been observed. The vaccines are safe, and no serious adverse effect associated with the vaccine has been demonstrated (Phillips et al., 2017).

Implementation strategies and their effectiveness

Vaccination of girls

The first and most adopted immunization policy against HPV has been vaccinating only girls before the onset of sexual activity (in most countries, around the 11th year of life) (Obel et al., 2015; Dorleans et al., 2010; de Vuyst et al., 2015). The rationale for this choice is to target cohorts that are virtually all HPV-naïve, but delaying the vaccination as long as possible to assure the longest vaccine protection possible, thereby avoiding any need for a booster dose. The first vaccination campaigns started in 2007 and 2008 in Australia and in some European countries; now, almost all industrialized countries include HPV in their routine vaccine schedule. This strategy has achieved high coverage in countries with active invitation and particularly in those with school-based campaigns (de Vuyst et al., 2015). Obviously, this strategy will show benefits, that is, cancer incidence reduction, only several years after the start of the vaccination campaigns, when these cohorts will reach the age where at least precancerous lesions are worth treating and when screening starts; the effect on cervical incidence will be observed even later.

Great emphasis has been given to equity in HPV vaccination for two reasons. The first is the intrinsic value given to equity of access to effective preventive measures, which all health systems recognize, particularly when vaccination is financed with public money. The second reason is the possible detrimental interaction of the inequity of access both to vaccination and to screening (Venturelli et al., 2017; Malagón et al., 2015). In the plausible hypothesis that those girls who are not vaccinated when they are 12 will also be those that do not participate in screening as adults, the impact of vaccination on the burden of disease in the population will be small. In fact, most invasive cervical cancers in many industrialized countries occur in the few women that are not screened or are under-screened (Sung et al., 2000; Leyden et al., 2005; Morrell et al., 2005; Bos et al., 2006; Andrae et al., 2008; Ingemann-Hansen et al., 2008; Lönnberg et al., 2010; Zucchetto et al., 2013). Furthermore, these women usually come from socioeconomically or culturally disadvantaged groups (Chiu, 2003; Giorgi Rossi et al., 2014); if the health system fails to vaccinate these women, it is missing the opportunity to really impact disease burden. Many authors have argued that the only way to be sure that vaccination is actually reaching a consistent part of these women is to have high population coverage, but results are inconsistent (Venturelli et al., 2017; Drolet et al., 2016).

Catch-up strategies

To accelerate the impact of vaccination campaigns, many countries have organized catch-up strategies, vaccinating girls aged 16 or even older. In most countries, these campaigns have achieved low coverage; in some cases, however, they have been quite successful (Pollock et al., 2014; Brotherton et al., 2015). These vaccinated cohorts of young women now make it possible to measure the effectiveness of the vaccine; the results are confirming the high protection of the vaccine against the targeted infection and related lesions, and in those areas with high coverage of the population, there is evidence of herd immunity (Drolet et al., 2015).

Vaccination of boys

Herd immunity is the main driver for the proposal of universal vaccination, that is, including boys and girls. With growing evidence of the involvement of HPV in a relevant proportion of head and neck cancers, in particular oropharynx (Plummer et al., 2016), it has become clear that the burden of vaccine-preventable disease in males is not negligible (de Martel et al., 2017). The vaccine has proven to be effective also on precancerous lesions of genital mucosae in males (Palefsky et al., 2011). Nevertheless, most models still predict that the largest benefit of vaccinating boys is the indirect effect on cervical cancer in women due to the reduction of circulating virus and to establishing of herd immunity faster (Brisson et al., 2011). The opportunity and cost effectiveness of vaccinating boys is still under debate, with the extreme heterogeneity of policies adopted in industrialized countries reflecting this uncertainty.

Given the very high incidence of HPV-related cancers in males having sex with males, there is consensus that vaccinating this group is opportune (Markowitz et al., 2014; Kirby, 2015; Sauvageau and Dufour-Turbis, 2016), even though implementing effective strategies to target high-risk populations without indirectly fostering discrimination or stigmatization is challenging.

Screening

The characteristics of cervical cancer's natural history have made screening with Pap test one of the most successful preventive interventions ever implemented in industrialized countries. As already discussed in the **Introduction** of this article, screening has completely changed the epidemiology of this disease in some countries, reducing both incidence and mortality, and making cervical cancer a rare disease. Nowadays there are three possible tests to screen for cervical cancer, according to WHO guidelines: Pap test, HPV test (alone or in parallel with Pap test, so-called co-testing) and the visual inspection of the cervix after application of acetic acid (VIA). VIA is recommended only for low-resource countries, as this type of screening is strongly influenced by the attempt to overcome the lack of human and technological resources. The strategies proposed for Pap test and HPV-based strategies have been developed for application in industrialized countries, with a few exceptions. The aim is to find the best balance between possible harms and benefits for women; sustainability is rarely an issue, given the very high cost effectiveness of cervical screening and that screening with very intensive protocols is already in place in most high-income countries.

In the following paragraph we will describe the recommendations given by the European Commission guidelines (Arbyn et al., 2008), with their recent update (Anttila et al., 2015), as an example of guidelines on how to implement organized screening programs, and of the most recent US guidelines as defined by the most influential scientific societies (ACS, ASCCCP, ASCP) (Saslow et al., 2012) and by the US Preventive Services Task Force (Moyer and US Preventive Services Task Force, 2012). For low-income countries, we report the 2013 recommendations of the World Health Organization (WHO, 2013).

Proof of efficacy and effectiveness

Pap test

The use of Pap test began to spread throughout most industrialized countries starting in the late 1960s, reaching very high population coverage by the end of the last century. Its introduction occurred in the absence of any experimental evidence of efficacy. Nevertheless, many observational studies demonstrated its effectiveness in reducing incidence and mortality just a few years after its introduction (IARC, 2005). The most convincing trend study on the effectiveness of Pap test screening reports the case of Finland, where organized screening was introduced in 1969, when opportunistic uptake of Pap test was still very low. Just 10 years after screening introduction, incidence decreased eightfold in the age class targets of screening (Anttila, 2007). The introduction of Pap test in many other countries was more gradual and the decrease in incidence was less dramatic. Nevertheless, most studies showed that an increase in Pap test coverage corresponded to a sharp decrease in cervical cancer incidence (Quinn et al., 1999; Serraino et al., 2015; Giorgi Rossi et al., 2015; Nygård et al., 2002). Case–control and cohort studies confirmed the trend studies (Sasieni et al., 2009; Ronco et al., 2005; Nieminen et al., 1999), and highlighted the additional protection of organized screening compared to opportunistic screening. (Ronco et al., 2005; Nieminen et al., 1999).

Some studies have also reported examples of ineffective screening with Pap test. Observational studies from Latin America showed very low impact on cervical cancer incidence (Herrero et al., 1992), mostly because of low participation by the target population (Hernández-Avila et al., 1998) and due to the low quality of cytology and follow-up procedures (Lazcano-Ponce et al., 1996). One large cluster randomized trial conducted in India found no reduction in cervical cancer incidence and mortality after only one Pap test compared to no intervention. However, the screening intervention in this trial was minimal and the authors suggested that the low effectiveness of Pap test and VIA could have been due to low compliance to follow up and treatment rates (Sankaranarayanan et al., 2009).

HPV test

HPV DNA in cervical cancer screening was first introduced as a triage test in cases of borderline (atypical squamous cells of undetermined significance, ASC-US) Pap tests (Solomon et al., 2001). A large body of evidence on HPV DNA test accuracy shows that it is much more sensitive, but less specific, than Pap test in detecting CIN2 or more severe lesions (Arbyn et al., 2012). Four large trials nested in population-based screening conducted during the first decade of this century tested whether the HPV test's higher sensitivity could also result in better prevention than Pap test screening (Naucler et al., 2007; Kitchener et al., 2009; Ronco et al., 2010; Rijkaart et al., 2012). In fact, the main endpoint of these four trials was the detection of high-grade lesions after the screening test.

Although none of these trials was powered to detect a difference in the incidence of invasive cancers after a negative screening test, an extremely rare event in regularly screened women, the pooled analysis of the four trials clearly showed a significant reduction in invasive cancer incidence in the HPV arm compared with the Pap test arm (Ronco et al., 2014). Furthermore, these trials were designed to gather information necessary to understand which screening algorithms could optimize the process, minimize colposcopy referral and the risk of overtreatment and maximize the preventive efficacy. Thus, the vast majority of women recruited in the experimental arm received both HPV and Pap test and were managed according to the results of both. The very small number of lesions found in women HPV-negative and Pap test-positive made it possible to definitely attribute cervical cancer incidence reduction to the HPV test and not to the combination of the two tests. On the other hand, some trials referred all HPV-positive women to colposcopy, regardless of Pap test results, while others referred only HPV- and Pap test-positive women to immediate colposcopy, the so-called triage strategy, and referred to short interval follow up women HPV-positive and cytology-negative. The reduction in CIN2 or more severe lesion at the following round was similar in those trials with immediate referral to colposcopy for all women and in those adopting a triage strategy (Ronco and Giorgi Rossi, 2017).

VIA

Visual inspection methods, including those with naked eye or with minimal magnification after acetic acid or Lugol, have been proposed as first-level screening test in low-resource settings because the cost of materials and reagents is very low and because no infrastructural resources are necessary. If included in a see-and-treat strategy, they can be used in a one-step screening that does not require follow up, an important consideration in remote rural areas.

Several studies have compared the accuracy of VIA versus HPV or cytology. Older studies, included in the systematic review conducted by the IARC (IARC, 2005), showed similar sensitivity and specificity of VIA compared with HPV test and similar sensitivity and lower specificity than cytology. However, the sensitivity of HPV test in these studies was implausibly low ($<80\%$) compared to what large population-based screening trials have now established. The recent systematic review by the WHO working group on cervical cancer prevention estimated a pooled sensitivity for HPV test of 95% versus 79% for VIA and HPV specificity of 84% versus 87% for VIA (WHO, 2013).

Apart from accuracy studies, one large randomized trial compared the effectiveness of one VIA, one HPV, or one cytology in a lifetime to no intervention at all. HPV was the only screening strategy that could reduce cervical cancer mortality with only one screening episode (Sankaranarayanan et al., 2009). As already commented for the Pap test, the researchers who conducted the study suggest that low compliance to treatment could explain the lack of effectiveness of VIA in this context. This could also explain the heterogeneity between this trial comparing VIA, Pap and HPV and the trials conducted in southern India a few years before, where VIA showed a 25% incidence reduction and 35% mortality reduction (Sankaranarayanan et al., 2007).

The protocols for screening now: Comparison of existing guidelines

High-income countries

Until 2010, according to guidelines developed for high-income countries the only recommended primary screening test was cytology, that is, the Pap test. It is still the most used test, even if most guidelines now recommend HPV as preferable primary screening test. The protocols now used for Pap test-based screening vary for starting age from 21 to 30 and for the interval from 1 to 5 years, with most guidelines recommending 3-year interval after a negative Pap test. The second-level test is colposcopy in order to guide biopsies. Treatment is only recommended for CIN2 or more severe lesions, while CIN1 should be followed up. Many guidelines recommend HPV test to triage ASC-US or borderline lesions. The trend is to introduce a less intensive protocol, primarily for two reasons: (1) the target populations in most industrialized countries has a very low prevalence after years of screening; (2) observational and modeling studies from Northern European countries have shown that screening programs with long intervals and very low colposcopy referral are extremely effective in reducing cancer risk (IARC, 2005).

With very few exceptions (Canadian Task Force on Preventive Health Care et al., 2013; Hamashima et al., 2010), the most recent guidelines in industrialized countries recommend HPV DNA test as primary screening. In some guidelines, Pap test-based and HPV-based strategies are considered comparable options (Moyer and US Preventive Services Task Force, 2012), while in other guidelines, HPV-based strategies are clearly identified as preferable (Anttila et al., 2015; Saslow et al., 2012).

Guidelines for the use of HPV are quite consistent regarding the starting age (not before 30) and interval for HPV-negative women (at least 5 years; **Fig. 3**). The main difference in the recommendations is the use of cotesting or a triage strategy. The European guidelines have adopted a triage strategy in which HPV is a stand-alone test, followed by cytology in case of positivity; women testing positive to cytology are referred to immediate colposcopy, while a follow up in 6 or 12 months is recommended for those with negative Pap test (Anttila et al., 2015). The US guidelines recommend cotesting with HPV and cytology in parallel; women who are HPV-positive and Pap test-negative are referred to repeat cotesting after 1 year, as are those who have negative HPV and low-grade cytology, while those with high-grade cytology, regardless of the HPV result, and those who are HPV-positive and have any kind of cytological abnormality are referred to immediate colposcopy (Saslow et al., 2012; Massad et al., 2013).

Low-income countries

In 2013, the WHO released new guidelines for screening, proposing a clear decision-making flowchart. According to these recommendations, whenever enough resources are available, HPV-based screening should be preferred to VIA, and cytology is recommended only if an ongoing program fulfilling the quality assurance criteria is in place (**Fig. 4**). VIA should be chosen only if there are no resources to implement HPV-screening or as second-level test after a positive HPV test. In this context, screen-and-treat approach is

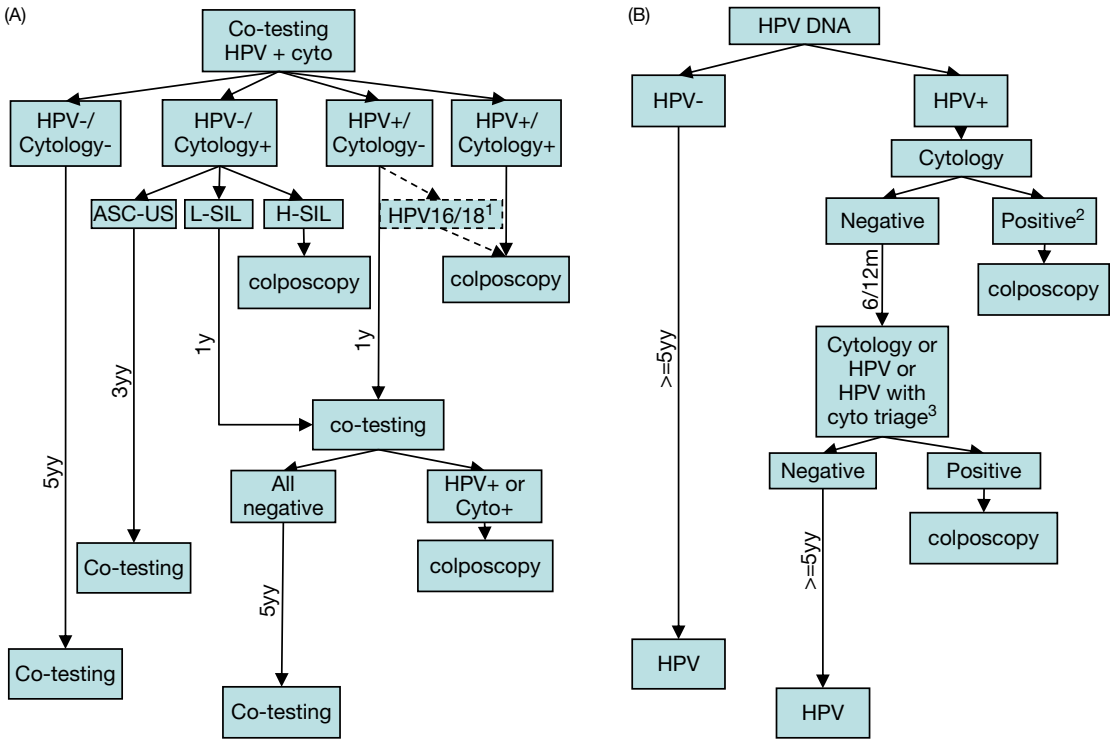

Fig. 3 Cervical cancer screening algorithms as recommended by US and European guidelines. (A) US algorithm according to USPSTF (Moyer and US Preventive Services Task Force, 2012) and multisociety guidelines by American Cancer Society, American Society for Colposcopy and Cervical Pathology, American Society of Clinical Pathology (Saslow et al., 2012). Management of HPV-negative/cytology-positive women is reported in the American Society for Colposcopy and Cervical Pathology guidelines (Massad et al., 2013). (B) European algorithm according to the 2015 supplements of the Quality Assurance Guidelines for Cervical Cancer Screening (Anttila et al., 2015). (1) Women with HPV 16 or 18 can be referred directly to colposcopy even if cytology is negative. (2) Positivity threshold can be set at abnormal squamous cells of undetermined significance (ASC-US) or at high-grade lesions only. Thus, immediate colposcopy or follow up are both possible options for borderline and low-grade lesions. (3) The following optimal managements are recommended for HPV-positive/cytology-negative women: cytology after 6/12 months; HPV followed by colposcopy if test positive not earlier than 12 months; HPV with cytology triage not earlier than 12 months.

recommended, that is, when there are no resources and facilities to perform a colposcopy and to obtain histological confirmation on biopsy, women who test positive can be treated immediately.

Treatment of precancerous lesions

As described previously, although the management of women with abnormal findings in cervical cancer screening is a complex process, when a clinically relevant lesion is found, it must be treated. In high-income countries, treatment should always follow a histological assessment on biopsy; see-and-treat approach during colposcopy is not recommended at all (Arbyn et al., 2008; Massad et al., 2013).

As explained above, CIN1 is no longer considered a precancerous lesion; treatment, therefore, is not recommended except for when there are some specific conditions, that is, cytological findings strongly suggesting the presence of a high-grade lesion that the biopsy has not captured or a lesion persisting >24 months (Arbyn et al., 2008; Massad et al., 2013). Treatment options should always be discussed with the woman.

Very few studies have directly measured the progression rate of CIN2, and even fewer that of CIN3. Only one unfortunate cohort of untreated CIN3 had a long enough follow up to measure a 30% probability of progression to cancer in 30 years (McCredie et al., 2008). For CIN2, some studies reporting the rate of regression with a relatively short follow up have inconsistent results (Uchimura et al., 2012; Moscicki et al., 2010b); certainly, the probability of regression is higher in women below 35 years of age (Ronco et al., 2006, 2008). CIN3 are considered lesions with a relevant risk of progression for which the balance between the benefit of preventing invasive cancer is definitely greater than the harms of the treatment. With a few exceptions, treatment is recommended by almost all guidelines for CIN2 as well. In some cases, strict follow up may be an option for young women because they have a higher probability of rapid regression and progression to cancer usually takes a long time.

The entire rationale for cervical cancer screening is based on the availability of very safe, noninvasive treatment options for precancerous lesions, making the balance between benefits and harms clearly in favor of the benefits, even when the probability of progression is quite low. Treatment options can be divided into ablative and excisional: in the former, as the tissue is destroyed, it is not available for postsurgery histological analysis; the latter maintains the integrity of the excised tissue. The most used

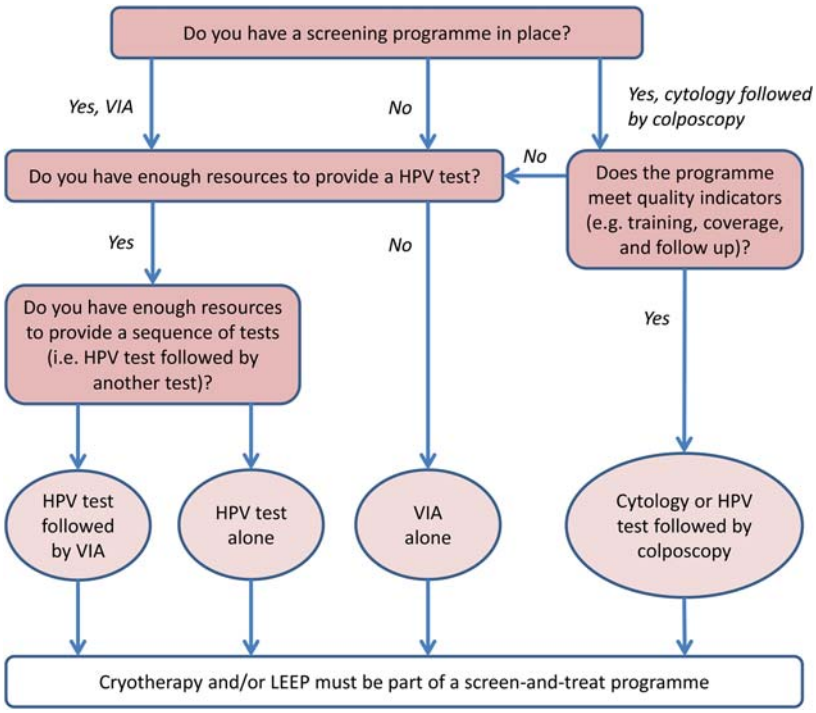

Fig. 4 Decision-making flowchart for screening program managers as defined by the WHO guidelines for screening and treatment of precancerous lesions for cervical cancer prevention. *Light pink bubbles* identify the screening strategy, from the left: HPV test followed by VIA for HPV-positive to triage those to be treated immediately and those to be rescreened after 1 year; HPV test alone and VIA alone are screen-and-treat strategies; cytology or HPV followed by colposcopy are strategies in which an assessment through colposcopy-guided biopsy is possible although not mandatory. No triage test is considered by these recommendations. HPV-negative women should be rescreened after at least 5 years; VIA or cytology-negative women should be rescreened after 3–5 years. From WHO. (2013). WHO guidelines for screening and treatment of precancerous lesions for cervical cancer prevention. Geneva: World Health Organization.

commonly techniques today are cryotherapy and laser ablation for ablative treatments, and large loop excision of transformation zone (LLETZ or LEEP, loop electrosurgical excision procedure), laser excisional conization, needle excision of the transformation zone (NETZ) and cold knife conization for the excisional treatments. These techniques are all effective, and a recent systematic review did not find strong differences in recurrence rate (IARC, 2005; WHO, 2014). The treatment-related morbidity is minimal and similar for all these outpatient procedures, except for cold knife conization, which is a more invasive technique and is now recommended only when other options are not feasible. However, these interventions have all proven to have an impact on pregnancy, doubling the probability of preterm birth. It is difficult to quantify precisely the effect of treatment on this outcome because CIN2 and 3 lesions are also associated with preterm birth; it is thus difficult to distinguish between the excess risk due to treatment and that related to the preexisting lesion itself. This excess risk is higher for cold knife conization, while the risk is very similar for other types of treatment. Clearly, the risk increases with the depth of the tissue excised (Kyrgiou et al., 2016).

Even though the harms of these treatments are very small compared to the consequence of an invasive cervical cancer, they are not negligible for women in their reproductive age. Therefore, the screening algorithm should minimize the probability of treating regressive lesions and false-positive lesions on biopsy, two different phenomena that nevertheless lead to the same kind of harms. False positives are an unavoidable consequence of any test; as for most tests, they are more frequent when the prevalence of the disease is low. Therefore, we can minimize false positives if we refer to colposcopy only those women with high prevalence of CIN2 or 3. In fact, it has been demonstrated that the probability of a false-positive biopsy, and thus of unnecessary treatment, increases when we refer to colposcopy women who have a very low probability of precancer, for example, HPV-negative women (Dalla Palma et al., 2008). The other cause of unnecessary treatment is over-diagnosis of regressive lesions. As mentioned previously, cervical cancer screening aims to treat precancer to avoid the incidence of cancer, and we accept that many of the lesions we treat will never become a cancer. Nevertheless, we should define protocols that try to minimize the diagnosis of regressive lesions to maintain the incidence of cancer as low as possible. Data from one of the European trials on HPV as primary screening, the ARTISTIC UK trial (Kitchener et al., 2009), clearly shows that lesions found in HPV-negative women are more regressive than HPV-positive lesions, at least when cytologists classify as positive a large proportion of the women, that is, they have low specificity. All these considerations led the working group that developed the European guidelines issued in 2015 (Anttila et al., 2015) to recommend against cotesting as primary screening strategy and to recommend an algorithm with HPV stand-alone followed by cytology triage. In this algorithm, only HPV-positive women are referred to colposcopy.

Prospective Vision

Screening in the Vaccine Era

Screening should be adapted to the new disease epidemiology that will emerge in those countries that have high vaccination coverage against HPV16/18 when cohorts of women who were vaccinated in their 12th year of life arrive at the target age of screening. For those countries that started vaccination campaigns in 2007/2008, this will happen in the early 2020s.

The main changes will be:

- >60% decrease in the prevalence of clinically relevant cervical lesions (Bruni et al., 2017; Li et al., 2011).
- About 25% decrease in low-grade and borderline lesions, which are the vast majority of abnormal Pap tests (Bruni et al., 2017; Li et al., 2011).
- As a consequence, a strong decrease in the positive predictive value of Pap test (Kiviat et al., 2008).
- Pap test sensitivity will also probably decrease because finding lesions among an enormous number of negative slides is as difficult as finding a needle in a haystack (Franco et al., 2009; Evans et al., 2011), the so-called prevalence bias.

Given that Pap test is still the only recommended screening test below the age of 30, this is an issue. It is worth to note that also the HPV test will decrease its positive predictive value, but much less than Pap test, since the positive predictive value of HPV test is not influenced by the prevalence of the disease (Giorgi Rossi et al., 2012).

On the other hand, we know that vaccinated women will have virtually no lesions from HPV16/18. These two HPV types are responsible for most cervical cancers overall, and in particular for early onset cancers, probably because their greater ability to transform the cell (Schiffman and Wentzensen, 2013) corresponds to a faster transition through the several progressive phases of the cancerogenic process and thus to a shorter lag time between infection and cancer. A lower absolute risk and longer lag time than in nonvaccinated women suggest that screening can safely be started later than today's protocols. The only recommendation produced to date according to an evidence-based process is to delay the age to start screening. This is based on the estimate that starting screening at age 30 in vaccinated women would maintain the same or slightly smaller number of prescreening cancers now observed in nonvaccinated women starting at 25 (Giorgi Rossi et al., 2017).

The same characteristics—low prevalence of disease and long lag time between infection and cancer—suggest that longer intervals should be adopted to maintain the same level of protection and to avoid overtreatment-related harms (**Fig. 5**).

The predicted low performance of Pap test and the probable increase of the screening starting age to 30 in those countries that now start screening at age 25 make the choice of HPV as primary test the most reasonable in vaccinated women.

The scientific and public health community is discussing whether changes in screening protocols should be applied to the entire target population (one-size-fits-all strategy) or only to vaccinated women (tailored strategy). Obviously, vaccination status is a strong predictor of cervical cancer risk and can define the best screening algorithm for each woman; in the framework of an organized screening program, information on administered vaccine doses should be linked with screening history to correctly invite vaccinated and nonvaccinated women according to two different algorithms (Giorgi Rossi et al., 2017).

On the other hand, herd immunity has been observed when coverage is high. Thus, beyond a certain level of vaccination coverage, the risk in nonvaccinated women will also be very low. Which coverage level and how long it should be sustained are subject matters for research. The answers are probably country-specific, as local sexual habits and social norms are important contributing factors (Baussano et al., 2016).

The here-described changes in screening protocols were developed to apply to women vaccinated before their sexual debut. For those vaccinated when they were already possibly HPV-infected, we know the vaccine is only effective in preventing new infections and has no effect on prevalent infections. To apply less intensive screening, algorithms should be designed that take this into consideration.

Finally, the introduction of 9-valent vaccine will further reduce cervical cancer risk in vaccinated women. How to screen, and whether it is opportune to do so, will be the next challenges of the scientific community.

Accelerating the Worldwide Control of Cervical Cancer

The proposed vaccination strategies, together with the very low diffusion of effective HPV-based screening in low-income countries, imply that the benefits of this revolution in cervical cancer prevention will only be enjoyed in the quite distant future. Trials of HPV vaccination in women aged up to 55 years have shown very high efficacy against HPV16/18-related precancer in women not infected by HPV16/18-DNA when vaccinated.

A group of authoritative researchers have advocated for extending routine vaccination programs to women of up to 30 years, paired with the use of HPV-screening with at least one test at age 30 years or older in low-resource countries and up to 5/6 screening episodes in high-income countries (**Fig. 5**) (Bosch et al., 2016).

Furthermore, HPV test has good accuracy also on self-collected samples (Arbyn et al., 2014), meaning that it is possible to develop new strategies to reach under-screened women, thereby increasing test coverage (Verdoodt et al., 2015).

All these strategies together have the potential to accelerate the decline in cervical cancer incidence and mortality in countries in Central and Eastern Europe, Latin America, Asia and Africa, where the burden of disease is higher because of the absence or low effectiveness of screening programs.

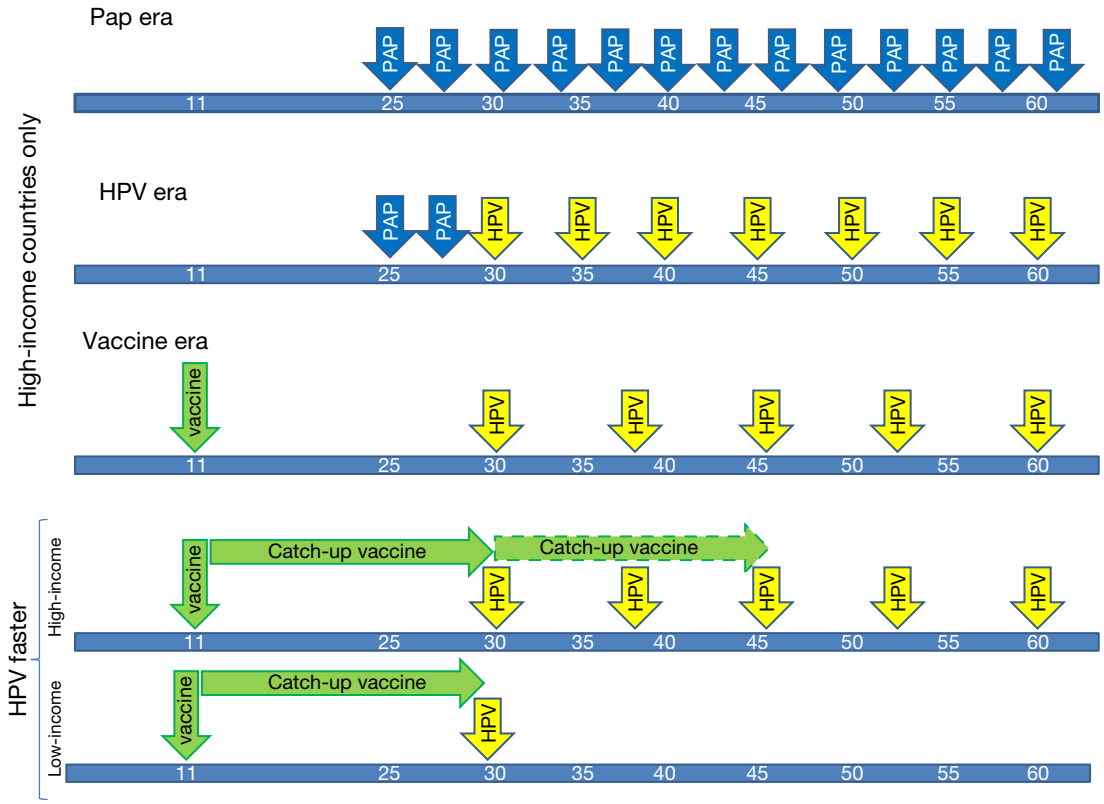

Fig. 5 The evolution of cervical cancer prevention. From programs based on Pap test requiring repeat test every 3 years, we now have HPV-based screening, recommended only after the age of 30, but with longer intervals (at least 5 years). In the near future, we will need to integrate vaccination and screening, with vaccinated women undergoing less intensive screening protocols which start later and which adopt longer intervals. Finally, the extension of more effective and faster strategies to reduce cervical cancer incidence in low-income countries as well, as proposed by Bosch et al. (2016) with the HPV faster program.

Most cost-effectiveness models suggest that the health benefits of extending the age of the vaccination target population (Jit et al., 2014; Brisson et al., 2016) have a very low cost, although this is still a subject matter of research. Nevertheless, this extension requires resources that are difficult to obtain in most of those settings that would reap the largest benefits; budget impact is thus much more an issue than is cost effectiveness.

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See also: Cancer Risk Reduction Through Lifestyle Changes.

References

Andrae, B., Kemetli, L., Sparén, P., et al., 2008. Screening-preventable cervical cancer risks: Evidence from a nationwide audit in Sweden. Journal of the National Cancer Institute 100 (9), 622.

Anttila, A., 2007. Cervical cancer screening is effective—The Finnish experience. Entre Nous 64, 26–28.

Anttila, A., Arbyn, A., de Vuyst, H., et al. (Eds.), 2015. European guidelines for quality assurance in cervical cancer screening, 2nd ed. Office for Official Publications of the European Union, Luxembourg. Supplements.

Arbyn, M., Anttila, A., Jordan, J., et al. (Eds.), 2008. European guidelines for quality assurance in cervical cancer screening, 2nd ed. Office for Official Publications of the European Communities, Luxembourg.

Arbyn, M., Ronco, G., Anttila, A., et al., 2012. Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. Vaccine 30 (Suppl 5), F88–99.

Arbyn, M., Verdoodt, F., Snijders, P.J., et al., 2014. Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: A meta-analysis. The Lancet Oncology 15, 172–183.

Azerkan, F., Zendehdel, K., Tillgren, P., Faxelid, E., Sparén, P., 2008. Risk of cervical cancer among immigrants by age at immigration and follow-up time in Sweden, from 1968 to 2004. International Journal of Cancer 123, 2664–2670.

Baussano, I., Elfström, K.M., Lazzarato, F., et al., 2013. Type-specific human papillomavirus biological features: Validated model-based estimates. PLoS One 8, e81171.
Baussano, I., Lazzarato, F., Brisson, M., Franceschi, S., 2016. Human papillomavirus vaccination at a time of changing sexual behaviour. Emerging Infectious Diseases 22, 18–23.
Beachler, D.C., Jenkins, G., Safaeian, M., et al., 2016. Natural acquired immunity against subsequent genital human papillomavirus infection: A systematic review and meta-analysis. The Journal of Infectious Diseases 213, 1444–1454.
Bos, A.B., Rebolj, M., Habbema, J.D., van Ballegooijen, M., 2006. Nonattendance is still the main limitation for the effectiveness of screening for cervical cancer in the Netherlands. International Journal of Cancer 119, 2372–2375.
Bosch, F.X., Robles, C., Díaz, M., et al., 2016. HPV-FASTER: Broadening the scope for prevention of HPV-related cancer. Nature Reviews. Clinical Oncology 13, 119–132.
Bravo, I.G., Felez-Sanchez, M., 2015. Papillomaviruses: Viral evolution, cancer and evolutionary medicine. Evolution, Medicine, and Public Health 2015, 32–35.
Bray, F., Carstensen, B., Møller, H., et al., 2005a. Incidence trends of adenocarcinoma of the cervix in 13 European countries. Cancer Epidemiology, Biomarkers & Prevention 14 (9), 2191.
Bray, F., Loos, A.H., McCarron, P., et al., 2005b. Trends in cervical squamous cell carcinoma incidence in 13 European countries: Changing risk and the effects of screening. Cancer Epidemiology, Biomarkers & Prevention 14, 677–686.
Brisson, M., van de Velde, N., Franco, E.L., Drolet, M., Boily, M.C., 2011. Incremental impact of adding boys to current human papillomavirus vaccination programs: Role of herd immunity. The Journal of Infectious Diseases 204, 372–376.
Brisson, M., Bénard, É., Drolet, M., et al., 2016. Population-level impact, herd immunity, and elimination after human papillomavirus vaccination: A systematic review and meta-analysis of predictions from transmission-dynamic models. Lancet Public Health 1, e8–e17.
Brotherton, J.M.L., Saville, A.M., May, C.L., Chappell, G., Gertig, D.M., 2015. Human papillomavirus vaccination is changing the epidemiology of high-grade cervical lesions in Australia. Cancer Causes & Control 26, 953–954.
Brotman, R.M., Shardell, M.D., Gajer, P., et al., 2014. Interplay between the temporal dynamics of the vaginal microbiota and human papillomavirus detection. The Journal of Infectious Diseases 210, 1723–1733.
Brown, D.R., Kjaer, S.K., Sigurdsson, K., et al., 2009. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16–26 years. The Journal of Infectious Diseases 199, 926–935.
Bruni, L., Barrionuevo-Rosas, L., Albero, G. et al. (2017). Human papillomavirus and related diseases in the world. Summary report 27 July 2017 Barcelona: ICO Information Centre on HPV and Cancer (HPV Information Centre). (Accessed 13/11/2017)
Burk, R.D., Harari, A., Chen, Z., 2013. Human papillomavirus genome variants. Virology 445, 232–243.
Campos, N.G., Rodriguez, A.C., Castle, P.E., et al., 2011. Persistence of concurrent infections with multiple human papillomavirus types: A population-based cohort study. The Journal of Infectious Diseases 203, 823–827.
Canadian Task Force on Preventive Health Care, Dickinson, J., Tsakonas, E., et al., 2013. Recommendations on screening for cervical cancer. CMAJ 185, 35–45.
Castellsagué, X., Bosch, F.X., Munoz, N., 2002. Environmental co-factors in HPV carcinogenesis. Virus Research 89, 191–199.
Castellsagué, X., Muñoz, N., Pitisuttithum, P., et al., 2011. End-of-study safety, immunogenicity, and efficacy of quadrivalent HPV (types 6, 11, 16, 18) recombinant vaccine in adult women 24–45 years of age. British Journal of Cancer 105, 28–37.
Castle, P.E., Gage, J.C., Wheeler, C.M., Schiffman, M., 2011a. The clinical meaning of a cervical intraepithelial neoplasia grade 1 biopsy. Obstetrics and Gynecology 118, 1222–1229.
Castle, P.E., Rodríguez, A.C., Burk, R.D., et al., 2011b. Long-term persistence of prevalently detected human papilloma-virus infections in the absence of detectable cervical precancer and cancer. The Journal of Infectious Diseases 203, 814–822.
Chiu, L.F., 2003. Inequalities of access to cancer screening: A literature review. Cancer Screening Series N° 1. NHS Cancer Screening Programmes, Sheffield.
Clarke, M.A., Wentzensen, N., Mirabello, L., et al., 2012. Human papillomavirus DNA methylationas a potential biomarker for cervical cancer. Cancer Epidemiology, Biomarkers & Prevention 21, 2125–2137.
Clifford, G.M., Gallus, S., Herrero, R., et al., 2005. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: A pooled analysis. Lancet 366, 991–998.
Cuzick, J., Mayrand, M.H., Ronco, G., Snijders, P., Wardle, J., 2006. Chapter 10: New dimensions in cervical cancer screening. Vaccine 24 (Suppl 3), S90–97.
Dalla Palma, P., Giorgi Rossi, P., Collina, G., et al., 2008. The risk of false-positive histology according to the reason for colposcopy referral in cervical cancer screening: A blind revision of all histologic lesions found in the NTCC trial. American Journal of Clinical Pathology 129, 75–80.
Doeberitz, M., Vinokurova, S., 2009. Host factors in HPV-related carcinogenesis: Cellular mechanisms controlling HPV infections. Archives of Medical Research 40, 435–444.
Doorbar, J., 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clinical Science (London, England) 110, 525–541.
Doorbar, J., Quint, W., Banks, L., et al., 2012. The biology and life-cycle of human papillomaviruses. Vaccine 30 (Suppl 5), F55–70.
Dorleans, F., Giambi, C., Dematte, L., et al., 2010. The current state of introduction of human papillomavirus vaccination into national immunisation schedules in Europe: First results of the VENICE2 2010 survey. Euro Surveillance 15 pii: 19730.
Drolet, M., Bénard, É., Boily, M.C., et al., 2015. Population level impact and herd effects following human papillomavirus vaccination programmes: A systematic review and meta-analysis. The Lancet Infectious Diseases 15, 565–580.
Drolet, M., Deeks, S.L., Kliewer, E., et al., 2016. Can high overall human papillomavirus vaccination coverage hide sociodemographic inequalities? An ecological analysis in Canada. Vaccine 34, 1874–1880.
Egawa, N., Doorbar, J., 2017. The low-risk papillomaviruses. Virus Research 231, 119–127.
Egawa, N., Egawa, K., Griffin, H., et al., 2015. Human papillomaviruses; epithelial tropisms, and the development of neoplasia. Virus 7, 386–390.
Elfstrom, K.M., Smelov, V., Johansson, A.L., et al., 2014. Long term duration of protective effect for HPV negative women: Follow-up of primary HPV screening randomised controlled trial. BMJ 348, g130.
Evans, K.K., Tambouret, R.H., Evered, A., Wibur, D.C., Wolfe, J.M., 2011. Prevalence of abnormalities influences cytologists' error rates in screening for cervical cancer. Archives of Pathology & Laboratory Medicine 135, 1557–1560.
di Felice, E., Caroli, S., Paterlini, L., et al., 2015. Cervical cancer epidemiology in foreign women in Northern Italy: Role of human papillomavirus prevalence in country of origin. European Journal of Cancer Prevention 24, 223–230.
Franco, E.L., Cuzick, J., Hildesheim, A., de Sanjosé, S., 2006. Chapter 20: Issues in planning cervical cancer screening in the era of HPV vaccination. Vaccine 24 (Suppl 3), S171–77.
Franco, E.L., Mahmud, S.M., Tota, J., Ferenczy, A., Coutlée, F., 2009. The expected impact of HPV vaccination on the accuracy of cervical cancer screening: The need for a paradigm change. Archives of Medical Research 40, 478–485.
GAVI Alliance Board, 2012. The announcement by the GAVI Alliance Board to support human papillomavirus (HPV) vaccines in the world's poorest countries. Vaccine 30 (Suppl 4), xii.
Giorgi Rossi, P., Ronco, G., 2013. Future of cervical cancer screening in Europe. Current Pharmaceutical Design 19, 1490–1497.
Giorgi Rossi, P., Franceschi, S., Ronco, G., 2012. HPV prevalence and accuracy of HPV testing to detect high-grade cervical intraepithelial neoplasia. International Journal of Cancer 130, 1387–1394.
Giorgi Rossi, P., Benevolo, M., Vocaturo, A., et al., 2013. Prognostic value of HPV E6/E7 mRNA assay in women with negative colposcopy or CIN1 histology result: A follow-up study. PLoS One 8, e57600.
Giorgi Rossi, P., Baldacchini, F., Ronco, G., 2014. The possible effects on socio-economic inequalities of introducing HPV-testing as primary test in cervical cancer screening programs. Frontiers in Oncology 4, 20.

Giorgi Rossi, P., Caroli, S., Mancini, S., et al., 2015. Screening history of cervical cancers in Emilia-Romagna, Italy: Defining priorities to improve cervical cancer screening. European Journal of Cancer Prevention 24, 128–134.
Giorgi Rossi, P., Carozzi, F., Federici, A., et al., 2017. Cervical cancer screening in women vaccinated against human papillomavirus infection: Recommendations from a consensus conference. Preventive Medicine 98, 21–30.
Giuliano, A.R., Lee, J.H., Fulp, W., et al., 2011a. Incidence and clearance of genital human papillomavirus infection in men (HIM): A cohort study. Lancet 377, 932–940.
Giuliano, A.R., Palefsky, J.M., Goldstone, S., et al., 2011b. Efficacy of quadrivalent HPV vaccine against HPV infection and disease in males. The New England Journal of Medicine 364, 401–411.
Gravitt, P.E., 2011. The known unknowns of HPV natural history. The Journal of Clinical Investigation 121, 4593–4599.
Guan, P., Howell-Jones, R., Li, N., et al., 2012. Human papillomavirus types in 115,789 HPV-positive women: A metaanalysis from cervical infection to cancer. International Journal of Cancer 131, 2349–2359.
Hamashima, C., Aoki, D., Miyagi, E., et al., 2010. The Japanese guideline for cervical cancer screening. Japanese Journal of Clinical Oncology 40, 485–502.
zur Hausen, H., 1999. Immortalization of human cells and their malignant conversion by high risk human papillomavirus genotypes. Seminars in Cancer Biology 9, 405–411.
Herfs M., Y. Yamamoto, A. Laury, X., et al. (2012). A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. Proceedings of the National Academy of Sciences of the United States of America 109, 10516–10521.
Hernández-Avila, M., Lazcano-Ponce, E.C., de Ruíz, P.A., Romieu, I., 1998. Evaluation of the cervical cancer screening programme in Mexico: A population-based case-control study. International Journal of Epidemiology 27, 370–376.
Herrero, R., Brinton, L.A., Reeves, W.C., et al., 1992. Screening for cervical cancer in Latin America: A case-control study. International Journal of Epidemiology 21 (6), 1050.
IARC, 2005. Handbooks of cancer prevention Vol. 10 Cervix cancer screening. IARC, Lyon.
IARC, 2007. Working group on the evaluation of carcinogenic risks to humans, vol. 90. IARC, Lyon.
Ingemann-Hansen, O., Lidang, M., Niemann, I., et al., 2008. Screening history of women with cervical cancer: A 6-year study in Aarhus, Denmark. British Journal of Cancer 98, 1292–1294.
International Collaboration of Epidemiological Studies of Cervical Cancer, 2006. Cervical carcinoma and reproductive factors: Collaborative reanalysis of individual data on 16,563 women with cervical carcinoma and 33,542 women without cervical carcinoma from 25 epidemiological studies. International Journal of Cancer 119, 1108–1124.
International Collaboration of Epidemiological Studies of Cervical Cancer, Appleby, P., Beral, V., et al., 2007. Cervical cancer and hormonal contraceptives: Collaborative reanalysis of individual data for 16,573 women with cervical cancer and 35,509 women without cervical cancer from 24 epidemiological studies. Lancet 370, 1609–1621.
Jit, M., Brisson, M., Portnoy, A., Hutubessy, R., 2014. Cost-effectiveness of female human papillomavirus vaccination in 179 countries: A PRIME modelling study. The Lancet Global Health 2, e406–14.
Joura, E.A., Giuliano, A.R., Iversen, O.E., et al., 2015. A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. The New England Journal of Medicine 372, 711–723.
Kalantari, M., Garcia-Carranca, A., Morales-Vazquez, C.D., et al., 2009. Laser capture microdissection of cervical human papillomavirus infections: Copy number of the virus in cancerous and normal tissue and heterogeneous DNA methylation. Virology 390, 261–267.
Kanodia, S., Fahey, L.M., Kast, W.M., 2007. Mechanisms used by human papillomaviruses to escape the host immune response. Current Cancer Drug Targets 7, 79–89.
Kelly, H., Mayaud, P., de Sanjose, S., 2015. Concomitant infection of HIV and HPV: What are the consequences? Current Obstetrics and Gynecology Reports 4, 213–219.
Kero, K., Rautava, J., Syrjanen, K., et al., 2017. Association of asymptomatic bacterial vaginosis with persistence of female genital human papillomavirus infection. European Journal of Clinical Microbiology & Infectious Diseases 36, 2215–2219.
Khan, M.J., Castle, P.E., Lorincz, A.T., et al., 2005. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. Journal of the National Cancer Institute 97, 1072–1079.
Kines, R.C., Thompson, C.D., Lowy, D.R., Schiller, J.T., Day, P.M., 2009. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proceedings of the National Academy of Sciences of the United States of America 106, 20458–20463.
Kirby, T., 2015. UK committee recommends HPV vaccination for MSM. The Lancet Oncology 16, e7.
Kitchener, H.C., Almonte, M., Thomson, C., et al., 2009. HPV testing in combination with liquid-based cytology in primary cervical screening (ARTISTIC): A randomised controlled trial. The Lancet Oncology 10, 672–682.
Kiviat, N.B., Hawes, S.E., Feng, Q., 2008. Screening for cervical cancer in the era of the HPV vaccine—The urgent need for both new screening guidelines and new biomarkers. Journal of the National Cancer Institute 100, 290–291.
Kjaer, S.K., Sigurdsson, K., Iversen, O.E., et al., 2009. A pooled analysis of continued prophylactic efficacy of quadrivalent human papillomavirus (types 6/11/16/18) vaccine against high-grade cervical and external genital lesions. Cancer Prevention Research (Philadelphia, Pa.) 2, 868–878.
Kyrgiou, M., Athanasiou, A., Paraskevaidi, M., et al., 2016. Adverse obstetric outcomes after local treatment for cervical preinvasive and early invasive disease according to cone depth: Systematic review and meta-analysis. BMJ 354, i3633.
Lazcano-Ponce, E.C., Rascón-Pacheco, R.A., Lozano-Ascencio, R., Velasco-Mondragón, H.E., 1996. Mortality from cervical carcinoma in Mexico: Impact of screening, 1980-1990. Acta Cytologica 40, 506–512.
Lehtinen, M., Paavonen, J., Wheeler, C.M., et al., 2012. Overall efficacy of HPV-16/18 AS04-adjuvanted vaccine against grade 3 or greater cervical intraepithelial neoplasia: 4-year end-of-study analysis of the randomised, duble-blind PATRICIA trial. The Lancet Oncology 13, 89–99.
Leyden, W.A., Manos, M.M., Geiger, A.M., et al., 2005. Cervical cancer in women with comprehensive health care access: Attributable factors in the screening process. Journal of the National Cancer Institute 97, 675–683.
Li, N., Franceschi, S., Howell-Jones, R., Snijders, P.J., Clifford, G.M., 2011. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. International Journal of Cancer 128, 927–935.
Lönnberg, S., Anttila, A., Kotaniemi-Talonen, L., et al., 2010. Low proportion of false-negative smears in the Finnish program for cervical cancer screening. Cancer Epidemiology, Biomarkers & Prevention 19, 381–387.
Maglennon, G.A., McIntosh, P.B., Doorbar, J., 2014. Immuno-suppression facilitates the reactivation of latent papillomavirus infections. Journal of Virology 88, 710–716.
Malagón, T., Drolet, M., Boily, M.C., Laprise, J.F., Brisson, M., 2015. Changing inequalities in cervical cancer: Modeling the impact of vaccine uptake, vaccine herd effects, and cervical cancer screening in the post-vaccination era. Cancer Epidemiology, Biomarkers & Prevention 24, 276–285.
Maran, A., Amella, C.A., di Lorenzo, T.P., et al., 1995. Human papillomavirus type 11 transcripts are present at low abundance in latently infected respiratory tissues. Virology 212, 285–294.
Markowitz, L.E., Dunne, E.F., Saraiya, M., et al., 2014. Human papillomavirus vaccination: Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR—Recommendations and Reports 63 (RR-05), 1–30.
Marks, M.A., Castle, P.E., Schiffman, M., et al., 2012. Evaluation of any or type-specific persistence of high-risk human papillomavirus for detecting cervical precancer. Journal of Clinical Microbiology 50, 300–306.
de Martel, C., Plummer, M., Vignat, J., Franceschi, S., 2017. Worldwide burden of cancer attributable to HPV by site, country and HPV type. International Journal of Cancer 141, 664–670.
Massad, L.S., Einstein, M.H., Huh, W.K., et al., 2013. 2012 ASCCP Consensus Guidelines Conference. 2012 updated consensus guidelines for the management of abnormal cervical cancer screening tests and cancer precursors. Journal of Lower Genital Tract Disease 17 (5 Suppl 1), S1–S27.
McBride, A.A., Warburton, A., 2017. The role of integration in oncogenic progression of HPV-associated cancers. PLoS Pathogens 13, 1006211.

McCredie, M.R., Sharples, K.J., Paul, C., et al., 2008. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: A retrospective cohort study. The Lancet Oncology 9, 425–434.
McLaughlin-Drubin, M.E., Munger, K., 2008. Viruses associated with human cancer. Biochimica et Biophysica Acta 1782, 127–150.
Meijer, C.J., Berkhof, J., Castle, P.E., Hesselink, A.T., Franco, E.L., Ronco, G., Arbyn, M., Bosch, F.X., Cuzick, J., Dillner, J., Heideman, D.A., Snijders, P.J., 2009. Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older. International Journal of Cancer 124 (3), 516–520. https://doi.org/10.1002/ijc.24010.
Mirabello, L., Yeager, M., Cullen, M., et al., 2016. HPV16 sublineage associations with histology-specific cancer risk using HPV whole-genome sequences in 3200 women. Journal of the National Cancer Institute 29, 108.
Mittal, S., Basu, P., Muwonge, R., et al., 2017. Risk of high-grade precancerous lesions and invasive cancers in high-risk HPV-positive women with normal cervix or CIN 1 at baseline-A population-based cohort study. International Journal of Cancer 140, 1850–1859.
Morrell, S., Taylor, R., Wain, G., 2005. A study of Pap test history and histologically determined cervical cancer in NSW women, 1997–2003. Journal of Medical Screening 12, 190–196.
Moscicki, A.B., Schiffman, M., Kjaer, S., Villa, L.L., 2006. Chapter 5: Updating the natural history of HPV and anogenital cancer. Vaccine 24 (Suppl 3), S3/42–51.
Moscicki, A.B., Ma, Y., Jonte, J., et al., 2010a. The role of sexual behavior and human papillomavirus persistence in predicting repeated infections with new human papillomavirus types. Cancer Epidemiology, Biomarkers & Prevention 19, 2055–2065.
Moscicki, A.B., Ma, Y., Wibbelsman, C., et al., 2010b. Rate of and risks for regression of cervical intraepithelial neoplasia 2 in adolescents and young women. Obstetrics and Gynecology 116, 1373–1380.
Moscicki, A.B., Ma, Y., Farhat, S., et al., 2014. Natural history of anal human papillomavirus infection in heterosexual women and risks associated with persistence. Clinical Infectious Diseases 58, 804.
Mousavi, S.M., Sundquist, K., Hemminki, K., 2012. Morbidity and mortality in gynecological cancers among first- and second-generation immigrants in Sweden. International Journal of Cancer 131, 497–504.
Moyer, V.A., US Preventive Services Task Force, 2012. Screening for cervical cancer. U.S. Preventive Services Task Force recommendation statement. Annals of Internal Medicine 156, 880–891.
Munoz, N., Hernandez-Suarez, G., Mendez, F., et al., 2009. Persistence of HPV infection and risk of high-grade cervical intraepithelial neoplasia in a cohort of Colombian women. British Journal of Cancer 100, 1184–1190.
Naucler, P., Ryd, W., Tornberg, S., et al., 2007. Human papillomavirus and Papanicolaou tests to screen for cervical cancer. The New England Journal of Medicine 357, 1589–1597.
Nieminen, P., Kallio, M., Anttila, A., Hakama, M., 1999. Organised vs. spontaneous Pap-smear screening for cervical cancer: A case-control study. International Journal of Cancer 83, 55–58.
Nygård, J.F., Skare, G.B., Thoresen, S.Ø., 2002. The cervical cancer screening programme in Norway, 1992–2000: Changes in Pap smear coverage and incidence of cervical cancer. Journal of Medical Screening 9, 86–91.
Nyitray, A.G., Carvalho da Silva, R.J., Baggio, M.L., et al., 2011. Six-month incidence, persistence, and factors associated with persistence of anal human papillomavirus in men: The HPV in men study. The Journal of Infectious Diseases 204, 1711–1722.
Obel, J., McKenzie, J., Buenconsejo-Lum, L.E., et al., 2015. Mapping HPV vaccination and cervical cancer screening practice in the Pacific region—strengthening national and regional cervical cancer prevention. Asian Pacific Journal of Cancer Prevention 16, 3435–3442.
Palefsky, J.M., Giuliano, A.R., Goldstone, S., et al., 2011. HPV vaccine against anal HPV infection and anal intraepithelial neoplasia. The New England Journal of Medicine 365, 1576–1585.
di Paola, M., Sani, C., Clemente, A.M., et al., 2017. Characterization of cervico-vaginal microbiota in women developing persistent high-risk human papillomavirus infection. Scientific Reports 7, 10200.
Peto, J., Gilham, C., Fletcher, O., Matthews, F.E., et al., 2004. The cervical cancer epidemic that screening has prevented in the UK. Lancet 364, 249–256.
Phillips, A., Patel, C., Pillsbury, A., Brotherton, J., Macartney, K., 2017. Safety of human papillomavirus vaccines: An updated review. Drug Safety. https://doi.org/10.1007/s40264-017-0625-z.
Plummer, M., de Martel, C., Vignat, J., et al., 2016. Global burden of cancers attributable to infections in 2012: A synthetic analysis. The Lancet Global Health 4, e609–e616.
Pollock, K.G., Kavanagh, K., Potts, A., et al., 2014. Reduction of low- and high-grade cervical abnormalities associated with high uptake of the HPV bivalent vaccine in Scotland. British Journal of Cancer 111, 1824–1830.
Prigge, E.S., von Knebel Doeberitz, M., Reuschenbach, M., 2017. Clinical relevance and implications of HPV-induced neoplasia in different anatomical locations. Mutation Research, Reviews in Mutation Research 772, 51.
Psyrri, A., DiMaio, D., 2008. Human papillomavirus in cervical and head-and-neck cancer. Nature Clinical Practice. Oncology 5, 24e31.
Pyeon, D., Pearce, S.M., Lank, S.M., Ahlquist, P., Lambert, P.F., 2009. Establishment of human papillomavirus infection requires cell cycle progression. PLoS Pathogens 5, e1000318.
Quinn, M., Babb, P., Jones, J., Allen, E., 1999. Effect of screening on incidence of and mortality from cancer of cervix in England: Evaluation based on routinely collected statistics. BMJ 318, 904–908.
Rijkaart, D.C., Berkhof, J., Rozendaal, L., et al., 2012. Human papillomavirus testing for the detection of high-grade cervical intraepithelial neoplasia and cancer: Final results of the POBASCAM randomised controlled trial. The Lancet Oncology 13, 78–88.
Rodríguez, A.C., Schiffman, M., Herrero, R., et al., 2010. Longitudinal study of human papillomavirus persistence and cervical intraepithelial neoplasia grade 2/3: Critical role of duration of infection. Journal of the National Cancer Institute 102, 315–324.
Ronco, G., Giorgi Rossi, P., 2017. Role of HPV DNA testing in modern gynaecological practice. Best Practice & Research: Clinical Obstetrics & Gynaecology pii:S1521-6934, 30119-0.
Ronco, G., Pilutti, S., Patriarca, S., et al., 2005. Impact of the introduction of organised screening for cervical cancer in Turin, Italy: Cancer incidence by screening history 1992-98. British Journal of Cancer 93, 376–378.
Ronco, G., Giorgi-Rossi, P., Carozzi, F., et al., 2006. Human papillomavirus testing and liquid-based cytology in primary screening of women younger than 35 years: Results at recruitment for a randomised controlled trial. The Lancet Oncology 7, 547–555.
Ronco, G., Giorgi-Rossi, P., Carozzi, F., et al., 2008. Results at recruitment from a randomized controlled trial comparing human papillomavirus testing alone with conventional cytology as the primary cervical cancer screening test. Journal of the National Cancer Institute 100, 492–501.
Ronco, G., Giorgi-Rossi, P., Carozzi, F., et al., 2010. Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: A randomised controlled trial. The Lancet Oncology 11, 249–257.
Ronco, G., Dillner, J., Elfstrom, K.M., et al., 2014. Efficacy of HPV-based screening for prevention of invasive cervical cancer: Follow-up of four European randomised controlled trials. Lancet 383, 524–532.
Ronco, G., Fiano, V., Grasso, C., et al., 2017. In: Association of HPV methylation with present and future high grade cervical lesions in the NTCC cohort. 31st International Papillomavirus Conference & Clinical and Public Health Workshops. International Papillomavirus Conference, Cape town.
Rositch, A.F., Koshiol, J., Hudgens, M.G., et al., 2013. Patterns of persistent genital human papillomavirus infection among women worldwide: A literature review and meta-analysis. International Journal of Cancer 133, 1271–1285.

de Sanjosé, S., Diaz, M., Castellsagué, X., et al., 2007. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: A meta-analysis. The Lancet Infectious Diseases 7, 453–459.
de Sanjose, S., Quint, W.G.V., Alemany, L., et al., 2010. Human papillomavirus genotype attribution in invasive cervical cancer: A retrospective cross-sectional worldwide study. The Lancet Oncology 11, 1048–1056.
de Sanjose, S., Brotons, M., Pavón, M.A., 2017. The natural history of human papillomavirus infection. Best Practice & Research. Clinical Obstetrics & gynaecology pii: S1521-6934, 30133-5.
Sankaranarayanan, R., Esmy, P.O., Rajkumar, R., et al., 2007. Effect of visual screening on cervical cancer incidence and mortality in Tamil Nadu, India: A cluster-randomised trial. Lancet 370, 398–406.
Sankaranarayanan, R., Nene, B.M., Shastri, S.S., et al., 2009. HPV screening for cervical cancer in rural India. The New England Journal of Medicine 360, 1385–1394.
Sasieni, P., Castanon, A., Cuzick, J., 2009. Effectiveness of cervical screening with age: Population based case-control study of prospectively recorded data. BMJ 339, b2968.
Saslow, D., Solomon, D., Lawson, H.W., et al., 2012. American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer. American Journal of Clinical Pathology 137, 516–542.
Sauvageau, C., Dufour-Turbis, C., 2016. HPV vaccination for MSM: Synthesis of the evidence and recommendations from the Québec Immunization Committee. Human Vaccines & Immunotherapeutics 12, 1560–1565.
Schiffman, M., Kjaer, S.K., 2003. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. Journal of the National Cancer Institute. Monographs (31), 14–19.
Schiffman, M., Wentzensen, N., 2013. Human papillomavirus infection and the multistage carcinogenesis of cervical cancer. Cancer Epidemiology, Biomarkers & Prevention 22, 553–560.
Schiffman, M., Doorbar, J., Wentzensen, N., et al., 2016. Carcinogenic human papillomavirus infection. Nature Reviews Disease Primers 2, 16086.
Schiller, J.T., Müller, M., 2015. Next generation prophylactic human papillomavirus vaccines. The Lancet Oncology 16, e217–e225.
Scott, M., Stites, D.P., Moscicki, A.B., 1999. Th1 cytokine patterns in cervical human papillomavirus infection. Clinical and Diagnostic Laboratory Immunology 6, 751–755.
Serraino, D., Gini, A., Taborelli, M., et al., 2015. Changes in cervical cancer incidence following the introduction of organized screening in Italy. Preventive Medicine 75, 56–63.
Shannon, B., Yi, T.J., Perusini, S., et al., 2017. Association of HPV infection and clearance with cervicovaginal immunology and the vaginal microbiota. Mucosal Immunology 10, 1310–1319.
Skinner, S.R., Szarewski, A., Romanowski, B., et al., 2014. Efficacy, safety, and immunogenicity of the human papillomavirus 16/18 AS04-adjuvanted vaccine in women older than 25 years: 4-year interim follow-up of the phase 3, double-blind, randomised controlled VIVIANE study. Lancet 384, 2213–2227.
Solomon, D., Schiffman, M., Tarone, R., ALTS Study Group, 2001. Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: Baseline results from a randomized trial. Journal of the National Cancer Institute 93, 293–299.
Sørbye, S.W., Arbyn, M., Fismen, S., Gutteberg, T.J., Mortensen, E.S., 2011. HPV E6/E7 mRNA testing is more specific than cytology in post-colposcopy follow-up of women with negative cervical biopsy. PLoS One 6, e26022.
Stanley, M.A., 2012. Epithelial cell responses to infection with human papillomavirus. Clinical Microbiology Reviews 25, 215–222.
Stanley, M., Pinto, L.A., Trimble, C., 2012. Human papillomavirus vaccine immune responses. Vaccine 30 (Suppl 5), F83–7.
Stubenrauch, F., Laimins, L.A., 1999. Human papillomavirus life cycle: Active and latent phases. Seminars in Cancer Biology 9, 379–386.
Sung, H.Y., Kearney, K.A., Miller, M., et al., 2000. Papanicolaou smear history and diagnosis of invasive cervical carcinoma among members of a large prepaid health plan. Cancer 88, 2283–2289.
Uchimura, N.S., Uchimura, T.T., Martins, J.P., Assakawa, F., Uchimura, L.Y., 2012. Evaluation of conservative management of high-grade cervical squamous intraepithelial lesion. Revista de Saude Publica 46, 466–471.
Venturelli, F., Baldacchini, F., Campari, C., et al., 2017. Association between mothers' screening uptake and daughters' HPV vaccination: A quasi-experimental study on the effect of an active invitation campaign. BMJ Open 7, e016189.
Verdoodt, F., Jentschke, M., Hillemanns, P., et al., 2015. Reaching women who do not participate in the regular cervical cancer screening programme by offering self-sampling kits: A systematic review and meta-analysis of randomised trials. European Journal of Cancer 51, 2375–2385.
de Vuyst, H., Howell-Jones, R., Levy-Bruhl, D., Giorgi Rossi, P., Franceschi, S., 2015. Implementation of vaccination against human papillomavirus in Europe. In: Anttila, A., Arbyn, M., de Vuyst, H., et al. (Eds.), European guidelines for quality assurance in cervical cancer screening, 2nd ed. Office for Official Publications of the European Union, Luxembourg, pp. 109–137. Supplements.
Walboomers, J.M., Jacobs, M.V., Manos, M.M., et al., 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. The Journal of Pathology 189, 12–19.
Wheeler, C.M., Castellsague, X., Garland, S.M., et al., 2011. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. The Lancet Oncology 13, 100–110.
WHO, 2013. WHO guidelines for screening and treatment of precancerous lesions for cervical cancer prevention. World Health Organization, Geneva.
WHO, 2014. WHO guidelines for treatment of cervical intraepithelial neoplasia 2–3 and glandular adenocarcinoma in situ: Cryotherapy, large loop excision of the transformation zone (LEEP/LLETZ), and cold knife conization. World Health Organization, Geneva.
WHO & IARC, 2012. IARC monographs on the evaluation of carcinogenic risks to humans. Biological agents. In: Volume 100B: A review of human carcinogens. IARC, Lyon.
Winer, R.L., Feng, Q., Hughes, J.P., et al., 2008. Risk of female human papillomavirus acquisition associated with first male sex partner. The Journal of Infectious Diseases 197, 279–822.
Winer, R.L., Hughes, J.P., Feng, Q., et al., 2011. Early natural history of incident, type-specific human papillomavirus infections in newly sexually active young women. Cancer Epidemiology, Biomarkers & Prevention 20, 699–707.
Yang, E.J., Quick, M.C., Hanamornroongruang, S., et al., 2015. Microanatomy of the cervical and anorectal squamocolumnar junctions: A proposed model for anatomical differences in HPV-related cancer risk. Modern Pathology 28, 994–1000.
Zucchetto, A., Ronco, G., Giorgi Rossi, P., et al., 2013. Screening patterns within organized programs and survival of Italian women with invasive cervical cancer. Preventive Medicine 57, 220–226.

Further Reading

Anon, 2014. Human papillomavirus vaccines: WHO position paper, October 2014. WHO Weekly Epidemiological Record 89, 465–492.
Ferlay, J., Soerjomataram, I., Ervik, M., et al., 2013. GLOBOCAN 2012 v1.2, cancer incidence and mortality worldwide: IARC CancerBase No. 11. International Agency for Research on Cancer, Lyon, France.
Lehtinen, M., Dillner, J., 2013. Clinical trials of human papillomavirus vaccines and beyond. Nature Reviews. Clinical Oncology 10, 400–410.

Relevant Websites

http://www.hpvcentre.net/index.php—HPV information centre.
http://www.iarc.fr/—International Agency for Research on Cancer.
http://www.who.int/reproductivehealth/topics/cancers/en/—World Health Organization, Cervical Cancer.
http://www.gavi.org/support/nvs/human-papillomavirus/—GAVI the vaccine alliance.
http://www.cervicalcanceraction.org/home/home.php—Cervical Cancer Action.
https://www.cancer.org/health-care-professionals/american-cancer-society-prevention-early-detection-guidelines/cervical-cancer-screening-guidelines.html—American Cancer Society.
http://www.asccp.org/asccp-guidelines—Society of Lower Genital Tract Disorders ASCCP guidelines (formerly American Society for Colposcopy and Cervical Pathology).

Chemoprevention of Cancer: An Overview of Promising Agents and Current Research

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Cancer diagnosis and treatment is distressing for patients and carries a high burden of morbidity and mortality. Preventable cases of cancer can be reduced through lifestyle changes and to augment this chemoprevention is becoming an increasingly vital area of research.

The principle of chemoprevention is to find a medication or dietary supplement which when given to certain groups of the population can reduce the risk of carcinogenesis before invasion. Optimum agents need to be well understood from a pharmacokinetic and safety perspective, be cost effective for broad usage and be well tolerated by patients.

A key draw back is chemoprevention agents must be taken for prolonged periods, creating a unique conundrum for clinicians in that within a population, for a proportion of people cancer may be delayed by a few years, yet this benefit may be outweighed by healthy patients who succumb to complications. Hence many agents that are studied are concurrently used for secondary prevention of other conditions and therefore may have multiple benefits to balance the risks (Fig. 1).

Here we give an overview of some of the potential chemoprevention agents (Table 1), with an emphasis on aspirin which has shown the most promise.

Statins

HMG-CoA reductase inhibitors act against the conversion of HMG CoA to mevalonate, thought to prevent disruption to Ras/Rho signaling proteins which in turn can affect normal cell differentiation, survival and growth. Statins also regulate the RAF–MAPK–ERK pathway which makes them proapoptotic, anti-inflammatory and immunomodulatory via both HMG-CoA independent and dependent pathways.

Statins have possible associations with colorectal (CRC), hepatocellular (HCC), gastric, esophageal, and advanced prostate cancers. Tsan et al. in a population-based cohort study of chronic hepatitis found a 53% reduction in risk of HCC in patients on statins. In a meta-analysis of 10 studies by Singh et al. (2013) the statin group showed a 37% reduction in HCC (adjusted OR 0.63; 95% CI 0.52–0.76) though the number needed to treat (NNT) was 5209 in East–Asian men.

Statins are well tolerated; common side effects include myalgia and deranged liver function tests. Concerns have been raised regarding an increased cancer risk in the elderly. Moreover a large epidemiological study into secondary effects of statins showed NNT (1266) to reduce esophageal cancer were markedly offset by number needed to harm (NNH) with severe liver derangement(136), moderate to severe myopathy (91 men 259 women) and renal impairment (434).

Statins are already widely used and are an attractive chemoprevention agent given their high efficacy at lowering all-cause mortality, particularly cardiovascular, and cerebrovascular. Persuading the public of a firm association with a specific cancer will require further research to justify the risks.

Metformin

The metabolic syndrome, sedentary lifestyle and high energy low complexity western diet have been linked with many cancers. Malignant cells are highly catabolic, and links between metabolic pathways, such as the AMPK/LKB1, and how these maybe affected by metformin chemoprevention are driving research. An energy stress response dependent on AMPK triggered by metformin is thought to reduce survival in cancer cell lines. A secondary effect of reducing proliferation of cells via inhibition of phosphoinositide 3-kinase/Akt/mammalian rapamycin signaling has been described.

Meta-analysis of retrospective data for diabetic patients showed an overall reduction in all cancers (31%), this was most evident for pancreatic and HCC. A nonsignificant association was seen with breast, prostate, and CRC cancers. Meta-analysis of studies looking at HCC risk showed a 50% reduction of risk in patients who had ever used metformin versus those who had never, even accounting for adjustments for other medications. Higurashi et al. performed a multicenter double-blind, randomized controlled trial of metformin in nondiabetic patients and showed a significantly lower colonic adenoma recurrence rate ($P = .034$, risk ratio 0.67 [95% CI 0.47–0.97]).

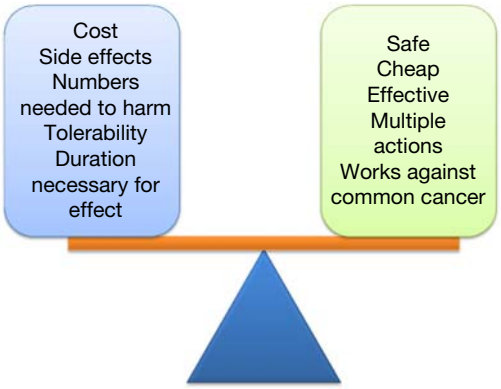

Fig. 1 Considerations when choosing a chemoprevention agent.

Table 1 Overview of agents discussed

Agent	*Cancer prevented*	*Risks*
Statins	CRC, HCC, gastric, esophageal, prostate	Liver injury, myopathy, renal derangement, increased cancer risk in the elderly
Metformin	Pancreatic, HCC, all cancers	Diarrhea, nausea, abdominal discomfort
Vitamin D	Breast, esophageal, CRC	Increased risk of adenocarcinoma of esophagus
Tamoxifen	ER positive breast cancer	VTE, Endometrial cancer, cataract formation
Aspirin	CRC, esophageal, hereditary CRC, breast, ovarian, pancreatic, prostate, lung	GI Bleeding, Intracranial hemorrhage, all bleeding

Metformin can be troubling for patients with many experiencing diarrhea, nausea, and abdominal discomfort. More serious adverse effects such as lactic acidosis are dose dependent and studies into optimum dosing are still needed.

The risk reduction of other conditions makes metformin a good agent which for the general public could seem appealing. The rising incidence of diabetes and risk of obesity related illnesses including cancers such as colorectal and postmenopausal breast cancer increase the case for its use in chemoprevention.

Vitamin D

The mechanism explored in in vitro settings suggests vitamin D levels can affect apoptosis, cell proliferation, and differentiation. Dietary vitamin D is hydroxylated to its' circulating form 25-hydroxyvitamin-D which can be measured in the circulation, the active form (1,25-HO-vitD) is created through a second hydroxylation allowing it to bind to VDR which results in a biological signaling cascade.

Vitamin D levels have been linked to reductions in breast, esophageal and CRC among others. Dietary vitamin D intake showed a possible relationship that was protective for squamous cell carcinoma of the esophagus but increased risk of adenocarcinoma (nonsignificant). Gandhini et al. through meta-analysis found an inverse relationship between 25-HO-vit D and CRC but could not show significant associations with breast or prostate cancer. They felt some bias could come from many case-control studies taking vitamin D levels after cancer diagnosis—when patients' diet, activity or sun exposure may have changed.

Side effects from vitamin D are few, however in some studies an adverse link has been raised, for example an increased association with adenocarcinoma of the esophagus. This seems to particularly relate to the use of supplementation, whereas studies looking at lifetime UV exposure seemed to be more favorable in outcome.

Vitamin D is certainly an attractive choice for patients, with few side effects as a supplement and when gained through sun exposure there is likely to be an association with physical activity, reducing other cancer risk factors such as sedentary behavior and obesity.

Tamoxifen

Tamoxifen produces an antiestrogen effect in breast tissue and weakly estrogenic effects in the bone and uterus. Estrogen normally binds with the estrogen receptor (ER) which causes CoA to bind with the receptor creating a transcription complex. The complex is then able to commence DNA transcription from an estrogen-receptive gene. Tamoxifen binds with the ER-α part of the receptor

preventing it from recruiting CoA molecules therefore causing an antiestrogen effect in breast tissue. However, where CoA levels are greater a weakly estrogenic effect occurs—in the uterus and bone—and through an association with fos/jun proteins stimulates the activating protein-1 (AP1) causing gene sequencing. Since these early mechanisms were described, studies into tamoxifen resistance have highlighted the importance of paired box-2 (PAX) in both signaling in breast tissue and the carcinogenesis in the endometrium.

Patients who have been treated with tamoxifen were found to have fewer contralateral breast lesions than other patients raising the possibility of a role as a chemoprevention agent. Meta-analysis of 9 trials by Cuziak et al., found a significant risk reduction in estrogen receptor positive breast cancer in both average and higher risk women who had no prior disease. They calculated a NNT of 42. There was a nonsignificant increased risk of ER negative cases, which they hypothesized could relate to mutation from ER sensitive to hormone resistance.

Significant adverse risks are associated with tamoxifen particularly venous thromboembolism (VTE) (reported up to twice as often as placebo) and endometrial cancer (2–5 times above the baseline rate). The risks are less for premenopausal women, with no increased risk of VTE or endometrial cancer, however in postmenopausal women there was an increase in VTE, Endometrial cancer and cataract formation.

As a treatment of breast cancer tamoxifen is well tolerated, the routine side effects are few and as chemoprevention for high risk women it is recommended by NICE and approved for use by the FDA in the United States. For the wider, "healthy" population, the more serious complications, particularly endometrial cancer, will be harder to justify.

Aspirin

The mechanism of chemoprevention via aspirin (Fig. 2) has been linked to prostaglandins, particularly PGE2, via COX signaling. Prostaglandin E2 has been associated with stimulating angiogenesis, increasing a cell's proliferation, migration potential, and invasiveness whilst modulating immunity and preventing apoptosis. In animal colonic models deletion of receptors for PGE2 resulted in reduced abnormal crypt formation and adenomas. Nan et al. through gene versus environment case-control study in CRC identified two genes which showed a greater prevention outcome with aspirin and NSAIDS that are functionally linked with increased PGE2 production. Inhibition by aspirin of COX-2-mediated platelet production of thromboxane has been hypothesized to affect tissues' response to damage caused by disruption by neoplasia resulting in reduced cell proliferation. COX-2 inhibition in epithelial cells occurs at higher doses than in platelets, however, chemopreventative effects have been shown at low doses too which supports platelet mediated mechanisms. Aspirin also reduces the cellular quantity of β-catenin through inactivation of protein phosphatase

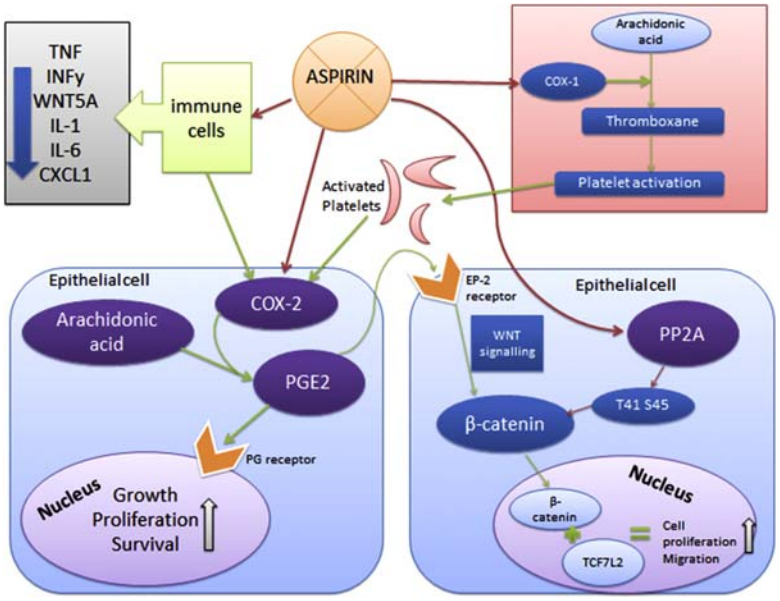

Fig. 2 Mechanism of aspirin chemoprevention. A diagram showing the pathways hypothesized to be affected by aspirin, *Green arrows* represent stimulation pathways and *red arrows* inhibitory. Aspirin affects pathways in four ways: affecting platelet activation (top right) by inhibiting the release of thromboxane; inhibiting the stabilization of β-catenin through inactivation of protein phosphatase 2A (PP2A) promoting ubiquitylation (bottom right panel);through inhibiting COX-2 mediated production of prostanoids (bottom left) by preventing COX-2 converting arachidonic acid to prostaglandin E2 (PGE2); and through the antiinflammatory effect of reducing circulating cytokines (*CXCL1*, C-X-C motif chemokine 1; *MIC1*, macrophage inhibitory cytokine 1; *IFN-γ*, interferon-γ; *IL*, interleukin; *PGT*, prostaglandin transporter; *sTNFR2*, soluble TNF receptor 2; *TNF*, tumor necrosis factor). Modified from a diagram by Drew, D. A., Cao, Y., Chan, A. T. (2016). Aspirin and colorectal cancer: The promise of precision chemoprevention. *Nature Reviews Cancer* **16**(*3*), 173–186. doi: 10.1038/nrc.2016.4.

Table 2 Current trials pending publication or completion

Status	*Trial name*	*Cancer target*	*Agent*	*Phase*	*Participants*	*Masking*	*Randomization*	*Completion date*	*Locations*
Completed	A randomized, double-blind, placebo-controlled, multicentre efficacy and safety study of toremifene citrate for the prevention of prostate cancer in men with high grade prostatic intraepithelial neoplasia (PIN)	Prostate	Toremifene	3	1590	Triple	Yes	Feb-10	35 Study locations
Completed	Physicians health study II: trial of vitamins in the chemoprevention of cancer, CVD and eye disease	Prostate, colorectal, all cancers	Vitamin E, C, multivitamin, beta carotene	N/a	14641	Factorial assignment	Yes	Jun-11	National Cancer Institute US
Completed	Phase III placebo-controlled chemoprevention study of oral cavity squamous cell carcinoma patients	Oral cancer	13-Cis retinoic acid	3	342	Single	Yes	Sep-12	National Taiwan University
Completed	A study to assess the efficacy and safety of enteric-coated acetylsalicylic acid in patients at moderate risk of cardiovascular disease (ARRIVE)	Colorectal cancer (secondary end point)	Aspirin	3	12546	Triple	Yes	Nov-16	Bayer, 673 International Centres
Completed	Phase I multiple-dose safety, pharmacokinetic and pharmacodynamics clinical study of nitric oxide releasing aspirin (NCX 4016)	Colorectal cancer	Nitric-oxide releasing acetylsalicylic acid	1	240	Double	Yes	Dec-16	Stony Brook University
Completed	The seafood (systematic evaluation of aspirin and fish oil) polyp prevention trial	Colorectal cancer	Aspirin	N/a	755	Double	Yes	Oct-17	University of Leeds, UK Centres
Active	A phase III, randomized, study of aspirin and esomeprazole chemoprevention in Barrett's metaplasia (AspECT)	Esophageal	Omeprazole, aspirin	3	2513	Open label	Yes	May-17	Oxford
Active	Pioglitazone for lung cancer chemoprevention	Lung cancer	Pioglitazone	2	391	Quadruple	Yes	Aug-17	Colorado/Tennessee
Active	Aspirin in reducing events in the elderly (ASPREE)	Colorectal cancer	Aspirin	4	19000	Quadruple	Yes	Jan-18	Berman Centre for Outcomes and Clinical Research
Active	Metformin hydrochloride in preventing breast cancer in patients with atypical hyperplasia or in situ breast cancer	Breast cancer	Metformin	3	Aim 128	Double	Yes	Feb-19	Alliance for Clinical Trials in Oncology, United States

Active	The HOT study: hormone replacement therapy opposed by low dose tamoxifen. A phase III trial of breast cancer prevention with low dose tamoxifen in HRT users	Breast cancer	Tamoxifen	3	1884	Triple	Yes	Dec-19	European Institute of Oncology
Active	International breast cancer intervention study	Breast cancer	Anastrozole		3864	Quadruple	Yes	Jan-22	Queen Mary London 75 study locations
Recruiting	Mesalamine for colorectal cancer prevention program in Lynch syndrome—MesaCAPP	Colorectal cancer	Mesalamine	2	Aim 540	Double	Yes	Sep-20	Medical University of Vienna
Recruiting	Targeted chemoprevention of gastric carcinogenesis in high risk populations	Gastric cancer	Eflornithine	2	Aim 300	Double	Yes	Dec-20	Colombia/Honduras/Puerto Rico
Recruiting	A trial looking at different doses of aspirin to prevent cancer in people who have Lynch syndrome (CaPP3)	Colorectal cancer	Aspirin	3	Aim 2000	Double	Yes	2023	CRUK and International
Recruiting	Assessment of the effect of a daily chemoprevention by low-dose aspirin of new or recurrent colorectal adenomas in patients with Lynch syndrome	Colorectal cancer	Aspirin	3	Aim 852	Quadruple	Yes	Dec-24	Hospital Avicenne France
Recruiting	Add-aspirin: A trial assessing the effects of aspirin on disease recurrence and survival after primary therapy in common nonmetastatic solid tumors	Breast, gastric, colorectal, esophageal, prostate	Aspirin	3	11000	Triple	Yes	Oct-26	University College London
Recruiting	Aspirin intervention for the reduction of colorectal cancer risk (ASPiRED)	Colorectal cancer	Aspirin	N/a	Aim 180	Double	Yes	Jul-28	Massachusetts General Hospital

2A—a protein which breaks down amino acid residues (T41 and S45) that are responsible for flagging β-catenin for ubiquitylation. β-catenin when it moves to the nucleus of a cell causes a sequence of gene expression resulting in increased cell migration and proliferation. Most of the cancers which seem to be prevented by aspirin have an inflammatory prodrome, such as Barrett's to esophageal, aspirin is thought to reduce circulating levels of inflammatory cytokines and proteins which have been linked with these cancers.

Parkin et al. estimated that if aspirin was taken for 10 years between the ages of 50–65 years a reduction in all cancers of 7%–10% could be achieved. The strongest associations appear to be with CRC, esophageal, gastric (all by around 1/3 reduction), prostate, breast, and lung cancers (10%–15% reduction). Rothwell et al. in meta-analysis of eight trials showed a significant reduction in 20 year cancer mortality verses placebo for all solid cancers, GI cancers and the benefit increased with age and duration of aspirin use. CRC cohort studies on large populations showed an inverse relationship between aspirin use and CRC incidence—with the Cancer Prevention Study II describing a reduction of 40% in regular aspirin users. The CAPP-2 randomized controlled trial in Lynch syndrome patients showed a significant reduction in CRC cancer risk in this population which was sustained after the end of the randomization to 4 years. Many studies show a reduction in esophageal cancer with aspirin, even reduction in Barrett's length and dysplasia progression. Further studies are underway to look at use in specific high risk groups such as in the AspECT trial—the largest RCT using PPI and aspirin in combination with end points of developing dysplasia and esophageal cancer. Aspirin for CRC chemoprevention has been endorsed by The US Preventative Services Task Force for use in 50–59 year olds with a 10% or greater cardiovascular 10 year risk profile and low bleeding risk, and also on an individual basis for 60–69 year olds, though emphasizing the need for further investigation into the mechanism of action.

The consequences of long term aspirin use are well understood, chiefly increased risk of bleeding due to the unselective nature of aspirin's COX inhibition. COX-1 inhibition reduces the secretion of prostaglandin E2 in the gastric mucosa, increasing ulceration and risk of upper gastrointestinal bleeding. Aspirin reduces thromboxane production in platelets hence reducing atherosclerotic risk but increasing bleeding risk. The risk of severe adverse effects increases dramatically after the age of 60. The international consensus paper suggested that given premalignant lesions often present in middle age- around age 50–60, commencing treatment between the ages of 40–50 years may help prevent most premalignant lesions before the risk of adverse events goes up. Cuzick et al. estimated that for 100 average risk 55 year olds taking aspirin for 10 years there would be 2.29 fewer myocardial infarctions, cancers, and strokes in men and 1.32 in women over 15 years, yet only 0.49 more GI bleeds in men and 0.25 in women. They also estimated a clear reduction in mortality from cancer which outweighed the mortality risk from GI bleeding (a ratio of 7:1 in the general population).

Aspirin is familiar to most lay populations, and has well studied risks meaning counseling healthy populations about it is very feasible. Many people understand its' use in cardiovascular prevention hence the step to use in cancer should be straight forward and well received. Patient preference has been studied in Barrett's esophagus patients with 76% open to the idea of using aspirin. The more knowledge that is gained through risk stratifying populations, as is already done in cardiovascular disease, the greater a case can be put to healthy subjects.

Conclusion

Cancer chemoprevention as a principle is an exciting prospect for researchers, clinicians and patients. There are significant draw backs at present, centered around the risk of causing adverse events and treatment associated mortality in an otherwise healthy population. Rather than a one pill fits all approach the future of chemoprevention is likely to mimic the principles of cardiovascular prevention by targeting higher risk populations. Further studies are ongoing to define these populations and gene profiling is being used to narrow down even more specifically (**Table 2**).

See also: Chemoprevention Trials.

Disclaimer

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Further Reading

Burn, J., Gerdes, A.M., MacRae, F., Mecklin, J.P., Moeslein, G., Olschwang, S., Bishop, D.T., 2011. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: An analysis from the CAPP2 randomized controlled trial. The Lancet 378 (9809), 2081–2087. https://doi.org/10.1016/S0140-6736(11)61049-0.

Cuzick, J., 2017. Preventive therapy for cancer. The Lancet Oncology. https://doi.org/10.1016/S1470-2045(17)30536-3.

Cuzick, J., Otto, F., Baron, J.A., Brown, P.H., Burn, J., Greenwald, P., Thun, M., 2009. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: An international consensus statement. The Lancet Oncology 10 (5), 501–507. https://doi.org/10.1016/S1470-2045(09)70035-X.

Cuzick, J., DeCensi, A., Arun, B., Brown, P.H., Castiglione, M., Dunn, B., Zwierzina, H., 2011. Preventive therapy for breast cancer: A consensus statement. The Lancet Oncology 12 (5), 496–503. https://doi.org/10.1016/S1470-2045(11)70030-4.

Cuzick, J., Sestak, I., Bonanni, B., Costantino, J.P., Cummings, S., DeCensi, A., Wickerham, D.L., 2013. Selective oestrogen receptor modulators in prevention of breast cancer: An updated meta-analysis of individual participant data. The Lancet 381 (9880), 1827–1834. https://doi.org/10.1016/S0140-6736(13)60140-3.

Cuzick, J., Thorat, M.A., Bosetti, C., Brown, P.H., Burn, J., Cook, N.R., Umar, A., 2015. Estimates of benefits and harms of prophylactic use of aspirin in the general population. Annals of Oncology 26 (1), 47–57. https://doi.org/10.1093/annonc/mdu225.

Dulai, P.S., Singh, S., Marquez, E., Khera, R., Prokop, L.J., Limburg, P.J., Murad, M.H., 2016. Chemoprevention of colorectal cancer in individuals with previous colorectal neoplasia: Systematic review and network meta-analysis. BMJ. https://doi.org/10.1136/bmj.i6188.

Higurashi, T., Hosono, K., Takahashi, H., Komiya, Y., Umezawa, S., Sakai, E., Nakajima, A., 2016. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: A multicentre double-blind, placebo-controlled, randomised phase 3 trial. The Lancet Oncology 17 (4), 475–483. https://doi.org/10.1016/S1470-2045(15)00565-3.

Hurtado, A., Holmes, K.A., Geistlinger, T.R., Hutcheson, I.R., Nicholson, R.I., Brown, M., Carroll, J.S., 2008. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. Nature 456 (7222), 663–666. https://doi.org/10.1038/nature07483.

Jankowski, J.A., Hawk, E.T., 2006. A methodologic analysis of chemoprevention and cancer prevention strategies for gastrointestinal cancer. Nature Clinical Practice. Gastroenterology & Hepatology 3 (2), 101–111. https://doi.org/10.1038/ncpgasthep0412.

Nan, H., Hutter, C.M., Lin, Y., Jacobs, E.J., Ulrich, C.M., White, E., Chan, A.T., 2015. Association of Aspirin and NSAID use with risk of colorectal Cancer according to genetic variants. JAMA 313 (11), 1133. https://doi.org/10.1001/jama.2015.1815.

Ricciardiello, L., Ahnen, D.J., Lynch, P.M., 2016. Chemoprevention of hereditary colon cancers: Time for new strategies. Nature Reviews. Gastroenterology & Hepatology 13 (6), 352–361. https://doi.org/10.1038/nrgastro.2016.56.

Singh, S., Singh, P.P., Roberts, L.R., Sanchez, W., 2014. Chemopreventive strategies in hepatocellular carcinoma. Nature Reviews. Gastroenterology & Hepatology 11 (1), 45–54. https://doi.org/10.1038/nrgastro.2013.143.

U.S. Preventive Services Task Force.n.d. Recommendation Statement: Aspirin to prevent cardiovascular disease and cancer U.S. Preventive Services Task Force [online] https://www.uspreventiveservicestaskforce.org/Page/Document/UpdateSummaryFinal/aspirin-to-prevent-cardiovascular-disease-and-cancer?ds=1&s=aspirin.

Chemoprevention Trials☆

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Introduction

Epithelial cancers have historically been a cause for frustration by both patients and practitioners. Despite decades of cancer research, the mortality rate from epithelial malignancies has improved only 1%–2% per year for both men and women (Siegel et al., 2017), largely driven by decreased tobacco use (Jemal et al., 2008). Although some patients will present with early stage disease, the majority of patients will present with locally advanced or metastatic disease. Surgical intervention with adjuvant or neoadjuvant treatment, including radiotherapy and/or chemotherapy, has offered some improvements in long-term survival rates. However, local and distant relapse remain unacceptably high in many cancers. Thus, novel approaches to these epithelial cancers are needed, specifically the prevention of carcinogenesis prior to the onset of invasive cancer (Meyskens, 1992).

Prevention strategies can occur at three broad levels: primary, secondary, and tertiary. A chemopreventive agent is a compound, either natural or synthetic, that prevents, reverses, or blocks the development of invasive cancer and can be considered at any level of prevention (Wattenberg, 1985). Primary prevention targets risk-reducing techniques in asymptomatic "normal" individuals and includes interventions such as diet and exercise. A high-risk individual such as one with a known inherited cancer syndrome who undergoes prophylactic surgery is also an example of primary prevention (e.g., BRCA carrier undergoing mastectomy). Treatments of individuals with premalignant findings to prevent progression to invasive cancer are considered secondary prevention. This can include surgical or ablative therapy for known precancerous conditions such as polypectomy for colonic adenomas or photodynamic therapy for Barrett's esophagus as well as topical chemopreventive therapies for actinic keratoses, the precursor to squamous cell carcinoma of the skin. Tertiary prevention involves reducing the risk of recurrence after established malignancy and is exemplified by studies targeting secondary primary tumors of the aerodigestive tract following definitive treatment.

Biologic Rationale for Chemoprevention

It is well established that advanced metastatic carcinomas are heterogonous invasive masses of genomically complex cells (Hanahan and Weinberg, 2011). Multiple dysregulated cellular-signaling pathways lead to the inevitable metastatic phenotype. The molecular heterogeneity observed occurs not only between patients with the same malignancy (interpatient heterogeneity) but also within metastatic sites of a given patient (intrametastatic heterogeneity). This complexity highlights the rational for targeting earlier neoplastic changes (i.e., intraepithelial neoplasia) prior to the onset of genomically diverse invasive carcinoma.

As a result of a variety of environmental and genotoxic insults, normal epithelium transforms to neoplasia most often via a stepwise accumulation of molecular and genetic aberrations over the course of many years. This sequential accumulation of abnormalities was typified by the model of colorectal tumorigenesis by Fearon and Vogelstein in 1992 (Fearon and Vogelstein, 1990) and has since been described in multiple tumors. Chemopreventive agents have the potential to interrupt or perhaps reverse these changes throughout the carcinogenic process.

Numerous potential molecular targets within neoplastic development are putative drivers. These include those that involve independence from growth signals (e.g., EGFR, PI3K), resistance to antigrowth signals (e.g., CDK, SMADs), evasion of apoptosis (e.g., mTOR, PTEN, *Ras*), immortal replicative potential (e.g., p53, pRb), angiogenesis (e.g., HIF-1a, VEGF, FGF), and tissue invasiveness and metastatic potential (e.g., E-cadherin, MAP-kinase) (Hanahan and Weinberg, 2011; Kelloff et al., 2006). Although inhibition of single transduction pathways has had clinical efficacy in the advanced cancer setting (e.g., anti-EGFR therapy in lung and colorectal cancers), the toxicity associated with complete inhibition of select pathways as well as the presence of bypass tracks (i.e., alternative proliferative pathways or downstream mutations) limits the applicability of single site blockade in complex tumors. This is highlighted by the failure of erlotinib, an anti-EGFR monoclonal antibody, to reduce oral cancer-free survival in high-risk individuals with oral premalignant lesions (William et al., 2016).

In addition to aberrations in signal transduction, dependence of cancer cells on a single oncogene (i.e., oncogene addiction) remains an important potential target. This is suggested by the inherent sensitivity of the addicted cell to blockade of the oncogene. For example, preclinical data support the antiproliferative effect of even partial inhibition of cyclin D1, a purported addicted oncogene in esophageal cancer (Weinstein, 2002; Jain et al., 2002). Further clinical study of manipulation of oncogene addiction and tumor suppressor hypersensitivity is ongoing (Weinstein et al., 2008; Jacobi et al., 2017).

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 https://doi.org/10.1016/B978-0-12-801238-3.96067-7

Other significant chemopreventive avenues have evolved from the improved understanding of the role of inflammation in neoplasia. Multiple studies support the effects of chronic inflammation on promoting carcinogenesis (Caruso et al., 2004; Philip et al., 2004). Nuclear factor-kappa β, a proinflammatory transcription factor, along with regulatory proteins (e.g., tumor necrosis factor-α, cyclooxygenase-2, inducible nitric oxide synthase) play an integral role in the development and progression of neoplasia and have been proposed to serve as therapeutic targets as well as potential biomarkers (Aggarwal, 2004). Additionally, states of chronic inflammation caused by inflammatory bowel disease (IBD) and Barrett's esophagus, or due to infections such as human papilloma virus (HPV) and hepatitis B (HBV) lead to prolonged inflammation that can progress to carcinogenesis (Shacter and Weitzman, 2002). Importantly, the development of vaccines for HPV and HBV has resulted in marked reduction in the incidence of cervical and hepatocellular carcinoma, respectively, representing the importance of targeting upstream molecular events prior to the development of frank carcinoma (Chang et al., 2009; Garland et al., 2016).

Drug Development of Chemopreventive Agents

Similar to drug development for other indications, translational efforts for chemopreventive agents follow a similar process. The chemoprevention drug-screening program outlined by the United States National Cancer Institute's Cancer Preclinical Drug Development Program (PREVENT) provides such a pragmatic approach involving a stage-gate process with go/no go decision points throughout the drug development process (Shoemaker et al., 2016). Ideally, preclinical testing includes biochemical prescreening assays, in vitro efficacy models, in vivo short-term screening, and animal efficacy prior to testing in human trials. Despite significant improvements in the preclinical sciences, the translation of findings from these models into clinical success in human chemoprevention trials has been limited. Although the reasons for this are multifactorial, murine/rodent models often consist of a genetically homogenous population that follows a strict dietary regimen in tightly controlled laboratory conditions. In contrast, even well-controlled human trials inherently include genetically diverse populations with varied dietary and environmental exposures that cannot be fully measured (Meyskens et al., 2016).

Similar to other oncologic therapeutics, evaluation of the clinical safety and efficacy of chemopreventive agents undergo phased testing. Understanding the nuances specific to prevention, as compared to treatment, are integral to the successful design and implementation of chemoprevention trials. These include the wide therapeutic index required of the agents, the long latency to neoplastic transformation, the necessity for prolonged adherence, and the complexity underlying risk evaluation (Shureiqi et al., 2000). Therapeutic index is driven by a combination of the agent's efficacy as well as its associated short- and long-term toxicities. Early phase studies are integral to understanding the optimal dosing, frequency, and acceptable toxicities prior to undertaking larger scale studies. The inherent principle of chemoprevention requires drug therapy in a largely asymptomatic patient and therefore greater understanding of detection, evaluation, and avoidance of toxicities is highly warranted. Such strategies include defining the minimally effective dose, evaluating the pharmacokinetic and pharmacodynamics of intermittent or combination therapies, and optimizing toxicity monitoring (Kelloff et al., 2006).

In latter phases, the ideal endpoint of chemoprevention trials is a reduction in cancer incidence. Because cancer incidence is extremely low among the general population with no known cancer risk factors, demonstrating a treatment-induced reduction in cancer incidence among the general population requires large randomized studies, which are expensive and time-consuming. Targeting high-risk populations and utilizing validated intermediate biomarkers can significantly reduce the time and resources required for chemoprevention trials.

Biomarkers can be defined as measurable and evaluable indicators of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic interventions. Currently, the most common biomarker utilized in chemoprevention studies is histopathologic intraepithelial neoplasia (IEN). Examples of histopathologic IEN include adenomas in colorectal cancers, leukoplakia in oral cancers, bronchial dysplasia in lung cancers, and cervical intraepithelial neoplasia in cervical cancer. The goal of such a biomarker is to be a true surrogate of a future invasive process. The ideal biomarker should be variably expressed throughout the carcinogenic process, detectable early in the course or carcinogenesis, modulation with intervention should accurately be associated with clinical benefits, and ideally should be able to be quantified directly, reliably, noninvasively, and with technical and cost feasibility. As histopathologic changes are a result of a multiple molecular, proteomic, and genomic changes, establishing a biomarker that accounts for this interplay is most ideal. For example, gene set enrichment analyses where genes in a critical pathway are mapped to determine which are affected and not affected in cancer tissue might allow to better target tissue-specific molecular aberrations (Mootha et al., 2003). Although significant progress is being made with advanced technologies such as the gene set enrichment analysis described; newer proteomic, imaging, and microarray techniques might allow for a truly practical, prognostic, and predictive biomarker for each organ system to be fully realized (Fabian and Kimler, 2016).

Clinical Trials Involving Chemoprevention

Cancer chemoprevention continues to evolve and its role in oncologic practice continues to expand. A multitude of chemopreventive agents have been studied in over 100 randomized trials over the past four decades. Some clinical activity has been demonstrated proving the potential utility of drug therapy for cancer prevention. Over 10 agents have been FDA approved for cancer risk reduction in select high-risk cohorts as well as the treatment of premalignant lesions (**Tables 1 and 2**). Although an exhaustive review of all chemoprevention trials is not possible in this article, a discussion regarding the major contemporary and historically significant chemoprevention trials by organ system is provided.

Table 1 FDA approved therapies for cancer risk reduction

Agent	*FDA indication and cohort*[a]
Tamoxifen	1. Reduction in risk of invasive breast cancers in women with DCIS following breast surgery and radiation. 2. Reduction in incidence of breast cancer in women at high risk for breast cancer ("high-risk" defined as women at least 35 years of age with a 5-year predicted risk of breast cancer 1.67%, as calculated by the Gail Model.)
Raloxifene	1. Reduction in the risk of invasive breast cancer in postmenopausal women at high risk for invasive breast cancer (high risk is defined as at least one breast biopsy showing lobular carcinoma in situ or atypical hyperplasia, one or more first-degree relatives with breast cancer, or a 5-year predicted risk of breast cancer $>1.66\%$ [based on the modified Gail mode].)
Cervarix (HPV Vaccine)	1. Female 9 through 25 years of age for the prevention of the following diseases caused by oncogenic human papillomavirus (HPV) types 16 and 18: • cervical cancer. • cervical intraepithelial neoplasia (CIN) Grade 2 or worse and adenocarcinoma in situ. • cervical intraepithelial neoplasia (CIN) Grade 1.
Gardasil9 (HPV Vaccine)	1. Girls and women 9 through 26 years of age for the prevention of the following diseases: • Cervical, vulvar, vaginal, and anal cancer caused by human papillomavirus (HPV) types 16, 18, 31, 33, 45, 52, and 58. • Genital warts (condyloma acuminata) caused by HPV types 6 and 11. And the following precancerous or dysplastic lesions caused by HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58: • Cervical intraepithelial neoplasia (CIN) grade 2/3 and cervical adenocarcinoma in situ (AIS). • Cervical intraepithelial neoplasia (CIN) grade 1 • Vulvar intraepithelial neoplasia (VIN) grade 2 and grade 3 • Vaginal intraepithelial neoplasia (VaIN) grade 2 and grade 3 • Anal intraepithelial neoplasia (AIN) grades 1, 2, and 3. 2. Boys and men 9 through 26 years of age for the prevention of the following diseases: • Anal cancer caused by HPV types 16, 18, 31, 33, 45, 52, and 58. • Genital warts (condyloma acuminata) caused by HPV types 6 and 11. And the following precancerous or dysplastic lesions caused by HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58: • Anal intraepithelial neoplasia (AIN) grades 1, 2, and 3.

[a]As per the FDA product label.

With permission, taken in part, from reference Maresso, K. C., Tsai, K. Y., Brown, P. H., et al. (2015). Molecular cancer prevention: Current status and future directions [Internet]. *CA: A Cancer Journal for Clinicians* **65**, 345–383. Available from: http://doi.wiley.com/10.3322/caac.21287 [cited 7 August 2017].

Table 2 FDA approved therapies for the treatment of precancerous lesions

Agent	*FDA indication*[a]
Imiquimod	1. Immunocompetent adults for the topical treatment of clinically typical, nonhyperkeratotic, nonhypertrophic actinic keratoses on the face or scalp.
Ingenol mebutate	1. Males and females for the topical treatment of actinic keratosis on the face, scalp, trunk, and extremities.
Diclofenac sodium	1. Males and females for the topical treatment of actinic keratoses.
Fluorouracil	1. Males and females for the topical treatment of multiple actinic or solar keratosis.
Photodynamic therapy with 5-aminolevulinic acid	1. Males and females for the topical treatment of minimally to moderately thick actinic keratoses of the face or scalp
Photodynamic therapy with photofrin	1. Males and females with high-grade dysplasia in Barrett's esophagus. Ablation of high-grade dysplasia in Barrett's esophagus who do not undergo esophagectomy
Bacillus-Calmette-Guerin	1. Males and females with carcinoma in situ of the urinary bladder. Intravesical use in the treatment and prophylaxis of carcinoma in situ of the urinary bladder and for the prophylaxis of primary or recurrence stage Ta and/or T1 papillary tumors following transurethral resection (TUS).
Valrubicin	1. Males and females with BCG-refractory carcinoma in situ. Intravesical therapy of BCG-refractory carcinoma in situ of the urinary bladder in patients for whom immediate cystectomy would be associated with unacceptable morbidity or mortality.

[a]As per the FDA product label.

With permission, taken in part, from reference Maresso, K. C., Tsai, K. Y., Brown, P. H., et al. (2015). Molecular cancer prevention: Current status and future directions [Internet]. *CA: A Cancer Journal for Clinicians* **65**, 345–383. Available from: http://doi.wiley.com/10.3322/caac.21287 [cited 7 August 2017].

Chemoprevention Trial Results by Cancer Type

Head and neck cancers

Currently, the primary risk factors for squamous cell carcinoma of the head and neck (SCCHN) include tobacco smoke, alcohol consumption, and HPV (Forastiere et al., 2001). Originally described in 1953 (Slaughter et al., 1953), field cancerization is the predilection of the surface epithelium of the upper aerodigestive tract, or field, to develop additional cancers in patients with known carcinogen-related SCCHN. The specific premalignant changes found in areas of carcinogen-exposed epithelium adjacent to tumors can progress concurrently to form second primary tumors (SPTs). SPTs are a leading cause of mortality in head and neck cancer and typifies the concept of field cancerization (Vokes et al., 1993). The reversal of oral premalignant lesions (oral IEN or dysplastic oral IEN as intermediate biomarkers of invasive cancers) and prevention of SPTs have been the focus of extensive research since the 1980s.

Despite significant preclinical rational (Uray et al., 2016) and early phase trial success (Hong et al., 1990; Hong et al., 1986; Lippman et al., 1993) of retinoids, the most commonly tested chemopreventive agent, multiple large randomized clinical trials of these agents have failed to generate an FDA approval for oral cancer prevention (van Zandwijk et al., 2000; Bolla et al., 1994; Papadimitrakopoulou et al., 2009; Khuri et al., 2006). Although retinoids have not yet proven effective for SCCHN cancer prevention, numerous correlative studies within these trials have led to the discovery of markers of cancer risk including loss of heterozygosity (LOH) profiles (Mao et al., 1996), EGFR copy number gain (Taoudi Benchekroun et al., 2010), genetic variation (Lee et al., 2011), and gene expression signatures (Saintigny et al., 2011). For example, a large single nucleotide polymorphism (SNP) analysis of a cohort within the Retinoid Head and Neck Second Primary Trial of 13-cis-retinoic acid found that patients carrying the common genotype of rs3118570 in the retinoid X receptor were at a greater than threefold increased risk and that this locus along with two other genetic loci (CDC25C:rs6596428 and JAK2:rs1887427) identified those that had a 76% reduction in SPT/recurrence with chemoprevention (Lee et al., 2011). This data suggests that pharmacogenetic analysis might allow for improved targeting of "higher-risk" populations in future retinoid chemoprevention trials.

The Erlotinib Prevention of Oral Cancer (EPOC) study represents the first chemoprevention trial that was based a priori on personalized molecular characterization (William et al., 2016). Erlotinib, an EGFR tyrosine kinase inhibitor, was chosen based on the preclinical data, suggesting that EGFR amplification is associated with oral premalignant lesions (OPLs) transformation (Taoudi Benchekroun et al., 2010) and that EGFR inhibition prevents oral dysplasia (Leeman-Neill et al., 2011). In this trial, patients with OPLs were first evaluated for LOH at select loci and were considered high risk if LOH was present at 3p14 and/or 9p21 in patients with a history of invasive cancer or LOH at 3p14 and/or 9p21 plus an additional site including 17p, 11p, 13q, 4q, or 8p and were low-risk if not meeting either of these criteria. High-risk LOH patients (N = 150) were randomized to erlotinib or placebo and the primary endpoint was oral cancer free survival (CFS). The 3-year CFS rates in erlotinib versus placebo-treated patients were 70% and 74%, respectively, and were not statistically significant ($P = .45$). The study, however, did validate LOH as a marker for oral cancer risk as the 3-year CFS rates for LOH-positive versus LOH-negative groups were 74% and 87%, respectively ($P = .01$).

HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) has increased in incidence by 225% from 1998 to 2004 and continues to rise (Chaturvedi et al., 2011). Approximately 7% of the adults aged 14–69 population harbor active oral HPV infection, however only 1% of woman and 3% of men have the high-risk HPV16 subtype (Gillison et al., 2012). Although it is not certain that HPV vaccinations have directly reduced OPSCC, secondary evaluation of a large HPV16/18 vaccine trial suggested that oral HPV prevalence 4 years after vaccination was significantly reduced as compared to control (Herrero et al., 2013).

Lung cancers

The rationale for prevention of lung cancer is similar to that in HNSCC. In both diseases, chronic exposure to tobacco is the major risk factor and dysplastic epithelial lesions are thought to represent a premalignant stage. Unfortunately, despite decades of work, a variety of agents including α-tocopherol (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994), β –carotene (Hennekens et al., 1996; Omenn et al., 1996), retinoids (van Zandwijk et al., 2000; Lippman et al., 2001), selenium (Karp et al., 2013), and a combination of multivitamins and minerals (Kamangar et al., 2006) were all ineffective or deleterious (e.g., beta carotene) in large randomized phase III trials. These studies exemplify the limitations of utilizing epidemiological evidence and secondary endpoints in the absence of strong preclinical rational when planning chemoprevention trials.

For example, three large randomized, double-blind, placebo-controlled trials of β-carotene were conducted primarily based on epidemiological data alone. The Alpha-Tocopherol, Beta Carotene (ATBC) Cancer Prevention study was a primary-prevention trial in which 29,133 Finnish male smokers received α-tocopherol 50 mg a day, β-carotene 20 mg a day, both, or placebo. These men were between 50 and 69 years of age and all smoked five or more cigarettes a day and were followed for 5–8 years. Lung cancer incidence, the primary endpoint, did not change with the addition of β-tocopherol alone, nor did overall mortality. However, both groups who received β-carotene supplementation (alone or with α-tocopherol) had an 18% increase in the incidence of lung cancer. There appeared to be a stronger adverse effect from β-carotene in those men who smoked more than 20 cigarettes a day. This trial raised the serious issue that pharmacologic doses of β-carotene could potentially be harmful in active smokers. The β-Carotene and Retinol Efficacy Trial (CARET) confirmed the results of the Finnish ATBC trial (Omenn et al., 1996). This trial tested the combination of β-carotene 30 mg and retinyl palmitate 25,000 IU against placebo in 18,314 men and women aged 50–69 years at high risk for lung cancer; 14,254 had at least a 20 pack-year smoking history and were either current or recent former smokers. This trial was stopped after 21 months because no benefit and lung cancer incidence, the primary endpoint, increased 28%

in the active intervention group. Overall mortality also increased 17% in this group. The Physicians Health Study included 22,071 healthy male physicians treated with β-carotene 50 mg on alternate days or placebo (Hennekens et al., 1996). In contrast to the ATBC and CARET, the use of supplemental β-carotene showed virtually no adverse effects, however no beneficial effects on cancer incidence or overall mortality during a 12-year follow-up were observed. Given these results, high-dose β-carotene is not recommended for chemoprevention of lung cancer.

Although not based on epidemiologic data, the ECOG 5597 trial of selenium supplementation was largely based on a secondary analysis endpoint observed in a skin cancer prevention trial suggesting reduced lung cancer incidence (Karp et al., 2013; Clark et al., 1996). In this trial, 1561 patients with resected stage I NSCLC were randomly assisted to selenium 200 μg versus placebo daily for 48 months. The study was stopped early due to futility and showed no benefit of selenium over placebo.

Given the inaccessibility of the lungs for repeated histologic evaluations, practical intermediate biomarkers remain an area of unmet need. An interesting avenue of research is the potential role of bronchial airway gene expression signatures as a measure of risk and as a treatment response biomarker. In a murine model, researchers performed transcriptome sequencing to analyze bronchial airway gene expression and found activation of PI3K and Myc signaling in normal bronchial epithelial cells with precancer lung squamous cell carcinoma lesions (Xiong et al., 2017). Most notably, the activation of PI3K and Myc was reversed by treatment with the PI3K Inhibitor XL-147 and pioglitazone, respectively. If validated, these and similar biomarker studies might provide improved tools to analyze the effect of chemopreventive agents in high-risk individuals. The negative randomized trials described earlier highlight the need for coordinated efforts integrating epidemiological data, validated intermediate biomarkers, and most importantly a strong biologic rational prior to undertaking large chemoprevention trials (Szabo et al., 2013).

Colorectal cancers

Colorectal cancer (CRC) is associated with premalignant lesions, including adenomatous polyps and dysplastic epithelium. Large polyps with villous elements and dysplastic epithelia are more likely to progress to carcinoma than small, tubular polyps. The premalignant stages of colon carcinogenesis present an evaluable process for chemoprevention trials. Based on the well-established role of the cyclooxygenase (COX) enzymes and inflammation in colorectal carcinogenesis (Janakiram and Rao, 2014), aspirin and NSAIDs have been most extensively studied. In addition, fiber supplementation, hormone replacement therapy, and calcium salts have also underwent considerable evaluation.

The effects of aspirin on primary prevention of colon cancer have been examined in two large population-based trials including the Physician's Health Study (PHS) and the Women's Health Study (WHS). In the PHS, there was no benefit observed for aspirin at 325 mg every other day in the primary prevention of CRC at 5 or 12 years of follow up (Stürmer et al., 2006). In contrast, the WHS of aspirin at 100 mg every other day found no reduction in CRC at 10 years but after a median of 18 years of follow up, there was a reduction in risk (HR 0.80), which was primarily for proximal cancers (Cook et al., 2013). Though the reason for the discrepant results is not fully established, the PHS study only tested 5 years of aspirin and longer duration of treatment might be needed given the relatively prolonged latency to CRC development (Chan et al., 2005). Further supporting the role of aspirin for primary CRC prevention comes from the pooled secondary analysis of four large European and British trials that evaluated the effect of aspirin on vascular events. This analysis included 14,033 patients and suggested that the 20-year incidence of colon, but not rectal cancer, was decreased by nearly 25% (HR 0.76) when given beyond four years and at a dose of at least 75 mg daily (Rothwell et al., 2010). This compelling evidence led the US Preventive Services Task Force (USPSTF) to recommend "low-dose aspirin use for the primary prevention of cardiovascular disease (CVD) and colorectal cancer in adults ages 50 to 59 years who have a 10% or greater 10-year CVD risk, are not at increased risk for bleeding, have a life expectancy of at least 10 years, and are willing to take low-dose aspirin for at least 10 years (Bibbins-Domingo, 2016)". For patients 60–69 years old, the USPSTF suggested consideration of low-dose aspirin after discussion of risks and benefits and for patients $\leq$49 or $>$70 years, there was insufficient evidence to recommend aspirin as chemoprevention. The USPSTF suggested utilizing the freely available ASCVD Risk Estimator from the ACC/AHA for estimating 10-year cardiovascular risk (Goff et al., 2013).

The role of aspirin as secondary prevention of recurrent adenomas after resection of adenomas is supported by a metaanalysis involving four clinical trials with 2967 randomly assigned patients (Cole et al., 2009). The risk of adenoma recurrence was lower for aspirin at any dose versus placebo (risk ratio [RR], 0.83), corresponding to an absolute risk reduction of nearly 7%. For advanced lesions, as defined as tubulovillous adenomas, villous adenomas, adenomas $\geq$1 cm in diameter, adenomas with high-grade dysplasia, or invasive cancer, the risk reduction was even greater (RR 0.72). These data suggest that aspirin is associated with a 20%–35% reduction in colonic adenomas and CRC incidence in average risk adults.

The role of aspirin in hereditary CRC syndromes has also been evaluated in a pragmatic series of studies within the Colorectal Adenoma/Carcinoma Prevention Programme (CAPP). CAPP1 randomized 133 patients with familial adenomatous polyposis (FAP) to 600 mg aspirin daily or placebo and found a nonsignificant trend suggesting reduction in polyp count (RR 0.77) (Burn et al., 2011). In CAPP2, 937 patients with hereditary nonpolyposis colon cancer (Lynch Syndrome) were similarly randomized and no significant effect on incidence of adenoma formation or carcinoma was observed with aspirin therapy (Burn et al., 2008). Notably, in a perprotocol secondary analysis, patients in CAPP2 who received aspirin 600 mg daily for at least 2 years had a nearly 60% reduction in colorectal cancer incidence (Burn et al., 2011). An ongoing study evaluating different doses of aspirin in Lynch Syndrome is ongoing (CAPP3) and eagerly awaited.

Similar to aspirin, COX-2 inhibitors and sulindac have also been extensively studied. Forms of the COX enzyme include COX-1, which is constitutively expressed, and COX-2, which is overexpressed in inflammatory cells and has been associated with colorectal adenomatous polyp and cancer formation. Most NSAIDs inhibit both enzymes, however inhibition of COX-2 specifically reduces

untoward side effects such as ulcers and gastritis. The Prevention of Colorectal Sporadic Adenomatous Polyps (PreSAP, $N = 1561$) and Adenomas Prevention with Celecoxib (APC, $N = 2035$) were large randomized placebo-controlled trials in patients with resected adenomas and both found significant 30%–50% reduction in recurrent polyp formation (Bertagnolli et al., 2006; Arber et al., 2006). However, both trials also observed a significant increase in adverse cardiovascular events (two- to threefold) precluding the routine use of this agent. Similarly, a smaller study of celecoxib in FAP showed significant benefit and led, in part, to the accelerated approval of celecoxib for patients with phenotypic expression of FAP (Steinbach et al., 2000). However, this indication was subsequently voluntary removed in 2011 by the manufacturer due to delays in completing follow up studies required under the approval. Additionally, despite three small ($N = 10–24$) studies of sulindac suggesting secondary preventive benefit of adenoma regression, number, and size in phenotypic FAP, a study of 41 genotypically confirmed, but not phenotypically affected, patients with FAP over four years failed to show sulindac prevented the development of adenomas compared with placebo (Giardiello et al., 2002). Given the established harm (cardiovascular and gastrointestinal) associated with long-term use of NSAIDs, these agents cannot currently be recommended for primary or secondary prevention of CRC.

Fiber in the prevention of colon cancer has been examined in several studies. The Polyp Prevention Trial studied 2079 patients with a history of colorectal adenomas randomized to receive counseling, a low-fat, high-fiber diet rich in fruits and vegetables, or to continue their current diet without counseling (Schatzkin et al., 2000). Colonoscopy after one and four years found no difference in the incidence of recurrent adenomas. Another study by the Phoenix Colon Cancer Prevention Physician's Network studied 1429 patients with a history of colorectal adenoma (Alberts et al., 2000). These patients were randomized to receive supplemental wheat bran, 2.0 or 13.5 g a day. Again, no difference in the incidence of recurrent adenomas was found between the two groups. Currently, there is no prospective evidence that fiber supplementation is effective for colorectal cancer prevention.

Hormone replacement therapy in women in the form of combined estrogen plus progestin was observed to reduce the risk of CRC by nearly 45% in the Women's Health Initiative (WHI). However, extended follow up of WHI suggested that with longer-term therapy, women receiving combination therapy had more advanced CRC diagnosis with a higher, albeit, nonstatistically significant increase in CRC morality rate (Simon et al., 2012). Given this data along with the negative data of unopposed estrogen (Lavasani et al., 2015), and the long-term cardiovascular concerns of HRT, HRT cannot be recommended for CRC prevention.

High intake of calcium salts has been associated with lower risk of colorectal cancer along with synergistic chemopreventive effects when combined with vitamin D (Grau et al., 2003; Pence, 1993). Despite the biologic rational and epidemiological data, a large randomized trial of 2259 patients receiving vitamin D3 1000 IU, calcium carbonate 1200 mg, both, or neither daily showed no difference in colorectal adenoma formation over a 3–5-year period (Baron et al., 2015). Another similar randomized trial of 36,282 postmenopausal women showed no benefit of calcium and vitamin D over placebo in the development of CRC during a seven-year period.

Nonmelanoma and melanoma skin cancers

Chemoprevention of skin cancers represents an ideal target organ for chemoprevention due to the sheer incidence in humans and practical accessibility of the skin. The vast majority of nonmelanoma skin cancers (NMSC) include cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC). cSCC accounts for 10%–20% of NMSC and occurs largely as a result of intraepithelial ultraviolet (UV)-induced damage which leads to a sequence of molecular changes, most often involving mutations of the p53 tumor suppressor gene (de Gruijl et al., 2001). cSCC has been the focus of the most chemoprevention trials due to the presence of the identifiable premalignant lesion, the actinic keratosis (AK) in 60%–70% of cSCC (Fernandez Figueras, 2017).

Currently, the primary strategy for lesion-directed therapy for a single or few AKs involves ablative modalities with curettage, cryosurgery, or electrodessication. Field-directed topical therapies, including 5-fluorouracil cream, imiquimod cream, diclofenac gel, ingenol mebutate gel, and delta-aminolevulinic acid photodynamic therapy have all been FDA-approved for the treatment of AKs (see **Table 2**). These topical agents have been extensively studied in randomized controlled trials; however, no large trials comparing the efficacy between these agents have been undertaken. Despite inherent limitations of cross trial comparisons, their effect on AK clearance and adverse effects are comparable (Haque et al., 2015; Gupta et al., 2012).

Retinoids, including retinol, acitretin, isotretinoin, and etretinate, have been the mainstay of oral chemoprevention trials directed at reduction of NMSCs. In a phase III trial, 2297 patients who were at moderate risk for skin cancer, including patients with a history of >10 AKs and ≤ 2 prior NMSCs, received oral retinol 25,000 IU or placebo daily for 5 years (Moon et al., 1997). Retinol treatment was effective in reducing the incidence of cSCC (HR 0.74), but not BCC. However, another much larger ($N = 22,071$) primary prevention trial of β-carotene compared with placebo showed no effect on cSCC or BCC (Frieling et al., 2000). Importantly, even in higher-risk patients with a history on NMSCs, three phase III trials that tested the effects of retinol (Levine et al., 1997), β-carotene (Greenberg et al., 1990), and low-dose isotretinoin (Tangrea et al., 1992) demonstrated that these retinoids had no effect on the incidence of SPTs.

The immunosuppression required for organ transplant patients is an established strong risk factor (65–250-fold) for the development of aggressive cSCC (Tufaro et al., 2015). The second-generation retinoid acitretin has shown some clinical benefit particularly in high-risk organ transplant recipients. For example, a randomized placebo-controlled trial of 44 renal-transplant patients showed that acitretin at 30 mg daily over a 6-month period reduced new cSCC and AKs compared with placebo (11% vs. 47% and 13% vs. 28%, respectively)(Bavinck et al., 1995). However, in another small trial ($N = 26$) of low (0.2 mg/kg daily) versus high-dose (0.4 mg/kg daily) acitretin, no difference in cSCC was observed (de Sévaux et al., 2003). Acitretin in nontransplant high-risk patients failed to

show benefit over placebo (Kadakia et al., 2012). Although systemic retinoids are used in high-risk patients in practice (Otley et al., 2006), neither acitretin nor any other systemic retinoid is FDA approved. Importantly, use of these agents is limited to the duration they are taken and rapid AKs/NMSC devolvement upon discontinuation can occur (George et al., 2002).

Despite preclinical data supporting the efficacy of COX-2 inhibition and the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO), neither agent in randomized placebo-controlled trials met their primary endpoint of reduction in AKs (Elmets et al., 2010) and NMSCs (Bailey et al., 2010), respectively. Nicotinamide (Vitamin B3) at 500 mg twice daily, which is protective against UV damage, was tested in a randomized placebo-controlled trial in 386 patients with a history of ≥ 2 NMSCs (Chen et al., 2015). There was a significant 23% reduction in new NMSC, with a slightly higher reduction in cSCC compared with BCC (30% vs. 20%). Although promising, longer-term safety and efficacy are needed prior to recommending nicotinamide for secondary prevention. Lastly, a phase II randomized placebo-controlled trial of vismodegib, an oral hedgehog inhibitor approved for locally advanced or metastatic BCC, at 150 mg daily showed significant chemopreventive action in patients with the highly penetrant nevoid basal cell carcinoma syndrome, however 54% of patients discontinued treatment due to toxicity (Tang et al., 2012).

Although significant advances in the field of immuno-oncology have changed the prognosis of advanced melanoma, no agent, including vitamin D, NSAIDs, sulindac, and lipid-lowering drugs, has yet been proven to successfully prevent neoplastic melanocytic transformation (Chhabra et al., 2017).

Breast cancer

Chemoprevention approaches in breast cancer focus on preventing breast cancer in high-risk patients. This strategy stemmed from the success of tamoxifen, a selective estrogen-receptor modulator (SERM), in reducing recurrent breast cancer by 40%–50% following definitive therapy for estrogen-receptor (ER) positive breast cancer (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al., 2011). Multiple large trials of tamoxifen compared with placebo in both high-risk and average risk women have been conducted since the 1990s. A 2013 meta-analysis revealed that compared to placebo tamoxifen decreased the incidence of invasive breast cancer, primarily as a reduction in ER positive breast cancer risk by 30% (RR 0.70) and a reduction in nonvertebral factures risk by 34% (RR 0.66) but had no impact on breast cancer-specific or all-cause mortality (Nelson et al., 2013). The long-term follow up of the STAR (Study of Tamoxifen and Raloxifene) trial found that the second-generation SERM raloxifene confers 78% efficacy of tamoxifen in breast cancer prevention, however was not associated with an increase in uterine cancer (Nelson et al., 2013; Vogel et al., 2010). Despite the chemopreventive benefits observed, a patient-level meta-analysis of 83,399 patients involved in SERM chemoprevention trials showed that multiple rare but serious risks occur including a slight increase in uterine cancer (105 vs. 63 events, HR 1.56) and thromboembolic disease (375 vs. 215, HR 1.73)(Cuzick et al., 2013). Based on these data, tamoxifen and raloxifene were FDA-approved for the primary prevention of breast cancer in high-risk population (see **Table 1**). High-risk in these studies was defined as women at least 35 years of age with a 5-year predicted risk of breast cancer $\geq 1.67\%$, as calculated by the Gail Model (Rockhill et al., 2001).

Aromatase inhibitors, which have also showed significant benefit over tamoxifen in the adjuvant breast cancer setting in postmenopausal women (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al., 2015), have also been evaluated for primary prevention in high-risk patients. Both exemestane (NCIC-MAP3 trial, $N = 4560$) and anastrozole (IBIS-II trial, $N = 3864$) as compared to placebo revealed significant reductions in invasive breast cancer by 50%–65% (Goss et al., 2011; Cuzick et al., 2014). As with SERMs, neither anastrozole or exemestane significantly reduced. Anastrozole and exemestane are not yet FDA-approved for risk reduction.

Despite the minimal negative effect on quality of life reported in the major phase III chemoprevention trials of SERMs and AIs, uptake of these agents in the clinic has been very low. For women aged 35 years or older with increased risk of breast cancer and low risk of adverse effects, the USPSTF provides a grade B recommendation and supports shared and informed decision making when offering tamoxifen or raloxifene. Both agents are effective in pre- and postmenopausal women (Nelson et al., 2013). For high-risk patients who wish to pursue chemoprevention, raloxifene is a reasonable choice if concerns for uterine cancer and thromboembolic disease predominate and tamoxifen if risk of breast cancer prevention predominates. To date, there are no proven therapies to prevent HER2-positive breast cancers, although trials are ongoing targeting anti-HER2 therapy for HER2-positive ductal carcinoma in situ and might provide rational for future chemoprevention studies.

Esophageal and gastric cancer

Esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) are the two predominate histologic subtypes of esophageal cancer. Though historically less common, the incidence of EAC has dramatically risen in the US and westernized countries and is suspected to be related to multiple risk factors including obesity and gastroesophageal reflux disease (Zhai et al., 2010; Drahos et al., 2016).

Given the precursor premalignant lesion (Barrett's esophagus) in EAC, secondary prevention utilizing endoscopic screening in high-risk patients as well as local ablative and excisional therapies for established high-grade Barrett's esophagus is the current clinical approach to reduce the risk of progression to EAC. The FDA approved porfimer sodium in conjunction with photodynamic therapy (PDT) in 2003 based on a randomized trial of this combination versus omeprazole alone in 208 patients with high-grade dysplasia (Overholt et al., 2005). Complete ablation of high-grade dysplasia and progression to invasive cancer was improved with porfimer sodium than with omeprazole alone (77% vs. 39% and 13% vs. 28%, respectively), however serious complications

occurred in 12% and strictures in 36%. Radiofrequency ablation with mucosal resection has replaced PDT due to the improved efficacy and safety (Shaheen et al., 2009). A randomized trial of celecoxib in patients with Barrett's esophagus showed no difference in regression of dysplasia as compared to placebo (Heath et al., 2007). A large study of 2500 patients (AspECT trial) comparing aspirin in combination with low or high-dose esomeprazole to esomeprazole alone and has completed accrual and results are eagerly awaited.

In contrast to EAC, ESCC remains prevalent in developing countries and is associated with tobacco and alcohol exposure as well as nutrient deficiencies and exposure to *N*-nitroso compounds. Multiple large randomized chemoprevention studies involving residents of Linxian, China tested the effects of combinations of agents such as retinol, riboflavin, zinc, selenomethionine, and vitamin E without a clear signal of benefit (Blot et al., 1993; Wang et al., 2013; Limburg et al., 2005). A 10-year follow up of the largest trial involving 29,584 patients suggested a 17% reduction in ESCC mortality in those aged <55 years, but an increase of 14% in mortality in those aged ≥ 55 years (Qiao et al., 2009). The interpretation of these studies is made difficult by the use of readily available vitamin supplements in the control arm, blurring potential differences from the treatment arm. This represents an inherent flaw in trials utilizing vitamin supplements and dietary interventions.

Worldwide, *Helicobacter pylori*, a Gram-negative microaerophilic bacterium, are frequently associated with noncardia gastric cancers. Although treatment of *H. pylori* with a combination of antibiotics and a proton pump inhibition ("triple therapy") is clearly effective, data to support a chemopreventive role of such therapy on decreasing gastric cancer occurrence are becoming increasingly realized. One large trial involving 3365 patients randomized patients to amoxicillin and omeprazole for two weeks and showed that at 15 years follow up the incidence of gastric cancer was reduced in those receiving antibiotic therapy (3% vs. 4.6%, odds ratio of 0.61) (Ma et al., 2012). COX-2 inhibition with celecoxib for 24 months was tested alone or following *H. pylori* treatment versus placebo in a randomized trial of 1024 patients with *H. pylori* and advanced gastric lesions (as defined as severe chronic atrophic gastric, intestinal metaplasia, indefinite dysplasia, or dysplasia) (Wong et al., 2012). Interestingly, regression of gastric lesions was increased by both celecoxib and anti-*H. pylori* treatment alone (53% vs. 41% and 59% vs. 41%, respectively), however celecoxib following anti-*H. pylori* treatment showed no benefit over placebo. A meta-analysis of aspirin from cardiovascular disease trials revealed that aspirin was associated with reduction in a variety of cancers including gastric cancer mortality if treated for >10 years (HR 0.42) (Rothwell et al., 2011).

Based on the available evidence, screening and local ablative therapies for high-risk Barrett's esophagus to prevent EAC and treatment of *H. pylori* in high-risk areas is recommended. Although data regarding NSAIDs and aspirin appear encouraging, large phase III studies are needed before firm recommendations can be made.

Bladder cancer

Urothelial carcinoma of the bladder is most commonly attributed to tobacco as well as environmental and occupational carcinogen exposures. Although a number of trials for primary and secondary chemoprevention have been conducted, none have proven effective with the exception of intravesical agents. Only Bacillus-Calmette-Guerin (BCG) and intravesical valrubicin in BCG-refractory patients are FDA-approved (**Table 2**). A comprehensive review of intravesical therapy to prevent recurrence of nonmuscle invasive bladder cancer will not be reviewed here. Instead, the results of major chemoprevention trials of oral agents will be discussed.

Previous studies using retinoids to prevent recurrence in bladder cancer patients have been small and limited by toxicity. Two trials with prolonged low-dose etretinate appeared to have some efficacy. One double-blind, placebo-controlled trial studied the preventive effect of etretinate in 30 patients with superficial bladder cancers (Alfthan et al., 1983). A reduction in recurrence was seen, however a larger multicenter randomized study of 90 patients observed significant toxicity including deaths from myocardial infarction precluding further study of this agent (Studer et al., 1995). Fenretinide, a better tolerated retinoid, failed to show any significant difference in recurrence rates over placebo in two randomized trials (Sabichi et al., 2008; Decensi et al., 2000). Notably, no benefit of retinoids as primary prevention of bladder cancer was observed in the ATBC trial (described in **Lung Cancers** section) or a meta-analysis of six case-control studies (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994; Steinmaus et al., 2000).

A number of other agents including vitamins, selenium, DFMO, and NSAIDs have been studied with largely disappointing results. Pyridoxine (vitamin B6) was hypothesized to counteract the high level of tryptophan metabolites observed in patients with bladder carcinoma. Despite encouraging results from a smaller trial, a larger European Organization for Research and Treatment of Cancer double-blind randomized phase III trial of 291 men with superficial bladder cancer showed no difference between pyridoxine and placebo with regards to recurrence rate or time to first recurrence (Newling et al., 1995). Notwithstanding promising preclinical rational, the phase III Selenium and Bladder Cancer Trial (SELEBLAT) randomized141 patients with noninvasive bladder cancer to selenium-yeast supplementation at 200 μg/day for 3 years and observed no significant decrease in recurrence compared with placebo (Goossens et al., 2016). Similarly, DFMO, a polyamine synthesis inhibitor, at 1 gram daily for 12 months failed to prevent recurrent bladder cancer compared with placebo (Messing et al., 2006). NSAIDs have also failed to show benefit in preventing secondary bladder cancer. In one trial patients were treated with celecoxib 200 mg twice daily for an average of 1.25 years with no difference in recurrence rate compared with the placebo group (Sabichi et al., 2011). Another larger double-blind phase III trial of celecoxib 200 mg twice daily for two years found no difference in recurrent disease and observed an increase in cardiovascular events (Kelly et al., 2015).

Currently, there is no role for any nonintravesical chemoprevention agent in the primary or secondary prevention of bladder cancer.

Cervical cancer

Following the regular implementation of the Papanicolaou (Pap) test for cervical cancer screening, a marked reduction in cervical cancer incidence and mortality in the US and worldwide was observed (Peirson et al., 2013). HPV is the chief etiologic agent for cervical cancer carcinogenesis. Although there are at least 118 HPV types, around 40 are specific to the anogenital epithelium and among these, several high-risk HPV types are considered carcinogenic including HPV types 16 and 18, which account for 70% of diagnoses (de Villiers et al., 2004). Both squamous cell carcinoma and adenocarcinoma of the cervix undergo well-recognized premalignant dysplastic stages (i.e., cervical intraepithelial neoplasia [CIN] 1–3 and adenocarcinoma in situ [AIS], respectively) that are accessible for chemoprevention study.

Primary prevention with HPV vaccination has markedly reduced the risk of cervical cancer and the development of CIN 2 and 3. The FUTURE II trial randomized 12,167 women between the ages of 15 and 26 to three doses of either HPV-6/11/16/18 vaccine or placebo, given at day 1, month 2, and month 6 with a primary composite endpoint of CIN 2 or 3, AIS, or cervical cancer related to HPV 16 or 18. At three years, the quadrivalent vaccine prevented the primary composite endpoint by 98% compared with 44% in the placebo group (FUTURE II Study Group, 2007). In a subsequent randomized trial of 14,215 women aged 16–26, the 9-valent HPV vaccine was equally effective at preventing cervical, vulvar, and vaginal cancers and warts as the quadrivalent vaccine (Joura et al., 2015). Other large trials of the bivalent HPV vaccine also showed similar efficacy in preventing CIN 2 or more severe disease due to HPV infection (Hildesheim et al., 2014). Quadrivalent HPV vaccination of 4065 boys and men aged 16–26 prevented infections with HPV-6/11/16/18 and led to 60% less genital lesions compared with placebo (Giuliano et al., 2011). These data led to the FDA approval of HPV vaccines: Gardasil9 for males and females as well as Cervarix for females (see **Table 1**).

Prior to the development of HPV vaccination, chemoprevention utilizing a variety of agents including interferon, folic acid, β-carotene, DFMO, and retinoids were tested with largely negative results. The efficacies of HPV vaccination along with the improved techniques of cervical screening remain the cornerstone of cervical cancer prevention.

Prostate cancer

The high incidence, long latency, dependence on androgen, and known intermediate biomarkers (high-grade prostatic intraepithelial neoplasia [HG-PIN] and prostate specific antigen [PSA]) of prostate cancer has led to numerous efforts focusing on chemoprevention. The 5-αreductase (5-AR) inhibitors and nutrients including selenium and vitamin E have been best studied.

The Prostate Cancer Prevention Trial (PCPT) was launched in 1993 with its principle endpoint being a reduction of biopsy-proven prostate cancer incidence. This double-blind placebo-controlled trial included 18,882 males aged 55 years and older who were randomized to finasteride, a 5-AR inhibitor, at 5 mg or placebo daily for seven years. The trial was closed early due to meeting the primary study endpoint with a 25% decrease in biopsy-proven prostate cancer, however controversy ensued due to the observed absolute increase in number and proportion of patients with high-grade (Gleason score $\geq$7) prostate cancers (Thompson et al., 2003). At 18 years of follow up, the risk of high-grade prostate cancer remained elevated in patients randomized to finasteride (3.5% vs. 3%) and no between-group difference in the rates of overall or cancer-specific survival was observed (Thompson et al., 2013). In another trial (REDUCE trial) testing the 5-AR inhibitor dutasteride in men with an elevated PSA of 2.5–10 ng/mL and a negative prostate biopsy showed a nearly 25% reduction in biopsy-proven prostate cancer. However, similar to PCPT, higher rates of high-grade prostate cancer were observed (Andriole et al., 2010).

Selenium and vitamin E also had been observed to decrease prostate cancer incidence in several early phase trials. Based on these early phase studies, the National Cancer Institute launched the SELECT trial which tested if selenium 200 μg/day, vitamin E 400 IU/day, both, or placebo in 35,533 men with PSA $\leq$SA 33 menbolenium 200 μg/day, exam (Klein et al., 2011). Study treatment was stopped after a three-year interim analysis suggested futility and vitamin E was noted to increase the risk of prostate cancer (HR 1.17). Similarly, selenium alone or in combination with vitamin E showed no chemopreventive benefit even in those with low selenium status. Importantly, selenium increased the risk of high-grade prostate cancer among those with high selenium status (Kristal et al., 2014).

Currently, the evidence to support any 5-AR inhibitor for prostate cancer chemoprevention is limited due to the potential for increasing high-grade prostate cancer and the lack of any demonstrable impact on mortality. Nutrients including selenium and vitamin E, above the recommended dietary intake, should not be endorsed for the chemoprevention of prostate cancer.

Hepatocellular carcinoma

HBV and hepatitis C virus (HCV) account for the major risk factors for hepatocellular carcinoma (HCC) due to the development of chronic liver disease. HBV-related HCC has declined with the increasing use of the HBV vaccination. In a 20-year follow-up study from Taiwan, where universal vaccination was first implemented in 1984, there was a nearly 70% risk reduction of HCC in vaccinated compared with unvaccinated cohorts (Chang et al., 2009). Although an effective HCV vaccine has yet to be created, observational data of antiviral therapy in the form of interferon or direct-acting antivirals might reduce the risk of HCV-related HCC, particularly those resulting in sustained viral response (Chou et al., 2014; Smith et al., 2012). Studies of retinoids in HCC have been described as well. A prospective randomized trial of 89 patients who were definitively treated for HCC received polyprenoic acid (600 mg daily) or placebo for 12 months. There was a significant decrease in recurrent HCC in the retinoid-treated group (27% vs. 49%) which lasted up to 199 weeks after randomization (Takai et al., 2005; Muto et al., 1996; Chu et al., 2010). Further studies are needed to verify these encouraging results.

Future Directions

Cancer Risk Models

Optimally, treatment of epithelial malignancies should move toward prevention. Since the early 1980s, many patients at high risk for epithelial cancers, mostly those with premalignant lesions or a personal history of epithelial malignancy, have been enrolled in chemoprevention trials (as described earlier). While this accounts for a substantial number of patients, the vast majority of epithelial malignancies arise in patients with no history of either of these risk factors. This particular population is, at present, generally unrecognizable and is therefore most often not entering chemoprevention trials. This highlights the need to develop risk models to define high-risk people from the general population.

Historically, cancer risk models have relied largely on risk factors obtained from population-level data. The Gail Model of breast cancer risk represents a relatively simple example of this and is related to multiple variables accounting for estrogen exposure (Rockhill et al., 2001). Although the Gail Model is validated in breast cancer, similar models for other cancers as well as more refined breast cancer risk assessments ideally require the incorporation of measurable objective biologic data. A simple example of such an objective risk assessment is diagnosing heredity breast and ovarian cancer syndrome via germ-line molecular testing of the BRCA-1 and BRCA-2 genes. This thereby allows identification of a high-risk cohort available for further study. However, for more common non-hereditary cancers it is likely that a combination of histologic, genetic, epigenetic, and proteomic analyses will elucidate those at highest risk. Utilizing transcriptome meta-analysis, scientists found specific gene signatures that drive HCC risk in cirrhosis (Nakagawa et al., 2016). Other studies utilizing novel technologies such as hierarchical gene clustering and computer-assisted imaging analysis (e.g., morphometric signatures) are ongoing (Gann et al., 2013; Ciriello et al., 2015). If validated, such comprehensive tools can be used to select the "highest-risk" patients for chemoprevention trials, thereby avoiding diluting any possible signal of efficacy by inclusion of lower-risk patients.

Summary

The ultimate goal of oncology is to prevent rather than treat morbid invasive cancer. Despite multiple chemoprevention trial failures described earlier, the successes of endocrine therapy in breast cancer primary prevention and HPV vaccinations in cervical cancer provide proof of principle that chemoprevention has the potential to significantly decrease the burden of cancer in society. Ongoing advances in our understanding of the molecular complexities driving carcinogenesis provide a plethora of new avenues to investigate. Current concerted multidisciplinary efforts such as the Precancer Atlas represent a major shift toward better defining the earliest features of neoplasia and exploiting all the technological advances in contemporary science to successfully undertake future precision chemoprevention trials (Spira et al., 2017).

See also: Cancer Survival and Survivorship. Chemoprevention of Cancer: An Overview of Promising Agents and Current Research. Hereditary Cancer Syndromes: Identification and Management.

References

Aggarwal, B.B., 2004. Nuclear factor-kappaB: The enemy within [Internet]. Cancer Cell 6, 203–208. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1535610804002442 [cited 2017 June 2019].

Alberts, D.S., Martinez, M.E., Roe, D.J., et al., 2000. Lack of effect of a high-fiber cereal supplement on the recurrence of colorectal adenomas [Internet]. The New England Journal of Medicine 342, 1156–1162. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10770980 [cited 24 June 2017].

Alfthan, O., Tarkkanen, J., Gröhn, P., et al., 1983. Tigason (etretinate) in prevention of recurrence of superficial bladder tumors. A double-blind clinical trial [Internet]. European Urology 9, 6–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6337054 [cited 25 June 2017].

Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers [Internet]. The New England Journal of Medicine 330, 1029–1035. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM199404143301501 [cited 24 June 2017].

Andriole, G.L., Bostwick, D.G., Brawley, O.W., et al., 2010. Effect of dutasteride on the risk of prostate cancer [Internet]. The New England Journal of Medicine 362, 1192–1202. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa0908127 [cited 25 June 2017].

Arber, N., Eagle, C.J., Spicak, J., et al., 2006. Celecoxib for the prevention of colorectal adenomatous polyps [Internet]. The New England Journal of Medicine 355, 885–895. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa061652 [cited 24 June 2017].

Bailey, H.H., Kim, K., Verma, A.K., et al., 2010. A randomized, double-blind, placebo-controlled phase 3 skin cancer prevention study of -difluoromethylornithine in subjects with previous history of skin cancer [Internet]. Cancer Prevention Research 3, 35–47. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20051371 [cited 25 June 2017].

Baron, J.A., Barry, E.L., Mott, L.A., et al., 2015. A trial of calcium and vitamin D for the prevention of colorectal adenomas [Internet]. The New England Journal of Medicine 373, 1519–1530. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26465985 [cited 24 June 2017].

Bavinck, J.N., Tieben, L.M., Van der Woude, F.J., et al., 1995. Prevention of skin cancer and reduction of keratotic skin lesions during acitretin therapy in renal transplant recipients: A double-blind, placebo-controlled study [Internet]. Journal of Clinical Oncology 13, 1933–1938. Available from: http://ascopubs.org/doi/10.1200/JCO.1995.13.8.1933 [cited 25 June 2017].

Bertagnolli, M.M., Eagle, C.J., Zauber, A.G., et al., 2006. Celecoxib for the prevention of sporadic colorectal adenomas [Internet]. The New England Journal of Medicine 355, 873–884. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa061355 [cited 24 June 2017].

Bibbins-Domingo, K., 2016. U.S. Preventive Services Task Force: Aspirin use for the primary prevention of cardiovascular disease and colorectal cancer: U.S. Preventive Services Task Force recommendation statement [Internet]. Annals of Internal Medicine 164, 836–845. Available from: http://annals.org/article.aspx?doi=10.7326/M16-0577 [cited 30 July 2017].

Blot, W.J., Li, J.Y., Taylor, P.R., et al., 1993. Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population [Internet]. Journal of the National Cancer Institute 85, 1483–1492. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8360931 [cited 25 June 2017].

Bolla, M., Lefur, R., Ton Van, J., et al., 1994. Prevention of second primary tumours with etretinate in squamous cell carcinoma of the oral cavity and oropharynx. Results of a multicentric double-blind randomised study [Internet]. European Journal of Cancer 30A, 767–772. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7917535 [cited 24 June 2017].

Burn, J., Bishop, D.T., Mecklin, J.-P., et al., 2008. Effect of aspirin or resistant starch on colorectal neoplasia in the Lynch syndrome [Internet]. The New England Journal of Medicine 359, 2567–2578. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa0801297 [cited 24 June 2017].

Burn J, Bishop DT, Chapman PD, et al.: A randomized placebo-controlled prevention trial of aspirin and/or resistant starch in young people with familial adenomatous polyposis [Internet]. Cancer Prevention Research (Philadelphia, Pa.) 4:655–65, 2011 Available from: http://cancerpreventionresearch.aacrjournals.org/cgi/doi/10.1158/1940-6207.CAPR-11-0106 [cited 24 June 2017]

Burn, J., Gerdes, A.-M., Macrae, F., et al., 2011. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: An analysis from the CAPP2 randomised controlled trial [Internet]. Lancet (London, England) 378, 2081–2087. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0140673611610490 [cited 24 June 2017].

Caruso, C., Lio, D., Cavallone, L., et al., 2004. Aging, longevity, inflammation, and cancer [Internet]. Annals of the New York Academy of Sciences 1028, 1–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15915584 [cited 2017 June 2019].

Chan, A.T., Giovannucci, E.L., Meyerhardt, J.A., et al., 2005. Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. [Internet]. JAMA 294, 914–923. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.294.8.914 [cited 24 June 2017].

Chang, M.-H., You, S.-L., Chen, C.-J., et al., 2009. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: A 20-year follow-up study [Internet]. Journal of the National Cancer Institute 101, 1348–1355. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19759364 [cited 19 June 2017].

Chaturvedi, A.K., Engels, E.A., Pfeiffer, R.M., et al., 2011. Human papillomavirus and rising oropharyngeal cancer incidence in the United States [Internet]. Journal of Clinical Oncology 29, 4294–4301. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21969503 [cited 30 July 2017].

Chen, A.C., Martin, A.J., Choy, B., et al., 2015. A phase 3 randomized trial of nicotinamide for skin-cancer chemoprevention [Internet]. The New England Journal of Medicine 373, 1618–1626. Available from: http://www.nejm.org/doi/10.1056/NEJMoa1506197 [cited 25 June 2017].

Chhabra, G., Ndiaye, M.A., Garcia-Peterson, L.M., et al., 2017. Melanoma chemoprevention: Current status and future prospects [Internet]. Photochemistry and Photobiology. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28295364 [cited 25 June 2017].

Chou, R., Dana, T., Bougatsos, C., et al., 2014. Screening for hepatitis B virus infection in adolescents and adults: A systematic review to update the U.S. preventive services task force recommendation [Internet]. Annals of Internal Medicine 161, 31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24861032 [cited 25 June 2017].

Chu, K.-J., ECH, L., Yao, X.-P., et al., 2010. Vitamin analogues in chemoprevention of hepatocellular carcinoma after resection or ablation—A systematic review and meta-analysis [Internet]. Asian Journal of Surgery 33, 120–126. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21163409 [cited 25 June 2017].

Ciriello, G., Gatza, M.L., Beck, A.H., et al., 2015. Comprehensive molecular portraits of invasive lobular breast cancer [Internet]. Cell 163, 506–519. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26451490 [cited 25 June 2017].

Clark, L.C., Combs, G.F., Turnbull, B.W., et al., 1996. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group [Internet]. JAMA 276, 1957–1963. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8971064 [cited 24 June 2017].

Cole BF, Logan RF, Halabi S, et al.: Aspirin for the chemoprevention of colorectal adenomas: Meta-analysis of the randomized trials [Internet]. Journal of the National Cancer Institute 101:256–66, 2009 Available from: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/djn485 [cited 24 June 2017]

Cook, N.R., Lee, I.-M., Zhang, S.M., et al., 2013. Alternate-day, low-dose aspirin and cancer risk: Long-term observational follow-up of a randomized trial [Internet]. Annals of Internal Medicine 159, 77–85. Available from: http://annals.org/article.aspx?doi=10.7326/0003-4819-159-2-201307160-00002 [cited 24 June 2017].

Cuzick, J., Sestak, I., Bonanni, B., et al., 2013. Selective oestrogen receptor modulators in prevention of breast cancer: An updated meta-analysis of individual participant data [Internet]. Lancet (London, England) 381, 1827–1834. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0140673613601403 [cited 25 June 2017].

Cuzick, J., Sestak, I., Forbes, J.F., et al., 2014. Anastrozole for prevention of breast cancer in high-risk postmenopausal women (IBIS-II): An international, double-blind, randomised placebo-controlled trial [Internet]. Lancet 383, 1041–1048. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24333009 [cited 25 June 2017].

Decensi, A., Torrisi, R., Bruno, S., et al., 2000. Randomized trial of fenretinide in superficial bladder cancer using DNA flow cytometry as an intermediate end point [Internet]. Cancer Epidemiology, Biomarkers & Prevention 9, 1071–1078. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11045790 [cited 25 June 2017].

Drahos, J., Xiao, Q., Risch, H.A., et al., 2016. Age-specific risk factor profiles of adenocarcinomas of the esophagus: A pooled analysis from the international BEACON consortium [Internet]. International Journal of Cancer 138, 55–64. Available from: http://doi.wiley.com/10.1002/ijc.29688 [cited 25 June 2017].

Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Davies, C., Godwin, J., et al., 2011. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: Patient-level meta-analysis of randomised trials [Internet]. Lancet (London, England) 378, 771–784. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0140673611609938 [cited 25 June 2017].

Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Dowsett, M., Forbes, J.F., et al., 2015. Aromatase inhibitors versus tamoxifen in early breast cancer: Patient-level meta-analysis of the randomised trials [Internet]. Lancet 386, 1341–1352. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26211827 [cited 25 June 2017].

Elmets, C.A., Viner, J.L., Pentland, A.P., et al., 2010. Chemoprevention of nonmelanoma skin cancer with celecoxib: A randomized, double-blind, placebo-controlled trial [Internet]. Journal of the National Cancer Institute 102, 1835–1844. Available from: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/djq442 [cited 25 June 2017].

Fabian, C.J., Kimler, B.F., 2016. Incorporating biomarkers in studies of chemoprevention [Internet]. Advances in Experimental Medicine and Biology 69–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26987531 [cited 25 June 2017].

Fearon, E.R., Vogelstein, B., 1990. A genetic model for colorectal tumorigenesis [Internet]. Cell 61, 759–767. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2188735 [cited 2017 June 2019].

Fernandez Figueras, M.T., 2017. From actinic keratosis to squamous cell carcinoma: Pathophysiology revisited [Internet]. Journal of the European Academy of Dermatology and Venereology 31, 5–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28263020 [cited 25 June 2017].

Forastiere, A., Koch, W., Trotti, A., et al., 2001. Head and neck cancer [Internet] The New England Journal of Medicine 345, 1890–1900. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMra001375 [cited 30 July 2017].

Frieling, U.M., Schaumberg, D.A., Kupper, T.S., et al., 2000. A randomized, 12-year primary-prevention trial of beta carotene supplementation for nonmelanoma skin cancer in the physician's health study [Internet]. Archives of Dermatology 136, 179–184. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10677093 [cited 25 June 2017].

FUTURE II Study Group, 2007. Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions [Internet]. The New England Journal of Medicine 356, 1915–1927. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa061741 [cited 25 June 2017].

Gann, P.H., Deaton, R., Amatya, A., et al., 2013. Development of a nuclear morphometric signature for prostate cancer risk in negative biopsies [Internet]. PLoS ONE 8, e69457. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23922715 [cited 25 June 2017].

Garland, S.M., Kjaer, S.K., Muñoz, N., et al., 2016. Impact and effectiveness of the quadrivalent human papillomavirus vaccine: A systematic review of 10 years of real-world experience [Internet]. Clinical Infectious Diseases 63, 519–527. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27230391 [cited 19 June 2017.

George, R., Weightman, W., Russ, G.R., et al., 2002. Acitretin for chemoprevention of non-melanoma skin cancers in renal transplant recipients [Internet]. The Australasian Journal of Dermatology 43, 269–273. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12423433 [cited 25 June 2017].

Giardiello, F.M., Yang, V.W., Hylind, L.M., et al., 2002. Primary chemoprevention of familial adenomatous polyposis with sulindac [Internet]. The New England Journal of Medicine 346, 1054–1059. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa012015 [cited 24 June 2017].

Gillison, M.L., Broutian, T., RKL, P., et al., 2012. Prevalence of oral HPV infection in the United States, 2009–2010 [Internet]. JAMA 307, 693. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22282321 [cited 30 July 2017].
Giuliano, A.R., Palefsky, J.M., Goldstone, S., et al., 2011. Efficacy of quadrivalent HPV vaccine against HPV Infection and disease in males [Internet]. The New England Journal of Medicine 364, 401–411. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa0909537 [cited 25 June 2017].
Goff, D.C., Lloyd-Jones, D.M., Bennett, G., et al., 2013. 2013 ACC/AHA guideline on the assessment of cardiovascular risk [Internet], Circulation. Available from: http://circ.ahajournals.org/content/early/2013/11/11/01.cir.0000437741.48606.98 [cited 30 July 2017].
Goossens, M.E., Zeegers, M.P., van Poppel, H., et al., 2016. Phase III randomised chemoprevention study with selenium on the recurrence of non-invasive urothelial carcinoma. The SELEnium and BLAdder cancer Trial [Internet]. European Journal of Cancer 69, 9–18. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0959804916324522 [cited 25 June 2017].
Goss PE, Ingle JN, Ales-Martinez JE, et al.: Exemestane for breast-cancer prevention in postmenopausal women [Internet]. The New England Journal of Medicine 364:2381–2391, 2011 Available from: http://www.ncbi.nlm.nih.gov/pubmed/21639806 [cited 25 June 2017]
Grau, M.V., Baron, J.A., Sandler, R.S., et al., 2003. Vitamin D, calcium supplementation, and colorectal adenomas: Results of a randomized trial [Internet]. Journal of the National Cancer Institute 95, 1765–1771. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14652238 [cited 24 June 2017].
Greenberg, E.R., Baron, J.A., Stukel, T.A., et al., 1990. A clinical trial of beta carotene to prevent basal-cell and squamous-cell cancers of the skin [Internet]. The New England Journal of Medicine 323, 789–795. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2202901 [cited 25 June 2017].
de Gruijl, F.R., van Kranen, H.J., Mullenders, L.H., 2001. UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer [Internet]. Journal of Photochemistry and Photobiology. B 63, 19–27. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11684448 [cited 25 June 2017].
Gupta, A.K., Paquet, M., Villanueva, E., et al., 2012. Interventions for actinic keratoses [Internet]. Cochrane Database of Systematic Reviews 12, CD004415. Available from: http://doi.wiley.com/10.1002/14651858.CD004415.pub2 [cited 25 June 2017].
Hanahan, D., Weinberg, R., 2011. Hallmarks of cancer: The next generation [Internet]. Cell 144, 646–674. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21376230 [cited 2017 June 2019].
Haque, T., Rahman, K.M., Thurston, D.E., et al., 2015. Topical therapies for skin cancer and actinic keratosis [Internet]. European Journal of Pharmaceutical Sciences 77, 279–289. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26091570 [cited 25 June 2017].
Heath, E.I., Canto, M.I., Piantadosi, S., et al., 2007. Secondary chemoprevention of Barrett's esophagus with celecoxib: Results of a randomized trial [Internet]. Journal of the National Cancer Institute 99, 545–557. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17405999 [cited 25 June 2017].
Hennekens, C.H., Buring, J.E., Manson, J.E., et al., 1996. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease [Internet]. The New England Journal of Medicine 334, 1145–1149. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM199605023341801 [cited 24 June 2017].
Herrero, R., Quint, W., Hildesheim, A., et al., 2013. Reduced prevalence of oral human papillomavirus (HPV) 4 years after bivalent HPV vaccination in a randomized clinical trial in Costa Rica [Internet]. PLoS ONE 8, e68329. Available from: http://dx.plos.org/10.1371/journal.pone.0068329 [cited 24 June 2017].
Hildesheim, A., Wacholder, S., Catteau, G., et al., 2014. Efficacy of the HPV-16/18 vaccine: Final according to protocol results from the blinded phase of the randomized Costa Rica HPV-16/18 vaccine trial [Internet]. Vaccine 32, 5087–5097. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0264410X14008275 [cited 25 June 2017].
Hong, W.K., Endicott, J., Itri, L.M., et al., 1986. 13-*cis*-Retinoic acid in the treatment of oral leukoplakia [Internet]. The New England Journal of Medicine 315, 1501–1505. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM198612113152401 [cited 24 June 2017].
Hong, W.K., Lippman, S.M., Itri, L.M., et al., 1990. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck [Internet]. The New England Journal of Medicine 323, 795–801. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM199009203231205 [cited 24 June 2017].
Jacobi, N., Seeboeck, R., Hofmann, E., et al., 2017. ErbB family signalling: A paradigm for oncogene addiction and personalized oncology [Internet]. Cancers (Basel) 9, 33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28417948 [cited 2017 June 2019].
Jain, M., Arvanitis, C., Chu, K., et al., 2002. Sustained loss of a neoplastic phenotype by brief inactivation of MYC [Internet]. Science 297 (80), 102–104. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12098700 [cited 2017 June 2019].
Janakiram, N.B., Rao, C.V., 2014. The role of inflammation in colon cancer [Internet]. In: Advances in experimental medicine and biology, pp. 25–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24818718 [cited 24 June 2017].
Jemal, A., Thun, M.J., Ries, L.A.G., et al., 2008. Annual report to the Nation on the Status of Cancer, 1975–2005, featuring trends in lung cancer, tobacco use, and tobacco control [Internet]. Journal of the National Cancer Institute 100, 1672–1694. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19033571 [cited 2017 June 2019].
Joura, E.A., Giuliano, A.R., Iversen, O.-E., et al., 2015. A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women [Internet]. The New England Journal of Medicine 372, 711–723. Available from: http://www.nejm.org/doi/10.1056/NEJMoa1405044 [cited 25 June 2017].
Kadakia, K.C., Barton, D.L., Loprinzi, C.L., et al., 2012. Randomized controlled trial of acitretin versus placebo in patients at high-risk for basal cell or squamous cell carcinoma of the skin (North Central Cancer Treatment Group Study 969251). Cancer 118, 2128–2137.
Kamangar, F., Qiao, Y.-L., Yu, B., et al., 2006. Lung cancer chemoprevention: A randomized, double-blind trial in Linxian, China [Internet]. Cancer Epidemiology, Biomarkers & Prevention 15, 1562–1564. Available from: http://cebp.aacrjournals.org/cgi/doi/10.1158/1055-9965.EPI-06-0316 [cited 24 June 2017.
Karp, D.D., Lee, S.J., Keller, S.M., et al., 2013. Randomized, double-blind, placebo-controlled, phase III chemoprevention trial of selenium supplementation in patients with resected stage I non-small-cell lung cancer: ECOG 5597 [Internet]. Journal of Clinical Oncology 31, 4179–4187. Available from: http://ascopubs.org/doi/10.1200/JCO.2013.49.2173 [cited 24 June 2017].
Kelloff, G.J., Lippman, S.M., Dannenberg, A.J., et al., 2006. Progress in chemoprevention drug development: The promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer—A plan to move forward [Internet]. Clinical Cancer Research 12, 3661–3697. Available from: http://clincancerres.aacrjournals.org/cgi/doi/10.1158/1078-0432.CCR-06-1104 [cited 2017 June 2019].
Kelly, J., Maynard, L., Mostafid, H., et al., 2015. Celecoxib for the treatment of non-muscle invasive bladder cancer (NMIBC): Results of the randomised BOXIT trial (CRUK/07/004) [Internet]. BJU International 115, 11–13. Available from: http://doi.wiley.com/10.1111/bju.13135 [cited 25 June 2017].
Khuri, F.R., Lee, J.J., Lippman, S.M., et al., 2006. Randomized phase III trial of low-dose isotretinoin for prevention of second primary tumors in stage I and II head and neck cancer patients [Internet] Journal of the National Cancer Institute 98, 441–450. Available from: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/djj091 [cited 24 June 2017].
Klein, E.A., Thompson, I.M., Tangen, C.M., et al., 2011. Vitamin E and the risk of prostate cancer: The selenium and vitamin E cancer prevention trial (SELECT) [Internet]. JAMA 306, 1549–1556. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2011.1437 [cited 25 June 2017].
Kristal, A.R., Darke, A.K., Morris, J.S., et al., 2014. Baseline selenium status and effects of selenium and vitamin E supplementation on prostate cancer risk [Internet]. Journal of the National Cancer Institute 106, djt456. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24563519 [cited 25 June 2017].
Lavasani, S., Chlebowski, R.T., Prentice, R.L., et al., 2015. Estrogen and colorectal cancer incidence and mortality [Internet]. Cancer 121, 3261–3271. Available from: http://doi.wiley.com/10.1002/cncr.29464 [cited 25 June 2017].
Lee, J.J., Wu, X., MAT, H., et al., 2011. Global assessment of genetic variation influencing response to retinoid chemoprevention in head and neck cancer patients [Internet]. Cancer Prevention Research (Philadelphia, Pa.) 4, 185–193. Available from: http://cancerpreventionresearch.aacrjournals.org/cgi/doi/10.1158/1940-6207.CAPR-10-0125 [cited 24 June 2017].
Leeman-Neill, R.J., Seethala, R.R., Singh, S.V., et al., 2011. Inhibition of EGFR-STAT3 signaling with erlotinib prevents carcinogenesis in a chemically-induced mouse model of oral squamous cell carcinoma [Internet]. Cancer Prevention Research 4, 230–237. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21163936 [cited 24 June 2017].

Levine, N., Moon, T.E., Cartmel, B., et al., 1997. Trial of retinol and isotretinoin in skin cancer prevention: A randomized, double-blind, controlled trial. Southwest Skin Cancer Prevention Study Group [Internet]. Cancer Epidemiology, Biomarkers & Prevention 6, 957–961. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9367070 [cited 25 June 2017].

Limburg, P.J., Wei, W., Ahnen, D.J., et al., 2005. Randomized, placebo-controlled, esophageal squamous cell cancer chemoprevention trial of selenomethionine and celecoxib [Internet]. Gastroenterology 129, 863–873. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16143126 [cited 25 June 2017].

Lippman, S.M., Batsakis, J.G., Toth, B.B., et al., 1993. Comparison of low-dose isotretinoin with beta carotene to prevent oral carcinogenesis [Internet]. The New England Journal of Medicine 328, 15–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8416267 [cited 24 June 2017].

Lippman, S.M., Lee, J.J., Karp, D.D., et al., 2001. Randomized phase III intergroup trial of isotretinoin to prevent second primary tumors in stage I non-small-cell lung cancer [Internet]. Journal of the National Cancer Institute 93, 605–618. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11309437 [cited 24 June 2017.

Ma, J.-L., Zhang, L., Brown, L.M., et al., 2012. Fifteen-year effects of *Helicobacter pylori*, garlic, and vitamin treatments on gastric cancer incidence and mortality [Internet]. Journal of the National Cancer Institute 104, 488–492. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22271764 [cited 25 June 2017].

Mao, L., Lee, J.S., Fan, Y.H., et al., 1996. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment [Internet]. Nature Medicine 2, 682–685. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8640560 [cited 24 June 2017].

Messing, E., Kim, K.M., Sharkey, F., et al., 2006. Randomized prospective phase III trial of difluoromethylornithine vs placebo in preventing recurrence of completely resected low risk superficial bladder cancer [Internet]. The Journal of Urology 176, 500–504. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022534706008305 [cited 25 June 2017].

Meyskens, F.L., 1992. Strategies for prevention of cancer in humans. [Internet]. Oncology (Williston Park) 6, 15–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1532731 [cited 2017 June 2019].

Meyskens, F.L., Mukhtar, H., Rock, C.L., et al., 2016. Cancer prevention: Obstacles, challenges, and the road ahead [Internet]. Journal of the National Cancer Institute 108. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26547931 [cited 19 June 2017].

Moon, T.E., Levine, N., Cartmel, B., et al., 1997. Effect of retinol in preventing squamous cell skin cancer in moderate-risk subjects: A randomized, double-blind, controlled trial. Southwest Skin Cancer Prevention Study Group [Internet]. Cancer Epidemiology, Biomarkers & Prevention 6, 949–956. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9367069 [cited 25 June 2017].

Mootha VK, Lindgren CM, Eriksson K-F, et al.: PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes [Internet]. Nature Genetics 34:267–73, 2003 Available from: http://www.nature.com/doifinder/10.1038/ng1180 [cited 24 June 2017]

Muto, Y., Moriwaki, H., Ninomiya, M., et al., 1996. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma [Internet] The New England Journal of Medicine 334, 1561–1568. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8628336 [cited 25 June 2017].

Nakagawa, S., Wei, L., Song, W.M., et al., 2016. Molecular liver cancer prevention in cirrhosis by organ transcriptome analysis and lysophosphatidic acid pathway inhibition [Internet]. Cancer Cell 30, 879–890. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27960085 [cited 25 June 2017].

Nelson, H.D., Smith, M.E.B., Griffin, J.C., et al., 2013. Use of medications to reduce risk for primary breast cancer: A systematic review for the U.S. Preventive Services Task Force [Internet]. Annals of Internal Medicine 158, 604–614. Available from: http://annals.org/article.aspx?doi=10.7326/0003-4819-158-8-201304160-00005 [cited 25 June 2017].

Newling, D.W., Robinson, M.R., Smith, P.H., et al., 1995. Tryptophan metabolites, pyridoxine (vitamin B6) and their influence on the recurrence rate of superficial bladder cancer. Results of a prospective, randomised phase III study performed by the EORTC GU Group. EORTC Genito-Urinary Tract Cancer Cooperative Group [Internet]. European Urology 27, 110–116. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7744151 [cited 25 June 2017].

Omenn, G.S., Goodman, G.E., Thornquist, M.D., et al., 1996. Effects of a combination of beta carotene and vitamin a on lung cancer and cardiovascular disease [Internet]. The New England Journal of Medicine 334, 1150–1155. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8602180 [cited 24 June 2017].

Otley, C.C., Stasko, T., Tope, W.D., et al., 2006. Chemoprevention of nonmelanoma skin cancer with systemic retinoids: Practical dosing and management of adverse effects [Internet]. Dermatologic Surgery 32, 562–568. Available from: http://doi.wiley.com/10.1111/j.1524-4725.2006.32115.x [cited 25 June 2017].

Overholt, B.F., Lightdale, C.J., Wang, K.K., et al., 2005. Photodynamic therapy with porfimer sodium for ablation of high-grade dysplasia in Barrett's esophagus: International, partially blinded, randomized phase III trial [Internet]. Gastrointestinal Endoscopy 62, 488–498. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0016510705023497 [cited 25 June 2017].

Papadimitrakopoulou, V.A., Lee, J.J., William, W.N., et al., 2009. Randomized trial of 13-*cis* retinoic acid compared with retinyl palmitate with or without beta-carotene in oral premalignancy [Internet]. Journal of Clinical Oncology 27, 599–604. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19075276 [cited 24 June 2017].

Peirson, L., Fitzpatrick-Lewis, D., Ciliska, D., et al., 2013. Screening for cervical cancer: A systematic review and meta-analysis [Internet]. Systematic Reviews 2, 35. Available from: http://systematicreviewsjournal.biomedcentral.com/articles/10.1186/2046-4053-2-35 [cited 25 June 2017].

Pence, B.C., 1993. Role of calcium in colon cancer prevention: Experimental and clinical studies [Internet]. Mutation Research 290, 87–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7694103 [cited 24 June 2017].

Philip, M., Rowley, D.A., Schreiber, H., 2004. Inflammation as a tumor promoter in cancer induction [Internet]. Seminars in Cancer Biology 14, 433–439. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1044579X04000495 [cited 2017 June 2019].

Qiao, Y.-L., Dawsey, S.M., Kamangar, F., et al., 2009. Total and cancer mortality after supplementation with vitamins and minerals: Follow-up of the linxian general population nutrition intervention trial [Internet]. Journal of the National Cancer Institute 101, 507–518. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19318634 [cited 25 June 2017].

Rockhill, B., Spiegelman, D., Byrne, C., et al., 2001. Validation of the Gail et al. model of breast cancer risk prediction and implications for chemoprevention [Internet]. Journal of the National Cancer Institute 93, 358–366. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11238697 [cited 30 July 2017].

Rothwell, P.M., Wilson, M., Elwin, C.-E., et al., 2010. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials [Internet]. Lancet (London, England) 376, 1741–1750. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0140673610615437 [cited 24 June 2017].

Rothwell PM, Fowkes FGR, Belch JFF, et al.: Effect of daily aspirin on long-term risk of death due to cancer: Analysis of individual patient data from randomised trials [Internet]. Lancet (London, England) 377:31–41, 2011 Available from: http://linkinghub.elsevier.com/retrieve/pii/S0140673610621101 [cited 25 June 2017]

Sabichi, A.L., Lerner, S.P., Atkinson, E.N., et al., 2008. Phase III prevention trial of fenretinide in patients with resected non-muscle-invasive bladder cancer [Internet]. Clinical Cancer Research 14, 224–229. Available from: http://clincancerres.aacrjournals.org/cgi/doi/10.1158/1078-0432.CCR-07-0733 [cited 25 June 2017].

Sabichi, A.L., Lee, J.J., Grossman, H.B., et al., 2011. A randomized controlled trial of celecoxib to prevent recurrence of nonmuscle-invasive bladder cancer [Internet]. Cancer Prevention Research (Philadelphia, Pa.) 4, 1580–1589. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21881030 [cited 25 June 2017].

Saintigny, P., Zhang, L., Fan, Y.-H., et al., 2011. Gene expression profiling predicts the development of oral cancer [Internet]. Cancer Prevention Research (Philadelphia, Pa.) 4, 218–229. Available from: http://cancerpreventionresearch.aacrjournals.org/cgi/doi/10.1158/1940-6207.CAPR-10-0155 [cited 24 June 2017].

Schatzkin, A., Lanza, E., Corle, D., et al., 2000. Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas [Internet]. The New England Journal of Medicine 342, 1149–1155. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10770979 [cited 24 June 2017].

de Sévaux, R.G.L., Smit, J.V., de Jong, E.M.G.J., et al., 2003. Acitretin treatment of premalignant and malignant skin disorders in renal transplant recipients: Clinical effects of a randomized trial comparing two doses of acitretin [Internet]. Journal of the American Academy of Dermatology 49, 407–412. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12963902 [cited 25 June 2017].

Shacter, E., Weitzman, S.A., 2002. Chronic inflammation and cancer [Internet]. Oncology (Williston Park) 16, 217–26, 229–232. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11866137 [cited 30 July 2017].

Shaheen, N.J., Sharma, P., Overholt, B.F., et al., 2009. Radiofrequency ablation in Barrett's esophagus with dysplasia [Internet]. The New England Journal of Medicine 360, 2277–2288. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa0808145 [cited 25 June 2017].
Shoemaker, R.H., Suen, C.S., Holmes, C.A., et al., 2016. The National Cancer Institute's PREVENT cancer preclinical drug development program: Overview, current projects, animal models, agent development strategies, and molecular targets [Internet]. Seminars in Oncology 43, 189–197. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26970137 [cited 19 June 2017].
Shureiqi, I., Reddy, P., Brenner, D.E., 2000. Chemoprevention: General perspective [Internet]. Critical Reviews in Oncology/Hematology 33, 157–167. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10789490 [cited 19 June 2017].
Siegel, R.L., Miller, K.D., Jemal, A., 2017. Cancer statistics, 2017 [Internet]. CA: a Cancer Journal for Clinicians 67, 7–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28055103 [cited 2017 June 2019].
Simon, M.S., Chlebowski, R.T., Wactawski-Wende, J., et al., 2012. Estrogen plus progestin and colorectal cancer incidence and mortality [Internet]. Journal of Clinical Oncology 30, 3983–3990. Available from: http://ascopubs.org/doi/10.1200/JCO.2012.42.7732 [cited 24 June 2017].
Slaughter, D.P., Southwick, H.W., Smejkal, W., 1953. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin [Internet]. Cancer 6, 963–968. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13094644 [cited 24 June 2017].
Smith, B.D., Morgan, R.L., Beckett, G.A., et al., 2012. Recommendations for the identification of chronic hepatitis C virus infection among persons born during 1945–1965 [Internet]. Morbidity and Mortality Weekly Report 61, 1–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22895429 [cited 25 June 2017].
Spira, A., Yurgelun, M.B., Alexandrov, L., et al., 2017. Precancer atlas to drive precision prevention trials [Internet]. Cancer Research 77, 1510–1541. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28373404 [cited 25 June 2017].
Steinbach, G., Lynch, P.M., Phillips, R.K., et al., 2000. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis [Internet]. The New England Journal of Medicine 342, 1946–1952. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM200006293422603 [cited 24 June 2017].
Steinmaus, C.M., Nuñez, S., Smith, A.H., 2000. Diet and bladder cancer: A meta-analysis of six dietary variables [Internet]. American Journal of Epidemiology 151, 693–702. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10752797 [cited 25 June 2017].
Studer, U.E., Jenzer, S., Biedermann, C., et al., 1995. Adjuvant treatment with a vitamin A analogue (etretinate) after transurethral resection of superficial bladder tumors. Final analysis of a prospective, randomized multicenter trial in Switzerland [Internet]. European Urology 28, 284–290. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8575494 [cited 25 June 2017].
Stürmer, T., Buring, J.E., Lee, I.-M., et al., 2006. Colorectal cancer after start of nonsteroidal anti-inflammatory drug use [Internet]. The American Journal of Medicine 119, 494–502. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0002934305010740 [cited 24 June 2017].
Szabo, E., Mao, J.T., Lam, S., et al., 2013. Chemoprevention of lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines [Internet]. Chest 143, e40S–e60S. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0012369213602883 [cited 24 June 2017].
Takai, K., Okuno, M., Yasuda, I., et al., 2005. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma [Internet]. Intervirology 48, 39–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15785088 [cited 25 June 2017].
Tang, J.Y., Mackay-Wiggan, J.M., Aszterbaum, M., et al., 2012. Inhibiting the hedgehog pathway in patients with the basal-cell nevus syndrome [Internet]. The New England Journal of Medicine 366, 2180–2188. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa1113538 [cited 25 June 2017].
Tangrea, J.A., Edwards, B.K., Taylor, P.R., et al., 1992. Long-term therapy with low-dose isotretinoin for prevention of basal cell carcinoma: A multicenter clinical trial. Isotretinoin-Basal Cell Carcinoma Study Group [Internet]. Journal of the National Cancer Institute 84, 328–332. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1738183 [cited 25 June 2017].
Taoudi Benchekroun, M., Saintigny, P., Thomas, S.M., et al., 2010. Epidermal growth factor receptor expression and gene copy number in the risk of oral cancer [Internet]. Cancer Prevention Research (Philadelphia, Pa.) 3, 800–809. Available from: http://cancerpreventionresearch.aacrjournals.org/cgi/doi/10.1158/1940-6207.CAPR-09-0163 [cited 24 June 2017].
Thompson, I.M., Goodman, P.J., Tangen, C.M., et al., 2003. The influence of finasteride on the development of prostate cancer [Internet]. The New England Journal of Medicine 349, 215–224. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa030660 [cited 25 June 2017].
Thompson, I.M., Goodman, P.J., Tangen, C.M., et al., 2013. Long-term survival of participants in the prostate cancer prevention trial [Internet]. The New England Journal of Medicine 369, 603–610. Available from: http://www.nejm.org/doi/10.1056/NEJMoa1215932 [cited 25 June 2017].
Tufaro, A.P., Azoury, S.C., Crompton, J.G., et al., 2015. Rising incidence and aggressive nature of cutaneous malignancies after transplantation: An update on epidemiology, risk factors, management and surveillance [Internet]. Surgical Oncology 24, 345–352. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0960740415300293 [cited 25 June 2017].
Uray, I.P., Dmitrovsky, E., Brown, P.H., 2016. Retinoids and rexinoids in cancer prevention: From laboratory to clinic [Internet]. Seminars in Oncology 43, 49–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26970124 [cited 24 June 2017].
de Villiers, E.-M., Fauquet, C., Broker, T.R., et al., 2004. Classification of papillomaviruses [Internet]. Virology 324, 17–27. Available from: http://linkinghub.elsevier.com/retrieve/pii/S004268220400220X [cited 25 June 2017].
Vogel, V.G., Costantino, J.P., Wickerham, D.L., et al., 2010. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 trial: Preventing breast cancer [Internet]. Cancer Prevention Research (Philadelphia, Pa.) 3, 696–706. Available from: http://cancerpreventionresearch.aacrjournals.org/cgi/doi/10.1158/1940-6207.CAPR-10-0076 [cited 25 June 2017].
Vokes, E.E., Weichselbaum, R.R., Lippman, S.M., et al., 1993. Head and neck cancer [Internet]. The New England Journal of Medicine 328, 184–194. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM199301213280306 [cited 24 June 2017].
Wang, J.-B., Abnet, C.C., Fan, J.-H., et al., 2013. The randomized Linxian Dysplasia Nutrition Intervention Trial after 26 years of follow-up: No effect of multivitamin supplementation on mortality [Internet]. JAMA Internal Medicine 173, 1259–1261. Available from: http://archinte.jamanetwork.com/article.aspx?doi=10.1001/jamainternmed.2013.6066 [cited 25 June 2017].
Wattenberg, L.W., 1985. Chemoprevention of cancer. [Internet]. Cancer Research 45, 1–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3880665 [cited 2017 June 2019].
Weinstein, I.B., 2002. Cancer. Addiction to oncogenes—The Achilles heal of cancer [Internet]. Science 297, 63–64. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1073096 [cited 2017 June 2019].
Weinstein, I.B., Joe, A., Felsher, D., 2008. Oncogene addiction [Internet]. Cancer Research 68, 3077–3080. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18451130 [cited 2017 June 2019].
William, W.N., Papadimitrakopoulou, V., Lee, J.J., et al., 2016. Erlotinib and the risk of oral cancer [Internet]. JAMA Oncology 2, 209. Available from: http://oncology.jamanetwork.com/article.aspx?doi=10.1001/jamaoncol.2015.4364 [cited 2017 June 2019].
Wong, B.C.Y., Zhang, L., Ma, J., et al., 2012. Effects of selective COX-2 inhibitor and *Helicobacter pylori* eradication on precancerous gastric lesions [Internet]. Gut 61, 812–818. Available from: http://gut.bmj.com/lookup/doi/10.1136/gutjnl-2011-300154 [cited 25 June 2017].
Xiong, D., Pan, J., Zhang, Q., et al., 2017. Bronchial airway gene expression signatures in mouse lung squamous cell carcinoma and their modulation by cancer chemopreventive agents [Internet]. Oncotarget 8, 18885–18900. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27935865 [cited 24 June 2017].
van Zandwijk, N., Dalesio, O., Pastorino, U., et al., 2000. EUROSCAN, a randomized trial of vitamin A and N-acetylcysteine in patients with head and neck cancer or lung cancer. For the EUropean Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperative Groups [Internet]. Journal of the National Cancer Institute 92, 977–986. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10861309 [cited 24 June 2017].

Zhai, R., Chen, F., Liu, G., et al., 2010. Interactions among genetic variants in apoptosis pathway genes, reflux symptoms, body mass index, and smoking indicate two distinct etiologic patterns of esophageal adenocarcinoma [Internet]. Journal of Clinical Oncology 28, 2445–2451. Available from: http://ascopubs.org/doi/10.1200/JCO.2009.26.2790 [cited 25 June 2017].

Further Reading

Rashid, S., 2017. Cancer and chemoprevention: An overview. Springier Publishing.
Cho, W.C. (Ed.), 2013. Cancer chemoprevention and treatment by diet therapy. Springer Publishing.
Drew, D.A., Cao, Y., Chan, A.T., 2016. Aspirin and colorectal cancer: The promise of precision chemoprevention. Nature Reviews. Cancer 16, 173–186.

Cholangiocarcinoma: Diagnosis and Treatment

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Abbreviations

^{18}F-FDG PET/CT 2-Deoxy-2-[fluorine-18]fluoro-D-glucose PET integrated with CT
ALP Alkaline phosphatase
CA 19-9 Carbohydrate antigen 19-9
CC Cholangiocarcinoma
CT Computed tomography
CEA Carcinoembryonic antigen
EUS Endoscopic ultrasound scan
ICC Intrahepatic cholangiocarcinoma
ICD-O International Classification of Diseases for Oncology
FISH Fluorescence in situ hybridization
FNA Fine-needle aspiration
FLR Future remnant liver
HBS Hepatobiliary scintigraphy
IDUS Intra-ductal ultrasound
MDCT Multidetector computed tomography
MRCP Magnetic resonance cholangiopancreatography
MRI Magnetic resonance imaging
OLT Orthotopic liver transplantation
PET Positron emission tomography
PTC Percutaneous transhepatic cholangiography

Definition and Classification

Cholangiocarcinoma (CC; ICD-O code: 8160/3; also called bile duct carcinoma and bile duct adenocarcinoma) is a term which encompasses a heterogeneous group of malignant tumors with pathological features of biliary tract differentiation, distinct from gallbladder cancer. It is presumed to arise from the intra- or extrahepatic biliary epithelium (cholangiocytes), although some studies have suggested that it may also arise directly from transdifferentiation of hepatocytes.

Cholangiocarcinoma is classified into subtypes based on its anatomical origin, with, however, some debate and even confusion in the nomenclature.

Traditionally, cholangiocarcinomas were divided into intrahepatic and extrahepatic. However, the boundary between intra- and extrahepatic biliary tree has been somewhat confused in the literature. Still, while the term "intrahepatic cholangiocarcinoma" seems to be well accepted, the use of the term "extrahepatic cholangiocarcinoma" and the nomenclature of the subtypes have been extensively discussed and revised in the past decade, and different terms referring to the same entities can be encountered in both clinical guidelines and in scientific literature.

Intrahepatic cholangiocarcinoma (ICC) is an intrahepatic malignancy with biliary epithelial differentiation. ICC can arise in any portion of the intrahepatic biliary tree, from the segmental and area ducts and their major branches to the smallest bile ducts and ductules. ICC arising from the small intrahepatic bile ducts is often called peripheral ICC but the International Classification of Diseases for Oncology (ICD-O) discourages the use of this term. CC originating from major intrahepatic ducts, including the hilum, has been called hilar CC. However, due to symptoms which are similar to those of extrahepatic CC, it has been classified as an extrahepatic lesion. Hilar tumors arising near the junction of the right and left hepatic duct are traditionally called Klatskin tumors. Current guidelines recommend classifying extrahepatic CC into two subtypes: perihilar and distal CC. However, the term "hilar cholangiocarcinoma" is still widely used. Moreover, one should bear in mind that distinguishing hilar and perihilar CC at advanced stages is usually controversial. All in all, the exact meaning (anatomical scope) of these two terms differs between different specialists.

Presentation and Diagnosis

Early CC symptoms are not specific and so most patients present with advanced disease, with extensive hepatic artery and/or portal vein infiltration by tumor and/or with distant metastases, which makes the definitive treatment impossible. A majority of them

present when jaundice or the sequelae of biliary obstruction appear. The degree of jaundice depends on the extent of biliary involvement. Abdominal pain, weight loss, fever, malaise, and/or hepatomegaly occur in half of the cases, usually in those with the advanced disease. Patients with unrelieved obstruction of large intrahepatic bile ducts may die from complications, such as liver failure or sepsis.

It has been proposed that CC can be diagnosed based on a combination of clinical presentation, blood serum testing, and imaging. However, as many of the criteria lack specificity, a pathological examination is recommended for definitive diagnosis in most patients.

The initial workup in case of suspected CC comprises blood serum testing. Elevated serum bilirubin and alkaline phosphatase (ALP) levels are common in CC patients. However, incomplete obstruction of the right or left hepatic ducts may lead to isolated elevation of ALP. Increased liver transaminase levels and prothrombin time are detected in case of a long-standing biliary obstruction but also cholestatic hepatocellular injury. Increased serum carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) levels are very common (up to 90%) but they are not specific to CC, either.

High-quality cross-sectional imaging plays a key role in the diagnostic process as well as in the preoperative workup. High-resolution contrast-enhanced multidetector computed tomography (MDCT) and magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRI/MRCP) are considered to be the most accurate.

For tissue diagnosis based on brush cytology, both endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC) have similar sensitivity and specificity. ERCP with brush cytology is particularly specific for the diagnosis of distal CC and has been the standard diagnostic method for years. However, it has a low sensitivity for some cellular patterns. Various techniques have been investigated to increase sensitivity of cytological testing. In particular, fluorescence in situ hybridization (FISH) has been reported to increase the detection sensitivity to up to 93%.

Endoscopic methods, such as endoscopic ultrasound scan (EUS), intra-ductal ultrasound (IDUS), and cholangioscopy, are used selectively in diagnosing and staging. Cholangioscopy offers direct visualization of biliary strictures and enables targeted biopsies, which increases both diagnostic sensitivity and specificity. IDUS enables detailed imaging of the bile ducts and periductal tissue and has been reported to improve diagnostic accuracy of ERCP. However, stents that are often required to drain obstructed bile ducts make the interpretation of IDUS results difficult. If this is the case, the use of EUS in combination with fine-needle aspiration (FNA) may be preferable. The latter is also useful to detect suspicious regional lymph node involvement. Radiological analyses are necessary for assessing the disease extent (locoregional or distant spread), staging, and resectability.

CCs have a locally aggressive behavior, presenting mainly with infiltration of contiguous structures (liver, hepatic artery and portal vein) and nodal involvement (up to 30% at diagnosis), microscopically invasive spread with neural, perineural and lymphatic involvement, and subepithelial extension. Intrahepatic CCs can be divided into mass-forming, periductal infiltrating, and intraductal growth types. Perihilar CCs can have exophytic (mass-forming) or intraductal macroscopic growth patterns. Over 90% of CCs are well differentiated and mucin-producing adenocarcinomas.

Epidemiology and Risk Factors

Burden

Globally, cholangiocarcinoma is a very rare tumor, accounting for 3% of all gastrointestinal malignancies and less than 1% of all cancers worldwide. However, intrahepatic CC is the second most common primary liver cancer (after hepatocellular carcinoma), with very high mortality rates. The exact incidence of different subtypes is difficult to estimate due to changing nomenclature and coding issues. However, a majority of CCs arise from the intrahepatic upper third of the biliary tract, including the hilum (50%–70% of all tumors). In countries for which incidence trends have been studies, the rates are reported to be rising.

The overall CC incidence shows striking geographical differences, with the highest incidence rates in northeastern Thailand where parasitic infections with endemic liver flukes lead to infestation of the billiary tree. CC occurs with roughly the same incidence in men and women, in most cases after 60 years of age.

Etiology and Risk Factors

Infections with liver flukes (*Clonorchis sinensis and Opisthorchis viverini)*, common in such areas of high CC incidence as Thailand (*O. viverini*) and China (*C. sinensis*), have been shown to be associated with an increased risk of CC, with a high proportion of CC patients being infected. Chronic infection with these parasites causes severe inflammation damage. Moreover, infections with hepatitis B or C virus have been shown to increase the risk of developing intrahepatic CC by contributing to the development of liver cirrhosis.

Several other medical conditions of the gastrointestinal tract have been associated with an increased risk of CC, such as inflammatory bowel disease (in particular chronic ulcerative colitis), primary sclerosing cholangitis, choledocholithiasis, and biliary stone disease. Elevated CC incidence rates have also been reported in patients with congenital anomalies of the intrahepatic and extrahepatic bile ducts (e.g. cysts, congenital dilation of the bile ducts, choledochal cyst, Caroli disease, congenital hepatic fibrosis, polycystic disease, abnormal pancreaticocholedochal junction).

A history of exposure to thorotrast, an alpha-particle emitter that was used as a radiopaque intra-arterial contrast medium between 1930 and 1955 is also a causative factor of CC, with latency period that may range from 25 to 48 years.

Finally, it has also been suggested that tobacco smoking, alcohol consumption and diabetes may contribute to the risk of intrahepatic CC.

Pathology and Genetics

The genetic background of CC is poorly defined. Gains and losses in chromosomal regions containing oncogenes and tumor suppressor genes (e.g., *EGFR*, *ERBB2*, *MAP2K2*/*MEK*) have been linked to the development of CC, while somatic mutations are relatively rare.

Mutations of *KRAS*, *TP53*, and *IDH1* seem to be the most common genetic alterations in intrahepatic CC. The incidence of *KRAS* mutations shows a striking geographical variation, ranging from 4% in Thai patients to as much as 100% in British patients, while Japanese have an intermediate prevalence (about 60%). This may reflect the prevalence of liver flukes. Indeed, the mutational profiles of CCs associated with *O. viverini* infection have been shown to differ from those of tumors not associated with these infections. Moreover, hotspot *KRAS* mutations have been shown to be associated with intrahepatic CC initiation in mouse models and with a proliferation subgroup of intrahepatic patients with poor prognosis. *TP53* mutations do not have any particular hotspot and coexist with *KRAS* mutations in a small proportion of tumors. Mutations in both *IDH1* and *2* have been frequently reported in CC, with some studies suggesting that they are more common in intrahepatic than in extrahepatic tumors. Two identified hotspots: *R132 IDH1* and *R172 IDH2*, promote cholangiocarcinogenesis through repression of hepatocyte differentiation. *IDH* and *KRAS* mutations as well *IDH* and *TP53* mutations seem to be mutually exclusive. Other most frequently altered genes include *ARID1A*, *BAP1*, *KDM6B*, and *SETD2*, interestingly all involved in chromatin remodeling (**Table 1**).

A recent comprehensive analysis of nearly 500 samples has shown that grouping intrahepatic CC patients by classifier mutations in the three most recurrently mutated genes (*TP53*-mutant, *KRAS*-mutant, *IDH1*-mutant, wild-type in the three genes (called "undetermined")) reveals distinct molecular and pharmacogenomic profiles. In *TP53*-driven tumors, G to A transversions were the most prevalent, including the ApT>NpG associated with aristolochic acid. *R249S* was enriched but the aflatoxin signature was not enriched like in hepatocellular carcinoma and, compared to other mutants, *R249S* was not associated with the HBV status or overall survival. C to T transitions were most prevalent in *KRAS*-mutant tumors, with a preference for an upstream purine (G or C), suggesting a role of the APOBEC signature. *IDH1*-mutant tumors were significantly enriched for C to A transversions. These mutations are related to oxidative stress and wild-type *IDH1* has been shown to protect cells from oxidative stress-induced damage. *TP53*-driven tumors were significantly enriched for *PTEN*, *RB1*, and *LATS2* mutations, whereas *BLAF-1* alterations were prevalent in *IDH1*-mutant tumors, *SMAD4* in the *KRAS* group, and *KDM6B* in undetermined tumors. Out of previously identified genes, *ARID1* alterations were present in all the groups except for *TP53*-driven tumors but they were significantly associated only with undetermined tumors. *BAP1* mutations were significantly associated with *IDH1*-mutant tumors. Interestingly, no chromatin

Table 1 Gene loci that are most frequently altered in cholangiocarcinoma

Pathway	*Gene (locus)*	*Alteration type*	*Clinical significance and other comments*
P13K/PTEN/Akt (control of cell metabolism)	*KRAS* (12p12.1)	Point mutations (hotspots)	In up to 12% of intrahepatic CCs
	PTEN (10q23)	Point mutations and deletions	Prevalent in *TP53*-driven intrahepatic tumors
p53 signaling (cell-cycle control)	*TP53* (17p13.1)	Point mutations (scattered)	In up to 20% of intrahepatic CCs; *TP53*-driven molecular signature associated with an in silico sensitivity to some RNA synthesis and mTOR inhibitors
	RB1 (13q14.2)	Mutations	Prevalent in *TP53*-driven intrahepatic tumors
Tyrosine kinase receptors	*FGFR2* (10q26.13)	Fusions	In 6%–15% of CC cases; associated with shorter overall survival
Citrate cycle	*IDH1* (2q34) and *IDH2* (15q26.1)	Point mutations (hotspots)	In up to 12% of intrahepatic CCs; also reported in extrahepatic tumors; mutations associated with sensitivity to dasatinib and PARP inhibitors; *IDH1*-driven molecular signature associated with an in silico sensitivity to microtubule modulators (e.g. taxanes)
Chromatin remodeling	*ARID1A* (1p36.11)	Point mutations, deletions, and insertions	
	BAP1 (3p21.1)	Point mutations, deletions, and insertions	Prevalent in *IDH1*-mutant intrahepatic tumors
	KDM6B (17p13.1)	Point mutations and deletions	
	SETD2 (3p21.31)	Point mutations	
Hippo signaling	*LATS2* (13q12.11)	Point mutations	Prevalent in *TP53*-driven intrahepatic tumors
TGF-ß signaling	*SMAD4* (18q21.2)	Point mutations and insertions	Prevalent in *KRAS*-driven intrahepatic tumors

remodeling genes were associated with the *TP53* group. Both *TP53*- and *KRAS*-driven profiles were associated with a worse overall survival and shorter recurrence-free time. The four molecular signatures were associated with an in silico sensitivity to different groups of anticancer drugs (**Table 1**).

Up to two thirds of all biliary tract carcinomas overexpress ERBB2, while membranous expression of E-cadherin, alpha-catenin, and beta-catenin is downregulated in a majority of intrahepatic CCs and this decreased expression has been shown to correlate with a high grade of intrahepatic CC. Moreover, bile acids have been demonstrated to further activate EGFR and increase the expression of oxidative agents. MET overexpression has also been shown in intrahepatic CCs, correlating with tumor grade (low expression in poorly differentiated tumors) and increased proliferation indices.

Furthermore, bcl-2, an antiapoptotic protein, has been reported to be overexpressed in intrahepatic CC, while telomerase activity is detected in up to 100% of cases. Chronic inflammation which is thought to contribute to tumor development is associated with the release of inflammatory cytokines that induce oxidative stress and DNA damage. These cytokines may also create an immune-suppressive environment that promotes tumor cell survival and proliferation by blocking apoptosis which would normally be induced by DNA damage.

Biomarkers

Early detection of CC is extremely difficult and no reliable screening biomarkers have been identified.

Most CC patients present with advanced disease and have a median survival of less than one year despite treatment with current standard chemotherapy. Even patients who undergo apparently curative resection have poor outcomes due to a high rate of tumor recurrence. The most reliable (statistically significant) prognostic markers include several clinicopathological criteria. Cachexia, poor performance status, serum bilirubin of 9 mg/dL or greater, multifocal disease, hilar or proximal sites, high tumor grade, sclerotic histology, liver invasion, lymph node involvement, and advanced stage have been shown to be significantly associated with a poor outcome. Liver cirrhosis has been associated with an increased risk of recurrence and a reduced survival following resection in intrahepatic CC patients.

The limited ability to reliably acquire tissue samples has resulted in an ongoing quest for CC blood serum biomarkers. In addition to liver-specific testing (see "Presentation and diagnosis"), serum CA 19-9 and CEA levels are used as a part of the diagnostic workup, with increased levels suggesting a gastrointestinal malignancy. However, these two markers are not specific to CC and their sensitivity is also variable. They might be more useful in surveillance for posttreatment recurrence but identifying more reliable and more specific biomarkers remains an urgent need.

Serum IgG4 levels may be used to rule out autoimmune cholangitis during the diagnostic workup of perihilar CC. However, defining clear cut-offs is an on-going challenge. Recently, a new test measuring the IgG4/IgG RNA ratio has been developed. It can, reportedly, distinguish IgG24-associated cholangitis from primary cholangitis and CC. However, its value still needs to be clinically evaluated. Cytokeratin- and mucin-based biomarkers (in particular CYFRA 21-1 and MUC-5) have also been reported to be of potential diagnostic value but they need to be evaluated in larger cohorts.

Management and Therapy

Complete tumor resection with negative margins is the only curative procedure for CC. However, definitive surgery can only be applied to selected patients with well-localized lesions in whom negative microscopic margins can be obtained ("R0 resection") while maintaining adequate liver remnant, vascular inflow/outflow, and biliary drainage. Correct determination of the tumor resectability is a major challenge of the preoperative workup, and requires multidisciplinary and multimodality evaluation.

Imaging plays a decisive role in evaluating tumor resectability. However, it can also lead to confusion due to overlapping appearances with other hepatobiliary diseases, including benign lesions. CT and MRI are commonly used in various combinations with cholangiographic analyses in preoperative planning. When well performed, the accuracy of MRI and CT in predicting resectability exceeds 75%. [^{18}F]-FDG PET-CT seems to have no additional value.

Defining anatomic location and ductal extent of the tumor is an important step in preoperative planning. The Bismuth-Corlette classification is probably the most widely adopted staging system. It classifies perihilar CCs into four subtypes based on their anatomic location within the biliary tree. However, it is mainly informative for surgeons for planning resection type and not to determine resectability since other parameters, such as distant metastases and vascular involvement, are not included. For optimal determination of resectability, patients with potentially resectable CC may undergo staging laparoscopy. However, recent studies question the overall diagnostic yield and accuracy of staging laparoscopy, mainly due to improved imaging techniques.

Since extended liver resections are often required, it is critical to assess the future remnant liver (FRL) preoperatively. CT-volumetric analysis is the standard technique used to this effect. However, as liver volume is not synonymous with liver function and function is not homogeneously distributed in the liver, FRL function assessment is additionally performed. 99mTc-mebrofenin hepatobiliary scintigraphy (HBS) is a validated quantitative dynamic liver function test, with mebrofenin, an iminodiacetic derivative, used as a tracer.

The presence of severe underlying medical comorbidities, distant metastasis, involvement of major vascular structures not amenable to reconstruction, bilateral segmental ductal involvement, unilateral segmental ductal extension with contralateral

vascular inflow involvement, and calculated inadequate future liver remnant (FLR) are generally considered as contraindications to surgical resection. The reported resectability rates show much variability between centers, ranging from 20% to 90%.

Many CC patients present with obstructive jaundice which has a negative effect on liver function, increases the risk of biliary infection and impairs cellular immunity, and may increase the in-hospital mortality by 10%. To improve patient outcomes, preoperative biliary drainage (PBD; biliary stenting) is recommended by many surgical teams. It reduces jaundice, improves liver function and the patient's condition, at the same time improving the ability of the liver to regenerate postoperatively. The impact of these effects is particularly high in patients with an insufficient FLR and preoperative drainage has been shown to improve outcomes especially in patients requiring extended resections. However, the drainage itself may induce severe complications and the optimal drainage method is still a subject of debate. Percutaneous transhepatic biliary drainage (PTBD) had been widely used and has an advantage of a direct access to the biliary duct. However, it has been shown to have no positive effect on postoperative morbidity and mortality and to increase potential risks. Currently, endoscopic biliary drainage (EBD) is the most commonly used in Western countries as having fewer complications and better outcomes, while nasobiliary drainage (ENBD) is favored in many Asian countries. All in all, each method comes with its own set of complications and the potential risks for the patient need to be carefully evaluated against potential benefits when taking a decision whether to perform preoperative drainage at all, with many specialists advocating that it is only recommended if the FLR is small. At the same time, more studies are needed to determine the most optimal drainage method. To avoid the postoperative liver dysfunction resulting from extended hepatic resection (insufficient FRL volume or function), portal vein embolization (PVE) may be used. The use of preoperative radiotherapy has also been proposed in order to prevent seeding metastases following biliary drainage. It has also been reported to have an effect on the tumor size and that it may relieve jaundice in patients without biliary stenting. However, its benefits remain controversial.

R0 resection requires an aggressive surgical approach combining bile duct resection with extended liver resection frequently accompanied with vascular reconstructions, and regional lymphadenectomy. These extended resections are associated with higher morbidity and mortality rates than experienced in liver resections without bile duct resection, probably because of the sequelae of obstructive jaundice. Overall, up to 50% of CC patients suffer from severe postoperative complications. Liver failure is a major complication after extensive hepatectomy and is a major cause of mortality in patients with perihilar CC. Other complications include, among others, biliary leakage, infections, bleeding complications, and cholangitis. The 30-day operative mortality rate may be as high as 25%. Surgical biliary tract bypass may be performed in patients whose tumors are found to be unresectable at operation.

Liver transplantation in CC patients remains controversial. Theoretically, orthotopic liver transplantation (OLT) offers the advantage of resecting all of the structures that may be affected by the tumor, for example the portal vein, bilateral hepatic ducts and atrophic liver lobes. However, it is often not considered appropriate because of the high incidence of local recurrence and no standardized criteria to select patients for transplantation. Generally, transplantation is sometimes considered for selected patients with localized disease. Chemoradiation prior to transplantation has been shown to improve survival, according to some opinions enough to make the transplant meaningful.

Systematic studies on the recurrence rates are scarce and rates from 50% to 100% have been reported. Patients with recurrent disease are not candidates for curative therapy and can only receive adjuvant therapy to improve long-term outcome. Adjuvant therapy has been advocated to reduce the high incidence of local recurrence, but it does not appear to improve survival after curative resection. The role of adjuvant radiotherapy remains unclear. Cholangiocarcinoma is radiosensitive but bile duct tolerance to radiation is limited, with biliary and duodenal stenosis being frequently induced complications.

Chemotherapy, as either first or second-line is of potential benefit. Different regimens in different clinical settings have been and are still being tested in clinical trials. The most recent (February 2018) US National Comprehensive Cancer Network (NCCN) guidelines recommend using fluoripyrimidine- or gemcitabine-based regimens in post-resection CC patients (both intrahepatic and extrahepatic). One of these two regimens, or the gemcitabine/cisplatin combination may be used in unresectable extrahepatic CC patients. The latter is also the treatment of choice for palliative treatment in case of metastatic extrahepatic disease.

See also: Hepatocellular Carcinoma: Pathology and Genetics.

Further Reading

Bridgewater, J., et al., 2014. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. Journal of Hepatology 60 (6), 1268–1289.
Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer.
Ganeshan, et al., 2012. Extrahepatic biliary cancer: New staging classification. World Journal of Radiology 4 (8), 345–352.
Lewis, H.L., et al., 2017. Current management of perihilar cholangiocarcinoma and future perspectives. Chirurgia (Bucur). 112 (3), 193–207.
International Agency for Research on Cancer, 2010. In: Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), WHO classification of tumors of the digestive system, 4th edn. Lyon, France.
National Comprehensive Cancer Network (NCCN). (2018) NCCN clinical practice guidelines in oncology. Hepatobilliary Cancers. Version 1.2018.
Nepal, C., et al., 2017. Genomic perturbations reveal distinct regulatory networks in intrahepatic cholangiocarcinoma. Hepatology. https://doi.org/10.1002/hep.29764.
Rassam, F., et al., 2018. Modern work-up and extended resection in perihilar cholangiocarcinoma: The AMC experience. Langenbeck's Archives of Surgery 403 (3), 289–307.
Zhang, W., Yan, L.-N., 2014. Perihilar cholangiocarcinoma: Current therapy. World Journal of Gastrointestinal Pathophysiology 5 (3), 344–354.

Chromatin Dynamics in Cancer: Epigenetic Parameters and Cellular Fate

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Glossary

Centromere Chromosomal region of constriction joining two sister chromatids to which the microtubules of the spindle attach during cell division, via the kinetochore. Coined in 1936 by Cyril Darlington. From Greek *centro-* for "center" and *-meros* for "part."

Chromatin Macromolecular complex of DNA, protein and RNA that makes up the chromosomes of eukaryotic cells. Coined in 1879 by Walther Flemming to describe protoplasm in cell nuclei. From Latinized form of Greek *khromat-* for "color", and the chemical suffix *-in*.

Epigenetics Coined by Conrad Waddington in 1942 in reference to Aristotle's theory of *epigenesis*, proposing that embryos develop progressively from an undifferentiated egg cell. From Greek *epi-*, meaning "over, outside of, around," and *-genetics*, the study of inherited traits. Epigenetic features refer to the "structural adaptation of chromosomal regions to register, signal or perpetuate altered activity states" Bird (2007).

Epigenome The ensemble of modifications that govern the regulation of the genome.

Histone chaperone Histone chaperones are defined mainly as factors that associate with histones and stimulate a reaction involving histone transfer without being part of the final product. In 1978 the term "molecular chaperone" by Ron Laskey was applied to nucleoplasmin, a protein enabling the storage of histones H3–H4 in *Xenopus* oocytes. Later, it has been extended to all factors that prevent incorrect interactions between histones and DNA and facilitate nucleosome assembly.

Histone Five types of basic proteins found in chromatin, namely H1, H2A, H2B, H3, and H4 and their variants. Coined in 1884 by Albrecht Kossel. From Greek *histos-*, meaning "warp, web", and *-one*, meaning "weaker".

Kinetochore complex of proteins associated with the centromere to which the microtubules of the spindle attach during cell division. Coined in 1934 by L.W. Sharp from the Greek *kinisis-* for "movement" and *-choros* for "place, region".

Nucleosome Fundamental repeating subunit of chromatin, made up of DNA wrapped around a single hetero-complex of histones. Coined in 1975 by Pierre Oudet to reflect their nuclear origin and in reference to the "*nu*" bodies described by Olins and Olins (1974).

Oncogene A gene with the potential to transform a cell into a tumor cell. Coined in 1969 by Robert Huebner and George Todaro. From Latinized form of Greek *onco-* meaning "tumor, mass" and *-gene* for "to produce".

Nomenclature

2-HG 2-hydroxyglutarate
5caC 5-carboxylcytosine
5fC 5-formylcytosine
5hmC 5-hydroxymethylcytosine
5hmU 5-hydroxymethyluracil
5mC 5-methylcytosine
8oxoG 8-oxoguanine
ADAADi Active DNA-dependent ATPase A domain inhibitor
ADS Alternative DNA structures
ALT Alternative lengthening of telomeres
AML Acute myeloid leukemia
ARCH Age-related clonal hematopoiesis
BET Bromodomain and extraterminal domain–containing
CCAN Constitutive-centromeric associated network
DIPG Diffuse intrinsic pontine glioma
DNMT DNA-methyltransferase
DSB DNA double-strand break
DSC DNA synthesis coupled
DSI DNA synthesis independent

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HAT Histone acetyl-transferase
HDAC Histone deacetylase
HDM Histone demethylase
HMT Histone methyl-transferase
HR Homologous recombination
ICB Immune checkpoint blockade
IDH Isocitrate dehydrogenase
iPSC Induced pluripotent stem cell
KMT Lysine methyltransferase
MEF Mouse embryonic fibroblast
NMC NUT-midline carcinoma
panNET Pancreatic neuroendocrine tumors
PRC2 Polycomb-repressive complex-2
PTM Posttranslational modification
SAM S-adenosyl methionine
TCGA The Cancer Genome Atlas
TKI Tyrosine kinase inhibitor
TSS Transcription start site
α-KG Alpha-ketoglutarate

Introduction

The Hierarchy of Chromatin Organization: A Versatile Modular Structure

Within the nucleus, eukaryotic genomes are organized as nucleoprotein complexes comprising DNA, RNA, and proteins. This complex, called chromatin, follows a hierarchical organization that ranges from the basic unit, the nucleosome, up to higher-level domains in the nuclear space in interphase and culminates with chromosome compaction in mitosis. Its repeated module, the nucleosome, comprises ~147 bp of double-stranded DNA wrapped around an octamer of histone proteins plus intervening linker DNA. It thus forms periodic arrays giving rise to the nucleofilament or "beads-on-a-string" characteristic observed in early electron microscopy experiments (Fig. 1A). Transcription factors, or various chromatin-associated proteins and RNA punctuate this organization and help in defining specific domains. The nucleofilament undergoes further coiling to give rise to higher-order chromatin structures (Fig. 1B). Notably, DNA can be modified, distinct histone variants can form specific core particles and histones can be modified. These features of chromatin organization in space and time thus define, for a given cell type, an epigenome that corresponds to a cell fate decision. Here, we will refer to epigenetic features as to the "structural adaptation of chromosomal regions to register, signal or perpetuate altered activity states" as defined by A. Bird in 2007. Notably, distinct nucleosome features such as histone composition and modifications along with specific positioning characterize regulatory regions of the genome, such as promoters, enhancers, coding regions, replication origins, telomeres, and centromeres. This versatility of chromatin in space and time allows switching and reversibility, a general characteristic of the modular protein–DNA interactions that constitute chromatin. Thus, chromatin functions as a modular molecular scaffold with epigenetic features that can change and adapt according to cellular states during development and lifetime, and in response to various environmental stimuli.

Epigenetic Regulators: Shaping Chromatin in the Nucleus

Specific chromatin factors, acting at various organizational levels, exert spatial and temporal control over gene expression and are referred to as epigenetic regulators (Fig. 1C).

DNA modifications: Covalent modifications of DNA bases, such as 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), play central roles in epigenetic regulation of gene expression. DNA-methyltransferases (DNMTs) are enzymes that catalyze the covalent addition of a methyl group to a cytosine base of DNA. DNA methylation is commonly associated with promoter silencing and transcriptional repression. On the other hand, 10–11 translocation (TET) enzymes, TET1, 2, and 3, sequentially convert 5mC to 5hmC, then 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Several additional DNA adducts, such as 8-oxoguanine (8oxoG) and 5-hydroxymethyluracil (5hmU), important for epigenetic regulation should also be considered and the discovery of novel modifications is growing.

Histone choice and chaperones: As key components of the nucleosome, the core histone proteins (H3, H4, H2A, and H2B), along with the linker histone H1, serve as the building blocks of the epigenome. There are multiple forms of the four core histones, called histone variants, originally characterized based on migration properties using Triton Acid Urea gel electrophoresis by S. Franklin and A. Zweidler in 1977. The differences in amino acid sequence for the core histones from their variants range from single residue

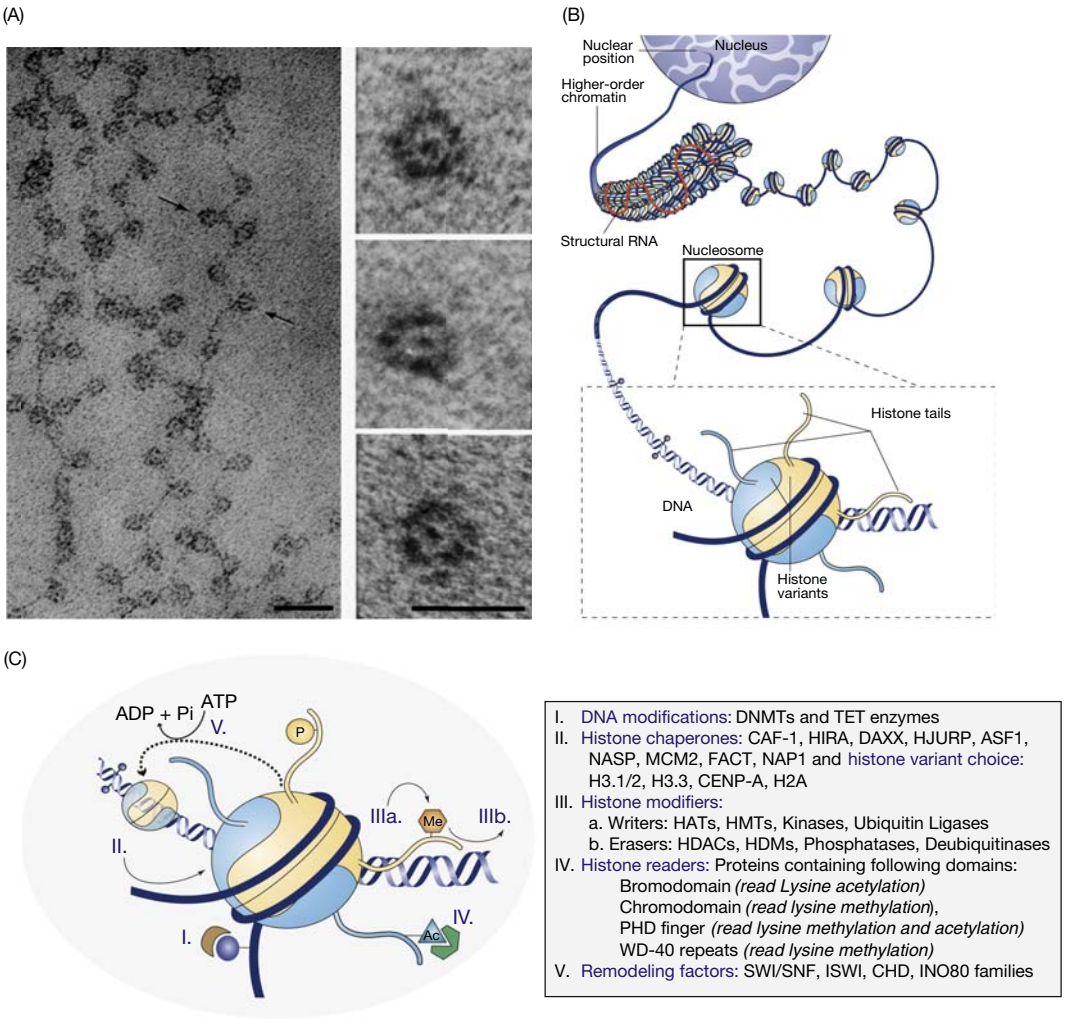

Fig. 1 Chromatin structure and organization: key regulators. (A) Electron micrographs. Left panel: low ionic-strength chromatin spread, the "beads on a string." Scale bar: 30 nm; Right panel: isolated mononucleosomes derived from nuclease-digested chromatin. Scale bar: 10 nm. (B) The hierarchy of chromatin organization—numerous epigenetic factors help organize chromatin in the 4D nucleus: along with the linker histone H1, DNA wrapped around one $(H3–H4)_2$ tetramer capped by two H2A–H2B dimers forms the nucleosome—the fundamental repeating unit of chromatin. DNA and histone tails can be modified. The presence of histone variants adds further complexity. Arrays of nucleosomes fold into higher-order chromatin structures, potentially guided by noncoding RNA. The nuclear localization of a given chromosomal domain represents an additional level of regulatory information. (C) Key chromatin regulators act at each hierarchical level. (A) From Olins, D. E., Olins, A. L. (2003). Chromatin history: Our view from the bridge. *Nature Reviews Molecular Cell Biology* **4**, 809–814—with permission; (B and C) Modified from Probst, A. V., Dunleavy, E., Almouzni, G. (2009). Epigenetic inheritance during the cell cycle. *Nature Reviews Molecular Cell Biology* **10**, 192–206.

alterations to greater than 50% divergence. Within the nucleus, the spatiotemporal dynamics of these various histone bricks are controlled by a specialized group of proteins called histone chaperones (Fig. 2). Thus, histones are never found alone as histone chaperones escort them throughout their cellular life.

Histone modifications—writers, erasers, and readers: While DNA is wrapped around the core histones, the N-terminal tails of histones remain exposed and undergo a variety of posttranslational modifications (PTMs). Individual and combined PTMs constitute what has been called a "histone code" with direct effects on the binding affinity of chromatin-interacting factors as well as DNA accessibility to various cell machineries. To place modifications, a series of enzymes have been identified as "writers," such as histone acetyl- and methyl-transferases (HATs and HMTs), including EZH2, the catalytic subunit of PRC2 (Polycomb repressive complex 2). To remove modifications, a series of enzymes have been identified as "erasers," such as histone deacetylases (HDACs) and demethylases (HDMs) (Fig. 1C). "Readers" that recognize specific chromatin PTMs comprise bromodomain and extraterminal domain–containing proteins (BET), chromodomain-, PHD finger- and WD40-containing complexes. They further recruit or stabilize factors at precise genomic loci to regulate nuclear functions. While a number of modifications are imposed on the nucleosome particle, several of them are also placed on histones before deposition. The dynamics of histone deposition and modifications should also be considered.

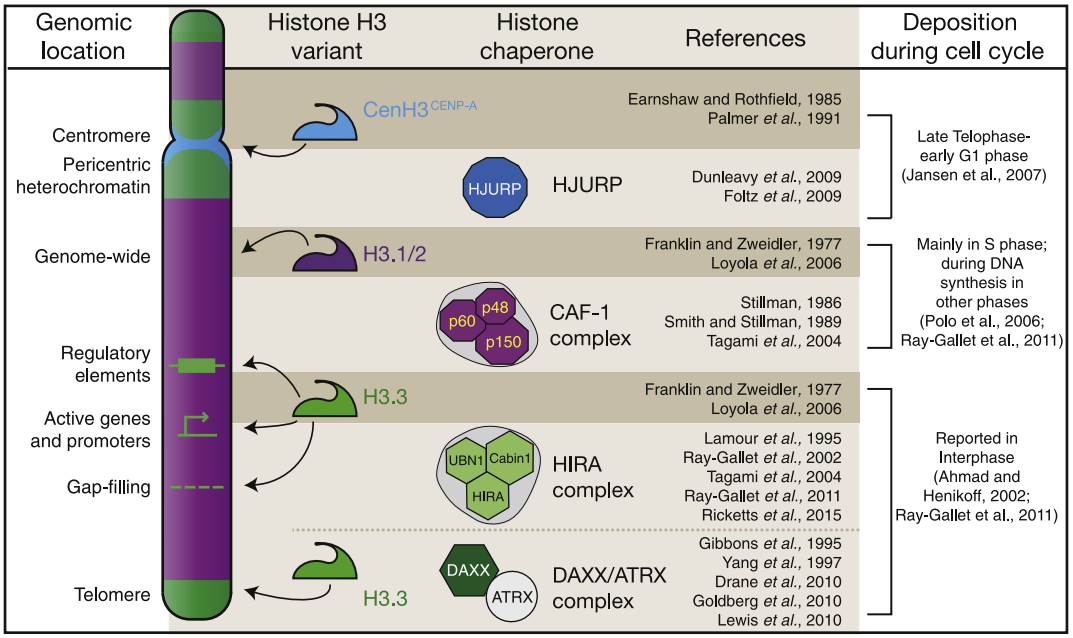

Fig. 2 Histone chaperones dedicated to distinct histone variants help their incorporation into chromatin at particular genomics locations and precise times during the cell cycle. Modified from Sitbon, D., Podsypanina, K., Yadav, T., Almouzni, G. (2017). *Shaping chromatin in the Nucleus: The bricks and the architects*. Cold Spring Harbor symposia on quantitative biology.

Chromatin remodelers and higher order chromatin: Chromatin remodelers are large, multisubunit, ATP-dependent molecular motors that regulate nucleosome structure, positioning and organization (Fig. 1C). These helicases share a common ATPase domain and can be classified into four subgroups based on their divergent domains: SWI/SNF, INO80, ISWI, and Mi2-CHD-NuRD complexes. Factors contributing to higher order structure include transcription factors, insulators, heterochromatin-associated proteins such as HP1, and noncoding RNA, while several others remain to be identified. While we describe the various factors individually, they are often found in combination, for example, a remodeler as a subunit of a larger complex. Also, note that each of the modifiers or remodeler complexes often contain a histone chaperone as a subunit or are associated with one of them.

Connections Between Epigenetic Regulators and Cancer Biology

Importantly, the relationships between epigenetic regulators, nuclear architecture and cancer biology have been stressed in a series of reviews. Indeed, several chromatin factors, such as histone modifiers and chromatin remodeling enzymes are disrupted or altered in cancers, including histone variants and chaperones. With the Cancer Genome Atlas (TCGA) reporting complete genomic and transcriptional data from several thousands of cancer patients and enabling the profiling of somatic tumor mutations, a series of potential chromatin-associated drivers of cancer have been identified. Nearly a quarter of the top 25 mutated genes, ranked by the frequency of alteration across all tumor types, encode chromatin-modifying enzymes—*KMT2C/MLL3*, *KMT2D/MLL2* (Lysine methyltransferases 2C, 2D), *ARID1A* (AT-Rich Interaction Domain 1A), *PBRM1* (Polybromo 1), *SETD2* (SET Domain Containing 2), *CREBBP* (CREB Binding Protein), and *SMARCA4/BRG1* (SWI/SNF-related Matrix-Associated Regulator of Chromatin A4). Indeed, one or more of the 12–15 subunits of SWI/SNF chromatin remodeling complexes are mutated in nearly 20% of all solid malignancies and, hence, represent attractive therapeutic targets. Importantly, individual SWI/SNF mutations vary in frequency in different cancer types, based on TCGA data. In addition to cancer development and maintenance, epigenetic factors have also been shown to correlate with patient response and clinical outcomes to current treatments. For example, specific chromatin regulators, including SWI/SNF chromatin remodelers, can serve as biomarkers for drug response (e.g., *SMARCB4* for docetaxel response or worse prognosis) and the histone chaperone CAF-1 holds prognostic value in renal, endometrial, and cervical carcinomas.

Genetic evidence supports a causal role for histone H3 mutations in aggressive brain cancers in children. Specifically, point mutations of H3 lysine 27 to methionine/isoleucine (H3K27M/I) or glycine 34 to arginine or valine (H3G34R/V) lead to critical changes in the histone methylation status and are found in 35%–80% of pediatric gliomas, depending on the subtype. Recently, recurrent mutations of histone H3 lysine 36 to methionine/isoleucine (H3K36M/I) or glycine 34 to tryptophan/leucine (H3G34W/L) have also been reported in specific bone cancers. Several instances of H1 loss-of-function mutations have been found in diffuse large B-cell/follicular. Although these particular histone mutations are linked to a subset of cancers, changes in histone levels are commonly observed in nearly all types of tumor.

So far, epidrugs have been used mainly in combinations with other cytotoxic treatments with the goal of sensitizing cancer cells as an end point (Table 1). Dynamic changes to the chromatin landscape over time can gradually or acutely influence specific

Table 1 Epidrugs in ongoing clinical trials for cancer treatment (December 2017)

Target[a]	*Epidrug*[b]	*National clinical trial (NCT) identifier*[c]	*Current status*[d]	*Delivery*[e]	*Cancer type*[f]	*Molecular selection (biomarker testing) for patient stratification*[g]
HMT inhibitors						
DOT-1L	EPZ-5676	NCT02141828	Phase 1	Monotherapy	Pediatric leukemia	*MLL*-rearranged
EED (PRC2 component)	MAK683	NCT02900651	Phase 1	Monotherapy	DLBCL/solid tumors	No
EZH2	CPI-1205	NCT02395601	Phase 1	Monotherapy	DLBCL	No
	Tazemetostat	NCT02889523	Phase 1/2	Combination	DLBCL	No
		NCT02601937	Phase 1	Monotherapy	Pediatric sarcoma	*SMARCB1*-negative or *SSX-SS18* fusion
		NCT03213665, NCT03155620	Phase 2	Both	Mixed	*EZH2*, *SMARCB1*, or *SMARCA4* mutation
		NCT02860286		Monotherapy	Mesothelioma	*BAP1*-deficient
		NCT02601950			Mixed	*SMARCB1-negative/EZH2 GOF mutation*
HDM inhibitors						
LSD1	GSK2879552	NCT02929498	Phase 2	Both	MDS	No
		NCT02177812	Phase 1	Monotherapy	AML	No
	IMG-7289	NCT02842827	Phase 1	Combination	AML/MDS	No
	INCB059872	NCT02712905	Phase 1/2	Combination	AML/MDS/solid tumors	No
LSD1 (off-target)	Tranylcypromine	NCT02717884, NCT02273102	Phase 1/2	Combination	AML/MDS	No
DNMT inhibitors						
pan DNMT	5-Azacytidine	EMEA/H/C/000978	*EMA approved (1st line)*	Monotherapy	MDS/AML/CML	No
		NDA 050794	*FDA approved (1st line)*	Monotherapy	MDS	
		NCT01386346	Phase 1	Combination	Esophageal	
		NCT00336063	Phase 1	Combination	Nasal/nasopharyngeal	Testing *EBV* promoter meth., histone acetyl.
		NCT02940483, NCT03206021	Phase 1	Monotherapy	Pediatric brain	No
		NCT01155583	Phase 1/2	Combination	MM	
		NCT02959437	Phase 1/2	Combination	Solid tumors	
		NCT02788201	Phase 2	ND	Urinary/bladder	COXEN model
		NCT02374099	Phase 2	Combination	Breast	No
		NCT02260440	Phase 2	Combination	Colorectal	
		NCT02828358	Phase 2	Combination	Infant ALL	*KMT2A* rearrangement; testing *LINE-1* meth.
		NCT02900560	Phase 2	Combination	Ovarian	No
		NCT03264404, NCT01845805	Phase 2	Combination	Pancreatic	
		NCT02178072	Phase 2	Monotherapy	Head/neck	HPV±
		NCT01281124	Phase 2	Monotherapy	Lung	Testing DNA meth., *mir29* expression

pan DNMT	5-Azacytidine	NCT01522976	Phase 2/3	Both	MDS/CML	Testing cytogenetic abnormalities
		NCT02319135, NCT03151408	Phase 3	Combination	AML	No
		NCT02951156	Phase 3	Combination	DLBL	
		NCT01566695	Phase 3	Combination	MDS	
	Decitabine	EMEA/H/C/002221	*EMA approved*	Monotherapy	AML	No
		NDA 21-790	*FDA approved*	Monotherapy	MDS	
		NCT02959164	Phase 1	Combination	Pancreatic/sarcoma	
		NCT02951728	Phase 1/2	Combination	DLBCL	
		NCT03250962	Phase 1/2	Combination	Hodgkin Lymphoma	
		NCT01876641	Phase 1/2	Combination	Melanoma	
		NCT03346642	Phase 1/2	Combination	Large B-cell Lymphoma	
		NCT02788201	Phase 2	ND	Urinary/Bladder	COXEN model
		NCT02634827	Phase 2	Combination	AML	*FLT3* mutation
		NCT02957968	Phase 2	Combination	Breast	*HER2-*
		NCT02664181	Phase 2	Combination	Lung	No
		NCT02159820	Phase 2/3	Combination	Ovarian	
		NCT01882660	Pre-operative	Monotherapy	Colorectal	
		NCT03026842	Phase 4	Monotherapy	AML	t8;21
	Epigallocatechin-3-gallate	NCT01317953	Phase 1	Monotherapy	Lung	No
	Hydralazine	NCT02446652	Phase 3	Combination	Cervix	No
BET inhibitors						
BRD2-4	AZD5153	NCT03205176	Phase 1	Monotherapy	Mixed	No
	I-BET762	NCT01587703	Phase 1	Monotherapy	Nut-midline Carcinoma	*BRD4-NUT* translocation
	I-BET762	NCT03150056	Phase 1	Combination	Prostate	No
	I-BET762	NCT02964507	Phase 2	Combination	Breast	*ER+*
	INCB054329	NCT02711137	Phase 1	Combination	Mixed	No
BRD2-4/T	BMS-986158	NCT02419417	Phase 1/2	Monotherapy	Mixed	No
	CC-90010	NCT03220347; NCT01949883	Phase 1	Monotherapy	Mixed	
	CPI-0610	NCT02158858	Phase 1	Monotherapy	AML/MDS	
	CPI-0610	NCT02157636	Phase 1	Monotherapy	MM	
	CPI-0610	NCT02986919	Phase 2	Monotherapy	MPNST	
	GS-5829	NCT02983604	Phase 1	Combination	Breast	*ER+ HER2-* breast cancer
	GS-5829	NCT02607228	Phase 1	Combination	CRPC	No
	GSK2820151	NCT02630251	Phase 1	Monotherapy	Solid tumors	
	RO6870810	NCT03068351	Phase 1	Both	MM	
	ZEN003694	NCT02711956	Phase 1	Combination	CRPC	
	ABBV-075	NCT02391480	Phase 1	Combination	Mixed	
BRD4	ODM-207	NCT03035591	Phase 1/2	Monotherapy	Solid tumors	No
PI3K and BRD4	SF1126	NCT03059147	Phase 1	Monotherapy	Liver	No
HDAC inhibitors						
HDAC1	Quisinostat	NCT02948075	Phase 2	Combination	Ovarian	No

(Continued)

Table 1 Epidrugs in ongoing clinical trials for cancer treatment (December 2017)—cont'd

Target[a]	*Epidrug*[b]	*National clinical trial (NCT) identifier*[c]	*Current status*[d]	*Delivery*[e]	*Cancer type*[f]	*Molecular selection (biomarker testing) for patient stratification*[g]
HDAC1/2	Romidepsin	022393/S-004	*FDA approved (2nd line)*	Monotherapy	CTCL/PTCL	No
		NCT02512172	Phase 1	Both	Colorectal	Microsatellite stable
		NCT02393794	Phase 1/2	Combination	Breast	*ER-/PR-/HER2-*
		NCT02788201	Phase 2	ND	Urinary/bladder	COXEN model
		NCT01796002	Phase 3	Combination	PTCL	No
HDAC3/4	Tasquinimod	NCT01743469	Phase 2	Monotherapy	Lung/ovarian/kidney/ gastric	No
HDAC6	ACY-241	NCT02635061	Phase 1	Combination	Lung	No
	Resminostat	NCT02400788	Phase 1/2	Combination	Liver	No
pan HDAC	Ricolinostat	NCT02632071	Phase 1	Combination	Breast	No
		NCT02091063	Phase 1/2	Monotherapy	Lymphoma	
	Abexinostat	NCT01543763	Phase 1	Combination	Mixed	No
	AR-42	NCT02282917	Phase 0	Monotherapy	Meningioma/schwannoma	No
	Belinostat	NDA 206256	*FDA approved (2nd line)*	Both	PTCL	No
		NCT02137759	Phase 2	Combination	Brain	MRSI-based
		NCT01686165	Phase 2	Combination	DLBL	No
		NCT02737046	Phase 2	Combination	T-cell leukemia/lymphoma	
	Entinostat	NCT02780804	Phase 1	Monotherapy	Pediatric brain	No
		NCT02915523	Phase 1/2	Combination	Ovarian/peritoneal/ fallopian	
		NCT01038778, NCT03024437	Phase 1/2	Combination	Renal	Testing lymphocyte levels
		NCT02708680	Phase 1/2	Combination	Breast	No
		NCT01305499	Phase 2	Combination	AML	
		NCT03018249	Phase 2	Combination	Endometrial	Testing PR, Ki67, and p21 levels
		NCT01928576	Phase 2	Combination	Lung	No
		NCT03179930	Phase 2	Combination	Lymphoma	
		NCT02115282	Phase 3	Combination	Breast	*ER+/PR+/HER2-*; testing lysine acetyl
	Givinostat	NCT01761968	Phase 2	Monotherapy	Bone marrow/blood	No
	Mocetinostat	NCT02282358	Phase 1/2	Monotherapy	Lymphoma	Acetyltransferase mutations (*CREBBP* or *EP300*)
		NCT02805660	Phase 1/2	Combination	Solid tumors	No
		NCT02954991	Phase 2	Combination	Lung	
	Panobinostat	NDA 205353	*FDA & EMA approved (3rd line)*	Combination	MM	No
		NDA 205353	*FDA & EMA approved (3rd line)*	Combination	MM	
		NCT02032810	Phase 1	Combination	Skin	

pan HDAC	Panobinostat	NCT02717455	Phase 1	Monotherapy	Pediatric brain	Testing *H3K27* mutations
		NCT01451268	Phase 1/2	Monotherapy	MDS/AML	No
		NCT01238692	Phase 2	Both	DLBL	Testing biomarkers
		NCT02506959	Phase 2	Combination	MM	No
		NCT01261247	Phase 2	Monotherapy	Lymphoma	Testing SNPs, cytokines, lymphocytes
	Pracinostat	NCT03151304, NCT01873703	Phase 2	Combination	MDS	NO
		NCT03151408	Phase 3	Combination	AML	
	SAHA (Vorinostat)	NDA 021991	*FDA approved (2nd line)*	Monotherapy	CTCL	No
		NCT02619253	Phase 1	Combination	Kidney/urinary/bladder	
		NCT01075113	Phase 1	Combination	Liver	
		NCT00336063	Phase 1	Combination	Nasal/nasopharyngeal	Testing *EBV* promoter meth., histone acetyl.
		NCT02349867	Phase 1	Combination	Pancreatic	No
		NCT00555399	Phase 1/2	Combination	Adult brain	
		NCT02538510	Phase 1/2	Combination	Head/neck	
		NCT02638090	Phase 1/2	Combination	Lung	
		NCT01294670, NCT01879085	Phase 1/2	Combination	Sarcomas	
		NCT02836548	Phase 1/2	Combination	Skin	*BRAF V600* mutant
		NCT02788201	Phase 2	ND	Urinary/bladder	COXEN model
		NCT02395627	Phase 2	Combination	Breast	No
		NCT02035137	Phase 2	Combination	CNS	
		NCT02316340	Phase 2	Combination	Colorectal	
		NCT00875056	Phase 2	Monotherapy	Lymphoma	
		NCT01175980	Phase 2	Monotherapy	Mouth	Testing *HR23B* expression, aneuploidy, polyploidy, whole exome, CGH array
		NCT01802333	Phase 3	Combination	AML	No
		NCT01554852	Phase 3	Combination	MM	
	Tefinostat (CHR-2845)	NCT02759601	Phase 1/2	Monotherapy	Liver	No
	Valproic acid	NCT02520115	Phase 1	Combination	Ovarian	Folate receptor induction
		NCT01898104	Phase 1/2	Combination	Colorectal	No
		NCT01622439	Phase 1/2	Combination	DLBL	
		NCT00879437, NCT01817751	Phase 2	Combination	Adult/pediatric brain	
		NCT02124174, NCT01342692	Phase 2	Combination	AML/MDS	
		NCT02068586	Phase 2	Monotherapy	Melanoma	
		NCT02068590	Phase 3	Combination	Head/neck	
	Vorinostat	NCT01045538	Phase 1/2	Combination	Gastric	No
		NCT01064921	Phase 1	Combination	Head/neck	
HAT inhibitors						
p300/CREBBP	Curcumin	NCT03072992	Phase 2	Combination	Breast	No
		NCT03192059	Phase 2	Combination	Cervical/endometrial/ uterine	

(Continued)

Table 1 Epidrugs in ongoing clinical trials for cancer treatment (December 2017)—cont'd

Target[a]	*Epidrug*[b]	*National clinical trial (NCT) identifier*[c]	*Current status*[d]	*Delivery*[e]	*Cancer type*[f]	*Molecular selection (biomarker testing) for patient stratification*[g]
p300/CREBBP	Curcumin	NCT02439385, NCT02439385	Phase 2	Combination	Colorectal	No
		NCT02100423	Phase 2	Combination	Leukemia/lymphoma	
		NCT02064673	Phase 3	Monotherapy	Prostate	
IDH inhibitors						
IDH1	Ivosidenib (AG-120)	NCT02074839, NCT03245424	Phase 1	Monotherapy	AML	*IDH1* mutation
		NCT03343197	Phase 1	Monotherapy	Brain	*IDH1* or *IDH2* mutation
		NCT02989857	Phase 3	Monotherapy	Cholangiocarcinoma	*IDH1* mutation
		NCT03173248	Phase 3	Combination	AML/MDS	
	BAY1436032	NCT02746081	Phase 1	Monotherapy	Solid tumor	*IDH1-R132X* mutation
		NCT03127735	Phase 1	Monotherapy	AML	
	IDH305	NCT02381886	Phase 1	Monotherapy	Mixed	*IDH1-R132* mutation
IDH1/2	Vorasidenib (AG-881)	NCT02492737	Phase 1	Monotherapy	AML/MDS	*IDH1* or *IDH2* mutation
		NCT03343197	Phase 1	Monotherapy	Brain	
IDH2	Enasidenib (AG-221)	NDA 209606	*FDA approved (2nd/3rd line)*	Monotherapy	AML	*IDH2* mutation
		NCT02577406	Phase 3	Monotherapy	Leukemia	

Abbreviations: *acetyl*, acetylation; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *CGH*, comparative genomic hybridization; *CML*, chronic myeloid leukemia; *CNS*, central nervous system; *COXEN*, co-expression extrapolation; *CRPC*, castration resistant prostate cancer; *CTCL*, cutaneous T-cell lymphoma; *DLBL*, diffuse large B-cell lymphoma; *DNMT*, DNA methyl-transferase; *EMA*, European Medicines Agency; *ER*, estrogen receptor; *FDA*, Food & Drug Administration (USA); *GOF*, gain of function; *HAT*, histone acetyl-transferase; *HDAC*, histone deacetylase; *HDM*, histone demethylase; *HMT*, histone methyl-transferase; *MDS*, myelodysplastic syndromes; *meth*, methylation; *mixed*, solid and hematologic malignancies; *MM*, multiple melanoma; *MPNST*, malignant peripheral nerve sheath tumors; *MRSI*, magnetic resonance spectroscopic imaging; *PR*, progesterone receptor; *PTCL*, peripheral T-cell lymphoma.

[a]Target: the epigenetic regulator (molecule(s) or complex(es)) directly affected by the drug.

[b]Epidrugs: all anticancer drugs in the list were selected from the human epigenetic drug database (hedds.org: December 2017) and others with reported epigenetic effects in current literature.

[c]National Clinical Trial (NCT identifier): unique reference from an online database (www.ClinicalTrials.gov) providing details about each trial (start date, study design, current status, etc.).

[d]Current status: the table reports the most advanced clinical trial per cancer type.

[e]Delivery: the administration of the drug as a single agent or in combination with other drugs.

[f]Cancer type: refers to the cancers where the epidrug is tested against a specific tissue/organ tumor, excluding trials for mixed cancer types (except if they use molecular stratification of patients or are the only representative trial for that epidrug).

[g]Molecular selection (biomarker testing) for patient stratification: details about mutations, molecular markers or other physiological features used to select target patients.

genome functions. Importantly, chromatin regulators can also affect the tumor microenvironment and immune system. Understanding the combination of effects and the dynamics of disease progression is of paramount importance. By interfering with specific epigenetic factors with known effects in space and time, in either the cancer cells or cells of the microenvironment, we may be able to alter precise chromosomal territories at defined moments in the cell cycle to adapt the treatment strategy to the type of tumor. This capacity to selectively manipulate genome regulation may provide us with the ability to combat some of the fundamental difficulties of cancer treatment.

Epigenetic Links to the Roadblocks of Cancer Treatment

In the past four decades, advances in the treatment of malignant disease have resulted in growing improvement in overall clinical outcomes. Nevertheless, fundamental problems persist which result in drug resistance, disease recurrence and, ultimately, patient mortality. To name the most critical ones, the roadblocks to patient cure include: clonogenic potential of cancer cells, tumor evolution, tumor microenvironment, inefficient immune surveillance, tumor heterogeneity, and drug-related side effects (Fig. 3A–F). Since multiple chromatin components have been shown to underpin one or several aspects of these pervasive complications, epigenetic targeting represents an important avenue to explore.

Clonogenic potential (Fig. 3A) represents the ability of a single cancer cell to reconstitute the entire tumor as if it were a stem cell. Theoretically, even a single remaining malignant cell following treatment can lead to disease recurrence if it harbors this clonogenic capacity. In fact, the principles of combining chemo- and radiotherapy conceived to combat minimal residual disease are still applied today. However, the "stemness" of residual cancer cells is reminiscent of the pluripotency displayed by normal stem cells. The differences between stem and differentiated cells, supported by changes to chromatin architecture during stem cell differentiation, has to be taken into account for rational drug design. In particular, it is intriguing that the plasticity of transitions between

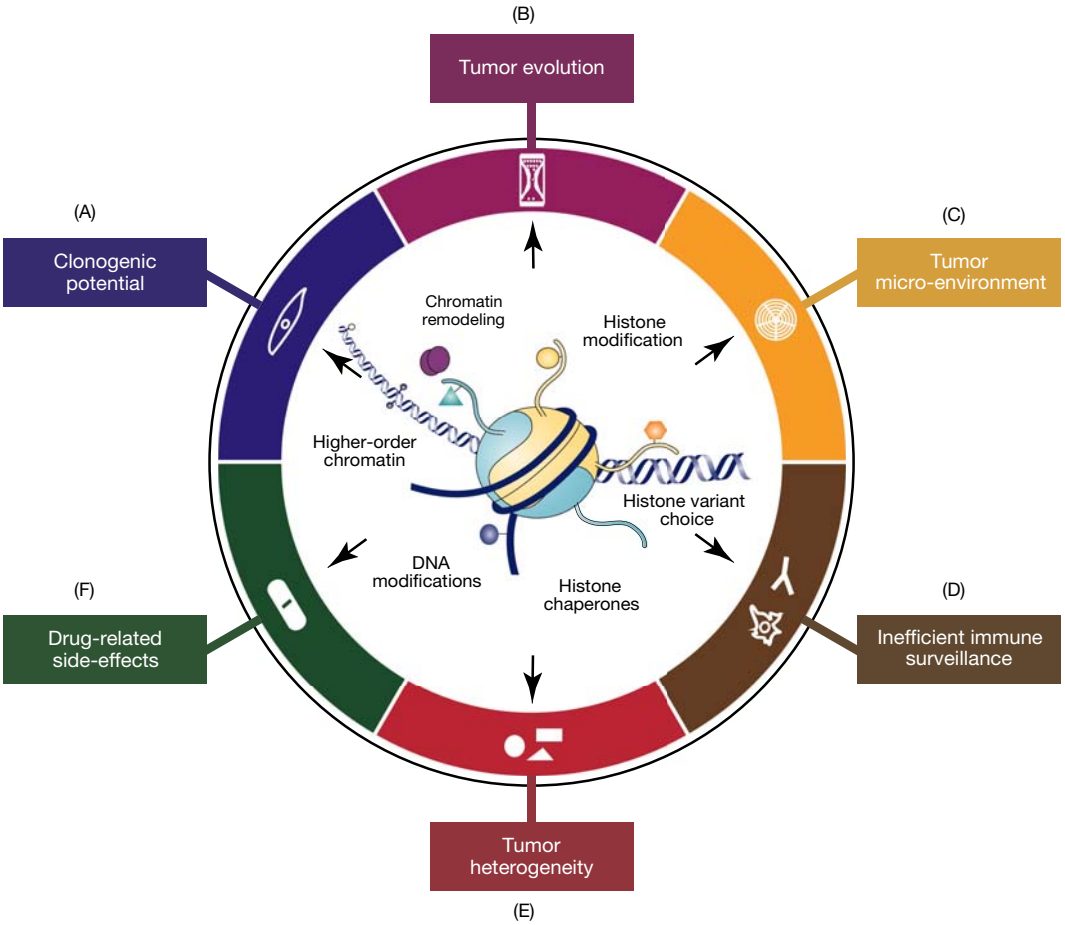

Fig. 3 Epigenetic Links to the Roadblocks of Cancer treatment. Starting from the scheme depicting the hallmarks of cancer, we place the building blocks of chromatin, the nucleosome, and its versatile forms as central players underpinning different roadblocks of cancer treatment (A–F). See section Epigenetic links to the Roadblocks of Cancer Treatment in text for details. Original artwork based on Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* ***100***, 57–70.

differentiated and undifferentiated states shows direct ties to the histone chaperone CAF-1, a chromatin factor found overexpressed in series of aggressive cancers. Indeed, CAF-1 knockdown combined with the modulation of transcription factors improves reprogramming of mouse fibroblasts to induced pluripotent stem cells (iPSCs) by several orders of magnitude and in embryonic stem cells it can enhance totipotency. Therefore, modulation of the expression of CAF-1 may have opposing effects on tumor growth and clonogenicity. Progress in understanding parameters involved in control of stemness at the chromatin level will help shed further light on the problem of residual disease in cancer.

Tumor evolution (Fig. 3B) occurs as the disease progresses from less aggressive to more malignant stages. Cancer cells divide in the context of genetic and *epi*genetic instability. As a result, a tumor is equipped with a vast repertoire of divergent subclones of varying fitness. Continuous tumor growth creates bottlenecks for survival, resulting in selection of individual subclones. As selective pressure mounts during metastasis or administration of treatment, (epi)genetic plasticity provides additional adaptability to the evolving tumor. This form of drug escape may be overcome by modulating the (epi)genome. For example, cancer cells resistant to tyrosine kinase inhibitors (TKI), such as gefitinib, show decreased levels of H3K4me2/3, correlated with poor prognosis. In turn, TKI-resistant tumors become susceptible to KDM5A (H3K4 demethylase) silencing or inhibition of KDM5A-associated HDACs, while the parental tumor cell lines remained unaffected. These results suggest that *epi*genetic factors such as H3K4 demethylase KDM5A likely underlie nongenetic tumor heterogeneity leading to resistance. This example underlines the capacity to exploit chromatin plasticity for overcoming drug resistance linked to (epi)genetic instability and malignant evolution.

The tumor microenvironment (Fig. 3C), a complex network of cells and extra-cellular factors surrounding a developing tumor, functions as the "ecosystem" for an expanding population of cancer cells. These nonmalignant tissues can be co-opted by the tumor to sustain its growth and can also physically block the access of anticancer drugs or the immune system to the tumor. In some instances, marked changes in expression of chromatin factors, such as histone PTMs and DNMTs, have been reported in cancer-associated fibroblasts in contrast to their normal counterparts, despite their nonmalignant genetic status. In breast cancers, these epigenetic changes have been shown to promote tumor invasiveness and malignancy. Increased understanding of epigenetic changes in cancer-associated stroma provides clues for how to overcome this roadblock to cancer treatment. However, the efficacy of currently available epigenetic therapies has not been specifically evaluated in cancer stroma. Describing the metabolic and epigenetic pathways connecting immune, stromal, and cancer cells could provide necessary clues to combat drug resistance linked to microenvironment.

Inefficient immune surveillance (Fig. 3D) is a failure of the immune system to recognize malignant tissue as abnormal or pathological. In the absence of immune recognition, the patient may not present with overt clinical symptoms and there is a paucity of tumor-specific antigens that can generate a proficient antitumor response. Recent experiments studying immune checkpoint blockade (ICB) indicated that tumor-specific effector T-cells are often kept inactive by de novo methylation programs that hinder clonal expansion and diversity of effector T-cells. In this case, an epigenetic strategy to reactivate the T-cell antitumor response could involve reversal of these DNA methylation changes. Indeed, examples of exposure to DNA-demethylating agents, such as decitabine, prior to ICB showed restoration of antitumor CD8 T-cell function, highlighting a role for epigenetic reprogramming in immune surveillance. Other methods could also participate in controlling T cell fate and T cell differentiation. This connection between the immune system and epigenetic regulators offers a number of possibilities that are just beginning to be explored.

Tumor heterogeneity (Fig. 3E) originates from the combined effects of: (1) genetic instability and selection of divergent mutational clones and (2) hierarchical development of a cancer cell population. Epigenetic pathways influence both of these causative factors. For example, aberrant DNA methylation is associated with an increased frequency of cytosine to thymidine (C > T) transitions, initiated by the spontaneous deamination of methylated cytosines, a mutational signature found in all cancer types and most cancer samples. Also, tumors develop as populations of mixed subclones at various stages of lineage commitment. As already mentioned above, factors like the histone chaperone CAF-1 and linker histone H1 are known to regulate lineage specification and cell fate determination. Indeed, cancer-specific chromatin patterns are reminiscent of those in embryonic stem cells and some adult stem cells. This example highlights the ability to manipulate chromatin factors, involved in cell fate decisions, to influence tumor heterogeneity.

Drug-related side effects (Fig. 3F) are commonly associated with cancer therapies, to the extent where patients may even perceive the treatment as being worse than the disease itself. Due to the fact that cancer originates from the host's own cells, classical anticancer drugs target properties that are shared by both tumor and normal cells. Therefore, the therapeutic window can be very narrow and epigenetic manipulation can provide interesting means to help direct a more selective targeting and limit drug-related side effects. For example, the use of the DNMT inhibitor, decitabine, which led to the preferential de-repression of gene promoters silenced only in cancer cells, has been reported as a disease-selective means to avoid affecting noncancer cells. Thus, targeting epigenetic pathways in the future could improve upon the selectivity and specificity of currently available anticancer drugs.

Chromatin Regulators in Cancer: Epi(genetic) Drug Targets

DNA- and Histone-Modifying Enzymes

Drugs inhibiting the enzymatic activities of DNA-modifying enzymes and PTM writers, readers and erasers that can provide cytotoxic effects with a strong potential in cancer therapy are listed (Table 1). Alterations in global DNA methylation levels are linked to aberrant changes in gene expression of oncogenes as well as tumor-suppressor genes. DNMT inhibitors, azacitidine and decitabine,

are cytosine-analogs that cannot be methylated resulting in the sequestration of DNMTs and subsequent global DNA hypomethylation. At high doses, DNMT inhibitors impair DNA synthesis or translation of proteins causing cell death.

Gain-of-function mutations in genes encoding isocitrate dehydrogenases (IDHs) are commonly associated with AML, breast and brain cancers. IDH mutations affect both DNA and histone methylation. *IDH1* and *IDH2* catalyze the production of alpha-ketoglutarate (α-KG), a key cofactor in TET-mediated conversion of 5mC to 5hmC and the HMT function of the Jumonji-C domain-containing family of enzymes. Mutations altering R132 (*IDH1*), R140 or R172 (*IDH2*) enable them to convert α-KG to 2-hydroxyglutarate (2-HG), which competitively interferes with α-KG-dependent epigenetic reactions such as DNA modification and histone-methylation. Thus, therapies targeting these specific chromatin-associated mutations are currently being tested in several clinical trials (**Table 1**).

At the histone level, the presence of acetyl group modifications on the histone tails has generally been correlated with gene activation. Different classes of HDACs catalyze the removal of acetyl groups from a variety of histone and nonhistone substrates. HDAC inhibitors, such as vorinostat, interfere with the active site of the enzyme resulting in hyperacetylated substrates and associated changes in gene expression and posttranslational regulation of nonhistone proteins. However, DNMT and HDAC inhibitors as mono-therapies have demonstrated limited clinical efficacy, especially against solid tumors. It remains unclear if this poor success was due to a lack of informed patient selection based on predictive biomarkers. This highlighted the need to choose patients by careful molecular stratification in future trials.

Careful selection of patient populations for drug testing showed promise in early clinical trials evaluating a new generation of epigenetic drugs, such as targeted inhibitors of BRD4 and its homologs (BET family proteins) or EZH2. BRD4 is a bromodomain-containing reader of acetylated-histones and a transcriptional coactivator, also implicated in DNA replication and repair. *BRD4* fusion with *NUTM1* encodes an oncogenic protein that defines highly aggressive NUT-midline carcinoma (NMC). This fusion leads to oncogenesis by aberrant recruitment of HATs and transcriptional activation of oncogenes, including *MYC*. As well, several lines of evidence demonstrate tumor cell addiction to functional BRD4 activity in acute myeloid leukemia (AML), which harbor translocations or mutations for the gene encoding the MLL1 histone lysine methyltransferase. Another epigenetic target includes the histone methyltransferase EZH2, catalytic subunit of the polycomb-repressive complex 2 (PRC2), which promotes transcriptional silencing and facultative heterochromatin formation. At the molecular level, EZH2 methylates lysine 27 of histone H3 (H3K27) using S-adenosyl methionine (SAM) as a cofactor. EZH2 gain-of-function mutations are associated with non-Hodgkins Lymphoma. At the same time, EZH2 inactivating mutations have also shown oncogenic properties. Thus, understanding the mechanisms of PRC2-mediated gene regulation may help treat patients with *EZH2* mutant cancers.

BET and EZH2 inhibitors are now in Phase II trials in molecularly selected patients, based on criteria described in **Table 1**. Current small-molecule BET inhibitors can be broadly classified as benzodiazepine-derivatives, namely JQ1 and its analog OTX015/MK-8625 (**Table 2**), and quinoline class drugs such as I-BET151 and I-BET762. BET inhibitors mimic acetyl-lysines and serve as competitive inhibitors of BET protein binding to chromatin. EZH2 inhibitors, such as tazemetostat, work as SAM-competitive small molecules that block HMT activity of the PRC2 complex. Moreover, PRC2 shows epigenetic antagonism with specific chromatin remodeling complexes. Hence, these interrelationships suggest that chromatin remodelers may also serve as potent drug targets.

Chromatin Remodeling Enzymes

ATP-dependent chromatin remodeling complexes such as SWI/SNF facilitate the exchange or displacement of nucleosomes. Although at least one alteration in SWI/SNF complexes is directly linked to oncogenesis, few therapeutic agents have been characterized against individual subunits. Currently, ADAADi (Active DNA-dependent ATPase A Domain inhibitor) has shown an ability to interfere with SMARCA4/BRG1, a core catalytic ATPase of the SWI/SNF chromatin remodeling complex. Previously, depletion of SMARCA4 was found to enhance sensitivity toward platinum-based chemotherapy and docetaxel. Despite their overlapping

Table 2 Epigenetic drugs outside of current clinical trials for cancer treatment

Status	*Class of epidrugs*	*Epidrug*
Ongoing and upcoming (noncancer)	DNMTi	Procainamide
	BETi	RVX-208
	HDACi	Resveratrol, sodium butyrate
Previous clinical trials for cancer treatment	DNMTi	Zebularine
	BETi	OTX015/MK-8625
	HDACi	CHR-3996, pivanex
Preclinical only	HMTi	3-Deazaneplanocin A, BIX-01294, CPI360, EI1, EPZ004777, GSK126, GSK343, and UNC0638
	HDMi	Bizine, clorgyline, GSK-J4, JIB-04, KDM5-C70, and pargyline
	BETi	CPI203, I-BET151, and JQ1
	HDACi	Trichostatin A, CG-1521, HC-toxin, ITF-A, and ITF-B
	HATi	C646

molecular functions, various SWI/SNF complexes display heterogeneity in their subunit composition. Synthetic lethal relationships have been reported between independent catalytic subunits such as SMARCA2 and SMARCA4. The loss of either gene is dispensable for viability while their combined loss results in cell death. Interestingly, isolated inactivation of SMARCA2 or SMARCA4 increases cisplatin sensitivity. Similarly, the structural subunit ARID1B is essential for survival of tumor cells lacking its mutually exclusive partner ARID1A. Thus, synthetic interdependencies of intracomplex subunits within chromatin remodeler assemblies provide druggable targets for anticancer therapy.

Importantly, SWI/SNF complexes display interdependent relationships with several pathways involving PRC2, PARP (important for single-strand DNA break repair), and PI3K/AKT (cell cycle regulators). This genetic crosstalk provides additional vulnerabilities in SWI/SNF-mutant tumors. The SWI/SNF chromatin remodeler and PRC2 repressive complexes show opposite effects on target gene expression, such that tumors defective for SWI/SNF subunits display increased PRC2 activation. This results in a PRC2 dependency that is only present when SWI/SNF is compromised. Inhibiting the EZH2 subunit of the PRC2 complex with tazemetostat selectively increased efficacy in patients negative for SMARCA4 or the core SMARCB1/INI1 subunit, in Phase I clinical trials. Although there are currently no ARID1B inhibitors, in preclinical studies ARID1A-deficient cancer cells have shown increased sensitivity to EZH2 inhibitors as well as PARP and PI3K/AKT inhibition. In addition, ARID1A and p53 interact with each other and act as codependent tumor suppressors in certain cancers, providing another example of SWI/SNF interplay with cell cycle regulation.

The increased efficacy brought about by the molecular selection of patients for treatment with epigenetic drugs suggests that educated selection of patients may be essential in the future. Indeed, one reason that the first generation of epigenetic therapies showed limited clinical effects is likely the absence of rational prescreening of patients. However, it is important to consider that while this stratification may need to be tumor-type specific, the characterization of additional biomarkers for these interdependent factors could have widespread applicability. Careful molecular selection would be especially helpful for those situations where the epigenetic targets were deemed undruggable but have demonstrated an interdependent relationship with a different factor for which drugs are already available. This also highlights the opportunity represented in characterizing new vulnerabilities in chromatin regulators that have been shown to play a role in DNA replication and repair or cell cycle progression.

New Potential Targets in Anticancer Therapy: Histones and Chaperones

As the foundation of chromatin structure, nucleosome composition and loading influences many aspects of cancer progression. A wealth of knowledge has identified histone variant-chaperone partnerships critical for DNA replication and repair, gene expression, cell proliferation, and differentiation (Fig. 2).

Histone Variants

To date, eight histone H3 variants have been identified in humans and the identification of homologous variants across species reveals a significant degree of evolutionary conservation. The most widely studied H3 variants (Fig. 2) can be classified based on two modes of incorporation into chromatin: DNA-synthesis coupled (DSC) or DNA-synthesis independent (DSI) pathways. In humans, the highest expression of the replicative variants H3.1 and H3.2 occurs during S phase of the cell cycle, facilitating deposition via the DSC pathway during DNA replication or DNA break repair involving DNA synthesis. Meanwhile, the DSI pathway involves incorporation of the "replacement" variant H3.3, expressed throughout all cell cycle phases. Importantly, the H3.3 variant also plays a significant role in transcription. Expression of CenH3 (histone H3-like centromeric protein A), called CENP-A in humans, is also independent of DNA synthesis. It is incorporated at centromeric sites during late mitosis (M)/early G1 phase while its expression peaks during the G2/M phases. CENP-A is the most divergent H3 variant and has a unique function, acting as the foundation for the constitutive-centromeric associated network (CCAN), which nucleates the kinetochore, and epigenetically determines the sites at which spindle microtubules attach during cell division. Without CENP-A this scaffold for cell division is unable to form and cells fail to segregate their chromosomes appropriately, resulting in cell death. CENP-A acts as one of many examples where specific histone variants play a significant role in the epigenetic control of cellular functions.

The H2A family also contains several different variants found in humans, including H2A.X, H2A.Z, macroH2A1, macroH2A2, H2A.F/Z, and H2A.Bbd. Briefly, H2A.Z is expressed throughout all cell cycle phases and preferentially incorporated at gene promoters and regulatory regions to promote transcription. Two non-allelic genes, *H2AFZ* and *H2AFV*, encode two distinct H2A.Z isoforms: H2A.Z.1 and H2A.Z.2, respectively. H2A.X, synthesized in both S and G1 phases, is a variant directly involved in the DNA double-strand break (DSB) repair pathway as well as several distinct functions involving sex chromosome inactivation, stem cell development, and maintenance of cellular senescence. MacroH2A1 is commonly associated with transcriptionally silent heterochromatin but at a subset of genes it is found near transcription start sites (TSSs) and CTCF-binding sites. MacroH2A1.1 and macroH2A1.2 are two alternatively spliced isoforms originating from the *H2AFY* gene. MacroH2A1.1 is expressed in differentiated cells, while macroH2A1.2 is found in embryonic stem cells and the early embryo.

Histone Chaperones

Histone chaperones escort histones throughout their cellular life. Some of them show very little selectivity and represent "casual" histone partners. In contrast, others associate with and are "dedicated" to a specific variant. They function as a network to ensure the

proper supply of histones in tune with the cellular demand. Dedicated chaperones play a crucial role for the incorporation of distinct histone variants into chromatin at particular locations and times throughout the cell cycle. The histone chaperone CAF-1, dedicated to the replicative H3 variants, deposits histones onto newly synthesized DNA through its association with PCNA, an accessory factor of DNA polymerase. The HIRA complex promotes incorporation of the H3.3 variant, independently of DNA synthesis, at gene bodies, promoters and regulatory elements. DAXX/ATRX ensures accumulation of H3.3 at peri-centromeric heterochromatin and telomeres. Finally, a dedicated chaperone HJURP ensures the timely incorporation of de novo CENP-A at centromeres in late mitosis/G1 in mammals.

The chaperone Asf1 is considered to act as a hand-over chaperone, upstream of other chaperones involved more directly in histone deposition. Asf1 deals with both parental and newly synthesized histones in the DSC and DSI pathways. NASP is another histone chaperone, with lower specificity, that acts as the emergency reservoir for soluble histones H3. Interestingly, MCM2, a DNA replication licensing factor and a component of the DNA helicase at the replication fork, also executes a histone chaperone function by favoring parental histone recycling. FACT is a histone chaperone that acts as a multifunctional heterodimer facilitating transcription, DNA replication and repair. FACT helps deposit the H2A–H2B dimer and $(H3–H4)_2$ tetramer onto DNA and also disrupts core histone–histone and histone–DNA interactions. A coordinated network of chaperones, along with other chromatin regulators, regulates chromatin dynamics during critical cellular processes such as cell differentiation and organism development and is disrupted in various diseases.

Importantly, a number of histone chaperones and variants are overexpressed or mutated in different cancer types. Among histone chaperones: CAF-1 is overexpressed in a series of aggressive cancers. *ASF1b*, one of the two human paralogs of the Asf1 chaperone, is overexpressed in a variety of tumor types. Considering histone variants: the increase in levels of CENP-A or its partner histone chaperone HJURP proves to be a key feature of tumor progression and poor prognosis. Perturbation of these factors to limit tumor growth represents opportunities that remain to be explored.

Changing Chromatin Factor Relationships—A Case of Cellular Adaptability

Experimental CENP-A overexpression is associated with altered genomic localization to the chromosome arms in addition to centromeric incorporation. Importantly, CENP-A and HJURP levels are higher in p53-deficient transformed cells compared to p53 wild-type tumors. Ectopic CENP-A deposition is carried out by DAXX instead of its cognate chaperone HJURP. Although DAXX acts as a casual chaperone for CENP-A in this scenario, it deposits it at locations normally occupied by its variant H3.3 (Fig. 2), namely sites of high histone turnover along the chromosome arms. As previously proposed, this inherent flexibility in chaperone-variant interactions could represent adaptability in the face of transformation-induced stress and cytotoxic chemo- and radiotherapies.

Context of Dosage Perturbation Matters

In p53-null mouse embryonic fibroblasts (MEFs), exogenous induction of HJURP or CENP-A alone does not act as a driver of transformation. Strikingly though, increased CENP-A and HJURP expression plays a critical role in the *maintenance* of the transformed state in p53-null cells. Experimental downregulation of HJURP in fully transformed p53-null cells results in cell death and loss of tumor viability in vivo while p53-proficient cells respond by growth arrest. This "epigenetic" addiction for HJURP in the context of p53-loss suggests that histone variants and their chaperones might act as crucial navigators of the neoplastic process instead of serving as classical drivers or simply passengers.

Targeting Histone Chaperones: The Architects of Chromatin

Given the epigenetic addiction to HJURP in p53-deficient transformed cells, interfering with de novo CENP-A deposition in cycling cells could represent an ideal pharmacological strategy. Since p53-proficient cells tolerated HJURP downregulation, it suggests that there is a therapeutic window for inhibitors of HJURP or its interaction with CENP-A in p53-null tumoral cells compared to non-tumoral quiescent cells (Fig. 4). Essentially, p53-HJURP codependence serves as an example of synthetic lethality due to epigenetic addiction. Several approaches to block HJURP can be envisaged to treat p53-deficient tumors.

These include interfering with:

(1) The N-terminal domain of HJURP that mediates protein–protein interactions with the CENP-A.
(2) Dimerization of HJURP, important for its activity, and potentially required to accommodate a CENP-A-H4 tetramer.
(3) Phosphorylation and DNA-binding of HJURP that is essential for timely CENP-A deposition.
(4) Complex formation with partners CENP-C, a key centromeric factor, or the MIS18 complex (consisting of MIS18BP1, MIS18α, and MIS18β), that are required for proper CENP-A incorporation.
(5) PLK1 kinase-dependent phosphorylation and recruitment of MIS18.

Beyond the example of HJURP, additional histone chaperones would also be worth exploring in future epigenetic therapies. For example, CAF-1, a reliable proliferative marker, acts as a molecular barrier to reprogramming of cell fate. The dual role of CAF-1 in maintaining stemness and promoting cell division may make it an attractive complementary target to existing cytotoxic therapies. The dynamics of CAF-1 posttranslational modifications could provide a means to target its proliferative role in tumors. For example,

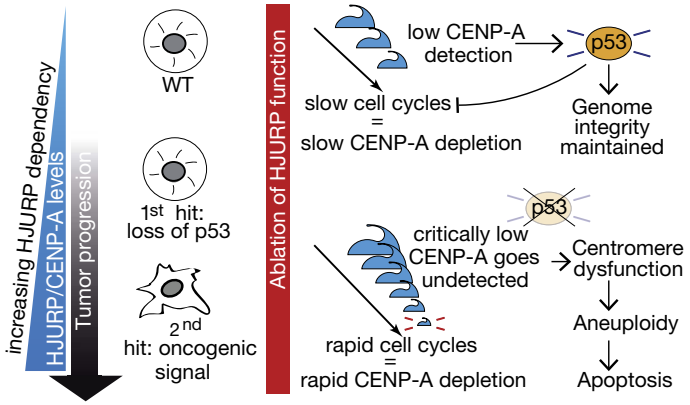

Fig. 4 The unbalance between histone chaperone and variants in p53-deficient tumors: an Achilles' heel in cancer cells. HJURP sustains cellular transformation in cells lacking p53. HJURP and CENP-A levels are increased in cells that lose p53 expression and increase even further following oncogenic transformation. When HJURP is depleted in wild-type cells with functional p53, p53 senses gradual CENP-A depletion and induces cell cycle arrest in order to maintain genome integrity. In cells lacking p53, HJURP depletion results in rapid CENP-A loss at centromeres, leading to centromere dysfunction, aneuploidy, and p53-independant apoptosis. From Filipescu, D., Naughtin, M., Podsypanina, K., Lejour, V., Wilson, L., Gurard-Levin, Z. A., Orsi, G. A., Simeonova, I., Toufektchan, E., Attardi, L. D. et al. (2017). Essential role for centromeric factors following p53 loss and oncogenic transformation. *Genes & Development* **31**, 463–480.

phosphorylation of the large subunit of CAF-1 (p150) by the replication kinase Cdc7-Dbf4 is important for stabilizing its monomeric state and promotes CAF-1 interaction with PCNA. By blocking this modification, CAF-1 function could be modulated during hyperproliferation. While hyperphosphorylation of the p60 subunit keeps CAF-1 inactive during mitosis, in interphase active CAF-1 comprises both hypophosphorylated and/or phosphorylated forms of p60. The phosphorylation of the p60 subunit is regulated by cyclin/Cdk kinase complexes and PP1 phosphatase. Importantly, the phosphorylated form of p60 is preferentially recruited to sites of UV-induced DNA damage. Therefore, therapeutic interference with the regulation of CAF-1 phosphorylation could hinder its nucleosome assembly activity during S-phase and at specific sites of DNA damage in combination with epidrug-treatments.

The proliferation-dependent isoform of the Asf1 histone chaperone, ASF1b, is prognostic of breast cancer outcomes. Thus, interfering with the histone supply function of Asf1 could also be promising. Like aforementioned chaperones, phosphorylation of free Asf1 by Tousled-like kinases (TLK) has been suggested to play a role in S phase progression by regulating Asf1-mediated histone delivery at sites of chromatin assembly. Inhibition of these kinases could potentially interrupt Asf1 activity in targeted cancer types. Furthermore, depletion of Asf1 (both a and b) activates the telomerase-independent telomere maintenance pathway ALT (alternative lengthening of telomeres) in primary and cancer cells. This suggests restoration of Asf1 function could provide a means to suppress the ALT pathway used by immortal cells to maintain their telomeres.

Loss-of-function mutations in the *DAXX* and *ATRX* genes are frequently reported in pancreatic neuroendocrine tumors (pan-NETs) and are also invariably linked to ALT. The ALT pathway is dependent on homologous recombination (HR). Thus, inhibitors against protein kinase ATR, a key regulator of HR, could prove to be effective in ALT-positive tumors carrying mutations in *ATRX* and/or *ASF1*. Considering another histone chaperone complex, FACT is overexpressed in nearly 20% of all breast cancers. FACT recognizes and binds to alternative DNA structures (ADS) leading to p53-activation. As such, curaxin, a FACT-complex anticancer drug that promotes ADS formation, could prove to be efficacious in treating p53-proficient cancers. Additional histone chaperones, such as MCM2, NASP, HIRA, etc., need to be examined for their roles in oncogenesis and tumor development and, thus, their suitability as potential epigenetic targets.

Targeting the Histone Variants: Core Components of Chromatin

Elevated CENP-A levels and recurrent H3.3/H3.1 K27, K36, and G34 mutations are examples of histone variants associated with particular types of cancer. Mutations of H3K27 residues in different histone H3 variants prevent methylation by PRC2 and have been strongly linked with pediatric brain tumors. A recent study, using model and primary patient-derived diffuse intrinsic pontine glioma (DIPG) cell lines, reported PRC2 dependency in H3K27M tumors. Notably, small-molecule EZH2 inhibitors were able to stop these tumor cells from growing. Thus, promising therapeutic opportunities can be envisaged using polycomb complex inhibitors. In addition, a 2014 screen by K. Funato and colleagues demonstrated that inhibitors against Menin, a transcriptional regulator and a member of the trithorax family histone methyltransferase complex, slowed brain tumor growth in mice. These findings highlight Menin as a potential target in patients with gliomas harboring the H3.3K27M mutation. In another study the same year by Hasizume et al. showed that GSKJ4, an inhibitor of H3K27 histone demethylation, extends survival in mice grafted with human H3K27M mutant brain tumors. Thus, pharmacological inhibition of histone K27 demethylation may hold promise in treating K27M pediatric gliomas.

Besides these histone mutants, variants of histone H2A should also be considered. For example, increased expression of H2A.Z isoforms confers poor prognosis in metastatic melanoma. Accordingly, H2A.Z.2 knockdown sensitized melanoma cells to treatment

with doxorubicin, JQ1 or PD325901 (inhibitor of MEK, a MAPK kinase). Expression of macroH2A1.1 decreases in several cancer types and the low macroH2A1.1 to macroH2A1.2 ratio negatively affects patient outcomes. For example, loss of macroH2A has been associated with progression of and osteosarcoma. This is likely due to the antiproliferative roles ascribed to macroH2A1.1 compared to the pro-proliferative role of macroH2A1.2. As well, previous studies have reported that macroH2A1.1 expression reduces PARP-1 protein levels and suppresses growth of lung and cervical cancer cells. This raises the possibility that PARP-1 inhibitors could have therapeutic value in cancers that display decreased macroH2A1.1 expression. In addition, phosphorylation of H2A.X is one of the first events after the occurrence of DSBs, which activates the DNA damage repair (DDR) response. Therefore, interfering with H2A.X could be highly complementary to existing cytotoxic therapies. Lastly, H2A.Z is overexpressed in hormone-resistant cancers. In prostate cancer cell lines, H2A.Z was shown to be downregulated upon overexpression of the histone deacetylase *SIRT1* (Sirtuin-1). Altogether, different histone variants display critical associations with clinical responses and disease progression in specific cancer types. In the future, the connection between histone variants and resistance to anticancer drugs will have to be extensively pursued.

Conclusions and Perspectives

With the advent of epigenetic targeting strategies and advanced knowledge in cancer biology, new therapeutic opportunities and challenges lie ahead. The merits of early pan-cancer interventions against HDACs and DNMTs were overshadowed by the absence of mechanism-based patient stratification criteria. In recent trials using molecularly chosen populations, second-generation drugs against carefully selected targets, EZH2 and BET family proteins, proved more effective. Therefore, a compelling argument can be made for selecting the right tumors in an effort to move away from the pan-cancer type treatments of the past. Understanding how targeting the epigenome could maximally reinforce our current therapies against the pervasive roadblocks of cancer still remains a challenge and there is a need to integrate not only the tumor but also its ecosystem in our therapeutic decisions. Treating carefully selected patient cohorts may also prove to be inadequate unless specific molecular biomarkers are used to choose these patients.

For the development of next generation epigenetic anticancer therapies, there is a pressing need not only to expand our arsenal of epigenetic targets but also to consider novel epigenetic-oncogene interactions (both synergistic and antagonistic). Histone variant-chaperone interplay represents one such druggable epigenetic relationship that needs to be considered. Indeed, histone chaperones could be promising anticancer targets for future pharmacological and clinical efforts. Not only the direct histone variant-chaperone interactions but also the protein–peptide, protein–nucleic acid, and enzymatic control that regulates these interactions in space and time could be targeted. Based on the encouraging example of HJURP-addiction in p53-null tumors, it could be of significant value to characterize additional histone chaperones for interdependencies with tumor suppressor genes and oncogenes. As appears to be the case for EZH2 where both up- and downregulation can have tumor-promoting effects, an important consideration for drug development will be the dosage of chromatin components. Moreover, histone variant choice underlies a key question: is it the means of histone incorporation by a histone chaperone (e.g., HJURP vs. DAXX in the case of CENP-A) and/or the choice of histone variant itself that matters for tumor growth and survival? A detailed examination of the fine-tuned plasticity in chaperone-variant interactions will be necessary to delineate contributions to beneficial adaptation during disease.

Prospective Vision

It has become increasingly evident that the effective treatment of cancer will require the combination of different approaches that counter the different hallmarks of cancer and overcome the roadblocks of current anticancer agents. In parallel, efforts should be made to assess large-scale, tissue-specific genomic, and epigenomic profiling to identify exceptional responders or entire subsets of patients where monotherapy against epigenetic factors will prove to be efficacious (e.g., ARID1B inhibition in ARID1A-deficient tumors). In cases of precancerous conditions, such as Age-related Clonal Hematopoiesis (ARCH), modulating the epigenome could even be considered to prevent progression to malignant neoplasia.

Conventional epigenetic drugs have aimed to block the catalytic activity of enzymatic chromatin modifiers and remodelers. However, noncatalytic domains may also play an important role. For example, compounds that compromise noncatalytic roles of PRC2 subunits, EZH2, SUZ12, and EED, may be required to obtain optimal antitumor benefits in SWI/SNF-defective cancers. Related to this, the "interfacial inhibitor concept" proposes that small-molecules can blockade interacting surfaces within large, macromolecular assemblies, as with topoisomerase enzymes, and unhinge the functional complex, effectively hindering its molecular activity. Similarly, histone chaperones are nonenzymatic factors that could be targeted by interfering with their regulation.

Epigenetic drugs could limit disease progression by crippling the oncogenic machinery of tumors and, potentially, also prevent recurrence in tumors that exploit chromatin pathways to develop resistance to existing therapies. This idea stems from the assumption that the epigenetic modifications responsible for tumor adaptation would remain reversible and sensitive to external manipulation, contrary to genetic changes. For example, loss of DNA methylation before immune checkpoint blockade can boost T cell renewal and enhances disease clearance in mouse models. This approach offers the promise of powerful treatments for chronic infections and cancer. More generally, the therapeutic promise of epigenome modulation could play a role beyond cancer with potential to treat other pathologies linked to defects in DNA metabolism or immunological disorders.

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References

Bird, A., 2007. Perceptions of epigenetics. Nature 447, 396–398.
Olins, A., Olins, D., 1974. Spheroid chromatin units (ν bodies). Science 183, 330–333.

Further Reading

Alexandrov, L.B., Jones, P.H., Wedge, D.C., Sale, J.E., Campbell, P.J., Nik-Zainal, S., Stratton, M.R., 2015. Clock-like mutational processes in human somatic cells. Nature Genetics 47, 1402–1407.
Attwood, K., Fleyshman, D., Prendergast, L., Paszkiewicz, G., Omilian, A.R., Bshara, W., Gurova, K., 2017. Prognostic value of histone chaperone FACT subunits expression in breast cancer. Breast Cancer (Dove Medical Press) 9, 301–311.
Barone, T.A., Burkhart, C.A., Safina, A., Haderski, G., Gurova, K.V., Purmal, A.A., Gudkov, A.V., Plunkett, R.J., 2017. Anticancer drug candidate CBL0137, which inhibits histone chaperone FACT, is efficacious in preclinical orthotopic models of temozolomide-responsive and -resistant glioblastoma. Neuro-Oncology 19, 186–196.
Burgess, R.J., Zhang, Z., 2013. Histone chaperones in nucleosome assembly and human disease. Nature Structural & Molecular Biology 20, 14–22.
Cheloufi, S., Elling, U., Hopfgartner, B., Jung, Y.L., Murn, J., Ninova, M., Hubmann, M., Badeaux, A.I., Euong Ang, C., Tenen, D., et al., 2015. The histone chaperone CAF-1 safeguards somatic cell identity. Nature 528, 218–224.
Filipescu, D., Naughtin, M., Podsypanina, K., Lejour, V., Wilson, L., Gurard-Levin, Z.A., Orsi, G.A., Simeonova, I., Toufektchan, E., Attardi, L.D., et al., 2017. Essential role for centromeric factors following p53 loss and oncogenic transformation. Genes & Development 31, 463–480.
Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., et al., 2010. Selective inhibition of BET bromodomains. Nature 468, 1067–1073.
Flynn, R.L., Cox, K.E., Jeitany, M., Wakimoto, H., Bryll, A.R., Ganem, N.J., Bersani, F., Pineda, J.R., Suva, M.L., Benes, C.H., et al., 2015. Alternative lengthening of telomeres renders cancer cells hypersensitive to ATR inhibitors. Science 347, 273–277.
Gentric, G., Mieulet, V., Mechta-Grigoriou, F., 2017. Heterogeneity in Cancer metabolism: New concepts in an old field. Antioxidants & Redox Signaling 26, 462–485.
Ghoneim, H.E., Fan, Y., Moustaki, A., Abdelsamed, H.A., Dash, P., Dogra, P., Carter, R., Awad, W., Neale, G., Thomas, P.G., et al., 2017. De novo epigenetic programs inhibit PD-1 blockade-mediated T cell rejuvenation. Cell 170, 142–157.e119.
Gurard-Levin, Z.A., Quivy, J.P., Almouzni, G., 2014. Histone chaperones: Assisting histone traffic and nucleosome dynamics. Annual Review of Biochemistry 83, 487–517.
Hammond, C.M., Stromme, C.B., Huang, H., Patel, D.J., Groth, A., 2017. Histone chaperone networks shaping chromatin function. Nature Reviews. Molecular Cell Biology 18, 141–158.
Ishiuchi, T., Enriquez-Gasca, R., Mizutani, E., Boskovic, A., Ziegler-Birling, C., Rodriguez-Terrones, D., Wakayama, T., Vaquerizas, J.M., Torres-Padilla, M.E., 2015. Early embryonic-like cells are induced by downregulating replication-dependent chromatin assembly. Nature Structural & Molecular Biology 22, 662–671.
Kim, K.H., Roberts, C.W., 2016. Targeting EZH2 in cancer. Nature Medicine 22, 128–134.
Kim, Y., Kim, H., Park, H., Park, D., Lee, H., Lee, Y.S., Choe, J., Kim, Y.M., Jeoung, D., 2014. miR-326-histone deacetylase-3 feedback loop regulates the invasion and tumorigenic and angiogenic response to anti-cancer drugs. The Journal of Biological Chemistry 289, 28019–28039.
Lacoste, N., Woolfe, A., Tachiwana, H., Garea, A.V., Barth, T., Cantaloube, S., Kurumizaka, H., Imhof, A., Almouzni, G., 2014. Mislocalization of the centromeric histone variant CenH3/CENP-A in human cells depends on the chaperone DAXX. Molecular Cell 53, 631–644.
Maze, I., Noh, K.M., Soshnev, A.A., Allis, C.D., 2014. Every amino acid matters: Essential contributions of histone variants to mammalian development and disease. Nature Reviews. Genetics 15, 259–271.
Mohammad, F., Weissmann, S., Leblanc, B., Pandey, D.P., Hojfeldt, J.W., Comet, I., Zheng, C., Johansen, J.V., Rapin, N., Porse, B.T., et al., 2017. EZH2 is a potential therapeutic target for H3K27M-mutant pediatric gliomas. Nature Medicine 23, 483–492.
Monteiro, F.L., Baptista, T., Amado, F., Vitorino, R., Jeronimo, C., Helguero, L.A., 2014. Expression and functionality of histone H2A variants in cancer. Oncotarget 5, 3428–3443.
Morel, D., Almouzni, G., Soria, J.C., Postel-Vinay, S., 2017. Targeting chromatin defects in selected solid tumors based on oncogene addiction, synthetic lethality and epigenetic antagonism. Annals of Oncology 28, 254–269.
Muller, S., Almouzni, G., 2017. Chromatin dynamics during the cell cycle at centromeres. Nature Reviews. Genetics 18, 192–208.
Narlikar, G.J., Sundaramoorthy, R., Owen-Hughes, T., 2013. Mechanisms and functions of ATP-dependent chromatin-remodeling enzymes. Cell 154, 490–503.
Safina, A., Cheney, P., Pal, M., Brodsky, L., Ivanov, A., Kirsanov, K., Lesovaya, E., Naberezhnov, D., Nesher, E., Koman, I., et al., 2017. FACT is a sensor of DNA torsional stress in eukaryotic cells. Nucleic Acids Research 45, 1925–1945.
Sato, T., Issa, J.J., Kropf, P., 2017. DNA Hypomethylating drugs in Cancer therapy. Cold Spring Harbor Perspectives in Medicine 7, a026948.
Siegel, R.L., Miller, K.D., Jemal, A., 2017. Cancer statistics, 2017. CA: A Cancer Journal for Clinicians 67, 7–30.
Vardabasso, C., Gaspar-Maia, A., Hasson, D., Punzeler, S., Valle-Garcia, D., Straub, T., Keilhauer, E.C., Strub, T., Dong, J., Panda, T., et al., 2015. Histone variant H2A.Z.2 mediates proliferation and drug sensitivity of malignant melanoma. Molecular Cell 59, 75–88.
Vardabasso, C., Hasson, D., Ratnakumar, K., Chung, C.Y., Duarte, L.F., Bernstein, E., 2014. Histone variants: Emerging players in cancer biology. Cellular and Molecular Life Sciences 71, 379–404.
Wang, C.Y., Filippakopoulos, P., 2015. Beating the odds: BETs in disease. Trends in Biochemical Sciences 40, 468–479.
Weinberg, D.N., Allis, C.D., Lu, C., 2017. Oncogenic mechanisms of histone H3 mutations. Cold Spring Harbor Perspectives in Medicine 7, a026443.
Wijdeven, R.H., Pang, B., van der Zanden, S.Y., Qiao, X., Blomen, V., Hoogstraat, M., Lips, E.H., Janssen, L., Wessels, L., Brummelkamp, T.R., et al., 2015. Genome-wide identification and characterization of novel factors conferring resistance to topoisomerase II poisons in Cancer. Cancer Research 75, 4176–4187.
Wilson, B.G., Wang, X., Shen, X., McKenna, E.S., Lemieux, M.E., Cho, Y.J., Koellhoffer, E.C., Pomeroy, S.L., Orkin, S.H., Roberts, C.W., 2010. Epigenetic antagonism between polycomb and SWI/SNF complexes during oncogenic transformation. Cancer Cell 18, 316–328.
Wilting, R.H., Dannenberg, J.H., 2012. Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance. Drug Resistance Updates 15, 21–38.
Wu, Q., Sharma, S., Cui, H., LeBlanc, S.E., Zhang, H., Muthuswami, R., Nickerson, J.A., Imbalzano, A.N., 2016. Targeting the chromatin remodeling enzyme BRG1 increases the efficacy of chemotherapy drugs in breast cancer cells. Oncotarget 7, 27158–27175.
Xu, W., Yang, H., Liu, Y., Yang, Y., Wang, P., Kim, S.H., Ito, S., Yang, C., Wang, P., Xiao, M.T., et al., 2011. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell 19, 17–30.

Chromosome Rearrangements and Translocations

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Glossary

Chromosome inversion A chromosome segment reversed end-to-end; developed when a single chromosome sustains at least two double strand breaks, followed by rearrangement and repair within itself. Chromosome inversions can either be paracentric (involving parts of a chromosome on one side of the centromere) or pericentric (involving parts of a chromosome either side of the centromere). Inversions can also either be unbalanced or balanced, dependent upon whether there is or is not a gain or loss of genomic content following chromosomal rearrangement.

Chromosome translocation The exchange of chromosomal material when breakage, rearrangement and repair occur between chromosomes (usually involving distinct chromosomes, but sometimes involving different loci within homologous chromosomes). Theoretically, translocations can occur between any loci on any two chromosomes, but many random exchanges produce translocations that are mitotically lethal because they are dicentric (have two centromeres) and cause chromosome breakage, or mitotically unstable because they are acentric (have zero centromeres) and are often lost. A translocation is reciprocal if the exchange of material is bidirectional and reciprocal if it is unidirectional. Translocations can also either be unbalanced or balanced, dependent upon whether there is or is not a gain or loss of genomic material following chromosomal rearrangement.

Chromothripsis The phenomenon where hundreds or thousands of clustered chromosome breaks occur simultaneously causing extremely complex structural rearrangements.

Copy-number alteration Duplications or deletions of chromosome sections resulting in the gain or loss of genetic material, respectively. Copy-number alterations can develop due to errors during both DNA replication and DNA repair, and the specific mechanisms differ dependent on the length of the duplication/deletion. Copy-number alterations can be the result of unbalanced chromosome inversions or translocations.

Cytogenetics A branch of genetics that studies the number, structure, and function of chromosomes; particularly focused on how chromosomes relate to cell behaviors during mitosis and meiosis.

Structural rearrangements A chromosomal abnormality resulting in a change to the normal chromosome structure, including translocations, inversions, duplications, deletions, and chromothriptic events.

Abbreviations

AEJ Alternative end joining
ALL Acute lymphoblastic leukemia
AML Acute myeloid leukemia
BFB Breakage fusion bridge
BIR Breakage induced replication
C-NHEJ Classical nonhomologous end joining
CGH Comparative genomic hybridization
CML Chronic myeloid leukemia
CNA Copy-number alteration
DSB Double strand break
FISH Fluorescent in situ hybridization
HR Homologous recombination
IR Ionizing radiation
MRN MRE11/Rad50/NBN
NHEJ Nonhomologous end joining
PML Promyelocytic leukemia
SCID Severe combined immunodeficiency syndrome
SNP Single nucleotide polymorphism
SSA Single strand annealing
V(D)J Variable diversity joining
WGS Whole-genome sequencing

Gene Names

ABL1 ABL Proto-oncogene 1 nonreceptor tyrosine kinase
AID Activation induced deaminase
ALK ALK receptor tyrosine kinase
BCL2 BCL2 apoptosis regulator
BCR BCR RhoGEF and GTPase activating protein
BLM Bloom syndrome RECQ like helicase
BRCA1 Breast cancer 1
CBFB Core-binding factor beta
BRCA2 Breast cancer 2
CDK4 Cyclin dependent kinase 4
DNA-PKcs DNA-dependent protein kinase catalytic subunit
EML4 Echinoderm microtubule associated protein like 4
ERCC1 ERCC excision repair 1 endonuclease noncatalytic subunit
ETO Eight twenty one protein
ETV6 ETS variant 6
FEN1 Flap structure-specific endonuclease 1
FOXO1 Forkhead box O1
FOXO3 Forkhead box O3
FOXO4 Forkhead box O4
HMGA2 High mobility group AT-hook 2
IGH Immunoglobulin H
INPP5D Inositol polyphosphate-5-phosphatase D
Ku70 70 kDa subunit of Ku Antigen
Ku80 86 kDa subunit of Ku Antigen
LigIII DNA Ligase 3
LigIV DNA Ligase 4
MDM2 MDM2 Proto-oncogene
MLL Myeloid/lymphoid mixed-lineage leukemia
MRE11 MRE11 homolog double strand break repair nuclease
MYC MYC Proto-oncogene bHLH transcription factor
NBN Nibrin (also known as NBS1)
PARP1 Poly(ADP-ribose) polymerase 1
PAX3 Paired box 3
PLZF Promyelocytic leukemia zinc finger
PML Promyelocytic leukemia
RB1 Retinoblastoma protein
RAD1 RAD1 checkpoint DNA exonuclease
RAD50 RAD50 double strand break repair protein
RAD51 RAD51 recombinase
RAD52 RAD52 homolog DNA repair protein
RAG Recombination activating gene
RARA Retinoic acid receptor alpha
RB1 RB transcriptional corepressor 1
RECQL4 RECQ-like helicase 4
RPA Replication protein A
RUNX1 Runt-related transcription factor 1 (aka AML1: Acute myeloid leukemia 1 protein)
TGFBR2 Tumor growth factor beta receptor 2
TP53 Tumor protein 53
WRN Werner syndrome RECQ like helicase
XRCC1 X-ray repair cross complementing 1
XRCC2 X-ray repair cross complementing 2
XRCC3 X-ray repair cross complementing 3
XRCC4 X-ray repair cross complementing 4

Units
bp Base pairs
kbp Kilo base pairs
Mbp Mega base pair

Introduction to Structural Rearrangements in Cancers

Cancers and Genome Instability

Cancers are complex diseases that arise as a result of genetic, epigenetic and environmental influences, or a combination thereof, which cause genetic or epigenetic aberrations in cells. When these alterations are pro-tumorigenic (i.e., they provide a survival advantage by fulfilling one of the 10 hallmarks of cancer, they are positively selected for in a manner analogous to Darwinian evolution at the cellular level. Therefore, pro-tumorigenic lesions accumulate over time driving oncogenesis and may eventually lead to cancer.

It is possible for such genetic and epigenetic changes to be acquired in cells because there is a very low level of genome instability in all cells. This instability exists because there is a significant energetic cost to maintaining a stable genome, alongside the fact that it is optimal to have high genome stability for proper functioning and faithful reproduction, but to also have slight genome instability to allow evolutionary changes to occur. Furthermore, genome instability can be increased by the acquisition of mutations followed by selection and clonal expansion of the fittest cells, and in this way a pro-tumorigenic cycle is established.

Structural Rearrangements

Many different DNA alterations can contribute to tumorigenesis, where one class is termed "structural rearrangements." These are genomic abnormalities where the structure of a chromosome is altered and they include, translocations (rearrangements of chromosome segments usually between nonhomologous chromosomes), inversions (reversal of the direction of a chromosome segment within a single chromosome) and copy-number alterations (CNAs) (deletions or duplications of chromosome segments). In addition, although extremely rare there are also complex structural rearrangements that involve more than two DNA breakpoints and the exchange of chromosomal DNA between two or more chromosomes. The most severe complex structural rearrangement is chromothripsis, where hundreds or thousands of chromosomal rearrangements develop in a localized genomic region during a single event.

Structural rearrangements can be respectively described as either unbalanced or balanced, dependent on whether the rearrangement results, or does not result, in the gain or loss of genomic content. For translocations, the descriptors nonreciprocal and reciprocal can be used, respectively referring to whether the translocation of chromosomal material is one-way or bi-directional between two chromosomes. Balanced rearrangements are more common than unbalanced and reciprocal rearrangements are more common than nonreciprocal. Furthermore, it is more common for structural rearrangements to develop within a single chromosome than between chromosomes and between loci with closer spatial proximity in the interphase nucleus.

Structural Rearrangements in Cancer Research and Clinics

Structural rearrangements are mainly identified by cytogenetics, which is concerned with the number, structure, and function of chromosomes as well as how chromosomal variations may affect cell function in health and disease. Studying structural rearrangements has been highly important in cancer research, and in fact, it was the discovery of recurring translocations in chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) patients that was the first evidence to conclusively demonstrate cancers as genetic disorders. Furthermore, the discovery that many nonrandom chromosomal aberrations are frequently associated with driving specific cancers, has shown the importance of structural rearrangements in tumorigenesis, as well as elucidating mechanisms through which they may be generated (i.e., errors during DNA repair or replication) or through which they may function (i.e., fusion proteins and deregulation of gene expression). Moreover, it has enabled structural rearrangements to be important diagnostic, prognostic and disease management indicators, as well as identifying novel targets for cancer therapies. This is particularly true for blood cancers, lymphomas, leukemias and myelomas, where the distinction between critical and irrelevant structural rearrangements is more clear, and consequently it has become routine in clinical practice to perform chromosomal analysis for blood cancer patients. In contrast, it has only become common practice to assess structural rearrangements in very few solid tumors, namely some sarcomas, prostate cancers and lung cancers where often only certain rearrangements are searched for. Hence, the field of cytogenetics and structural rearrangements is much more advanced for hematological malignancies than solid tumors, and thus this article will largely use structural rearrangements in blood cancers as examples, in particular frequently recurring balanced chromosome translocations which are most well researched.

This article will focus on the origin of structural rearrangements, their functional consequences toward tumorigenesis, the methods that enable their detection, and the research and medical advancements that come from studying them.

The Origin of Structural Rearrangements in Cancer

Like other forms of genome aberrations, structural rearrangements develop due to errors during DNA replication and DNA repair. There is a multitude of different mechanisms that can result in structural rearrangements, however the most important mechanisms are those that involve the acquisition of DNA double strand breaks (DSBs). This is because faulty DSB repair is a common cause of all types of structural rearrangements, and in fact, the incorrect repair of two DSBs which happen at close spatial and temporal proximity in the interphase nucleus is the only means through which chromosome inversions and translocations can develop, as well as being the major cause of cancer associated CNAs.

Sources of DSBs

For DNA DSBs to develop, the phosphate backbones of two complementary DNA strands need to break simultaneously. DSBs can originate from both endogenous and exogenous causes.

Firstly, many DSBs occur spontaneously due to a variety of endogenous factors: for example, natural metabolite by-products in cells (e.g., reactive oxygen species), chromosome nondisjunction during cell division, excision of transposable elements, physical or mechanical stress on DNA (e.g., mitotic spindle stress on dicentric chromosomes), and inadvertent action of nuclear enzymes of lymphoid cells (e.g., RAG complex and AID). In addition, intentional cellular processes are an endogenous source of site-specific DSBs, where examples of such processes include homologous recombination to produce gametes, V(D)J recombination in lymphocytes to assemble immunoglobulin antigen receptor genes and T cell receptor genes, and chromatin condensation and expansion via topoisomerases and helicases. Moreover, DSBs can also result from natural DNA impediments that cause DNA replication to pause or stop (e.g., unusual DNA and chromatin structures, transcription machinery, or collisions with DNA binding proteins.

In contrast, major exogenous sources of DSBs include ionizing radiation (IR), cigarette smoke, and anticancer drugs (e.g., cross-linking agents like Cisplatin and Mitomycin C, alkylating agents like Methyl methanosulfonate and Temozolomide), topoisomerase inhibitors like Etoposide, replication inhibitors like Aphidicolin and hydroxyurea, and radiomimetic compounds like bleomycin and phleomycin).

In addition to all of the above, which are direct sources of DNA strand breaks, it is important to note that DSBs can also result when other forms of DNA damage are not repaired properly, such as base mismatches, uracil bases, abasic sites, pyrimidine dimers, and chemically modified bases (e.g., alkyl groups and O-6-methylguanine) or when some DNA lesions are encountered during DNA replication, for example strand cross-links and damaged nucleotides.

Detection and Response to DSBs

Once a DSB is generated, the lesion has to be detected rapidly before it is passed on to daughter cells, because otherwise it could have serious consequences including blood disorders and a range of cancers. Following detection, the cell needs to decide whether the damage can be repaired or whether it is too extensive, because this determines whether the appropriate response would be DNA damage repair, senescence (prevention of further cell cycling in affected cells) or apoptosis (induce programmed cell death in affected cells). It is not yet clear how this decision is made by cells, but it is known that TP53 (Tumor protein p53), RB (Retinoblastoma protein), and other tumor suppressor proteins play an integral role in this process. Furthermore, it is understood that this decision is made very rapidly, typically within a few minutes of DSB generation.

DSB recognition and the subsequent cell signaling are initiated by the two broken ends of a double stranded DNA molecule, because unlike telomeric ends of DNA molecules, broken ends are not hidden by telomeric extensions and telomere associated proteins. As a result, broken ends at DSBs are recognized and attract several proteins that orchestrate the appropriate response, by recruiting additional proteins involved in repair, cell senescence or apoptosis (**Fig. 1**).

Altogether, the signaling cascades from DSB recognition through to completion of the DSB response are very complex. This is because: DSB recognition pathways differ dependent on cell type and DSB origin, there is a great number of proteins that can be involved in the recognition, repair, senescence and apoptosis processes, and there are many different repair mechanisms that can resolve DSBs, where the particular proteins that respond to the broken ends determines which repair pathway will take place.

Some commonalities amongst all repair mechanisms are that significant modifications occur to chromatin, histones and other proteins both at the site of the DSB as well as extending outwards (e.g., poly(ADP-ribosylation), ubiquitination, sumoylation, acetylation, and phosphorylation), and that the gap between the two broken ends is bridged. In some circumstances the DSB may be repaired faithfully without any errors, but in other cases the repair can result in aberrations from the original DNA molecule, with some of the most severe consequences being structural rearrangements.

DSB Repair Mechanisms and Generation of Structural Rearrangements

The mechanisms that produce structural rearrangements from DSBs can be divided into two broad classes, homology-based and nonhomology-based, which respectively do and do not require extensive homology to enable DSB repair. For instance, homology-based mechanisms require homologous template sequences approximately 97% similar to one another, whereas nonhomology-based mechanisms might only require a few to zero base pairs of homology. Consequently, homology-based

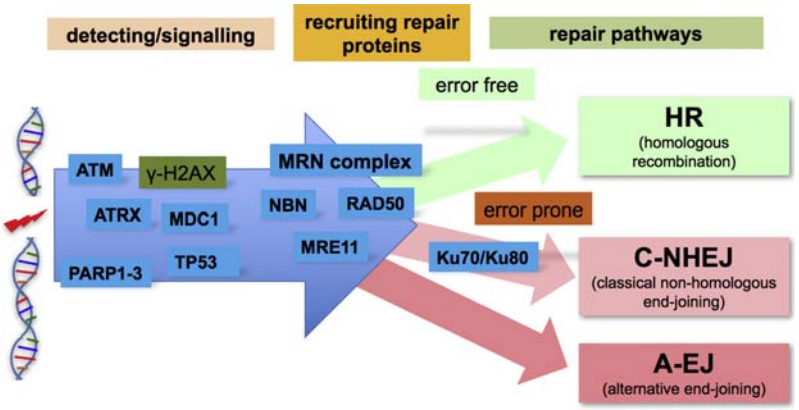

Fig. 1 Detection and resolution of DSBs through different repair pathways. The resolution of DSBs involves the detection of the DSB, protein signaling pathways, recruitment of proteins involved in DSB repair and filling in the gap between the two break points. This example shows these steps and depicts both error-prone (*red*) and relatively error-free (*green*) pathways. The MRN complex is composed of MRE11, Rad50, and NBN.

mechanisms are typically error-free whereas nonhomology-based mechanisms are more error-prone. Respectively, this means that the DSB repair usually produces a DNA sequence that is either identical or different to the original DNA sequence before the DSB occurred. Therefore, it would be more common for nonhomology-based mechanisms to join DSB ends incorrectly and generate structural rearrangements than it would for canonical homology-based mechanisms. Interestingly, in cancers proteins involved in DNA repair mechanisms are frequently mutated and become faulty. Subsequently increasing genomic instability and the acquisition of structural rearrangements. In fact, the mutation of DNA repair pathways is so essential that a two-step model has been proposed for the predisposition of structural rearrangements to tumorigenesis, whereby there must both be an error in DSB repair mechanisms and a failure to initiate cell cycle arrest in order for cancers to develop.

Homology-based mechanisms

Homologous recombination (HR)

HR is a DSB repair mechanism that operates during the S and G2 phases of the mitotic cell cycle in response to DNA damage. This is because HR requires a homologous sister chromatid (second intact copy of the affected chromosome) to use as a DNA repair template, and in mitosis these are more accessible during and shortly after nuclear DNA replication. HR also takes place during the meiotic cell cycle where it occurs in prophase 1 until metaphase 1 to generate crossing overs. Because HR is very cell cycle specific, the cyclin-dependent Kinases that mediate cell cycle progression are important regulators of HR.

HR begins with the MRE11/RAD50/NBN (MRN) complex binding to the broken ends of the DSB. Here MRE11 excises nucleotides from the 5′ ends leaving 3′ single strand DNA overhangs at the DSB break points. Next, the 3′ overhangs are coated with RPA, and then RPA is replaced by RAD51 in a reaction promoted by mediator proteins, such as XRCC2, XRCC3, RAD52, and BRCA2, amongst others. This produces the Rad51-single stranded DNA-nucleoprotein filament which then invades at the homologous site on the double stranded homologous sister chromatid. At this site a Holliday junction develops and the homologous sister chromatid is used as a template to perform DNA repair. Once the Holliday junction is resolved the DNA repair is complete.

Although canonical HR is very efficient, defective HR is known to play a role in the generation of structural rearrangements in disease, such as Downs syndrome (trisomy 21), hereditary breast and ovarian cancers (*BRCA1* and *BRCA2* mutations), Bloom's syndrome (*BLM* mutations), Werner's syndrome (*WRN* mutations), and Rothmund–Thompson syndrome (*RECQL4* mutations).

Mutations in HR genes can contribute to cancers by causing homologous chromatids to misalign during DSB repair, such that the DSB and subsequent Holliday junctions are also misaligned. The subsequent cross over would therefore be unequal as it would occur at nonallelic regions on the homologous chromosomes. This can enable the transfer of genetic material between homologous chromosomes, potentially causing deletion, duplication or inversion of the chromosomal region which may favor cancer development. Secondly, mutations in HR genes can cause nondisjunction to occur where homologous chromatids do not segregate properly during anaphase, resulting in chromosome breakage or aneuploidy (abnormal number of chromosomes in a cell), both of which can contribute to cancer development. For example, mitotic nondisjunction is known to play a role in retinoblastoma when chromosome 13 with wildtype *RB1* is lost through nondisjunction and the remaining chromosome 13 has a mutant *RB1*. Lastly, HR gene mutations can cause HR to be downregulated, consequently increasing the frequency of more error prone DSB repair pathways, which ultimately increases the acquisition of tumorigenic structural rearrangements and genomic instability. Interestingly, the genomic instability caused by mutations in HR genes actually makes cancer cells more susceptible to chemotherapeutic drugs, because these cells have an impaired ability to tolerate the induction of many DSBs at once.

Single strand annealing (SSA)

SSA is an alternate HR pathway which repairs DSBs between two DNA repeats that are less than 25 kbp (kilo base pairs) apart and oriented in the same direction. Unlike the classical HR pathway described above, SSA is unique in that it does not require a separate

homologous chromosome or DNA sequence for the DSB repair. Instead, because SSA only occurs in repeat regions, it merely uses one of the unbroken repeats as a homologous template to resolve the DSB. Because the required template for SSA is always present, SSA does not preferentially occur in any particular part of the cell cycle.

The first step in SSA is to perform single stranded DNA resection at the site of the DSB, generating complementary 3′ overhanging free DNA ends either side of the breakpoint. The overhanging strands are coated by RPA to prevent them adhering to themselves, and then Rad52 assists the alignment of the 3′ overhangs so that the complementary repeat sequences can anneal and form a single repeat. After annealing is complete, any remaining single stranded nonhomologous flaps (remnants from the 3′ overhangs) are reselected by Rad1 and ERCC1. Lastly, DNA synthesis fills in any gaps and ligation produces a continuous double stranded DNA molecule and DSB repair is complete.

Altogether, SSA typically deletes one repeat alongside any sequences between the two repeats, and such deletions cause structural rearrangements and can be tumorigenic. SSA always results in a CNA, and translocations or inversions develop when SSA occurs between two unlinked repeats, but this is highly uncommon due to the requirement for two DSBs to occur at the same time.

Breakage induced replication (BIR) template switching

BIR template switching occurs when the DNA repair machinery from another DSB repair mechanism unexpectedly switches to its homologous chromosome as a template to complete the DSB repair. Therefore, the repair proteins involved depend on the original DSB repair mechanism prior to template switching. BIR template switching is most common when errors occur during the repair of a single DSB, and BIR has not been shown to preferentially occur during any particular phase of the cell cycle.

As in nonallelic HR, because the broken end of the DSB invades a nonallelic region of the homologous chromosome during BIR template switching, it subsequently results in the transfer of genetic material between the respective chromosomes to result in chromosome inversions, translocations and CNAs which can potentially be tumorigenic. Furthermore, in cancers BIR template switching and the subsequent structural rearrangements often increase in frequency due to defective or downregulated cohesins (i.e., because cohesins function to reduce interchromosomal strand invasion).

Nonhomologous-based mechanisms

Nonhomologous end joining (NHEJ)

Classical and alternate NHEJ pathways are the major repair pathways for DSBs in cells, and they are respectively referred to as Classical-NHEJ (C-NHEJ) and alternative end joining (AEJ). C-NHEJ is more efficient than AEJ and C-NHEJ utilizes all of the classical NHEJ factors whereas AEJ lacks one or more NHEJ components as well as utilizing nonclassical factors. These mechanisms also differ in when they predominantly occur during the cell cycle, although it is interesting that NHEJ mechanisms function at least at low levels throughout all cell cycle stages. C-NHEJ is most prevalent in the G1 phase when cells are growing but not yet ready to replicate their nuclear DNA because it does not require an attack template from a homologous sister chromatid. In contrast, AEJ pathways are backup survival mechanisms that occur when C-NHEJ is defective or less active, and AEJ is most frequent during S phase of the cell cycle.

The first step in C-NHEJ is DSB recognition and signaling by the MRN complex, followed by binding of Ku70 and Ku80 to the broken ends to prevent the broken ends drifting apart. Next, the coiled coil domains of Rad50 (component of the MRN complex) extend outwards to connect the broken ends to one another, the MRN complex performs some DNA resection at the broken ends and finally the broken ends are joined together by Lig IV and XRCC4. Other important proteins in C-NHEJ include, DNA-PKcs (functions as a molecular sensor for DNA damage and cooperates with Ku70/Ku80), Cernunnos (may serve as a bridge between XRCC4 and other factors), Artemis nuclease (crucial for processing hairpin structures that form during V(D)J recombination), Metnase (methylates histones to open chromatin to process DNA ends), and BRCA1 (interacts with many DNA repair proteins and is known to be important for certain types of NHEJ, but the specific role of BRCA1 is currently unclear).

In contrast to C-NHEJ, AEJ has a requirement for small regions of microhomology between 5 and 25 bp (Base Pairs), it is more heavily dependent on the MRN complex, it lacks several C-NHEJ factors (e.g., Ku70, Ku80, XRCC4 or LigIV) and it involves several other factors (e.g., PARP1, LigIII, FEN1, and XRCC1, amongst others). The first step of AEJ is the recognition and signaling of a DSB by the MRN complex, followed by the identification of microhomologous sequences upstream or downstream of the DSB. The microhomologous regions are then used as the bases for which to align the strands with mismatched ends, and following ligation of the broken DNA strands hanging ends result. Next, any overhanging nucleotides are resected and finally any missing base pairs are filled in (both by the MRN complex). Because AEJ ligates the DNA strands without checking for homology it causes deletions flanking the original DSB, as it removes any bases required to align the two broken DNA strands.

Because C-NHEJ and AEJ are both error prone they commonly result in structural rearrangements. CNAs almost always result from these repair mechanisms because small insertions and deletions frequently occur during C-NHEJ and always occur during AEJ. Furthermore, these mechanisms can incorrectly repair DSBs to cause chromosome inversions and translocations, where some models suggest that AEJ does this at a higher frequency, but other systems support that C-NHEJ plays a greater role. Furthermore, if any of the NHEJ factors are mutated the propensity of structural rearrangements increases, and consequently mutations in NHEJ are known to cause several conditions, such as hereditary breast and ovarian cancer syndrome (*BRCA1* mutations), LigIV syndrome (*LIGIV* mutations), Cernunnos-Severe Combined Immunodeficiency Syndrome (SCID) (*Cernunnos* mutations), Artemis-SCID (*Artemis* mutations), Ataxia Telangiectasia (*ATM* mutations), and Fanconi anemia (mutations in multiple genes). Additionally, significant upregulation of NHEJ factors is observed in several cancers and thus thought to play a role in tumorigenesis, for example breast cancers (*FEN1 LIGIII* and *MRE11* genes), prostate cancers (*FEN1* and *NBS1* genes), lung cancers (*FEN1* and

XRCC1 genes), stomach cancers (*FEN1* gene), pancreatic cancers (*FEN1* gene) head and neck cancers (*NBS1* gene), squamous cell carcinomas of the oral cavity (*NBS1* gene), Ewing Sarcoma (*PARP1* gene), germ cell tumors (*PARP1* gene), tyrosine kinase-activated leukemias (*PARP1* gene), CML (*LIGIII* gene), multiple myeloma (*LIGIII* gene) and neuroblastomas (*FEN1* and *PARP1* genes). Lastly, it is also known that because C-NHEJ is crucial for V(D)J recombination, somatic recombination and class switch recombination in lymphocytes, dysfunctional C-NHEJ is a major source of structural rearrangements in lymphoid cancers and other disorders (e.g., Nijmegen breakage Syndrome, Fanconi anemia, and Bloom's syndrome).

Breakage fusion bridge (BFB) cycle

The BFB cycle is a mechanism of genomic instability that occurs during anaphase as a result of telomere loss, dicentric chromosome formation, and mechanical and physical mitotic spindle stress.

For instance, if a single chromatid lost its telomeric repeats at one end and the DSB was not repaired prior to DNA replication, then the original chromatid plus its duplicate would both lack telomeric repeats. Without telomeres to prevent chromosome fusion, the two sister chromatids would join to form a single dicentric chromosome. During anaphase of the cell cycle, the two centromeres of the dicentric chromosome would be pulled in opposite polar directions to form a bridge shaped structure until the centromeres were pulled so far away from one another that a DSB resulted. If this DSB does not occur at the exact location where the two chromosomes originally fused, then structural rearrangements would result (e.g., a deletion from one chromosome will result in an inverted duplication on the other). Because the BFB cycle produces two chromosomes lacking telomeres at their broken ends the cycle could continue to produce further duplication, deletion, inversion, and translocation of chromosomal material.

The BFB cycle has been observed to play a role in the production of tumorigenic structural rearrangements in some cancers, where it produces giant abnormal chromosomes (cancer associated neochromosomes) in which oncogenes are highly amplified (e.g., *CDK4*, *HMGA2*, and *MDM2*). Furthermore, sometimes these neochromosomes can have fragments from many different chromosomes stitched together, due to the BFB cycle occurring following chromothripsis events (thousands of simultaneous DSBs). Although BFB-mediated neochromosomes only occur in approximately 3% of cancers, they are very common in certain rare cancers (e.g., in 90% of parosteal osteosarcomas).

Polymerase slippage

Unlike the above nonhomologous-based mechanisms, polymerase slippage is a mechanism of replication errors rather than of DSB repair. Polymerase slippage is most predominant when DNA/RNA synthesis is beginning or when it is stalled and resumed (e.g., following the collapse of a replication fork).

Polymerase slippage occurs when small scale tandem repeats in the genome disrupt DNA/RNA polymerase unclamping and reclamping during DNA/RNA synthesis. Specifically, such repeats may cause the DNA/RNA polymerase complex to reclamp at the wrong position (i.e., shift a few bases backwards or forwards). Respectively, this can cause duplication or deletion of bases in repeat regions and therefore result in small CNAs (where duplications are more common than deletions).

Polymerase slippage can take place in both coding and noncoding regions, and in coding regions it can result in the generation of abnormal proteins. If polymerase slippage occurs in critical conserved regions of tumor suppressor genes or oncogenes, it can play a role in tumorigenesis. For example, it has been shown that RNA polymerase slippage is responsible for the conversion of TGFBR2 between its tumor suppressive form and oncogenic role during colorectal cancer development, by introducing a small frameshift mutation in a microsatellite region that deletes its transmembrane and intracellular kinase domains.

Determining how a structural rearrangement developed

It is possible to determine which DNA repair mechanism was likely responsible for the generation of a structural rearrangement in cancer. Firstly, key DNA repair genes could be sequenced to observe if there are any mutations, and if so the mutated repair gene would implicate a specific repair pathway(s) (e.g., *LIGIV*, Cernunnos, and Artemis mutations correspond to faulty NHEJ). However, if a gene is common to many different repair pathways then its mutation would not be sufficient to implicate a specific pathway (e.g., mutations in the components of the MRN complex).

Another approach is to analyze the DNA sequence at the site of a structural rearrangement and obtain clues from any abnormalities at rearrangement boundaries or from the type of rearrangement present. For example, small insertions or deletions at the boundaries of the rearrangement would suggest that NHEJ had taken place, and in lymphocytes if there are heptamer or nonamer consensus sequences close to the breakpoints it would suggests that NHEJ had occurred as part of somatic recombination. Alternatively, multiple rearrangements or aneuploidies would suggest that multiple rounds of the BFB cycle had happened, whereas evidence of chromothripsis would suggests that replication fork stalling followed by BIR Template Switching may have ensued.

Consequences of Structural Rearrangements

Possible Outcomes of Incorrect Repair of DSBs

Multiple simultaneous DSBs generate free double stranded DNA ends that, regardless of the particular DSB repair mechanism, can either be repaired correctly by rejoining fragments in their original order, or misrepaired by joining fragments together in the incorrect order to produce structural rearrangements. The more DSBs exist locally at any given time, the more DNA free ends will be present (e.g., two DSBs = four free ends, three DSBs = six free ends etc.), and the more complex the potential misrepair outcomes

can be. This is because with an increasing number of DNA free ends, the possible combinations for how DNA fragments can be incorrectly joined together increases in a nonlinear fashion, and with more than two simultaneous DSBs complex structural rearrangements can result. Interestingly, with multiple DSBs, it is much more common for structural rearrangements to be intrachromosomal rather than interchromosomal due to a range of steric constraints restricting the movement of chromosome ends to a radius of approximately 1 μm (e.g., DNA packaging, chromatin packaging, and nuclear chromosome arrangement).

Because it is more likely for two DSBs to occur at the same time in close proximity in the interphase nucleus than three or more DSBs, this article will focus on the outcomes from the misrepair of two concurrent DSBs.

Two DSBs on the same chromosome arm

The misrepair of two simultaneous DSBs on the same chromosome arm can produce either a deletion or an inversion (Fig. 2A). A deletion results when the chromosomal fragment flanked by two DSBs joins end-to-end with the fragment itself producing a circular acentric chromosome, and the two remaining chromosomal fragments fuse to produce a chromosome with an interstitial deletion between the location of the DSBs. Because the acentric chromosome lacks a centromere it will be lost during anaphase when chromosomes are pulled to opposite spindle poles. Alternatively, a paracentric inversion results when the chromosomal fragment between the two breakpoints is inverted and rejoined in the opposite direction to its original position.

Two DSBs on different arms of a homologous chromosome

The misrepair of two simultaneous DSBs on different arms of a homologous chromosome can also produce either a deletion or an inversion (Fig. 2B). A deletion results when the ends of the centromere-containing fragment fuse to one another to produce a ring chromosome, and the two telomere-containing fragments fuse to one another to produce an acentric chromosome. The acentric chromosome will be lost during subsequent cell divisions causing deletions of the two distal fragments. In contrast, a pericentric inversion results when the two distal fragments fuse to the opposite chromosome positions from their original orientation.

Two DSBs on the same arm of two homologous chromosomes

The misrepair of two simultaneous DSBs on the same arm of two different homologous chromosomes will produce translocations that can have either of two outcomes. The first outcome is that a dicentric and acentric chromosome are respectively produced by the two centromere-containing fragments fusing and the two remaining fragments fusing (Fig. 2C). The acentric chromosome will be lost during subsequent cell divisions and result in distal deletions of chromosomal material, and the dicentric chromosome will break during anaphase causing duplications/deletions of chromosomal content if the breakpoint is not exactly where the two chromosome fragments originally fused. The breakage of the dicentric chromosome may also be lethal. Alternatively, the two centromere-containing fragments can fuse to the two distal acentric fragments, but in the incorrect homologous chromosome, producing interstitial deletions or duplications (Fig. 2C).

Two DSBs on two nonhomologous chromosomes

The misrepair of two simultaneous DSBs on two nonhomologous chromosomes can produce translocations with two different outcomes. Again, an acentric and dicentric chromosome may each be produced by fusing of the two centromere containing fragments and acentric fragments resulting in a distal deletion and possibly cell death during cell division. Alternatively, a balanced translocation can result when the two acentric fragments fuse to the centromere containing fragments of the wrong nonhomologous chromosome.

Driver and Passenger Structural Rearrangements

It is now well understood that chromosome inversions, CNAs and especially translocations can all have functional effects toward tumorigenesis. Such structural rearrangements may either be "driver" or "passenger" in nature; respectively, causing or contributing to cancer development, or being produced as a by-product of cancer-related genome instability. However, it is very difficult to determine the driver or passenger status of a rearrangement.

The two main types of driver rearrangements are those that produce a fusion gene and those that lead to the transcriptional deregulation of breakpoint adjacent genes. Passenger rearrangements do not possess tumorigenic functional consequences, and likely candidates are rearrangements that are not recurring, do not produce obvious fusion products and are not in the vicinity of tumor suppressor genes and proto-oncogenes.

Types of tumorigenic functional consequences from structural rearrangements

There are many different types of functional consequences that can result from structural rearrangements in cancers, from gene duplications or gene deletions of tumor suppressor genes or oncogenes, through to complex functional consequences where multiple genes can be impacted at once and novel oncoproteins can be generated. This section will focus on the more complicated functional consequences of structural rearrangements with chromosome translocations as examples, however the same principles can apply for inversions, deletions and complicated rearrangements.

One complex consequence of structural rearrangements is the production of fusion genes that produce tumor promoting fusion proteins. Fusion genes are generated when the translocation breakpoints occur in two gene loci, typically in introns, that result in a translocation of chromosome material between the two loci. Consequently, fusion genes produce a fusion transcript that contains

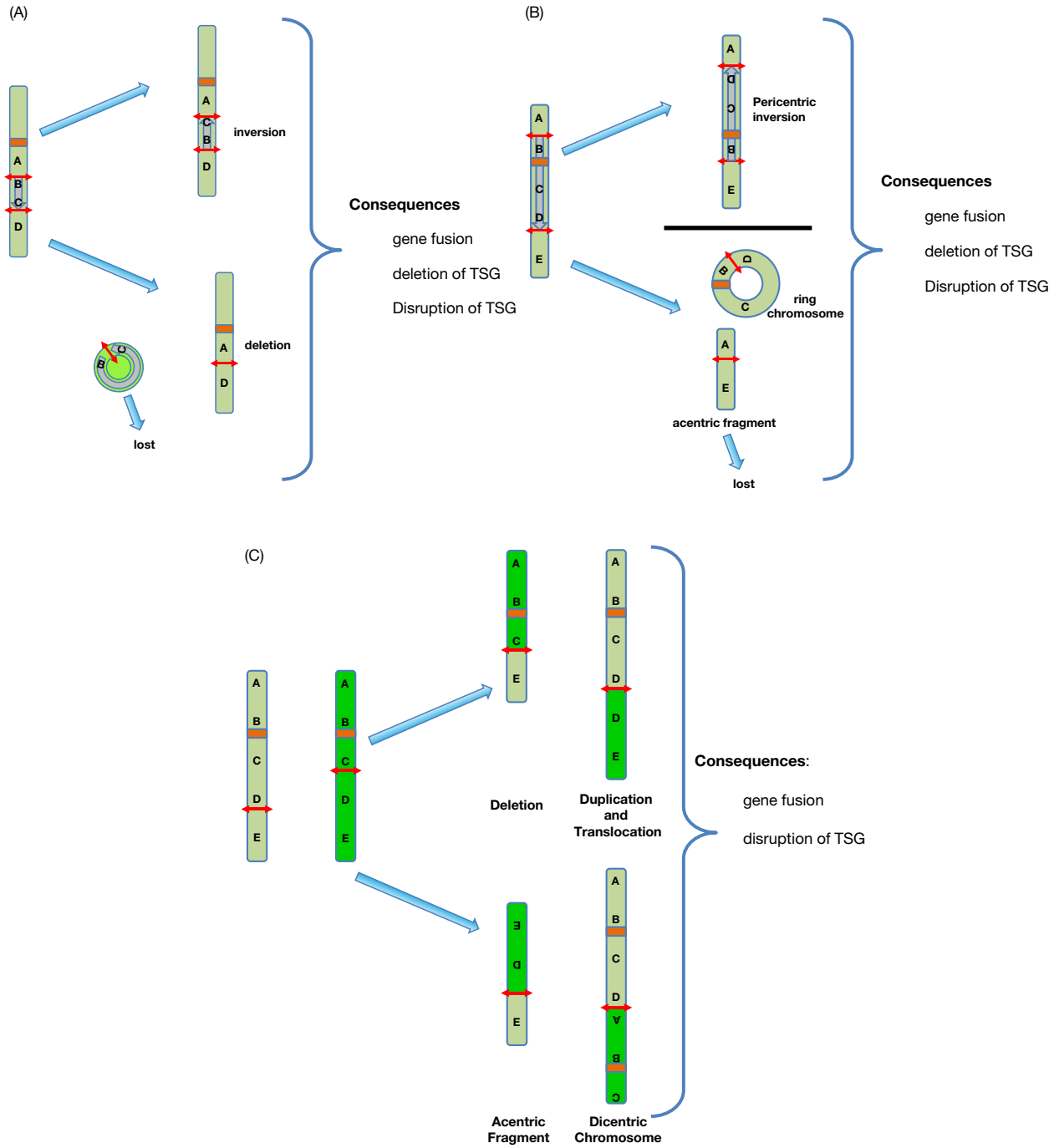

Fig. 2 Possible types of structural rearrangements that can result from the misrepair of two simultaneous DSBs depending on the location of the DSBs. (A) exhibits the possible outcomes (paracentric inversion or deletion and an acentric fragment) resulting from the misrepair of two simultaneous DSBs on the same arm of a homologous chromosome. (B) exhibits the possible outcomes (pericentric inversion or ring chromosome and an acentric fragment) from the misrepair of two simultaneous DSBs on different arms of homologous chromosomes. (C) exhibits the possible outcomes (deletion alongside duplication or a dicentric chromosome and an acentric fragment) from the misrepair of two simultaneous DSBs on the same arm of nonhomologous chromosomes (and these possible outcomes are the same as those from the misrepair of two simultaneous DSBs on different arms of nonhomologous chromosomes—shown in **Fig. 3**). It should be noted that ring chromosomes, dicentric chromosomes and acentric fragments are all frequently mitotically unstable and result in anueploidies.

coding exons from the first gene at the 5′ end and coding exons from the second gene at the 3′ end. Only in-frame fusion transcripts that do not disturb the reading frames for either gene will result in the translation of chimeric fusion proteins, consisting of an N-terminal protein region from the first gene and a C-terminal protein region from the second. For instance, the t(9;22)(q34;q11) translocation (in this case, the derivative chromosome 22 is also known as the Philadelphia chromosome) result

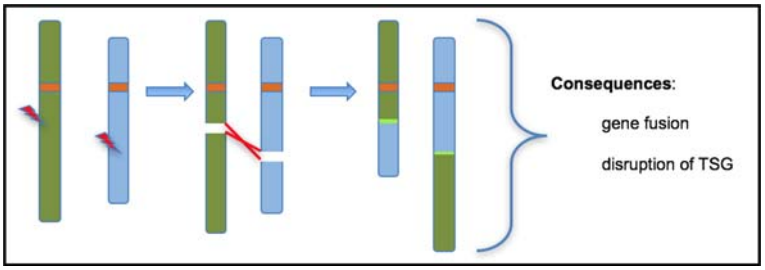

Fig. 3 Possible functional consequences that can result from structural rearrangements. Misrepair of two or more simultaneous DSBs results in the production of structural rearrangements which can have functional consequences. This example shows how two simultaneous DSBs on different arms of nonhomologous chromosomes can produce balanced translocations with either a fusion gene and/or deregulated gene expression as functional consequences.

in a fusion protein of N-terminal BCR and the C-terminal ABL1 (BCR/ABL1) in 90% of CML patients as well as in many acute lymphoblastic leukemia (ALL) patients).

In addition to a single chimeric fusion protein being produced, two reciprocal fusion proteins can be produced from reciprocal translocations. In this case, one of the fusion proteins may be dominant in driving tumorigenesis and the other reciprocal fusion protein is irrelevant, but in other cases both fusion proteins play a role (e.g., PLZF/RARA and RARA/PLZF frequently found in acute promyelocytic leukemiaPML).

Another consequence of a translocation can be the transcriptional deregulation of a gene close to the breakpoint by the juxtaposition of a strong enhancer. Examples of this are the t(8;14)(q24;q32) translocation associated with Burkitt's lymphomas and the t(14;18)(q32;q21) translocation commonly observed in follicular lymphomas. These translocations both involve the IGH intron enhancer and/or locus control regions. Respectively, these translocations result in the overexpression of *MYC* and *BCL2*, causing uncontrolled cell division, increased cell survival, and malignant transformation.

It should be noted that in cases where a balanced translocation leads to the formation of a fusion gene there will always be the disruption of one normal allele of the genes involved in the fusion. Thus, if one or both of the genes have a normal function that suppresses growth it can be speculated that in addition to the formation of two reciprocal fusion genes there will be haploinsufficiency of a tumor suppressor gene. This is, for example, the case in the t(12;21)(p13;q22) translocations in ALL that affects *ETV6* and *RUNX1*. RUNX1 and ETV6 are transcription factors that suppress cell division and encourage cell differentiation in hematopoiesis. Their loss-of-function can be tumorigenic in blood cells.

In addition, it is suggested that tumor suppressor gene functions may be disrupted by fusion proteins which interfere with protein–protein interactions (e.g., fusions between INPP5d and ABL1 caused by t(2;9)(q37;q34) translocations in ALL by altering the protein–protein interactions of the tumor suppressive INPP5D).

Lastly, it should be noted that many of the complex functional consequences of structural rearrangements outlined above may also lead to increased genomic instability (e.g., through upregulation of cell division, production of DSB causing agents like reactive oxygen species, or through knocking out the functions of essential tumor suppressor genes etc.).

Altogether, chromosomal translocation and other rearrangements can either produce fusion proteins, result in deregulation of gene expression or both. Interestingly, it is much more common for structural rearrangements resulting in chimeric fusion proteins to occur in myeloid malignancies and some solid tumors, whereas rearrangements resulting in deregulated gene expression are more typical of lymphoid tumors. Furthermore, for lymphoid cancers the rearrangements generally involve one of the immunoglobulin loci or the T cell receptor loci, as a consequence of faulty somatic recombination (**Table 1**).

Methods for the Detection of Structural Rearrangements in Cancer

Structural rearrangements have been detected and studied for a very long time, since the early 1900s, by observing changes to chromosome structure under light microscopes. However, structural rearrangements in human cancers were not detected until the 1960s and 1970s when a recurrent small chromosome was identified in the leukemic cells from CML patients (named the Philadelphia chromosome after the city where it was discovered). Later with the advent of chromosomal banding techniques the Philadelphia chromosome was determined to be the result of a translocation, the t(9;22)(q34;q11) translocation. Since, the methods for detecting structural rearrangements in cancers have been greatly improved and there are many genetic and cytogenetic techniques that detect different types and sizes of structural rearrangements.

Cytogenetic Methods

Cytogenetics is a branch of genetics that studies the number, structure and function of chromosomes. Cytogenetics is particularly focused on how chromosomes relate to cell behaviors, especially during mitosis and meiosis.

Table 1 Examples of commonly recurring structural rearrangements in different cancer types

Breakpoints	*Functional consequences*	*Frequently associated cancer(s)*
Inv(2)(p21;p23)	Produces an in-frame fusion protein EML4/ALK, constitutive tyrosine kinase signaling and uncontrolled cell proliferation	Nonsmall cell lung cancers
T(2;9)(q37;q34)	Produces an in-frame fusion protein (INPP5d/ABL1), deregulated gene expression through disruption of protein–protein interactions of INPP5D, and cancer transformation	ALL
T(2;13)(q36;q14)	Produces an in-frame fusion protein PAX3/FOXO1, deregulated gene expression through transcriptional activation, increased cell growth, increased cell survival, increased cell motility, and decreased cell differentiation	Alveolar rhabdomyosarcomas
T(6;11)(q21;q23) and T(X;11)(q13;q23)	Produce in-frame fusion proteins (FOXO3/MLL and FOXO4/MLL, respectively), deregulated gene expression through gene transactivation, increased cell stemness, and cancer transformation	Alveolar rhabdomyosarcomas and leukemias
T(8;14)(q24;q32)	Causes juxtaposition of breakpoint adjacent genes (MYC and IGH), overexpression of MYC, uncontrolled cell proliferation, and increased cell survival	Burkitt's lymphomas
T(8;21)(q22;q22)	Produces an in-frame fusion protein AML1/ETO, aberrant recruitment of epigenetic modifiers that regulate myelomonocytic development. Decreased differentiation and increased uncontrolled cell proliferation	AML
t(9;22)(q34;q11)	Produces an in-frame fusion protein BCR/ABL1, constitutive tyrosine kinase signaling, and uncontrolled cell proliferation	ALL, AML, and CML
T(12;21)(p13;q22)	Produces an in-frame fusion protein RUNX1/ETV6, haploinsufficiency of both genes, dysregulation of hematopoiesis, increased cell proliferation, and less influence toward cell differentiation	Leukemias and lymphomas
T(14;18)(q32;q21)	Causes juxtaposition of breakpoint adjacent genes (IGH and BCL2), overexpression of BCL2, uncontrolled cell proliferation, and increased cell survival	Follicular lymphomas
t(15;17)(q22;q21)	Produces two functional in-frame fusion proteins (PLZF/RARA and RARA/PLZF), deregulated gene expression, decreased granulocyte differentiation and uncontrolled cell proliferation	AML and PML

Eleven examples of structural rearrangements found frequently in cancers, with information on their breakpoints, associated genes, functional consequences (fusion genes and/or deregulated gene expression) and associated cancers (mostly hematological malignancies).

Standard cytogenetic methods

Standard cytogenetics uses a variety of chromosomal preparation techniques that determine the karyotype of cells under a light microscope, specifically attention is paid to chromosome number, chromosome length, position of the centromeres and the chromosome banding patterns, as well as any other distinctive characteristics. Karyotyping is achieved through chromosome banding techniques, which use dyes that produce horizontal bands across chromosomes when observed under a light microscope (**Fig. 4**). The banding patterns for a given chromosome pair is unique, enabling chromosomes of otherwise similar size to be distinguished from one another (**Fig. 4**). Alterations to the typical banding pattern of a given chromosome can identify chromosome breakpoints or large translocations, inversions and CNAs. Generally, chromosome banding techniques stain metaphase chromosomes, and typically around 20 cells are analyzed per sample.

Quinacrine banding was the first chromosome banding technique but it is now not as widely used as Giemsa banding. Reverse banding produces the opposite black and white banding pattern from what is seen using quinacrine and Giemsa banding and it is a useful technique for staining the distal ends of chromosomes. High resolution banding is a method that stains chromosomes at prophase or early metaphase when they are not completely condensed, because this produces many more chromosome bands and therefore allows more detailed karyotyping and the detection of less obvious aberrations. However, high resolution banding cannot be achieved in most tumor samples. There are also banding methods that only stain certain chromosomal regions, for instance, C-banding stains constitutive heterochromatin which is typically localized around the centromere, and nucleolar organizing regions banding stains satellites and stalks of acrocentric chromosomes which have centromeres very near one end of the chromosome. In general standard banding techniques have a resolution of only about 5–10 Mbp (mega base pairs). This means that any structural rearrangements smaller than the resolution limit cannot be detected confidently.

Molecular cytogenetic methods

Molecular cytogenetics is a field that combines molecular biology and cytogenetics, by utilizing a series of probe hybridization techniques to visualize one or more specific genomic regions (e.g., fluorescent in situ hybridization (FISH), array comparative genomic hybridization (array-CGH), and single nucleotide polymorphism (SNP) arrays).

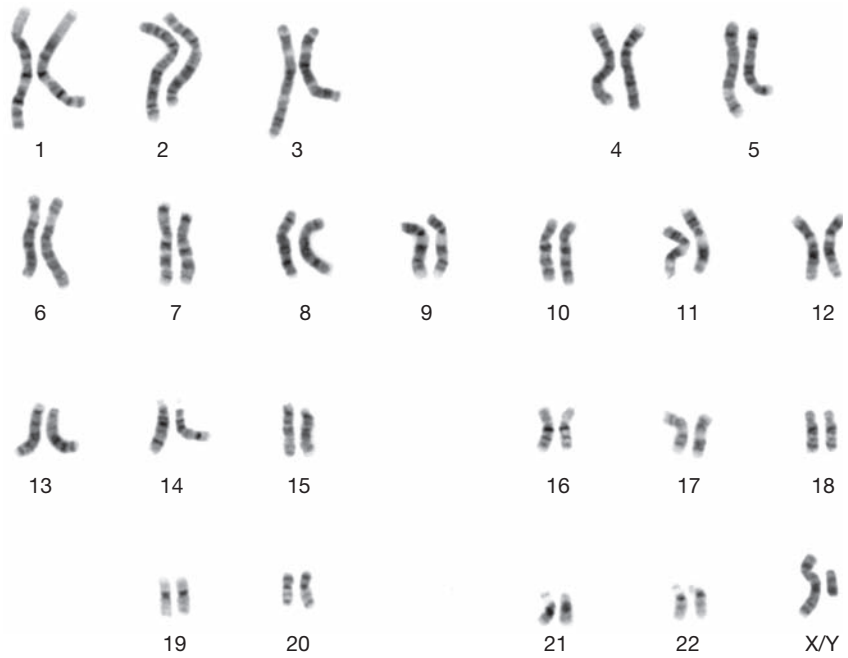

Fig. 4 Karyotype of a healthy human male using Giemsa banding. Twenty-three pairs of Giemsa banded chromosomes from an XY healthy human male, showing the unique black and white banding patterns for each chromosome pair that enables specific chromosomes and chromosome regions to be identified.

FISH uses fluorescently-labeled single stranded nucleotide probes to detect the presence or absence of specific DNA sequences on chromosomes (Fig. 5) or the presence or absence of specific RNA targets in cells. Fluorescence microscopy and computational analysis are used to identify whether any probes hybridized to the sample and if so where they hybridized in the genome or in the cell. Hence, FISH can be used to detect chromosome translocations, chromosome inversions and large CNAs. In oncology, FISH is typically carried out on approximately 100–200 cells to identify tumor cells even in residual disease and to observe the heterogeneity seen in tumors. FISH can be performed with different types of probes, for instance, probes binding the length of a chromosome can detect the gain or loss of whole chromosomes or large chromosomal regions, shorter probes can be used to hybridize more specifically to detect smaller CNAs, inversions or translocations. The extent to which probes overlap can be controlled to alter the resolution at which features can be detected, and different combinations of fluorescent probe colors can produce specific secondary colors. While the resolution of FISH can be as high as a few kbps, but typically is a few 100 kbp, it will only yield information regarding the region that is covered by the probe. Thus a typical FISH experiment will only assay a tiny fraction (less than 0.01%) of the genome and alterations that are outside this region will be invisible to the assay.

Like FISH, array-CGH also uses fluorescently-labeled single stranded nucleotides probes and fluorescence microscopy to detect chromosomal changes. However, in this method the tumor DNA itself is fluorescently labeled in one color and, together with a fluorescently labeled reference DNA, which has a different color, hybridized to an array of probes (e.g., bacterial artificial chromosome clones) that represents the whole genome (Fig. 6). Array CGH is only able to detect CNAs and is blind to balanced rearrangements. Array-CGH can detect alterations across an entire genome (with good coverage) and at a much greater resolution than chromosome banding and FISH, as low as 2 kbp. The resolution is dependent on the size and number of the cloned DNA fragments of the array used. Thus, alterations can be detected in greater detail and the aberrations can be mapped directly onto the genomic DNA sequence.

SNP (single nucleotide polymorphism) arrays are another type of DNA microarray which were originally designed to detect SNPs in the genome only, but can now be used to detect large chromosome abnormalities through identification of CNAs across genomes. SNP arrays involve the fragmentation and fluorescent labeling of sample DNA, followed by application to an SNP array chip. The SNP array chip has approximately a million different probes attached to it, which are roughly 25 nucleotides in length and are designed to bind to a portion of DNA sequence harboring the polymorphic SNP site. Fluorescence microscopy alongside computational analysis systems then detects, reports and interprets the hybridization signals. In this way, the SNPs present in a sample and the subsequent genotype can be determined, and if the intensity of the fluorescent signal for each of these SNPs is compared between multiple samples then CNAs can be inferred. In oncology, SNP arrays are used to detect thousands of CNAs across the genome, and comparisons between tumor and nontumor samples from the same patient can give an indication as to whether a particular CNA is germline-inherited or somatic in origin, as well as detecting loss of heterozygosity and regions of chromosomes that have been duplicated or deleted. Amazingly, SNP arrays have been reported to be over 99.5% accurate in SNP genotyping and CNA detection, with differences in probe and chip design influencing the accuracy and potential of a given SNP array experiment.

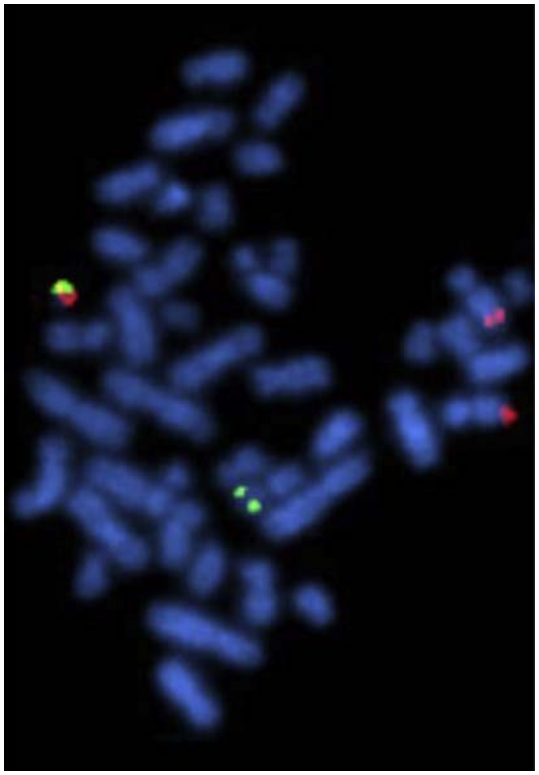

Fig. 5 FISH used to detect the t(9;22) translocation in AML. A metaphase cell positive for the t(9;22)(q34;q11) translocation in the leukemic cells from an AML patient. The chromosomes can be seen in *blue*, and the *green* and *red* spots on chromosome 22 (upper left) indicate a positive result for the BCR/ABL gene fusion.

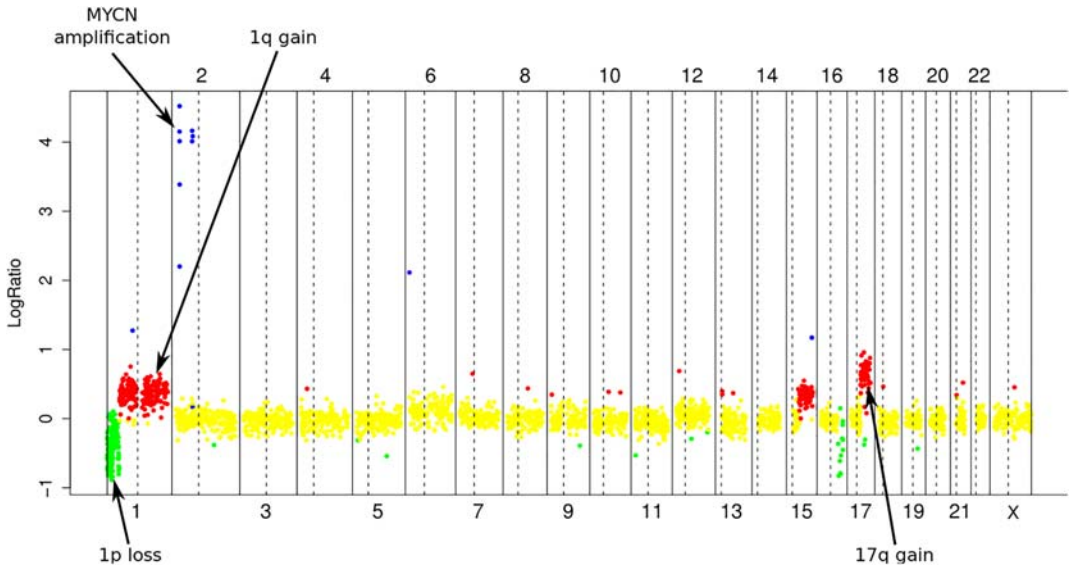

Fig. 6 Array-CGH copy number profile of a IMR32 Neuroblastoma cell line. Differences between the reference and test samples indicate copy number alterations in the IMR32 Neuroblastoma cell line, where regions of green indicate deletions, regions of *blue* indicate duplications and regions of *red* indicate gains and regions of *yellow* indicate copy number neutrality. The top left label shows an amplification of the *MYCN* proto-oncogene, the top right label and the bottom left labels respectively show the gain of chromosome regions 1q and 17q, and the bottom left label indicates a loss of chromosome region 1p.

Advances in the field of cytogenetics are focused on molecular cytogenetics techniques in particular, including automated analysis systems for calculating the results from FISH and other array methods, and improving the resolution of such methods to detect less conspicuous abnormalities.

Genetic Methods

DNA sequencing methods

DNA sequencing techniques determine the precise order of nucleotides in a DNA molecule. Whole-genome sequencing (WGS) uses a number of next-generation sequencing (NGS) methods that sequence entire genomes and they may thus be used to identify structural rearrangements across a genome. For the detection of structural rearrangements, the most efficacious WGS NGS approach is to combine mate pair sequencing and short-insert paired-end sequencing.

In mate pair sequencing, DNA is sheared into fragments of a desired length, often between 2 and 5 kbp. However this poses an issue because it is not feasible to sequence long fragments over 1 kbp using NGS. To combat this, the long fragments are biotinylated at their ends and circularized, and then the DNA rings are fragmented into approximately 400–600 bp. Next the biotin-containing fragments are extracted and the result is fragments short enough to be sequenced which still contain the two ends of the initial long fragment. Because the distance between these two ends was between 2 and 5 kbp depending on the original fragmentation parameters, if they are sequenced and mapped to a reference genome and found to be much closer together or further apart than they should be or even map to different chromosomes, then this indicates that a structural rearrangement has taken place.

In short-insert paired-end sequencing, DNA is fragmented into much shorter insert sizes between 200 and 800 bp, and adapter molecules containing primers are attached to the ends of the fragmented molecules. Because the primers in the adapters are different at each end, this allows bi-directional sequencing of the DNA fragments, which provides greater sequencing accuracy than sequencing from a single end because it generates greater coverage when aligned to a reference genome.

Both of these methodologies complement one another in the detection and evaluation of structural rearrangements. This is because mate pair Sequencing enables more comprehensive detection of large structural rearrangements, and paired-end sequencing provides more detailed sequencing data filling in any gaps that may be present in the mate pair sequencing genome coverage, as well as being particularly useful in highly repetitive regions. When the data from each of these DNA sequencing methods is analyzed together it facilitates determination of: the genomic loci of structural rearrangements, the size of the rearrangements, the altered DNA sequence following each rearrangement, the genes involved in each rearrangement, and an estimation of the impact of each rearrangement. Together, this gives WGS methods an advantage of having unprecedented sensitivity and resolution of structural and mutational changes. However, it should be noted that mate-pair sequencing is technically challenging and that WGS for the detection of structural rearrangements in tumor samples is a costly and highly experimental approach at present and far from being used routinely.

When Specific Methods are Utilized

The methods used to identify structural rearrangements differ dependent on the size and type of rearrangements to be detected. For instance, standard cytogenetics and FISH are typically used for studying balanced structural rearrangements; whereas molecular cytogenetics, particularly Array-CGH and SNP arrays, are generally used for studying smaller unbalanced structural rearrangements. DNA sequencing methods are not commonly used to investigate structural rearrangements because they are too expensive and difficult to use routinely for this purpose.

Overall there is a need to develop better methods for detecting structural rearrangements in cancers, particularly for DNA sequencing approaches, because it is common for fewer methods to be utilized to detect structural rearrangements in cancer research and routine diagnostics, thus sacrificing important detail in exchange for cheaper, quicker and simpler analysis. Further, currently structural rearrangements are mainly detected using standard cytogenetics. This is mostly done in liquid tumors (i.e., fCML, AML, and ALL, not so much for chronic lymphocytic leukemia, multiple myeloma, nonhodgkin lymphoma, and hodgkin lymphoma). In most solid tumors structural rearrangements are not routinely characterized with the exception of sarcomas and some prostate and lung cancers. Lastly, it should also be acknowledged that there is only a limited resolution achievable by standard and molecular cytogenetic techniques. So even in those cancers where structural rearrangements are often investigated, there are probably many more small-scale structural rearrangements, especially small inversions or CNAs, that are not identified by these methodologies and are thus not routinely diagnosed.

Medical Implications From Studying Structural Rearrangements in Cancer

The primary objective of detecting and evaluating structural rearrangements in cancers is to use the results for clinical management, with the most important aim being the improvement of patient survival. Notably, there are many structural rearrangements that have produced beneficial medical implications, with the t(9;22) translocation and the resulting BCR/ABL fusion protein being a prime example.

Firstly, structural rearrangements can function as disease biomarkers in cancer diagnosis, where the presence of BCR/ABL fusion alongside histopathological features can be used to diagnose CML. In addition, structural rearrangements can be used as biomarkers

for other applications; for instance, the level of the BCR/ABL fusion transcript is used as a measurement to guide prognosis and treatment success, as well as influencing clinical decisions regarding disease management strategies.

Furthermore, the BCR/ABL fusion protein resulting from Philadelphia chromosomes exemplifies how researching structural rearrangements can improve anticancer therapies by enabling personalized treatment approaches. This is because BCR/ABL results in constitutive tyrosine kinase signaling, and small molecule inhibitors of tyrosine kinase activity of BCR/ABL fusion protein (e.g., Imatinib, Dasatinib, and Nilotinib) have been developed. In fact, these drugs have been so efficacious that they have reduced the number of CML cells until the point at which a patient is declared to be in molecular remission with no detectable BCR/ABL fusion transcript, and they have greatly increased the survival time of many CML patients. Presently, studies are underway to investigate whether tyrosine kinase inhibitor treatment in CML patients can be stopped when certain criteria are met. First results indicate that about 50% of the CML patients can be considered cured and remain in permanent remission even after tyrosine kinase inhibitor treatment has ceased. Thus the study of chromosomal structural rearrangements which started in the early 1970s with Janet Rowley's pioneering cytogenetic work and her discoveries of the t(9;22) and t(8;21) translocations have now resulted in the first permanent cures of a blood cancer without chemotherapy just using targeted therapies.

Conclusion and Prospective Vision

Future research will aim to resolve many of the unanswered questions surrounding structural rearrangements in cancer; such as, which regions of the genome are most susceptible to structural rearrangements, what the exact mechanisms are behind common structural rearrangements, why certain rearrangements are seen in some cancers but not others, and how often the presence of structural rearrangements results in cancer development… amongst many more. Further, future research will aim to increase the capacity of genetic and cytogenetic methods to detect structural rearrangements, and it is anticipated that such technological advancements especially cheaper and more accurate NGS techniques will enable the identification of many new structural rearrangements in cancers and further increase the understanding on the genetics and biology surrounding structural rearrangements. Additionally, methodological improvements alongside increased routine assessment of structural rearrangements, will also likely further enhance the medical implications of structural rearrangements in oncology clinics, perhaps in helping with faster and more efficient screening of such rearrangements in patients and facilitating the improvement of anticancer therapies through individualized medicine.

See also: Chronic Myelogenous Leukemia: Pathology, Genetics, Diagnosis, and Treatment. Genetic Instability.

Further Reading

Aparicio, T., Baer, R., Gautier, J., 2014. DNA double-strand break repair pathway choice and cancer. DNA Repair 19, 169–175.
Beroukhim, R., Mermel, C.H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Mc Henry, K.T., 2010. The landscape of somatic copy-number alteration across human cancers. Nature 463 (7283), 899.
Das, K., Tan, P., 2013. Molecular cytogenetics: Recent developments and applications in cancer. Clinical Genetics 84 (4), 315–325.
Forment, J.V., Kaidi, A., Jackson, S.P., 2012. Chromothripsis and cancer: Causes and consequences of chromosome shattering. Nature Reviews. Cancer 12 (10), 663.
Glover, T.W., Wilson, T.E., Arlt, M.F., 2017. Fragile sites in cancer: More than meets the eye. Nature Reviews. Cancer 17 (8), 489.
Grade, M., Difilippantonio, M.J., Camps, J., 2015. Patterns of chromosomal aberrations in solid tumors. In: Chromosomal instability in cancer cells. Springer International Publishing, Cham, pp. 115–142.
Hasty, P., Montagna, C., 2014. Chromosomal rearrangements in cancer: Detection and potential causal mechanisms. Molecular & Cellular Oncology 1 (1), e29904.
Mitelman, F., 2000. Recurrent chromosome aberrations in cancer. Mutation Research 462 (2), 247–253.
Nambiar, M., Kari, V., Raghavan, S.C., 2008. Chromosomal translocations in cancer. Biochimica et Biophysica Acta 1786 (2), 139–152.
Nambiar, M., Raghavan, S.C., 2011. How does DNA break during chromosomal translocations? Nucleic Acids Research 39 (14), 5813–5825.
Nickoloff, J.A., De Haro, L.P., Wray, J., Hromas, R., 2008. Mechanisms of leukemia translocations. Current Opinion in Hematology 15 (4), 338.
Rowley, J.D., 1973. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature 243 (5405), 290–293.
Wan, T.S., 2014. Cancer cytogenetics: Methodology revisited. Annals of Laboratory Medicine 34 (6), 413–425.

Relevant Websites

Atlas Genetics Oncology. (2017). http://atlasgeneticsoncology.org—Atlas Genetics Oncology.
OMIC Tools. (2017). https://omictools.com/mitelman-database-of-chromosome-aberrations-and-gene-fusions-in-cancer-tool—Mitelman Database of Chromosomal Aberrations and Gene Fusions in Cancer.
Wikipedia. (2017). https://en.wikipedia.org/wiki/Chromosomal_inversion—Chromosomal inversion.
Wikipedia. (2017a). https://en.wikipedia.org/wiki/Chromosomal_rearrangement—Chromosomal rearrangement.
Wikipedia. (2017b). https://en.wikipedia.org/wiki/Chromosomal_translocation—Chromosomal translocation.
Wikipedia. (2017c). https://en.wikipedia.org/wiki/Copy-number_variation#Types_and_chromosomal_rearrangements—Copy-number variation.
Wikipedia. (2017d).https://en.wikipedia.org/wiki/Cytogenetics#Human_abnormalities_and_medical_applications— Cytogenetics.
Wikipedia. (2017e). https://en.wikipedia.org/wiki/DNA_repair—DNA repair.

Wikipedia. (2017f). https://en.wikipedia.org/wiki/Homologous_recombination—Homologous recombination.
Wikipedia. (2017g). https://en.wikipedia.org/wiki/Microhomology-mediated_end_joining—Microhomology mediated end joining.
Wikipedia. (2017h). https://en.wikipedia.org/wiki/Non-homologous end_joining—Nonhomologous end joining.
Wikipedia. (2017i). https://en.wikipedia.org/wiki/Replication_slippage—Replication slippage.
Wikipedia. (2017j). https://en.wikipedia.org/wiki/Structural_variation—Structural Variation.

Chronic Lymphocytic Leukemia: Pathology and Genetics

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Nomenclature

CBA Chromosome banding analysis
CNA Copy number alterations
CLL Chronic lymphocytic leukemia
CLPD Chronic lymphoproliferative disorders
FISH Fluorescent in-situ hybridization
GEP Gene-expression profiling
IGHV Immunoglobulin heavy chain variable region
MBL Monoclonal B-cell lymphocytosis
mCLL CLL with mutated IGHV genes
RT Richter's transformation
SLL Small lymphocytic lymphoma
SNP Single nucleotide polymorphism
uCLL CLL with unmutated IGHV genes
WES whole exome sequencing
WGS Whole genome sequencing
WHO World Health Organization

Introduction

Chronic lymphocytic leukemia (CLL) is a malignancy of $CD5^+$ B-cells characterized by the accumulation of small, mature-appearing neoplastic lymphocytes in the blood, bone marrow and lymphoid tissues, thereby resulting in lymphocytosis, marrow infiltration, lymphadenopathy and splenomegaly. The vast majority of CLL cases are thought to originate from monoclonal B-cell lymphocytosis (MBL), a precursor state that differs from CLL essentially by the concentration of circulating tumor cells. CLL is biologically and clinically very heterogeneous, with some patients never requiring any therapy and others displaying an aggressive course with limited response to therapy.

Definition

The World Health Organization (WHO) requires the presence of a CLL-type monoclonal B-cell population of $\geq 5 \times 10^9$/L in the peripheral blood for the diagnosis of CLL. Small lymphocytic lymphoma (SLL) is the same disease but the term SLL refers to cases with blood involvement of $< 5 \times 10^9$/L with documented nodal, splenic, or other extramedullary involvement. The WHO also defines monoclonal B-cell lymphocytosis (MBL) as the presence of the same type of B-cell population of $< 5 \times 10^9$/L in the absence of lymphadenopathy, organomegaly, or other extramedullary disease.

Epidemiology

CLL is the most prevalent form of leukemia. In the USA it is estimated that approximately 20,000 new cases will be diagnosed during 2017, representing the 1.2% of all new cancer cases in that country. The age-adjusted incidence rate is 4.7 cases per 100,000 men and women per year, with a clear difference between men (6.4 cases/year) and women (3.3 cases/year). In Europe, the incidence rate is similar (4.9 cases/year), with a similar sex distribution (5.9 for males and 4.0 for females). The median age at diagnosis is 70 years, while the median age at death is 80 years.

One of the most notorious epidemiological characteristic of CLL is its racial diversity. Within the USA, the incidence rate is highest in Caucasian males (6.8 cases/year) and females (3.6 cases/year) compared with African Americans (5.1 and 2.5 cases/year for males and females, respectively), while Asian Americans and Pacific Islanders have the lowest incidence (1.6 and 0.7 cases/year for males and females, respectively). For unknown reasons, the incidence of CLL is very low in the Far East, including China and Japan,

where it is estimated to occur at a frequency that is approximately 10% of that seen in Western countries. Genetic rather than environmental factors have been invoked for these differences, since Japanese who settled in Hawaii do not have a higher incidence than those living in Japan. The incidence of CLL is higher than expected among relatives of patients with the disease. Recent genome-wide association studies have identified common risk single nucleotide polymorphisms associated with sporadic CLL. The risk of CLL associated with each of these variants is however modest at best. Although families with CLL provide evidence for genetic susceptibility, no rare alleles of large effect have thus far been discovered.

Clinical Presentation

Patients with CLL usually are asymptomatic at the time of diagnosis. A small proportion of patients consult a physician because of painless swelling of lymph nodes, which may wax and wane, but does not disappear. $<5\%$ of patients present with the general symptoms including one or more of the following: (i) unintentional weight loss $\geq 10\%$of body weight within the previous 6 months; (ii) fevers of $>38\,^{\circ}C$ for ≥ 2 weeks without evidence of infection; (iii) drenching night sweats without evidence of infection; and (iv) extreme fatigue. In some patients, the disease presents with features of acquired immunodeficiency (e.g. infections), autoimmune phenomena such as hemolytic anemia, thrombocytopenia or pure red cell aplasia, or exaggerated reactions to arthropod stings or bites.

The most common finding on physical examination is lymphadenopathy, usually located in cervical, supraclavicular and axillary sites. The spleen may also be enlarged and, as is the case with enlarged lymph nodes, the splenomegaly is usually painless. Enlargement of the liver may also be rarely noted at diagnosis. In those cases, the liver is usually nontender and firm. Infiltration of the skin (leukemia cutis) is seen in $<5\%$ of cases. In contrast to other lymphoid malignancies, gastrointestinal and meningeal involvement are rarely seen in CLL.

Laboratory Abnormalities

The hallmark of the disease is an increased white blood cell count with a high percentage of small, mature-looking lymphocytes (i.e. lymphocytosis). The lymphocyte count is extremely variable, ranging from 10 to 200–300 $\times 10^9$/L. In patients diagnosed with CLL on routine analysis, anemia is found in $<10\%$ of the patients. Importantly, anemia is not always due to the infiltration of the bone marrow by the disease; other causes such as autoimmunity, iron, folic acid or vitamin B_{12} deficiency may account for it and should be investigated in all patients with anemia. Likewise, a marked thrombocytopenia (i.e. $<20 \times 10^9$/L) should raise the possibility of an immune origin, particularly in the absence of anemia. Hypogammaglobulinemia is frequent (30% of patients) and tends to worsen over the course of the disease. Serum immunofixation can demonstrate a monoclonal band, usually of the immunoglobulin M type, in around 10–15% of patients. A positive direct antiglobulin test is observed in around 5% of the patients at the time of diagnosis, with clinically apparent autoimmune hemolytic anemia being less frequent.

Morphology

Peripheral Blood and Bone Marrow

Before the advent of immunophenotyping, most chronic lymphoproliferative disorders (CLPD) with leukemic expression were diagnosed as CLL, but advances in the morphological evaluation of peripheral blood and lymphoid tissues, immunohistochemistry, flow cytometry, cytogenetics and molecular genetics have facilitated the differential diagnosis. Morphologically, leukemic CLL cells are characteristically small, mature-looking lymphocytes with scanty cytoplasm and a dense nucleus with clumped chromatin (Fig. 1). CLL cells are extremely fragile and can become partially broken during the preparation of blood smears; these cells are known as 'basket', 'smudge' of 'Gumprecht cells' which, although not specific, are typical of CLL (Fig. 1). Depending on the morphologic features of the tumor cells, two variants are recognized: typical and atypical CLL. In typical CLL $>90\%$ of leukemic lymphocytes have the previously mentioned morphology. In atypical CLL, an increased number of atypical cells (prolymphocytes, centrocytes, centroblasts, large stimutated cells, etc.) is observed in the lymphocyte differential count. Atypical CLL can be further subdivided in CLL with increased prolymphocytes (CLL/PL), when the percentage of prolymphocytes ranges from 11% to 55%, and CLL mixed cellularity, when the atypical lymphocytes represent $>15\%$ of all lymphocytes. CLL with atypical morphology is associated with trisomy 12 and it is often necessary to request additional tests to rule out other lymphoproliferative disorders.

Lymph Nodes and Bone Marrow

Neither bone marrow or lymph node histological examination is required for CLL diagnosis, but it may be useful in difficult or atypical cases. Involved lymph nodes show a characteristic morphological picture with a diffuse effacement of the architecture by a population of small lymphocytes with scant cytoplasm, round nuclei, clumped chromatin and absent nucleoli. In some cases, lymph nodes may be only partially involved by the disease, with tumor cells infiltrating the areas between reactive follicles or surrounding these follicles with a perifollicular pattern. Most lymph nodes have a variable number of pale areas called proliferation centers (formerly named pseudofollicles) (Fig. 2). These areas have a continuum of small to larger cells with broader cytoplasm and

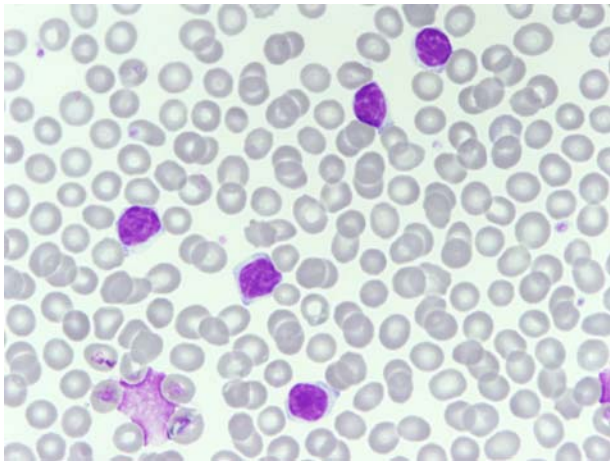

Fig. 1 CLL in peripheral blood. Peripheral blood smear showing increased number of small-sized lymphocytes with partially aggregated chromatin. Notice also one 'smudge' or 'Gumprecht' cell (May-Grünwald-Giemsa, $\times 1000$).

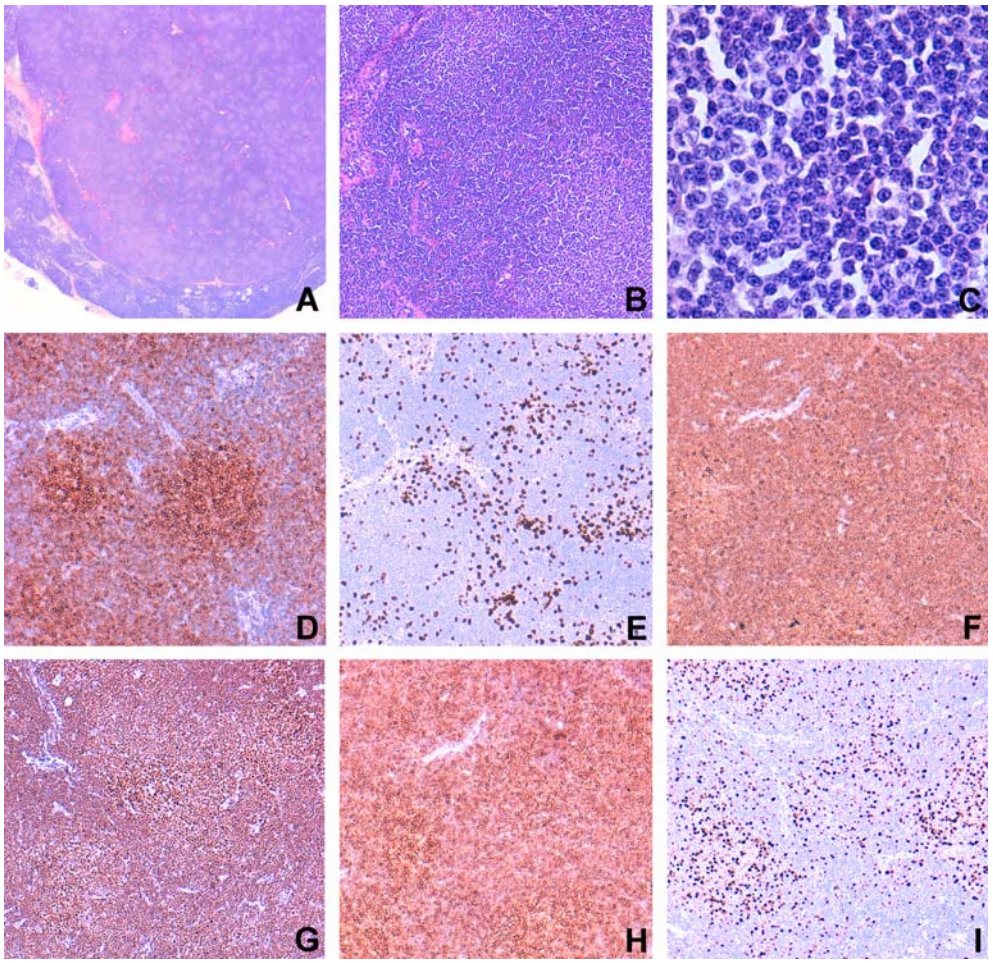

Fig. 2 CLL morphology and phenotype in a lymph node. (A) The lymph node at low power shows pale areas corresponding to proliferation centers (H&E, $2\times$); (B) The lymph node shows an effacement of the architecture by a diffuse proliferation of small cells (H&E, $10\times$); (C) The tumor cells are small with round nuclei, clumped chromatin and scarce cytoplasms. Occasional cells have larger nuclei with evident central nucleoli corresponding to prolymphocytes (H&E, $40\times$); (D) CD20 immunohistochemical staining shows the diffuse positivity of tumor cells, which is stronger in the cells of the proliferation centers; (E) CD3 is positive in infiltrating reactive T cells; (F) CD5, in contrast, is diffusely positive in tumor cells. Occasional cells with stronger staining correspond to infiltrating reactive T-cells that have a similar distribution as CD3 positive cells; (G) LEF1 is diffusely positive in CLL cells with a nuclear staining pattern; (H) CD23 is diffusely positive in CLL cells and it is stronger in the cells of the proliferation centers; I: The expression of the proliferation antigen KI67 is higher in tumor cells of the proliferation centers than in cells from diffuse areas.

round nuclei with central nucleoli corresponding to prolymphocytes and paraimmunoblasts. These proliferation centers are characteristic of CLL and not present in any other lymphoid neoplasms. A few mitotic figures are usually found in proliferation centers. In some cases, proliferation centers may be large, becoming confluent and occupying an area broader than a 20× field. They may have also a higher number of proliferating cells with a Ki67 index >40% and >2.4 mitosis per proliferation center. These cases have been called "accelerated" or "histologically aggressive CLL" and are associated with unfavorable genetic alterations. These patients usually have clinical manifestations of progressive disease and a clinical outcome that is intermediate between typical CLL and Richter's transformation.

The bone marrow is virtually always infiltrated by the same population of cells with interstitial, nodular and/or diffuse patterns. Proliferation centers are less common than in lymph nodes but may be seen in some cases. In the spleen, tumor cells tend to expand white pulp nodules, but may also infiltrate the red pulp. Proliferation centers may be seen, but are usually less prominent and frequent than in lymph nodes.

Immunophenotype

Flow Cytometry

Immunophenotyping is essential for the diagnosis of CLL. Immunophenotyping usually distinguishes B- from T-cell disorders, establishes the monoclonal nature of the proliferation by showing immunoglobulin light chain restriction and defines the characteristic profile of CLL cells. CLL cells are phenotypically very different from normal mature and precursor B-cells. Typically, their levels of surface immunoglobulin, CD20, CD22, CD79b, CD81, and FMC7 are low or absent compared to normal B-cells. They also co-express CD5, CD43, and ROR1, and overexpress CD23 and CD200 (Fig. 3 and Table 1). The differential antigenic expression between CLL and normal B-cells has allowed the design of flow cytometry tests able to quantify minimal residual disease with a sensitivity ≤0.01%.

Since there is no single marker exclusively expressed on CLL cells, a composite immunophenotype that integrates five markers into a scoring system helps distinguish CLL form other CLPD (Matutes score). In this score, one point is given for each of the following markers: (i) positive CD5 expression, (ii) positive CD23 expression, (iii) weak kappa/lambda chain expression, (iv) negative or weakly positive CD79b expression, and (v) negative FMC7 expression. Patients with CLL usually score of 4 or 5 points, while patients with other lymphoproliferative disorders usually score 0–2 points.

Beyond its diagnostic and therapeutic value, immunophenotypic analysis has been shown to be useful for the estimation of prognosis. Expression of CD38, ZAP70 and CD49d are all predictors for an unfavorable outcome in terms of treatment-free interval and overall survival. Although there is an overlap on the expression of these markers and the IGHV mutational status (see below), some studies have demonstrated that they have independent prognostic value. High sensitive flow cytometry assays, able to detect at least a CLL cell in 10.000 normal cells, are used to assess minimal residual disease in patients receiving intensive therapy and its presence is associated with a worse outcome.

Immunohistochemistry

Immunophenotype may be studied in tissues using immunohistochemical methods (Fig. 2). Tumor cells are diffusely positive for the mature B-cell markers CD20 and CD79a with coexpression of CD5 and CD23. CD20 and CD23 expression is stronger in cells

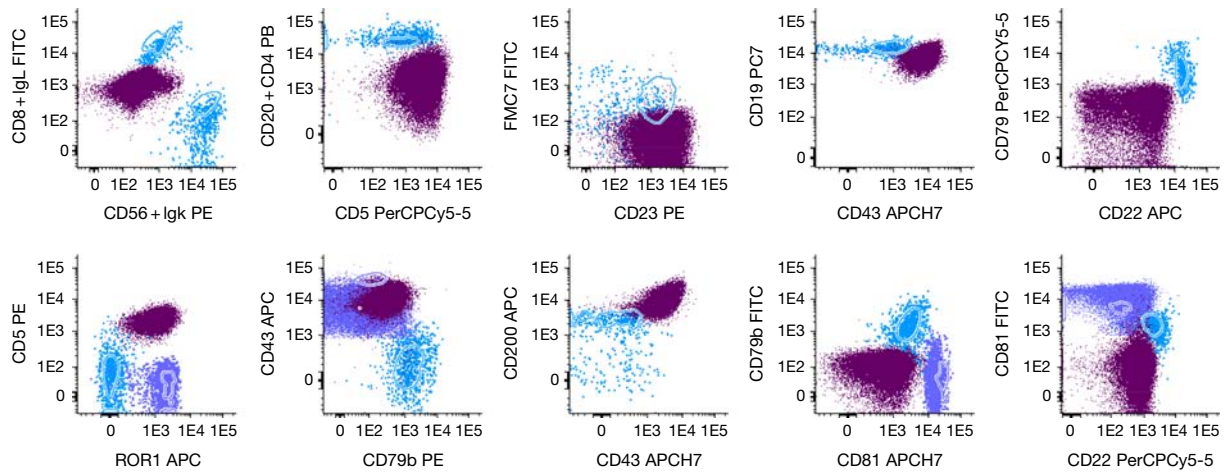

Fig. 3 Phenotypic profile of CLL cells in peripheral blood. Dot plots of B-cells from the peripheral blood and bone marrow of a patient with CLL stained with different combinations of antibodies. In each plot a countour showing the reactivity of the normal mature B-cells (*cyan*) or B-cell precursors (*light violet*) is depicted. CLL are painted in dark magenta, residual normal mature B cells in cyan and normal B-cell precursors in light violet.

Table 1 Immunophenotypic profile of B-cell lymphoproliferative disorders with leukemic expression

	CLL	*MCL*	*LPL*	*B-PLL*	*FL*	*HCL*	*MZL*
CD19	++	++	++	++	+	++	++
mIg[a]	+	++	+ or ++	++	++	++	++
CD20	+	++	+ or ++	++	++	+++	++
CD22[a]	+	++	+ or ++	++	++	+++	++
CD79b[a]	+ or ++	++	+ or ++	++	++	++	++
CD5[a]	++	++	- or +	- or +	−	−	−
CD23[a]	+++	−	- or +	- or +	+	−	+
CD200	+++	−	+	- or +	+	+++	+
CD43	+	- or +	−	- or +	−	−	- or +
CD10	−	−	−	−	+	−	−
CD103	−	−	−	−	−	+	−
CD123	−	−	−	−	−	+	−
CD25	- or +	−	−	+	−	+	- or +
CD11c	- or +	−	−	−	- or +	+	- or +
FMC7	−	+	+	+	+	+	+

CLL, chronic lymphocytic leukemia; *MCL*, mantle-cell lymphoma; *LPL*, lymphoplasmacytic lymphoma; *B-PLL*, B-cell prolymphocytic leukemia; *FL*, follicular lymphoma; *HCL*, hairy-cell leukemia; *MZL*, marginal zone lymphoma.
[a]Indicates antigens included in the immunophenotypic score for CLL.

located in the proliferations centers (Fig. 2). The CD5 staining of neoplastic B-cells is weaker than that of T-cells, and this double intensity is helpful in identifying the tumor subpopulation. Proliferation centers contain numerous T-cells, most of which are $CD4^+$, and in some cases a fine network of dendritic cells. Cells in proliferation centers have increased expression of the proliferation-associated marker Ki67, co-expression of survivin and MUM1/IRF4. These cells may also express MYC protein, NOTCH1, and cyclin D1 in up to about 30% of cases, but they are not associated with the associated genomic alterations. TP53 protein expression may be found in >30% of the cells in CLL with *TP53* mutations. This finding may suggest the presence of mutations, but should not substitute cytogenetic or molecular tests.

Monoclonal B-Cell Lymphocytosis

In the absence of lymphadenopathy, organomegaly, cytopenias and clinical symptoms, the presence of up to 5×10^9/L monoclonal B-lymphocytes in blood is defined as monoclonal B-cell lymphocytosis (MBL). MBL represents a miscellaneous category ranging from patients with "low-count" clonal B-cell populations ($< 0.5 \times 10^9$/L), detected through general population studies, to patients with absolute "high-count" ($\geq 0.5 \times 10^9$/L) lymphocytosis that are referred for consultation. This is important because low count MBL has significant differences from CLL, an extremely limited, if any, chance of progression, and, until new evidence is provided, does not require routine follow-up outside of standard medical care. In contrast, high-count MBL requires routine follow-up, and has very similar biomarkers as early stage CLL. MBL is classified into three categories on the basis of phenotype: (1) CLL-type, when the cells have the typical CLL phenotype; (2) atypical CLL-type, when the clonal cells have strong CD20 and/or surface immunoglobulin, while CD23 may be negative; (3) non–CLL-type, which is considered when clonal cells are CD5 negative and usually have a stronger expression of CD20 and surface immunoglobulins. In atypical-CLL and non-CLL-type MBL, additional cytogenetic and molecular studies are recommended to rule out other leukemic lymphoid neoplasms, particularly mantle cell lymphoma and splenic marginal zone lymphoma.

The prevalence of MBL is closely associated with the sensitivity of the flow cytometry test used. Initial studies using basic B-cell panels revealed that the MBL prevalence was 0.6%, but this figure reached 5–12% in adults over 60 years when a more complex and sensitive flow cytometry test was used. The highest reported prevalence of CLL-like MBL occurs in first-degree relatives of CLL patients. Studies in both the UK and USA showed a high prevalence of CLL-like MBL (13.5–18%) in individuals with a family history of CLL who had normal blood counts. Indeed, a fourfold increased prevalence of MBL was observed in families with a genetic predisposition to CLL compared to the general population.

High-count MBL and early stage CLL seem to share the same immune system perturbation and in particular the increased risk of infections. Conversely, it has been reported that patients with MBL frequently have a personal history of pneumonia, meningitis and even infectious episodes among their siblings or children, suggesting that exposure to infectious agents may trigger immune events leading to MBL. High-count MBL does not always progress to CLL, a phenomenon that occurs in only 1–2% cases per year, but disease progression may occur over time and therefore lifelong monitoring is recommended for these cases, similar to what is current practice for patients with early stage CLL.

Another important question is whether all CLL cases are preceded by MBL or if some CLL cases develop de novo. To address this question, a study evaluated 77,469 healthy adults who were enrolled in a US Cancer Screening Trial. Among 45 participants who

were subsequently diagnosed with CLL during the study and had a prediagnostic peripheral blood sample available, MBL was present in 44 (98%) patients, suggesting that virtually all CLL cases are preceded by MBL.

Although confirmatory studies are necessary, the concept of tissue-based MBL of CLL type is in discussion as there is a subset of cases with lymph node involvement by CLL cells that do not appear to have a significant rate of progression. In one retrospective study, lymph nodes with CLL/SLL in which proliferation centers were not observed and patients in whom lymphadenopathy was <1.5 cm based on computed tomography scans were the best candidates for this new diagnostic category of tissue-based MBL.

Richter's Transformation

Richter's transformation (RT) represents the development of an aggressive lymphoma, most commonly a diffuse large B-cell lymphoma in a patient previously (or simultaneously) diagnosed with CLL. The diagnosis needs to be substantiated through the biopsy of a lymph node or another lymphoid tissue. Rarely, RT presents in peripheral blood or bone marrow smears in the form of large immunoblasts with reticular chromatin, prominent nucleoli and basophilic cytoplasm admixed with small CLL lymphocytes. In this context, immunophenotyping is equally important as these cells tend to have a CLL phenotype with minor deviations. RT is reported to occur in 3–5% of patients with CLL, with a 10-year-cumulative incidence of around 10%. Clinical and laboratory clues that should raise the possibility of RT include sudden worsening of the general status, appearance of B symptoms, enlarging lymph nodes or spleen and dramatically increasing serum lactate dehydrogenase. Risk factors predicting for the development of RT are high CD38 expression, unmutated and stereotyped IGHV genes and presence of *NOTCH1* mutations. In 80% of cases, the transformation occurs in the CLL clone, which usually acquires *TP53*, *CDKN2A*, and *MYC* abnormalities and/or increased number of chromosomal alterations. The prognosis of these patients is poor. In the remaining 20% of patients, the large-cell lymphoma arises in a CLL-unrelated B-cell clone, and these patients have a similar prognosis compared with patients with de novo diffuse large B-cell lymphoma.

There are infrequent cases ($<1\%$) of CLL transformation into Hodgkin's lymphoma. The disease predominates in older men and the most common histological subtype is mixed cellularity. Most cases originate in CLL with mutated IGHV genes and are Epstein-Barr virus (EBV) positive. The diagnosis of Hodgkin's lymphoma requires the presence of diagnostic cells associated with an appropriate microenvirontment of reactive T and inflammatory cells. Some CLL cases may have isolated Reed-Sternberg cells, frequently EBV positive, but without this microenvirotnment. These cases should not be diagnosis as Hodgkin's lymphoma. The presence of EBV-positive Reed-Sternberg cells or Hodgkin's lymphoma may be associated with treatments including fludarabine.

Differential Diagnosis with Other Lymphoproliferative Disorders

Usually, the diagnosis of CLL is straightforward after the morphologic evaluation of peripheral smears and determination of the leukemic phenotype by flow cytometry. However, other chronic lymphoproliferative disorders (CLPD) with leukemic expression can have overlapping morphological and immunophenotypical features and in those cases genetic and molecular tests can be useful in the differential diagnosis.

Morphology

CLL with atypical morphology is characterized by a variable percentage ($>10\%$) of atypical lymphocytes (e.g. prolymphocytes, lymphoplasmacytoid cells, centrocytes, centroblasts or activated lymphocytes) resembling other lymphoid neoplasms such as follicular lymphoma, mantle cell lymphoma, lymphoplasmacytic lymphoma and marginal zone lymphoma. The presence of $>55\%$ prolymphocytes in the blood arbitrarily separates CLL from B-cell prolymphocytic leukemia, a completely separate disorder.

In lymph nodes the presence of proliferation centers is very characteristic of CLL. However, in the few cases in which these structures are absent the differential diagnosis may need to be established with other small B-cell lymphomas. Atypical CLL cells infiltrating lymph nodes may have irregular nuclei that suggest mantle cell lymphoma. Some CLL cases may have residual reactive germinal centers without mantle zones similar to those seen in mantle cell lymphoma. The expression of LEF1 and lack of cyclin D1 would facilitate the CLL diagnosis.

Immunophenotyping

Immunophenotyping of peripheral blood cells is necessary to make the differential diagnosis (**Table 1**). The characteristic phenotype of CLL is shown in **Figs. 2** and **3**. The dim expression of B-cell markers and the coexpression of CD5 are infrequent in other CLPD. The positivity of CD10 helps to distinguish CLL from follicular lymphoma as it is negative in the former and usually positive in the latter. The expression of CD43 and overexpression of CD23 and CD200 may provide further information in the differential diagnosis of CLL and other CLPD. For instance, these three antigens tend to be negative or dimly expressed in MCL, another $CD5^+$ CLPD. CD200 is a glycoprotein belonging to the Ig superfamily and this antigen is being considered as a target for therapy in

$CD200^+$ tumors, including CLL, but also in rheumatoid arthritis and other autoimmune disorders. The differential diagnosis with lymphoplasmacytic lymphoma is sometimes difficult as it could express a CLL-like phenotype. The analysis of the plasma cell compartment and molecular tests may be useful. Finally, the receptor tyrosine-like orphan receptor one (ROR1) is an embryonic glycoprotein highly and specifically expressed on the membrane of malignant, but not normal B-cells. In particular, ROR1 is highly expressed in CLL and hairy-cell leukemia, intermediately expressed in mantle-cell lymphoma and diffuse large B-cell lymphoma and scarcely expressed in follicular lymphoma. Moreover, ROR1 expression in CLL patients is not influenced by therapy. In contrast, normal B-cells have no significant expression of ROR1. Several studies are currently testing the diagnostic and therapeutic use of monoclonal antibodies targeting this molecule.

Cytogenetics and Molecular Biology

In contrast with what is observed in lymphoma, reciprocal balanced chromosomal translocations are rare in CLL. Indeed, fluorescent in-situ hybridization (FISH) tests investigating the presence of t(11;14)(q13;q32), leading to cyclin D1 rearrangement, are useful for distinguishing CLL from mantle-cell lymphoma. Since CLL does not display specific chromosomal aberrations, cytogenetics and FISH are generally performed for prognostic purposes. Chromosomal deletions and amplifications can be detected in up to 90% of patients. The most frequent genetic abnormalities in patients with CLL are del13q14 as isolated abnormality (14–60% of cases), del11q22-q23 (10–32%), trisomy 12 (11–18%), del 17p13 (3–27%) and del6q (2–9%), depending on the time-point at which the study is performed, and whether or not the disease is resistant to therapy. These structural abnormalities will be discussed in detail in the following section.

Similarly, CLL is not characterized by specific gene mutations. *MYD88* L265P mutation is detected in near all lymphoplasmacytic lymphoma and in only 3% of CLL. In the appropriate clinical, morphological and phenotypic context, the determination of *MYD88* L265P mutation is useful in the differential diagnosis of CLL and this type of lymphoma.

Genetics of CLL

Immunogenetics

CLL can be divided into two main subsets with different clinical behavior based on the presence or absence of mutations in the variable region of the immunoglobulin genes, reflecting the stage of normal B-cell differentiation from which they originate. CLL cells expressing unmutated IGHV genes (uCLL) arise from B-cells that have not undergone differentiation in germinal centers. CLL cells with mutated IGHV (mCLL) originate from post-germinal center B-cells. Of note, these IGHV mutations occur in normal B-cells during an immune response to antigens and should not be considered pathological. Patients with uCLL typically have a more aggressive disease than those with mCLL in terms of time to first treatment, overall survival and also progression-free survival after therapy.

Another interesting aspect of CLL immunogenetics is that the repertoire of immunoglobin molecules produced by CLL patients in general is considerably more limited than that of normal controls, reflecting the biased use of certain IGHV genes that have restricted somatic mutation and limited junctional and heavy–light chain combinatorial diversity. Moreover, in one third of patients the tumor expresses immunoglobulin 'stereotypes', which are stretches of the variable region that can also be identified in the immunoglobulins produced by the CLL cells of other patients. This limited immunoglobulin diversity provides compelling evidence that CLL cells are selected based on the binding activity of their surface immunoglobulin, which plays a crucial part in its pathogenesis. There are hundreds of stereotyped subsets, each defined by a unique VH CDR3 motif, and are classified as major (containing 20 or more sequences) or minor subsets. As of today, 19 different major subsets have been identified, representing 41% of al stereotyped cases and 12% of all CLL cases. Examples are subset #1, which is defined by the IGHD6–19 and IGHJ4 genes, subset #2 (IGHV3–21) and subset #4 (IGHV4–34). Interestingly, these subsets have different clinical characteristics. For instance, patients from subset #4 have a very indolent clinical course, even better than that of patients carrying del13q14, while patients from subsets #1 and #2 have a very aggressive disease with a prognosis similar to patients with *TP53* aberrations.

Structural Abnormalities

The importance of structural abnormalities in the outcome of patients with CLL is well established. Initial efforts were made using conventional chromosome banding analysis (CBA), but this technique is able to identify abnormalities in only 50% of patients, probably due to the low mitotic rate of CLL cells. These results were consistently improved with the advent of FISH so that, with a relatively low number of probes, genomic aberrations can be found in 80% of patients. In view of these results, FISH became the gold-standard method for cytogenetic assessment and has remained so to date even though it only evaluates specific genomic regions and not the entire genome. In parallel, the results of CBA could be improved when tumor cells were incubated with novel mitogens (e.g. DSP30 and IL2), which are capable of detect aberrations in 80% of patients, including 20–35% of those who have "normal" FISH results. Moreover, single-nucleotide polymorphism (SNP)-arrays also interrogate the entire genome and could be a suitable alternative to FISH for detecting deletions or amplifications. SNP-arrays do not detect balanced rearrangements (e.g. translocations), but these are rare in CLL. Irrespective of the technique used, the most common structural aberration in patients with CLL are:

- Del13q14, which is present in 50–60% of cases. This deletion, affecting the microRNAs miR15-a and miR16–1, reduces the expression of these genes, which are implicated in the regulation of apoptosis and the cell cycle. In addition, translocations involving 13q14 with a variety of chromosomal partners are also relatively frequent and generally disrupt the miR15-a/miR16–1 locus as well. When del13q14 is the only cytogenetic aberration, it is usually associated with early, non-progressive disease and favorable prognosis.
- Trisomy 12 is generally considered the second most frequent aberration in patients with CLL, occurring in 15–20% of patients, although the gene involved remains unknown. It often appears as a unique cytogenetic alteration but it can also be associated with others. Importantly, both del13q14 and trisomy 12 are nowadays associated with a favorable/intermediate prognosis and considered early CLL-founding events, since they are usually present in most tumor cells. Trisomy 12 is associated with atypical morphology and immunophenotype (e.g. FMC7 positivity, CD23 and CD43 negativity, strong surface immunoglobulin, CD20 and CD22 expression). It is also associated with *NOTCH1* mutations, particularly when trisomy 12 is not part of a complex karyotype.
- Deletions of chromosome 11q22-q23 may imply loss of the *ATM* and/or *BIRC3* function. They are usually identified in younger, male patients with bulky lymphadenopathy and aggressive disease. The disease is usually less responsive to conventional monotherapy and tends to relapse early. It is statistically associated with the presence of *SF3B1* mutations.
- Del17p13 alters the function of the *TP53* gene. Most patients with 17p deletion also have *TP53* mutations on the other allele, but 2–5% of patients present mutations of *TP53* in absence of del17p13. Both del17p13 and *TP53* mutations are associated with progressive disease resistant to conventional therapy, although there is also some degree of heterogeneity. In about a third of cases, *ATM*, *TP53* and *BIRC3* genes are inactivated by either point mutations or mutations plus copy number neutral loss of heterozygosity. Both del11q22-q23 and del17p13 are initially subclonal events that expand over time as a function of their proliferative advantage or environmental pressures (e.g. therapy).
- Novel candidate CLL driver genes recently identified in regions of copy number alterations (CNA) as detected by SNP arrays are *ZNF292* (6q15 loss), *SP140*/*SP110* (2q37 loss), *SMARCC1*/*SETD2* (3p21 loss), *NFKB2* (10q24 loss) and *MGA* (15q15.1 loss).

Since these structural abnormalities may coexist, a hierarchical model was devised in which patients are assigned to one of the following groups (from unfavorable to favorable prognosis): (i) del17p13; (ii) del11q22-q23 in the absence of del17p13; (iii) trisomy 12 in the absence of del17p13 or del11q22-q23; and (iv) isolated del13q14. Patients without any of the previous abnormalities have a prognosis that is intermediate between those with trisomy 12 and those with del13q14. However, this and other models combining chromosomal alterations and mutations need to be confirmed in larger cohorts of patients.

Genomic complexity, either defined as complex karyotype by CBA or increased CNA by SNP arrays has been consistently associated with adverse clinical outcome. Genomic complexity frequently co-exists with *TP53* disruption and modulates the poor prognosis of the latter. More recently, extreme genomic complex alterations called chromothripsis have been identified in 2–3% of patients. In chromothripsis focal regions of the genome undergo extensive chromosome fragmentation and reorganization causing structural genomic complexity which is also associated with *TP53* disruption and poor outcome. It has also been associated with *SETD2* inactivation, a gene that has been shown to cooperate with chromosomal aberrations in other types of leukemia.

Translocations are rare in CLL, but cases of t(14;19)(q32;q13) involving the immunoglobulin heavy chain gene and BCL3 loci, or even t(14;18)(q32;q21), involving the immunoglobulin heavy chain locus and BCL2, have been observed. Of note, the use of novel mitogens has increased the detection of chromosomal abnormalities as detected by conventional cytogenetics.

Mutations in Coding Regions

All cancers arise as a result of somatically acquired changes in the tumor cell genome. This does not mean, however, that all these abnormalities are involved in cancer development and, indeed, it is likely that some make no contribution at all. It is, therefore, of utmost importance to distinguish 'driver' from 'passenger' mutations. Driver mutations are causally implicated in oncogenesis, confer growth advantage and are positively selected by the microenvironment of the tissue in which the cancer arises. Passenger mutations are not selected, do not confer growth advantage and therefore do not contribute to cancer development. Passenger mutations are found within cancer genomes because somatic mutations without functional consequences often occur during cell division by different mechanisms. Thus, any given cell acquiring a driver mutation will already harbor innocuous passenger mutations within its genome, which will be carried along and therefore will be present in all tumor cells. In general, CLL is a very heterogeneous disease from a genomic point of view, in clear contrast to other diseases such as hairy cell leukemia and Waldenström's macroglobulinemia, where *BRAF* and *MYD88* mutations, respectively, are almost diagnostic.

The advent of next generation sequencing (NGS) techniques together with potent bioinformatic pipelines has fuelled cost- and time-effective studies of cancer genomes. The first NGS-based study performed in patients with CLL was the whole genome sequencing (WGS) of four patients including two uCLL and two mCLL cases. This study identified *NOTCH1*, *MYD88*, *XPO1* and *KLHL6* as potential CLL drivers, a hypothesis that was validated by targeted sequencing of additional samples. The results from this study and those from an independent cohort confirmed the role of *NOTCH1* activating mutations as a CLL driver. Moreover, *NOTCH1* mutations are more common in uCLL compared to mCLL, and associated with resistance to rituximab therapy, shorter survival and high risk of transformation to aggressive lymphoma (RT). *MYD88* mutations, on the other hand, had been previously observed in a proportion of cases of diffuse large B-cell lymphoma and virtually all cases of WM. In CLL, *MYD88* mutations are associated with young age, mCLL and a normal life expectancy. Finally, the role of *XPO1* mutations remains unclear due to the

low frequency at which it appears in CLL patients (< 2%), even though selective protein inhibition appears promising for the treatment of CLL and other malignancies.

This initial study has ben followed by whole genome and exome sequencing (WES) of larger cohorts comprising around 1000 patients in total. These studies confirmed the role as CLL drivers of previously described genes (e.g. *TP53* and *ATM*) but also revealed other recurrently mutated genes not previously reported, including *SF3B1*, *CHD2*, *POT1*, *FBXW7*, *DDX3X* and *BIRC3*. In one cohort, thirty-six significantly mutated genes were identified together with 23 additional genes that were either significantly mutated in one subgroup (uCLL or mCLL), had truncating mutations, or were infrequent but had been described in other malignancies. The most recurrently mutated genes were *NOTCH1* (12.6%), *ATM* (11%), *SF3B1* (8.6%), *BIRC3* (8.8%), *CHD2* (6%), *TP53* (5.3%) and *MYD88* (4%). Novel CLL drivers were also identified, including *ZNF292*, *ARID1A*, *ZMYM3* and *PTPN11*, and also less frequent mutations in well-known oncogenes (*KRAS* and *NRAS*), tumor suppressor genes (*CDKN1B* and *CDKN2A*) and transcription factors (*IKZF3*). In the other cohort, 44 recurrently mutated genes and 11 recurrently CNAs were identified. Some examples, not included in the previous list, are: *MGA* (3.2%) and *FUBP1* (1.7%), both of which modulate MYC expression together with *PTPN11*; *MAP2K1* (2%), which belongs to the MAPK-ERK pathway together with *KRAS*, *NRAS* and *BRAF*; and *RPS15* (4.3%), a component of the S40 ribosomal unit. *SF3B1* mutations cause aberrant splicing of specific genes and are more frequent in patients with aggressive disease. Other candidate genes such as *POT1* and *CHD2* have been further validated as bona fide CLL drivers by functional studies. Truncating mutations in *DDX3X* or *BIRC3* have been associated with refractoriness to therapy and adverse outcome.

Globally, these mutations could be grouped into eight main signaling pathways: B-cell receptor signaling (*MYD88*, *IRAK1*, *TLR2*, *IRF4*, *CARD11*), cell cycle regulation (*PTPN11*, *KRAS*, *NRAS*, *BRAF*, *CDKN1B*, *CDKN2A*), apoptosis (*TP53*, *BAX*), DNA damage response (*ATM*, *TP53*, *POT1*, *CHEK2*), chromatin remodeling (*ARID1A*, *SETD1A*, *ZMYM3*, *IKZF3*, *ASXL1*, *CHD2*), NF-kB signaling (*TRAF3*, *BIRC3*, *NFKB2*), NOTCH signaling (*NOTCH1*, *FBXW7*), and RNA metabolism (*MGA*, *ZNF292*, *SF3B1*, *RPS15*, *DDX3X*, *XPO1*, *FUBP1*).

Mutations in Noncoding Regions

The role of non-coding mutations in cancer pathogenesis is starting to emerge after decades of controversy. A recent WGS study of CLL found recurrent mutations in two regions of interest. The first was located in the 3′-UTR of *NOTCH1* in 2% of patients, suggesting that in about 20% of CLL tumors with *NOTCH1* mutations, these are located in non-coding regions. The mutations create new splicing sites and result in the deletion of the protein's PEST domain, thus increasing its stability in a similar way as previously described for the typical p.P2514fs*4 mutation. The second mutational region was found in 8% of patients and is located in a small intergenic region of chromosome 9p13. Several functional studies confirmed that this region contains an active enhancer for *PAX5*, a critical transcription factor for B-cells whose locus is located 330 kilobases away from this region. Remarkably, tumor cells with this mutation had a significantly reduced protein expression of *PAX5* compared to wild-type CLL or even normal B-cells. Subsequent studies revealed that this mutation is also present in other lymphoid malignancies such as diffuse large B-cell lymphoma and follicular lymphoma, raising the possibility that *PAX5* enhancer mutations might constitute driver events contributing to the development of these tumors.

Genomic Evolution

As most tumors, CLL is composed of a variety of subclones with diverse genomic aberrations. These subclones may evolve differently over the years in response to intrinsic (microenvironment) or extrinsic (therapy) pressures in a process that has been termed "clonal evolution". The first evidence of clonal evolution in CLL came from studies using FISH and SNP arrays. However, it was the digital quantification allowed by NGS that confirmed and extended this concept. According to these studies, the so-called "clonal" mutations are found in all or almost all tumor cells and probably constitute founder alterations (e.g. trisomy 12, del13q14, *MYD88* mutations), whereas "subclonal" mutations are probably acquired over the course of the disease (e.g. mutations in *TP53*, *ATM*, *SF3B1*, *NRAS*) and their prevalence correlates with the number of treatments received by the patient. In line with this, direct pairwise relationships have identified that, for instance, del11q22-q23 or trisomy 12 usually precede the acquisition of *ATM* and *NOTCH1* mutations, respectively. Of note, therapy may eradicate dominant clones and select subclones containing driver mutations, which would eventually propel disease progression and treatment refractoriness in most cases. Clonal evolution can be, however, very heterogeneous, including dominant clones that remain stable over time, but also subclones that switch dominance before and after treatment. Of special interest, several studies have shown that small subclones carrying *SF3B1* mutations may evolve without treatment pressure and become the dominant tumor population at progression. In this regard, ultra-deep NGS analysis allows for the detection of very small subclones harboring these mutations (down to 0.3% of all tumor cells), which are invisible by traditional Sanger sequencing and non-detected in previous WGS/WES studies. In one study, patients with small *TP53* mutated subclones only detectable by NGS had the same poor outcome compared to patients with clonal *TP53* mutations detected by Sanger sequencing. The same approach was applied to other recurrently mutated genes (*NOTCH1*, *SF3B1*, *BIRC3*, *POT1*, *XPO1*, etc.), and the same conclusion was reached: subclonal mutations in these genes may have prognostic impact in CLL. In contrast, for some genes the proportion of tumor cells carrying the mutation is prognostically relevant. As such, a number of studies have revealed that high-frequency *NOTCH1* mutations shorten patients' survival, whereas low-frequency mutations do not. Moreover, ultra-deep targeted NGS using a panel of 28 genes plus SNP arrays, all molecular tests performed after one simple DNA extraction, were able to identify

one driver abnormality in 86% of patients with the disease (almost as high as what you would achieve using WES or WGS). All together, these results have led to the progressive incorporation of NGS panels in the prognostic evaluation of patients with CLL.

A great example of how different subclones may evolve depending on therapy comes from the expanding role of ibrutinib in CLL. Ibrutinib is a Bruton tyrosine kinase inhibitor that has been approved for patients with relapsed/refractory CLL and also for those with *TP53* disruption. Shortly after this agent was introduced into the CLL therapeutic armamentarium, several studies revealed that relapse on this agent is predominantly due to acquired mutations in the *BTK* or in the *PLCG2* gene, the enzyme immediately downstream of BTK. Of note, clinical resistance is preceded by a prolonged period of asymptomatic expansion of *BTK*- or *PLCG2*-mutated clones, suggesting that, perhaps, these patients could be switched to alternative agents before the patient develops refractory disease or even Richter's transformation.

Gene Expression (Transcriptome)

More than a decade ago, microarray gene expression profiling (GEP) studies were crucial for the characterization of the different gene signatures observed in patients with CLL. These seminal studies were responsible, among many other things, for the identification of ZAP70 expression as one of the best surrogate markers for IGHV mutational status; or ROR1 as one of the most CLL-specific surface antigens. Subsequent GEP studies were also of utmost importance in the delineation of the cellular origin of CLL and characterization of the different anatomic compartments of the disease. However, the transcriptional landscape of CLL at high resolution has remained elusive until the development of novel technologies for deep RNA sequencing such as RNAseq. Using this technology, global transcriptome signatures were identified, including transcriptional elements such as transposable elements, long non-coding RNAs and pseudogenes, all of which are by definition invisible to GEP microarrays.

Epigenomic Profile

The advent of NGS and genome-wide methylation arrays has recently allowed investigators to explore the whole genome methylation pattern of CLL and normal B-cell subsets. CLL cells harbor a global hypomethylation in gene bodies, which correlates imperfectly with gene expression. Moreover, there is a striking correlation between the methylation pattern of uCLL and normal naïve B cells, and also between mCLL and normal memory B cells. These results supported the idea that naïve B-cells could potentially be the cell of origin of uCLL, whereas mCLL arises from memory B-cells, a conclusion also reached by an independent research group using gene expression profiling and similar genome wide methylation analysis. In addition, the authors could classify CLL patients according to their methylation profile into 3 groups with a distinct clinical outcome: naïve B-cell like, intermediate and memory B-cell like. These 3 groups had different IGHV mutational status, IGHV usage and other molecular characteristics such as *NOTCH1* and *SF3B1* mutations, and their diverse prognostic impact was validated in two independent cohorts.

Integrating Genomic, Epigenomic and Transcriptomic Data

In the last decade, NGS technology has provided scientists with a wealth of information that requires careful interpretation. For instance, WGS revealed that each patient with CLL or MBL tends to bear from 240 to 5416 point base substitutions or small insertions/deletions, yielding an average of 0.87 mutations per megabase. This mutation rate is low compared to melanoma or lung carcinoma, but in agreement with previous estimates of less than one mutation per megabase for leukemias. Moreover, there are marked differences in the genomic and epigenomic pattern according to the tumor subtype (uCLL vs. mCLL), thus reflecting the molecular mechanisms implicated in their respective development. The number of mutations (excluding the immunoglobulin loci) is higher in mCLL than in uCLL even though the number of driver mutations is significantly lower in the former compared with the latter. In other words, mCLL accumulates a significantly higher proportion of passenger mutations, and not necessarily driver mutations, as they pass through the germinal centre. Indeed, the proportion of mCLL cases with at least one driver mutation identified is 83% compared to 96% for uCLL cases. Equally, CLL and MBL patients have a comparable number of mutations, but a significantly different number of driver mutations, which is consistent with the notion that progression from MBL to CLL is generally determined by the increasing accumulation of driver alterations.

On the other hand, NGS and SNP arrays have identified a variety of CLL drivers associated with adverse prognosis. Interestingly, apart from having specific driver genes mutated, what confers a particularly dismal prognosis to patients is their accumulation, so that an increasing number of driver alterations correlates with a progressively shorter time to first treatment and overall survival. Moreover, epigenomics and genomics are clearly linked in CLL. The three epigenetic subgroups defined according to the methylome appear to have a distinct genomic profile as well. Patients from the naïve B-cell like subgroup generally had a higher tumor burden at diagnosis, as measured by beta$_2$-microglobulin, and almost exclusively unmutated *IGHV* genes, with preferential use of the VH1–69 gene. Phenotypic markers linked with adverse outcome, such as high CD38, ZAP70 or CD49d expression were more frequent in this subgroup, and this also applies to some genomic aberrations such as trisomy 12, del11q22–23 and *NOTCH1* and *ATM* mutations. In contrast, patients included in the memory B-cell like subgroup were portrayed as bearing mutated *IGHV* genes in almost all cases (frequently the VH4–34 gene) together with the absence of poor prognostic markers. Interestingly, though, patients categorized as 'intermediate', also have specific genomic aberrations. These patients, apart from having an intermediate outcome, bear unmutated *IGHV* genes in 22% of cases and, when they are mutated, the mutation rate is low (median 4%) compared to the memory B-cell like subgroup (median 7%). These tumors preferentially use the IGHV1–18, IGHV3–21 and IGHV3–23 genes and the incidence of

SF3B1 and *MYD88* mutations is remarkably high compared to the naïve B-cell like or memory B-cell like subgroups, thus confirming that this subset of patients deserves being separated from the other two subsets.

Applying this Knowledge into Current and Future Medical Practice

Since a good proportion of patients with CLL have a normal life expectancy, and clinical trials have not shown to date any advantage for initiating treatment in asymptomatic patients, the gold-standard of care for the majority of patients at diagnosis is careful observation without therapy. Once the disease becomes symptomatic, patients are usually treated with DNA-damaging agents such as fludarabine, cyclophosphamide or chlorambucil in combination with monoclonal antibodies (i.e. chemoimmunotherapy). More recently, BCR inhibitors such as ibrutinib or idelalisib, or BCL2 antagonists such as venetoclax, have been added to the treatment armamentarium after promising results obtained in clinical trials. Despite these advances, CLL remains incurable with current therapeutic options and new agents are desperately needed for patients with high-risk disease.

New genomic developments could improve patient outcome in two ways: (1) allowing for a more precise prognostic estimation and, (2) identifying novel therapeutic targets. The first aspect has already been put into practice in a variety of publications evaluating the prognostic value of these new driver genes in combination with classical markers, and also as predictors of response to therapy. Examples of these are:

- Patients with *TP53* abnormalities are unlikely to respond to conventional chemotherapeutic agents and clearly benefit from the use of ibrutinib or idelalisib. Indeed, both agents have been approved by the European Medicines Agency for patients with CLL and *TP53* disruption (del17p13 or TP53 mutations).
- Patients with *NOTCH1* mutations do not benefit from the addition of rituximab to the FC (fludarabine plus cyclophosphamide) backbone, which still remains the gold standard frontline therapy for younger and fitter patients.
- Patients harboring *SF3B1* and *NOTCH1* mutations have very short progression-free survival after chemoimmunotherapy (i.e. rituximab, fludarabine and cyclophosphamide), probably suggesting that they should be considered for inclusion into appropriate clinical trials whenever possible.
- Both complex karyotype and del17p13 are associated with disease progression on ibrutinib, suggesting that markers that predict poor prognosis with standard therapies also confer risk with novel therapies, albeit a delayed risk. Moreover, as previously mentioned, disease progression on ibrutinib is very frequently preceded by the emergence of *BTK*- or *PLCG2*-mutated clones.
- Patients with mCLL devoid of *TP53* abnormalities or del11q22–23 obtain very good responses and a very prolonged progression-free survival with conventional chemoimmunotherapy (i.e. fludarabine, cyclophosphamide and rituximab).

The second important message from NGS studies is that the prognostic value of these aberrations is not only evident when they are detectable using conventional Sanger sequencing, but also when they are only present in very small subclones, which could change the way we approach molecular studies in our daily practice. The uncovering of intra-tumor heterogeneity and the description of the Darwinian process of clonal evolution leading to the expansion of resistant subclones upon therapy, initially proposed by Nowell in 1976, could change the way we approach CLL therapy. Indeed, half of all driver mutations are subclonal, and the clone composition remains stable over time for most untreated patients, probably owing to the slow growing capacity of CLL cells. In contrast, however, most patients requiring therapy experience a clonal evolution characterized by the expansion of highly fit clones and, perhaps, conventional therapy should be combined with agents targeting the subclonal driver(s) that could potentially expand upon disease relapse. In this regard, genomic studies have identified a number of pathways that could be potential targets for therapeutic intervention:

- B-cell receptor (BCR) signaling: The importance of BCR signaling in CLL biology is well established since all B-cells require these signals for their survival and proliferation. As such, pharmacological inhibition of several proteins downstream the BCR (Syk, BTK, PI3K) is remarkably effective in this disease. Some of these genes (i.e. BTK and PLCG2) may acquire mutations upon BTK inhibition. Moreover, point mutations affecting key molecules belonging to this and other tightly related pathways (e.g. Toll-like receptors) have also been identified (*MYD88*, *IRAK1*, *TLR2*, *IRF4*, *CARD11*). As already stated, the BTK inhibitor ibrutinib and the PI3K inhibitor idelalisib have approved for therapy of patients with CLL.
- Cell cycle regulation: CLL cells overexpress a variety of cyclins and cyclin-dependent kinases, mostly through BCR activation, but also through point mutations in cyclin-dependent kinase inhibitors (*CDKN1B*, *CDKN2A*) and other similar molecules (*PTPN11*). Pharmacological inhibition of cyclin-dependent kinases has been tested in CLL with mixed results, mostly due to safety concerns (not lack of efficacy), but novel oral inhibitors (e.g. palbociclib) have been approved in solid tumors and hold promise for CLL. Furthermore, the MAPK/ERK pathway is disrupted in a proportion of patients with CLL, with mutations identified in *KRAS*, *NRAS*, or *BRAF*. Interestingly, specific inhibitors of *BRAF* or *MEK* have been already approved for a number of solid tumors and could be of interest. Also, multikinase inhibitors of the MAPK/ERK pathway, such as sorafenib, have showned a remarkable anti-CLL activity in vitro.
- Apoptosis: As in most malignancies, CLL cells try to evade apoptosis either by disrupting DNA damage response (mutations in *ATM*, *TP53*, *POT1*, or *CHEK2*), disrupting pro-apoptotic molecules (mutations in *BAX*) or overexpressing anti-apoptotic molecules (BCL2 or MCL1). Over the past years, several BCL2 inhibitors have been developed and one of them, venetoclax, has remarkable efficacy in CLL, including patients with *TP53* and *ATM* disruption and has been already approved by the US Food and Drug Administration for the treatment of CLL.

- NOTCH signaling: This pathway is involved in cell-fate decisions during development through its nuclear translocation and transcription of target genes including *HES1* and *MYC*. In CLL, NOTCH1 is constitutively expressed thereby promoting apoptosis evasion and cell survival. In some patients, this is due to *NOTCH1* mutations which stabilize the protein. Some agents, called gamma-secretase inhibitors, are able to induce apoptosis in vitro and inhibit the constitutive Notch activation that is characteristic of *NOTCH1*-mutated CLL cells. Moreover, MYC itself could be a potential target thanks to the novel Bromodomain and Extra-Terminal motif (BET) protein inhibitors, which are currently being investigated in many different lymphoid malignancies.
- RNA metabolism: The identification of mutations in the spliceosome-RNA processing machinery (*SF3B1*, *RPS15*, *DDX3X*) has led to the development of spliceosome modulators. These agents induce selective cytotoxicity of CLL cells in vitro when compared with normal lymphocytes or cells from other B-cell malignancies.
- NF-kB signaling: The NF-kB signaling pathway is an important therapeutic target since it is constitutively activated in CLL through the canonical and alternative (non-canonical) pathways. In some patients this is due to point mutations (*TRAF3*, *BIRC3*, *NFKBIE*) or structural abnormalities (*NFKB2*), and pharmacological blockade of this pathway (e.g. using proteasome inhibitors or direct IKK inhibitors) leads to anti-CLL activity in vitro. Moreover, BET inhibitors block downstream oncogenic NF-kB-driven transcriptional programs in in vitro and in vivo lymphoma models. Of note, TRAF3 serves as a negative regulator of the non-canonical NF-kB signaling pathway and targets NIK for constant ubiquitination and degradation. As a result, *TRAF3*-mutated tumors result in non-canonical NF-kB activation through NIK overexpression and, indeed, NIK has emerged as a very attractive target for therapy.

Conclusions

CLL is the most common leukemia in adults in the developed world. The diagnosis of CLL requires the phenotypic analysis of tumor cells. Small lymphocytic lymphoma is the same disease with tissue involvement and low tumor cell counts in peripheral blood. Morphology and immunophenotype are the initial, and usually sufficient, diagnostic investigations. In cases with morphological atypical features, additional tests such as molecular genetics and/or histology are required to confirm the diagnosis. Some other markers, such as CD38 and ZAP70 expression, are also useful for prognostic purposes. A monoclonal population immunophenotypically identical to that found in CLL or other CLPD can be detected in up to 3–10% of healthy people. However, only those with 'high-count' MBL behave as early stage CLL and need to be clinically followed, while avoiding cumbersome and anxiety-triggering medical examinations in those with minute ('low-count') monoclonal populations.

Regarding the genetics of the disease, several research groups have discovered significant prognostic associations coupled with new driver genes and signaling pathways whose role in cancer was previously unknown or poorly understood. Massive scale epigenomics and transcriptomics have supplied the foundations for the cellular origin of the disease, and recent studies have uncovered non-coding mutations as drivers for the disease. We are now in a position to compose a fully integrated model of biological factors that could be assimilated into daily practice, even though the list of genes that should be evaluated remains undefined. Moreover, some drivers could be targeted pharmacologically, even when present at subclonal level, thus avoiding clonal selection and disease refractoriness. This outburst of genomic knowledge is just starting to expand into the clinic, but in the coming years, as our understanding broadens and ongoing technological innovation propels new achievements, we will certainly learn how to apply it in our daily practice.

Further Reading

Falchi, L., Keating, M.J., Marom, E.M., Truong, M.T., Schlette, E.J., Sargent, R.L., Trinh, L., Wang, X., Smith, S.C., Jain, N., Estrov, Z., O'Brien, S., Wierda, W.G., Lerner, S., Ferrajoli, A., 2014. Correlation between FDG/PET, histology, characteristics, and survival in 332 patients with chronic lymphoid leukemia. Blood 123, 2783–2790.

Giné, E., Martinez, A., Villamor, N., López-Guillermo, A., Camos, M., Martinez, D., Esteve, J., Calvo, X., Muntañola, A., Abrisqueta, P., Rozman, M., Rozman, C., Bosch, F., Campo, E., Montserrat, E., 2010. Expanded and highly active proliferation centers identify a histological subtype of chronic lymphocytic leukemia ("accelerated" chronic lymphocytic leukemia) with aggressive clinical behavior. Haematologica 95, 1526–1533.

Kipps, T.J., Stevenson, F.K., Wu, C.J., Croce, C.M., Packham, G., Wierda, W.G., O'Brien, S., Gribben, J., Rai, K., 2017. Chronic lymphocytic leukemia. Nature Reviews Disease Primers 3, 17008.

Kulis, M., Heath, S., Bibikova, M., Queirós, A.C., Navarro, A., Clot, G., Martínez-Trillos, A., Castellano, G., Brun-Heath, I., Pinyol, M., Barberán-Soler, S., Papasaikas, P., Jares, P., Beà, S., Rico, D., Ecker, S., Rubio, M., Royo, R., Ho, V., Klotzle, B., Hernández, L., Conde, L., López-Guerra, M., Colomer, D., Villamor, N., Aymerich, M., Rozman, M., Bayes, M., Gut, M., Gelpí, J.L., Orozco, M., Fan, J.B., Quesada, V., Puente, X.S., Pisano, D.G., Valencia, A., López-Guillermo, A., Gut, I., López-Otín, C., Campo, E., Martín-Subero, J.I., 2012. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. Nature Genetics 44, 1236–1242.

Landau, D.A., Carter, S.L., Stojanov, P., McKenna, A., Stevenson, K., Lawrence, M.S., Sougnez, C., Stewart, C., Sivachenko, A., Wang, L., Wan, Y., Zhang, W., Shukla, S.A., Vartanov, A., Fernandes, S.M., Saksena, G., Cibulskis, K., Tesar, B., Gabriel, S., Hacohen, N., Meyerson, M., Lander, E.S., Neuberg, D., Brown, J.R., Getz, G., Wu, C.J., 2013. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell 152, 714–726.

Landau, D.A., Tausch, E., Taylor-Weiner, A.N., Stewart, C., Reiter, J.G., Bahlo, J., Kluth, S., Bozic, I., Lawrence, M., Böttcher, S., Carter, S.L., Cibulskis, K., Mertens, D., Sougnez, C.L., Rosenberg, M., Hess, J.M., Edelmann, J., Kless, S., Kneba, M., Ritgen, M., Fink, A., Fischer, K., Gabriel, S., Lander, E.S., Nowak, M.A., Döhner, H., Hallek, M., Neuberg, D., Getz, G., Stilgenbauer, S., Wu, C.J., 2015. Mutations driving CLL and their evolution in progression and relapse. Nature 526, 525–530.

Landgren, O., Albitar, M., Ma, W., Abbasi, F., Hayes, R.B., Ghia, P., Marti, G.E., Caporaso, N.E., 2009. B-cell clones as early markers for chronic lymphocytic leukemia. New England Journal of Medicine 360, 659–667.

Nadeu, F., Delgado, J., Royo, C., Baumann, T., Stankovic, T., Pinyol, M., Jares, P., Navarro, A., Martín-García, D., Beà, S., Salaverria, I., Oldreive, C., Aymerich, M., Suárez-Cisneros, H., Rozman, M., Villamor, N., Colomer, D., López-Guillermo, A., González, M., Alcoceba, M., Terol, M.J., Colado, E., Puente, X.S., López-Otín, C., Enjuanes, A., Campo, E., 2016. Clinical impact of clonal and subclonal *TP53, SF3B1, BIRC3, NOTCH1*, and *ATM* mutations in chronic lymphocytic leukemia. Blood 127, 2122–2130.

Puente, X.S., Beà, S., Valdés-Mas, R., Villamor, N., Gutiérrez-Abril, J., Martín-Subero, J.I., Munar, M., Rubio-Pérez, C., Jares, P., Aymerich, M., Baumann, T., Beekman, R., Belver, L., Carrio, A., Castellano, G., Clot, G., Colado, E., Colomer, D., Costa, D., Delgado, J., Enjuanes, A., Estivill, X., Ferrando, A.A., Gelpí, J.L., González, B., González, S., González, M., Gut, M., Hernández-Rivas, J.M., López-Guerra, M., Martín-García, D., Navarro, A., Nicolás, P., Orozco, M., Payer, Á.R., Pinyol, M., Pisano, D.G., Puente, D.A., Queirós, A.C., Quesada, V., Romeo-Casabona, C.M., Royo, C., Royo, R., Rozman, M., Russiñol, N., Salaverría, I., Stamatopoulos, K., Stunnenberg, H.G., Tamborero, D., Terol, M.J., Valencia, A., López-Bigas, N., Torrents, D., Gut, I., López-Guillermo, A., López-Otín, C., Campo, E., 2015. Non-coding recurrent mutations in chronic lymphocytic leukemia. Nature 526, 519–524.

Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Dürig, J., Küppers, R., 2012. Cellular origin and pathophysiology of chronic lymphocytic leukemia. Journal of Experimental Medicine 209, 2183–2198.

Swerdlow, S.H., Campo, E., Pileri, S.A., Harris, N.L., Stein, H., Siebert, R., Advani, R., Ghielmini, M., Salles, G.A., Zelenetz, A.D., Jaffe, E.S., 2016. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood 127, 2375–2390.

Chronic Myelogenous Leukemia: Pathology, Genetics, Diagnosis, and Treatment

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Abbreviations

ALP Alkaline phosphatase
AP Accelerated phase
BP Blast phase (blast crisis)
CBC Complete blood count
CD Cluster of differentiation
CML Chronic myelogenous leukemia
CP Chronic phase
DLI Donor lymphocyte infusion
FDA US Food and Drug Administration
FISH Fluorescence in situ hybridization
HSCT Hematopoietic stem cell transplantation
ICD-O International Classification of Diseases for Oncology
LSCs Leukemic stem cells
NCCN US National Comprehensive Cancer Network
RT-PCR Reverse transcriptase polymerase chain reaction
TKI Tyrosine kinase inhibitor
WBC White blood cells
WHO World Health Organization

Definition and Classification

Chronic myelogenous leukemia (CML), *BCR-ABL* positive (ICD-O code: 9875/3) is a myeloproliferative neoplasm that originates from an abnormal pluripotent bone marrow stem cell and is consistently associated with the *t(9;22)(q34.1;q11.2)* reciprocal chromosomal translocation which generates the *BCR-ABL1* fusion gene located on the so called Philadelphia (Ph) chromosome. The *BCR-ABL1* is found in all myeloid lineages as well as in some lymphoid cells and endothelial cells.

CML, *BCR-ABL* positive is also called CML, Philadelphia chromosome positive; CML, *t(9;22)(q34;q11)*; chronic granulocytic leukemia, Philadelphia chromosome positive; chronic granulocytic leukemia, *t(9;22)(q34;q11)*; chronic granulocytic leukemia, *BCR/ABL*; and chronic myeloid leukemia.

Epidemiology and Risk Factors

Burden

CML accounts for 15%–20% of adult leukemias. It affects 1–2 individuals per 100,000 annually, with a slight male predominance. The incidence increases with age: the disease is rare in children (<0.1 case per 100,000 population) and is most frequent in the elderly (over 2.5 cases per 100,000). No significant geographical variations in the CML incidence have been reported, however patients in areas of lower socioeconomic status tend to be younger at disease onset.

CML which has reached the blast phase used to be associated with a dismal outcome. However, with the introduction of targeted therapies and more accurate monitoring methods, the 10-year survival rates for CML patients have risen to approximately 80%–90%.

Etiology and Risk Factors

The etiology of CML is largely unknown. Except for acute radiation exposure (such as in atomic bomb survivors), no environmental or lifestyle factors have been identified. There seem to be no familial predisposition, either.

Presentation and Diagnosis

The natural clinical course of untreated CML consists of two or three phases: chronic phase (CP) and blast phase (BP; also called blast crisis) which are usually—but not always—separated by accelerated phase (AP; sometimes also called acute phase because of its resemblance to acute leukemia).

Most patients are diagnosed in CP which usually has an insidious onset. Nearly 50% of newly diagnosed CP-CML cases are asymptomatic and are discovered incidentally with abnormally high white blood cell (WBC) counts at routine blood testing and/or with splenomegaly at physical examination. Common findings at presentation are nonspecific fatigue, malaise, night sweats, and weight loss, and they may occur long after the disease onset. Loss of energy and reduced exercise tolerance may appear during CP after several months. Many patients have symptoms related to enlargement of the spleen, with about half of the patients presenting with palpable splenomegaly. The enlarged spleen may encroach on the stomach and cause early satiety and decreased food intake leading to weight loss. Spleen infarction may manifest as "gripping" pain in the left upper abdominal quadrant. Splenomegaly may also be associated with a hypermetabolic state manifesting as low-grade fevers, excessive sweating, and chronic fatigue. The spleen size usually correlates with the peripheral blood granulocyte counts (the largest spleen in patients with the highest WBC counts). Hepatomegaly, usually asymptomatic, may also occur but it is less common. It usually results from the extramedullary hematopoiesis occurring in the spleen. Visual changes, seizures, cerebral or myocardial infarctions, and priapism may occur in patients with extremely high WBC counts (over 300,000 cells/μL) as manifestations of leukostasis (abnormal WBC clumping) and hyperviscosity. The retina may show papilledema, venous obstruction, and hemorrhages on funduscopy.

Untreated CP-CML inevitably progresses to blast crisis (via AP or without it) within 3–5 years. About 5% of patients are diagnosed in AP or BP (without a recognized CP). Bleeding, petechiae, and ecchymoses are frequently prominent symptoms of AP-CML. Fever is usually associated with infections. Bone pain and fever, very large spleen, and/or increased bone marrow fibrosis are common harbingers of the blast phase. Typical symptoms of the blast crisis are due to increasing anemia, thrombocytopenia, basophilia, a rapidly enlarging spleen, and failure of the usual medications to control leukocytosis and splenomegaly.

The initial workup consists of a physical examination, including palpation of the spleen and the liver, and a complete blood count (CBC) with a differential and a chemistry profile. Patients should also be tested for infections with hepatitis viruses.

CML can usually be diagnosed based on the peripheral blood findings combined with the detection of the Philadelphia chromosome and/or the BCR/ABL1 fusion protein and transcript using cytogenetic and molecular techniques (fluorescence in situ hybridization (FISH) with dual *BCR/ABL1* probes and reverse transcriptase polymerase chain reaction (RT-PCR), respectively). Bone marrow aspiration is necessary to obtain sufficient material for karyotyping and morphological evaluation which allow to determine the CML phase. For patients diagnosed with advanced-phase disease (AP or BP), flow cytometry to determine the cell lineage and mutational analyses are recommended. In a majority of patients, bone marrow biopsy is not necessary for diagnosis. However, it remains an important alternative in case of atypical peripheral blood findings or when a cellular aspirate cannot be obtained.

The major initial finding at the blood test is neutrophilic leukocytosis, with neutrophil left shift (increased proportion of immature neutrophils) and WBC counts commonly exceeding 30,000 cells/μL at diagnosis. Mild eosinophilia and basophilia are often present already in CP and increase with the disease progression to advanced phases. Increased platelet levels (thrombocytosis) are common at presentation, with the counts that may exceed 1,000,000 cells/μL. Thrombocytopenia is uncommon in CP. Other laboratory abnormalities include hyperuricemia which reflects high bone marrow cell turnover, as well as markedly elevated serum vitamin B-12–binding protein correlated with the degree of leukocytosis (this protein is synthesized by granulocytes).

At cytogenetic and molecular analyses, the *BCR-ABL1* fusion is found in all myeloid lineages as well as in some lymphoid cells and endothelial cells, in all CML phases. The presence of additional chromosomal abnormalities in Ph-positive cells ("clonal evolution") is associated with the disease progression to AP and/or BP. Of note, very low levels of *BCR-ABL1* transcripts are detected in approximately 30% of healthy individuals, with an incidence which increases with age. The risk of developing CML is extremely low in these individuals and they do not require treatment.

Disease progression manifests with a declining performance status, constitutional signs such as fever and weight loss, recurrent bone pain, and symptoms related to increasing anemia, thrombocytopenia, basophilia, increasing WBC counts with increasing proportion of blasts, and enlarging spleen. The disease status should be reevaluated in this setting.

The clinical and morphological boundaries between different CML phases are not very sharp, and the diagnostic criteria may vary between different centers. In particular, some investigators insist that a cut-off of 30% (and not 20% as defined by the World Health Organization, WHO) sets the boundary of the blast crisis, and this threshold has been used in many clinical trials, including those which led to the approval of tyrosine kinase inhibitor (TKI) therapy for treatment of CML patients. However, using either of the two cut-offs does not seem to have any prognostic significance for BP-CML patients. The diagnostic criteria for the three CML phases defined by WHO are summarized in **Table 1**. As the established hematological/cytogenetic criteria, in particular in AP-CML patients, are not sufficient for accurate prognostication, provisional criteria of response to therapy (mainly treatment with TKIs) have been proposed. They seem to improve the prognostic utility of the established hematological/cytogenetic criteria but this remains to be clinically validated.

Table 1 Diagnostic criteria for different chronic myelogenous leukemia (CML) phases as defined by the World Health Organization (WHO)

Chronic phase (CP) CML	*Accelerated phase (AP) CML*	*Blast phase (BP) CML*
	Established hematological/cytogenetic criteria	
The presence of the Philadelphia chromosome and/or the BCR/ABL1 fusion protein or transcript confirmed by cytogenetic and molecular techniques		
<10% of blasts in the bone marrow or peripheral blood WBC	10% < blasts <20% in the bone marrow or peripheral blood WBC; Persistent thrombocytopenia (platelets $<100 \times 10^9$/L) or thrombocytosis (platelets $>1000 \times 10^9$/L) unresponsive to therapy; Increasing WBC count and spleen size unresponsive to therapy; Cytogenetic evidence of clonal evolution (the presence of additional chromosomal aberration in Ph-positive cells); 20% or more basophils in the peripheral blood* Large clusters or sheets of small abnormal megakaryocytes associated with marked reticulin or collagen fibrosis in biopsy specimens presumptive of AP diagnosis; usually associated with one of the above-listed criteria	At least 20% of blasts in the bone marrow or peripheral blood WBC; Extramedullary blast proliferation (e.g., osteolytic bone lesions or lymphadenopathy); Large foci or clusters of blasts in bone marrow biopsy
	Provisional response-to-TKI therapy criteria	
	Hematological resistance (i.e. failure to achieve a complete hematological response) to the first TKI treatment Any grade of resistance to two sequential TKI treatments Occurrence of two or more new mutations in the *BCR-ABL1* fusion gene during TKI therapy	

TKI, tyrosine kinase inhibitor; *WBC*, white blood cells.
*The presence of any of these criteria confirms the diagnosis.

Pathology and Genetics

Pathology

CML is characterized by the clonal expansion of terminally differentiated myeloid cells originating from a leukemic stem cell. The peripheral blood smear in CML patients shows a typical leukoerythroblastic blood picture, with circulating immature cells from the bone marrow. Alkaline phosphatase (ALP) in granulocytes is virtually absent and stains very low or negative by immunohistochemistry.

In chronic phase CML, bone marrow specimens show hypercellularity with marked granulocytic proliferation and a maturation pattern similar to that found in the normal blood, including expansion at the myelocyte stage. There is no significant dysplasia. Blasts usually account for <5% of the bone marrow cells (diagnostic criterion for the CP-CML: < 10% blasts). A substantially decreased proportion of erythroid precursors is usually observed. Up to 50% of cases show moderate to marked megakaryocytic proliferation, with normal or slightly decreased megakaryocyte counts. Megakaryocytes are smaller than normal and have hyposegmented nuclei ("dwarf megakaryocytes") but they are not true micromegakaryocytes like those observed in myelodysplastic syndromes. Eosinophil and basophil counts are usually elevated, and pseudo-Gaucher cells are common. This is often observed in bone marrow biopsy sections as a thicker layer of immature granulocytes around the bone trabeculae (5–10 cells compared to 2–3 cells in samples from healthy individuals). In 30%–40% of cases, increased megakaryocyte counts correlate with moderate to marked reticulin fibrosis which may also be associated with splenomegaly. Infiltration of the red pulp cords in the spleen by granulocytes, both mature and immature, leads to the spleen enlargement. A similar infiltrate can be observed in hepatic sinuses and portal areas. In most cases, blasts have a myeloid phenotype. Lymphoid blasts may also be seen but some data suggest that the presence of any lymphoblasts, whether in CP or AP, may predict an imminent lymphoblastic BP rather than a myeloid blast crisis.

The expression of CD7 on CD34-positive cells in CP-CML has been suggested to be an adverse prognostic factor, whereas the presence of a normal CD34-positive stem cell population which do not express abnormal markers (such as CD7, CD56, or CD11b) predicts a better response to TKI treatment.

The disease progression is associated with increasing numbers of circulating blasts, increasing numbers of abnormal megakaryocytes, and the occurrence of dysplasia. In accelerated phase, dysplastic changes may be observed in any of the myeloid lineages. Clusters of small megakaryocytes (including micromegakaryocytes similar to those observed in myelodysplastic syndromes) may appear and may be associated with significant reticulin and/or collagen fibrosis which is best visible in biopsy sections. The proportion of blasts is increased (diagnostic AP-CML criterion: 10% < blasts <20%), which can be highlighted by immunohistochemical staining for CD34.

In a majority of BP-CML cases (up to 70%), the blast lineage is myeloid, with blasts displaying a phenotype which is indistinguishable from that of acute myeloid leukemia. The lineage may include neutrophilic, monocytic, megakaryocytic, basophilic, eosinophilic, and/or erythroid blasts in any combination. Most of the remaining cases of blast crisis (20%–30%) are lymphoid, in which the blasts (lymphoblasts) usually have immunophenotypic characteristics of pre-B cells or have biphenotypic features (myeloid and B lymphoid). Megakaryocytic or undifferentiated blast crises may occur in up to 10% of patients. Sequential lymphoblastic and myeloblastic crises have also been reported. The blast lineage may be morphologically obvious. However, the blasts are often primitive and antigens of more than one lineage are frequently expressed. Therefore, cytochemical analysis and immunophenotyping (preferably using flow cytometry) of the blasts are recommended. Extramedullary blast proliferations may be of myeloid, lymphoid, or mixed phenotype. Sheets of blasts occupying focal but substantial areas of the bone marrow (e.g., an entire intertrabecular space) visible in marrow biopsy specimens strongly suggest BP diagnosis even if the rest of the bone marrow suggests CP.

Genetics

The defining genetic characteristics of CML is the *t(9;22)(q34.1;q11.2)* reciprocal chromosomal translocation. This translocation fuses sequences of the breakpoint cluster region (*BCR*) gene on chromosome 22 with regions of the Abelson murine leukemia (*ABL1*) gene on chromosome 9, generating the Philadelphia chromosome *der(22)t(9,22)* harboring the fused *BCR-ABL1* oncogene. About 5%–10% of patients have variant translocations which involve another (third) chromosome or even two extra chromosomes in addition to chromosomes 9 and 22, or a cryptic translocation of chromosomes 9q34.1 and 22q11.2. The *BCR-ABL1* fusion gene is present in all cases and can be detected by FISH and/or RT-PCR. However, the cryptic fusion is not detectable by routine cytogenetic analysis.

Depending on the site of the chromosome 22 breakpoint and the size of the resulting fused gene, different fusion proteins are produced, impacting the phenotype of the disease. In a majority of CML patients, the breakpoint is in the major BCR (M-BCR) region, spanning exons 12–16 and resulting in the production of an abnormal p210 BCR-ABL1 fusion protein with an increased tyrosine kinase activity. In rare cases, the breakpoint occurs in the μ-BCR region (exons 17–20), generating a gene encoding for a larger p230 fusion protein. Patients with this translocation may show prominent neutrophilic maturation and/or conspicuous thrombocytosis. Breaks in the minor breakpoint region (m-BCR; exons 1–2) resulting in the production of a short p190 protein (frequent in Ph-positive acute lymphocytic leukemia) are very rare in CML and are associated with increased monocyte counts, resembling chronic monomyelocytic leukemia. However, low p190 transcript levels may be detected in up to 90% of CML patients due to alternative *BCR* splicing.

The BCR-ABL1 fusion oncoprotein has an increased tyrosine kinase activity and plays a central role in the CML pathogenesis. It promotes cell growth and replication, and inhibits apoptosis, by constitutively activating targets in several signaling pathways, including the JAK/STAT (cell growth and differentiation), PI3K/Akt (cell metabolism), and the Ras/MEK pathway. This influences leukemogenesis by creating a cytokine-independent cell cycle with aberrant apoptotic signals in response to cytokine withdrawal. The chromosomal translocation leading to the *BCR-ABL1* fusion and the production of a hyperactive tyrosine kinase is believed to be both necessary and sufficient to initiate CP-CML. As leukemic hematopoiesis in CP-CML, with the exception of the very primitive leukemic stem cells, is dependent on the tyrosine kinase activity the disease is easily controllable by tyrosine kinase inhibitors. However, subclones of leukemic progenitor cells resistant to TKIs may emerge with time. These clones usually carry additional point mutations in the *BCR-ABL1* gene which prevent the binding of the TKI to its target by changing the amino acid sequence of the target tyrosine kinase domain. Second- and third-generation TKIs have been developed to overcome this acquired resistance to the initial TKI treatment (see section "**Management and Therapy**"). *BCR-ABL1* copy number gains may also confer resistance to TKIs.

Disease progression from CP to advanced phase (AP or BP) is associated with clonal evolution. Other chromosomal abnormalities (in addition to the Ph-chromosome which persists) appear in approximately 80% of patients entering AP or BP. These may include the so-called major route karyotypic abnormalities: an additional or double Ph-chromosome, isochromosome 17q, and gains (usually trisomies) of chromosomes 8 or 19. The presence of these abnormalities at diagnosis is usually associated with a worse prognosis.

The molecular bases of the transformation are poorly known. AP and BP have similar molecular characteristics, suggesting that the bulk of genetic changes occurs at transition from CP to AP-CML. Mutations in a number of genes encoding epigenetic regulators (e.g. *ASXL1*, *EZH2*, and *TET2*), proteins involved in cytokine signaling (e.g. *JAK2*), transcription factors (e.g. *RUNX1* and *SETBP1*), and those encoding cell-cycle regulators (e.g. *CDKN2A*, *TP53*, and *MYC*) have been reported. However, genome-wide analysis data are missing and the role of these mutations in the transformation is neither elucidated, nor established. Activation of the β-catenin signaling enhancing the self-renewal activity of granulocyte-macrophage progenitor cells has been suggested as one of the key events in the evolution towards blast crisis. Hyperactivation of the hedgehog signaling through SMO overexpression may also play a role.

An important issue in understanding the pathogenesis and clinical course of CML is the persistence of leukemic stem cells (LSCs). These cells are not eliminated by currently available TKI treatments which have a strong antiproliferative effect in LSCs but induce only modest levels of apoptosis. Quiescent LSCs are particularly resistant to TKI-induced apoptosis. Since several studies have shown that TKIs do inhibit kinase activity in LSCs, the resistance of these cells to TKIs seems to be BCR-ABL1-independent. Various intracellular regulatory mechanisms which contribute to CML LSC maintenance despite medication have been proposed. These include in particular the JAK/STAT, NF-kB, and β-catenin signaling pathways as well as the cell-cycle regulators: Myc, p53, and SIRT2. Further studies are needed to understand the comprehensive network of interactions between different molecules and pathways, and potentially refine targeting of future therapies.

Management and Therapy

CML management is a model example of a successful implementation of targeted therapies into clinical practice. With the introduction of tyrosine kinase inhibitors (TKIs) as the mainstay of CML treatment, the prognosis of CML patients has substantially improved, with the survival rates approaching those of the general population. In particular, CP-CML has become relatively easy to control by medication. BP-CML, however, remains to be associated with a poor prognosis.

The response of CML patients to therapy is defined at three levels: hematological, cytogenetic, and molecular level. In other words, CML treatment aims at hematological, cytogenetic, and molecular remission of the disease. The hematological remission is defined as normalization of CBC and physical examination findings (in particular reversing splenomegaly and hepatomegaly if they were present). Cytogenetic remission means no Philadelphia chromosome-positive cells detected by cytogenetic methods, whereas molecular remission is defined as no *BCR-ABL1* fusion transcripts detected by RT-PCR. However, with the increasing sensitivity of the molecular detection methods, the definition of the latter may become somewhat confusing and requires precise definitions of the detection cut-offs. Moreover, as current therapies fail to eliminate clones of leukemic stem cells which give rise to relapse, it has been suggested that obtaining prolonged treatment-free remission rather than complete disease eradication (as measured on a molecular level) should be set as a goal of CML therapy.

Several TKIs are available for the treatment of CML patients. The first TKI which was approved for the primary treatment of CML patients was imatinib mesylate (imatinib; Gleevec®). Imatinib competitively interferes with ATP binding to the kinase domain of the ABL kinase, thus preventing phosphorylation of tyrosine residues on its substrates. Imatinib generics also exist but their efficacy has been questioned.

Second-generation TKIs were initially developed for the treatment of patients resistant to imatinib. These include dasatinib (Sprycel®), nilotinib (Tasigna®), and bosutinib (Bosulif®). Dasatinib and bosutinib inhibit both SRC and ABL kinase activity, whereas nilotinib is an imatinib analogue. All the three agents induce a more rapid and deeper molecular response than imatinib. However, their impact on overall survival rates is only marginally better than that of imatinib and they are associated with more severe toxicities. Dasatinib and nilotinib are approved by the US Food and Drug Administration (FDA) also for the primary treatment of CP-CML patients, while bosutinib is approved for treatment of any phase CML patients who are resistant or intolerant to other TKI therapies, including imatinib.

A third-generation TKI, ponatinib (Iclusig®), was developed to specifically target *BCR-ABL1* with the *T315I* mutation which confers high-level resistance to imatinib and all second-generation TKIs. Surprisingly, it was found to be clinically effective against BCR-ABL1 with any mutation arising during treatment with other TKIs and it induces the highest response rates after failure of any second-generation TKI treatment. However, it is associated with severe toxicities, in particular cardiovascular complications (including fatal myocardial infarctions and heart failure) even in patients without known cardiovascular risk factors, and severe hepatotoxicity.

Acquired resistance to TKIs which develops during treatment, at least in part due to point mutations in the *BCR-ABL1* gene (see section "**Genetics**"), is a recurrent problem in the management of CML patients. CML with the most common mutations which appear under imatinib treatment can be effectively treated by some of the second-generation TKIs. The *V299 L* and *F317I BCR-ABL* mutations confer relative resistance to dasatinib, the *V299 L* mutation to bosutinib, and the *Y253H*, *E255V/K*, and *F359 V/C* mutations make the leukemic cells resistant to nilotinib. The *T315I* mutation confers high resistance to all of the TKIs but the third-generation drug ponatinib.

Management of CP-CML

The choice of the first-line TKI treatment for the CP-CML patients is based on the risk score, patient's age, comorbidities and ability to tolerate therapy which are weighed against the toxicity profile of the potential TKI treatment.

Risk stratification of patients newly diagnosed with CP-CML is usually performed using either the Sokal, or Hasford (Euro) scoring system. The Sokal scoring takes into account patient's age, spleen size, platelet count, and the proportion of blasts in the peripheral blood. The Hasford system additionally includes the eosinophil and basophil counts in the peripheral blood. The European Treatment and Outcome Study (EUTOS) score which takes into account the percentage of basophils and eosinophils in the blood and the spleen size seems to be more accurate. However, its predictive value has not yet been validated in clinical trials and many clinicians prefer to use the other scores, even if they were validated with older treatments (chemotherapy) and not with TKIs.

Intermediate- and high-risk patients have been shown to have higher rates of molecular response and lower risk of disease progression when treated with second-generation TKI therapy. Therefore, dasatinib, nilotinib, or bosutinib (depending on the national clinical guidelines and health policies by country) are the first-degree recommendation in these patients. Imatinib remains an alternative in case of clinically significant intolerance to these treatments. Imatinib is also frequently the frontline treatment of choice for low-risk CML patients, even though in some countries second-generation TKIs are preferred, after a careful consideration of the toxicity profiles. Dasatinib or bosutinib may be preferred in patients with a history of arrhythmias, heart disease, pancreatitis, or hyperglycemia, whereas for patients with a history or lung disease or deemed to be at risk of developing pleural effusions, nilotinib or bosutinib may be the best options.

Cytopenias (anemia, neutropenia, and thrombocytopenia) resulting from hematological toxicity of TKI treatments are managed with transient interruptions of the TKI therapy and dose modifications. These controlled treatment pauses should not be confused with patient-decided nonadherence to therapy which may lead to undesirable clinical outcomes. Patients should be educated that

any treatment interruptions should be done only under strict medical control and that their adherence to therapy is of critical importance. Frequent follow-up visits and monitoring of response to treatment are also helpful to this effect.

Monitoring response to primary TKI treatment is key to planning further therapy. Assessing molecular response using the so-called response milestones quantitatively defined by levels of *BCR-ABL1* transcripts detectable with RT-PCR at defined time points following the treatment is highly recommended. International Scale (IS) has been proposed to standardize molecular monitoring across different laboratories. Patients who do not meet thus defined response milestones following primary therapy, are treated with adequate second-line treatment. Patients with appropriate response to the primary treatment used to be deemed to continue the treatment indefinitely. However, the possibility to stop the primary TKI treatment in a subset of patients achieving deep molecular response has been recently widely debated. Based on results of some clinical studies, the US National Comprehensive Cancer Network (NCCN) expert panel states that TKI discontinuation is feasible, preferably in a clinical trial setting, in some carefully selected patients who achieved deep molecular remission and maintained it for at least 2 years.

Myelosuppressive agents, which used to be the mainstay of treatment to convert CML patients from uncontrolled to normalized presentation, are now used exclusively to reduce WBC counts and in CP-CML patients with hyperleukocytosis and to alleviate acute symptoms of leukostasis, hyperviscosity, and tissue infiltration while waiting for a molecular confirmation of the CML diagnosis and definitive treatment. The most common of the myelosuppressive agents is hydroxyurea, an inhibitor of deoxynucleotide synthesis. WBC counts can also be rapidly and safely lowered by leukapheresis using a cell separator.

Management of Advanced Phase (AP or BP) and Relapsed CML

Advanced phase disease, especially blast crisis, has an adverse prognosis. The prognosis of patients who progressed from CP-CML to advanced-CML while on TKI treatment is particularly poor.

TKI therapy is the recommended first-line treatment for all patients with a de novo diagnosed AP-CML and BP-CML. Imatinib induces favorable hematological and cytogenetic response rates in these patients. Dasatinib, nilotinib, bosutinib, and ponatinib may have an activity in imatinib-resistant CML or in imatinib-intolerant patients. Treatment regimens combining TKI therapy with chemotherapy, such as imatinib with decitabine or cytarabine-based chemotherapy, have been shown to be effective in AP-CML patients by some studies, however the sample sizes were small. Combining imatinib or dasatinib with HyperCVAD regimen (alternated cycles of hyperfractionated cyclophosphamide/vincristine/adriamycin/dexamethasone and high-dose methotrexate/cytarabine) is also effective in patients with lymphoid blast crisis, in particular when followed by allogeneic hematopoietic stem cell transplantation (HSCT).

An alternative for patients with CML resistant to multiple TKIs and for those with the *T315I* mutation is omacetaxine. Being an inhibitor of RNA synthesis, its activity is not affected by mutations in the *BCR-ABL1* gene. Omacetaxine (Synribo®) is approved for treatment of CML patients who have not responded to at least two TKIs. It can also be used as a bridge to HSCT in patients who do not respond to or tolerate any of the TKIs. However, omacetaxine is not equally popular in clinical practice in all Western countries.

Allogeneic HSCT, while no longer used for CP-CML patients, remains the appropriate first-line treatment for the rare patients who present with BP-CML and for those with the *T315I BCR-ABL1* mutation or other mutations in the fused gene which confer resistance to all TKIs, as well as for the extremely rare patients intolerant to all currently available TKIs. It is also often recommended as a second-line treatment in other advanced-phase CML patients.

The prognosis of CML patients who have progressed to advanced-phase disease while on TKI therapy for CP-CML is considerably worse than of those with a de novo advanced-phase CML. Allogeneic HSCT (if possible) and enrolment in a clinical trial are the recommended options for these patients. A treatment with an alternate TKI (a TKI that was not used in previous treatments) or with omacetaxine (if used) is beneficial as a "bridge" to HSCT. TKI in combination with chemotherapy used for acute lymphocytic leukemia or steroids is recommended for patients with BP-CML, whether myeloid or lymphoid, whereas chemotherapy regimens used in acute myeloid leukemia are suitable for CML patients with myeloid blast crisis only. In patients with lymphoid BP, central nervous system involvement may occur. It should be managed according to the standards for acute myeloid or lymphoblastic leukemia. Of note, TKI therapy has not been optimized for patients with central nervous system involvement. Dasatinib may be the best choice as it has been reported to cross the blood-brain barrier.

Donor lymphocyte infusion (DLI) as well as imatinib treatment induce durable molecular response in patients with CML who relapsed following allogenic HSCT. Some studies have suggested that combining these two treatments is even more effective. However, overall, better response rates are obtained in patients who relapsed in chronic phase than in those relapsing in advanced phase. The data on the use of other TKIs for treatment of relapsed CML are still limited. Interferon alfa may be used in combination with TKIs for treatment of refractory cases.

Pediatric CML

CML is rare in children and no specific clinical guidelines exist. Many pediatric oncologists follow the guidelines designed for adults, even though clinical manifestations and host factors in children are different. So far, imatinib and dasatinib are the only TKIs approved for the primary treatment of children with CML by FDA. There is little data on the safety and efficacy of other TKIs in children. The use of risk scores has not been validated in childhood CML patients, either, and their use to guide treatment decisions is not recommended.

Prospective Vision

The key to increasing the proportion of CML patients achieving durable treatment-free remission is understanding and overcoming the resistance of leukemic stem cells to the existing treatments. Several potential molecular targets other than the BCR-ABL1 kinase have been identified, including members of the JAK/STAT and p53 pathways, Wnt signaling molecules as well as epigenetic modulators. Among those, the efficacy of inhibiting the JAK2 kinase, a downstream target of the BCR-ABL1 kinase, with ruxolitinib is currently under investigation in clinical trials. Targeting inflammatory signaling also seems a promising direction. Identification, isolation and precise molecular characterization of leukemic stem cells that persist after primary TKI treatment using single-cell technologies, so far remaining a challenge, would help to identify other potential targets.

Further Reading

Bhatia, R., 2017. Novel approaches to therapy in CML. Hematology. American Society of Hematology. Education Program 2017 (1), 115–120.
Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th ed. Wolters Kluwer, Philadelphia.
Jabbour, E., Kantarjian, H., 2018 Mar. Chronic myeloid leukemia: 2018 update on diagnosis, therapy and monitoring. American Journal of Hematology 93 (3), 442–459.
National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Chronic Myeloid Leukemia. Version 4, January 24, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site.
International Agency for Research on Cancer, 2017. In: Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. (Eds.), *(2017) International Agency for Research on Cancer*. WHO classification of tumours of hematopoietic and lymphoid tissues. Revised 4th edn.

Relevant Website

Clinical trial information at the US National Library of Medicine, National Health Institutes—https://clinicaltrials.gov/.

Colorectal Cancer: Diagnosis and Treatment

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Abbreviations

5-FU 5-Fluorouracil
AFI Autofluorescence imaging
APR Abdominoperineal resection
CapeOX Capecitabine with oxaliplatin
CEA Carcinoembryonic antigen
CRC Colorectal carcinoma
CT Computed tomography
CTC CT colonography
DRE Digital rectal examination
EGFR Epidermal growth factor receptor
EUS Endoscopic ultrasound
FDA US Food and Drug Administration
FICE Flexible spectral imaging color enhancement
FIT Fecal immunochemical test
FLOX Bolus 5-FU/LV with oxaliplatin
FNA Fine-needle aspiration
FOLFOX 5-FU/LV with oxaliplatin
MMR Mismatch repair
MRI Magnetic resonance imaging
MSI Microsatellite instability
MSI-H High levels of microsatellite instability
MSI-L Low levels of microsatellite instability
MSS Microsatellite-stable
NBI Narrow band imaging
NCCN US National Comprehensive Cancer Network
LAR Low anterior resection
LV Leucovorin
PET Positron emission tomography
SBRT Stereotactic body radiation therapy
TME Total mesorectal excision
VEGF Vascular endothelial growth factor

Definitions

Carcinoma of the colon and rectum (colorectal carcinoma, CRC) is a malignant neoplasm which originates in the large bowel and invades through the muscular mucosae into the submucosa. Over 90% of CRCs are adenocarcinomas.

Presentation and Diagnosis

Most CRCs are located in the sigmoid colon and rectum but a proportion of CRCs located more proximally increases with age. Two-thirds of colorectal cancers occur in the left colon and one-third in the right colon, although women more often develop right-sided tumors. About 20% of CRCs develop in the rectum.

Patients commonly present with hematochezia and anemia due to bleeding from the tumor. Right-sided colonic lesions are often asymptomatic. Most typical symptoms—if they appear—include dull and ill-defined abdominal pain, bleeding, and symptomatic anemia causing weakness, fatigue, and weight loss. Left-sided lesions commonly result in changes in bowel habits, bleeding,

gas pain, decrease in stool caliber, constipation, and colonic obstruction. In some cases, only distant metastases, in particular those to the liver, cause the initial symptoms in which case patients present with some liver dysfunction.

Colonoscopy is currently the gold standard to detect colonic polyps and CRC. It allows to observe the mucosal surface of the entire large bowel and has a high sensitivity for detecting cancer and all classes of precancerous lesions. It also has an advantage of a single-session diagnosis and treatment, and—together with the fecal immunochemical test (FIT)—is the cornerstore of screening for CRC. Detection of non-protruding lesions can be improved by using contrast dyes (chromoendoscopy) or confocal endoscopy. In addition, other electronic image enhancement techniques, such as narrow band imaging (NBI), autofluorescence imaging (AFI), I-scan, and flexible spectral imaging color enhancement (FICE), have been shown to improve the accuracy of polyp characterization but their added value to the detection of polyps is still being evaluated.

Capsule colonoscopy has been approved by the US Food and Drug Administration (FDA) for imaging the proximal colon in patients with previous incomplete colonoscopies and more recently for patients who need colorectal imaging but who are not candidates for colonoscopy or sedation. As it is non-invasive, it allows to avoid the risks associated with colonoscopy, such as perforation or bleeding. However, the bowel preparation is more extensive than for colonoscopy.

Computed tomography (CT) colonography (CTC; sometimes also called virtual colonoscopy) has replaced double-contrast barium enema for colorectal imaging. It has been shown to achieve high levels of colonic polyp detection. However, it has limitations in detection of flat and serrated lesions, and it is less sensitive than colonoscopy for detecting polyps smaller than 1 cm. Moreover, associated radiation exposure is usually viewed as a disadvantage of this technique.

Biopsies of all suspicious lesions should be taken for pathological evaluation. This can be done during colonoscopy or using CT-guided fine-needle aspiration (FNA).

Cross-sectional CT imaging, magnetic resonance imaging (MRI), and transrectal ultrasonography may be used to assess the depth of tumor invasion and the possibility of regional and distant metastases. Chest, abdomen, and pelvis CT or MRI with contrast may identify small lung, liver, or intraperitoneal metastases. Positron emission tomography (PET) is also used to determine whether an anatomic lesion of unclear origin is malignant and to assess the spread of the disease.

Management and Therapy

The only universally accepted potentially curative treatment for colorectal cancer is surgery. It frequently follows an endoscopic removal (snare polypectomy, endoscopic mucosal resection, or submucosal dissection) and a pathological review of a primary malignant colonic polyp. In patients with invasive cancer in a pedunculated or sessile polyp (adenoma), no additional surgery is required provided that the polyp has been completely resected and has favorable histological features (low grade, no angiolymphatic invasion and negative margins). However, colectomy should be considered in patients with a completely resected sessile polyp with unfavorable histological features even if the resection margins are clear.

Patients presenting with resectable invasive CRC require a complete staging workup, with the pathological tissue review, total colonoscopy, complete blood counts and chemistry profile, carcinoembryonic antigen (CEA) levels, and baseline CT scans of the chest, abdomen, and pelvis. Elevated preoperative CEA levels indicate that CEA may be used as a prognostic marker for post-operative surveillance as metastases of primary tumors associated with elevated CEA are also likely to give elevated CEA measurements. However, variable specificity and sensitivity of this approach remains a major limitation. Endoscopic ultrasound (EUS) significantly improves the preoperative assessment of the depth of invasion of large bowel tumors, and especially rectal tumors. A combination of EUS to assess rectal tumor extent and digital rectal examination (DRE) to determine mobility should enable precise planning of surgical treatment and identifying patients who may benefit from preoperative chemoradiation. Transrectal biopsy of perirectal lymph nodes can often be taken with EUS guidance. MRI with concomitant administration of rectal contrast might be superior to EUS in the identification of perirectal lymph node metastases.

Curative surgery should excise the tumor with wide margins and maximize regional lymphadenectomy so that at least 12 lymph nodes are available for pathological evaluation. For resectable non-metastatic colon cancer, the preferred surgical procedure is colectomy with *en bloc* removal of regional lymph nodes. The extent of colectomy is defined by the tumor location. It should be such that both the portion of the bowel and the arterial arcade containing regional lymph nodes be resected. Other nodes should also be removed or at least biopsied. In order to be curative, the resection should be complete, including removal of all involved lymph nodes. Obstructing tumors in the right colon are usually managed by primary resection and primary anastomosis. Obstructing tumors in the left colon may be managed with initial decompression (proximal colostomy) or stent insertion followed by resection of the tumor and deferred closure of the colostomy. Recent trends, however, favor extending resection and primary anastomosis to include obstructing tumors in the transverse, descending, and even sigmoid colon.

For rectal tumors, several surgical approaches are possible. Transanal excision is sufficient for selected small low-grade tumors without lymph node involvement. Transanal endoscopic microsurgery (TEM) may facilitate tumor excision through the anus for lesions that can be properly identified in the rectum and some data suggest that it may be associated with better outcomes than transanal excision. In both cases, appropriate tumor margins should also be removed. In all other cases, more invasive transabdominal resection is performed. Depending on the tumor localization, this may be low anterior resection (LAR), proctectomy with total excision of the mesorectum (TME) and coloanal anastomosis, or abdominoperineal resection (APR). TME involves an en bloc removal of the mesorectum, including associated vascular and lymphatic structures, fatty tissue, and mesorectal fascia with the tumor.

Minimally invasive approaches may be alternatives to both open colectomy and invasive surgical excision of rectal tumors. Laparoscopic colectomy has been shown to be equally effective as a staging and therapeutic approach, while shortening the hospital stay and reducing the use of pain medication. Robotic colectomy, even though more expensive and more time-consuming, may also offer substantial advantages.

For patients who have unresectable tumors or are medically unfit for surgery, chemotherapy or chemoradiation is recommended, possibly with the goal of converting the lesion to a resectable state. Patients with resectable high-stage colonic disease (T4 tumors) may be given pre-operative neoadjuvant therapy in an attempt to downstage the tumor. For rectal cancer patients, pre-operative radiotherapy or chemoradiation have been shown to be beneficial.

Adjuvant chemotherapy is recommended for post-surgical patients with stage III (lymph node involvement) or high-risk stage II disease. A number of combined regimens have been and are being tested in clinical trials. The combinations that have shown most clinical benefit in stage III patients and are currently most commonly used are the following: 5-fluorouracil (5-FU) and leucovorin (LV) with oxaliplatin (FOLFOX), bolus 5-FU/LV with oxaliplatin (FLOX), and capecitabine with oxaliplatin (CapeOX, also called XELOX or CAPOX). However, given the plethora of constantly evolving incoming data, there is no international consensus so as to the best regimens. In the same line, the panel of the US National Comprehensive Cancer Network (NCCN) clearly endorses the concept that including CRC patients for treatment in clinical trials should have a priority over therapies considered standard or generally accepted.

The use of adjuvant therapy in stage II patients is more controversial. Some trials have shown that stage II patients with high levels of microsatellite instability (MSI-H) or a deficiency in the expression of mismatch repair proteins (MMRs) are less likely to benefit from adjuvant therapy with fluoropyrimidine alone as well as from 5-FU therapy following surgery than those with low MSI (MSI-L) or those being microsatellite-stable (MSS). However, other studies have shown that the MSI is of a purely prognostic value and does not predict response to treatment. A number of genetic assays depicting characteristic molecular signatures of the tumors have also been designed in an attempt to identify patients at high risk of recurrence. However, their added predictive value for selecting patients more likely to benefit from adjuvant therapy has not been established and none of these tests is currently recommended for routine use by clinical guidelines. Awaiting more clinical trials, the current clinical practice is to offer adjuvant therapy only to selected high-risk patients with stage II disease. The NCCN panel lists FOLFOX, FLOX, and capecitabine with or without oxaliplatin as possible choices, however with 2a category of evidence strength and no preference for any of these options.

Metastases are frequent in CRC patients, with about 85% appearing within 3 years and nearly all within 5 years from initial surgery. The most common metastasis site is the liver, followed by the lungs. 80%–90% of metastatic CRC patients develop unresectable liver metastases (more often metachronous than synchronous). For patients with resectable metastases, surgical resection remains the standard of care, usually combined with perioperative systemic therapy. If resection is not possible, image-guided ablation or stereotactic body radiation therapy (SBRT) are reasonable options. Numerous systemic treatment regimens combining previously mentioned chemotherapeutics with biological agents, like inhibitors of vascular endothelial growth factor (VEGF; e.g., bevacizumab) or endothelial growth factor receptor (EGFR; e.g., cetuximab), are applied in the treatment of both advanced and disseminated metastatic disease, many of them with promising results. However, identifying and validating molecular biomarkers that would allow to reliably stratify patients according to their responsiveness to particular treatment combinations is clearly the need for the future clinical practice.

See also: Colorectal Cancer: Pathology and Genetics.

Further Reading

Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), 2010. WHO Classification of tumors of the digestive system, 4th edn. International Agency for Research on Cancer, Lyon, France.

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.

National Comprehensive Cancer Network (NCCN). NCCN clinical practice guidelines in oncology. Colon Cancer. Version 12018 – January 18, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site.

National Comprehensive Cancer Network (NCCN). NCCN clinical practice guidelines in oncology. Rectal Cancer. Version 4. 2017 – January 18, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Vega, P., et al., 2015. Colorectal cancer diagnosis: Pitfalls and opportunities. World Journal of Gastrointestinal Oncology 7 (12), 422–433.

Colorectal Cancer: Pathology and Genetics

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Glossary

Adenoma Benign epithelial neoplasm of the colorectum, frequently presenting as a polyp.
Aberrant crypt focus Focus of colorectal mucosa with disturbance of crypt architecture, earliest stage of carcinogenesis.
APC Adenomatous polyposis coli gene.
BRAF v-Raf murine sarcoma viral oncogene homolog B.
Chromosomal instability (CIN) Pathway of colorectal cancer development characterized by chromosomal changes (loss, gain or rearrangement).
Colonoscopy Endoscopic examination of the mucosa of the colon.
CpG island methylator phenotype (CIMP) Colorectal cancer characterized by high frequency of gene promoter CpG island methylation.
CTNNB Gene encoding β-catenin.
Desmoplastic Cancer type characterized by extensive development of(fibrous) tumor stroma.
Exophytic Cancer growth type characterized by expansion into the lumen of the affected organ.
Hyperchromasia Characteristic of high basophilic staining intensity of cancer cell nuclei.
KRAS Proto-oncogene corresponding to the oncogene first identified in Kirsten rat sarcoma virus and encoding a p21 GTPase.
Microsatellite instability (MIN) Pathway of colorectal cancer development characterized by high gene mutation rate, as reflected in instability of microsatellite DNA.
Mismatch repair DNA repair mechanism targeting correcting errors of DNA replication.
Mitotic activity Measure of cellular growth activity of a cancer.
MLH1 DNA mismatch repair gene mutL homolog 1.
MSH2 DNA mismatch repair gene mutS homolog 2.
MSH6 DNA mismatch repair gene mutS homolog.
Morphotype Histopathological variant of a cancer.
MUTYH Gene encoding MYH glycosylase, which is involved in the repair of DNA.
NRAS Neuroblastoma RAS viral oncogene homolog.
NSAID Non-steroidal anti-inflammatory drug.
NTRK1 Gene encoding neurotrophic receptor tyrosine kinase.
PD1 Checkpoint protein on T cells blocking them from being activated.
PD-L1 Activating ligand for PD1.
PIK3CA Gene encoding phosphoinositide-3-kinase, a tyrosine kinase receptor.
Pleomorphism Variation is size and shape of cancer cells.
PMS2 DNA mismatch repair gene postmeiotic segregation 2.
Polypectomy Endoscopic removal of colorectal polyp.
Polyp Tumefaction in the shape of a pedunculated mass.
Polyposis Condition characterized by the occurrence of multiple polyps in the colorectum.
POLE DNA polymerase epsilon.
POLD1 DNA polymerase delta1.
pTEN Gene encoding the phosphatase and tensin homolog protein.
SMAD4 Mothers against decapentaplegic homolog 4, gene encoding a signaling molecule in the TGF-β pathway.
TGF-β pathway Signaling pathway triggered by the TGFbeta1 receptor.
TP53 Tumor associated gene.
Wnt pathway Signaling pathway triggered by activation of the frizzled receptor.

Definition

Colorectal cancer is the term used for a malignant epithelial neoplasm in the colon or the rectum. As a rule, this is an adenocarcinoma. Even though the term colorectal cancer suggests that adenocarcinoma of the colorectum is a single disease entity, in reality,

the term covers a variety of conditions differing in etiology, biological behaviour, treatment and prognosis. Colorectal suggests that carcinomas of the colon and of the rectum are similar and effectively in terms of morphology and molecular mechanisms involved this is true. However, the therapeutic approach of rectal cancer differs significantly from that of colon cancer, primarily due to the quite different anatomical setting of the rectum (as it is embedded in the floor of the minor pelvis with genitourinary organs in close proximity). Furthermore, it has become clear that cancer in the right colon (cecum, ascending colon) is different in terms of molecular characteristics and response to targeted treatment from that in the left colon (from the splenic flexure down). Most colorectal cancers are sporadic, but about 20% of colorectal cancers arise in the context of a familial syndrome or as a complication of inflammatory bowel disease, and these cancers have distinct characteristics. Colorectal cancer is one of the most closely studied cancer types and the different signaling pathways involved in the development of colorectal cancer have been elucidated and their association with diagnosis, treatment and outcome clarified. This has resulted in the recognition that colorectal cancers develop in different patterns, which are known as the chromosomal instability (CIN), microsatellite instability (MIN), and CpG island methylator phenotype (CIMP) pathways. Results of recent molecular profiling studies have clearly established molecular and genetic heterogeneity of colorectal cancer beyond these established categories.

Burden

Colorectal cancer is among the most frequent of human cancers with an estimated 1.4 million new cases of CRC occurred worldwide in 2012, representing about 9.4% of all new cancers. Globally, in women colorectal cancer is the third most frequent cancer (after cancer of the breast and uterine cervix) and in men the fourth (after cancer of the lung, prostate, and stomach). However, the age-standardized incidence varies >25-fold around the world. In well-developed industrialized countries of North America, Europe, Australia, New Zealand, and Japan the incidence is around 40–60 per 100,000, but this is much lower in Africa and most Asiatic countries.

A striking difference exists between high and low incidence countries in the average age at which colorectal cancer is diagnosed (Fig. 1). In high incidence countries around 5% of cases are diagnosed before the age of 40 while in low incidence countries, rates in this age category as high as 35% have been reported. There is a trend for the incidence rate in low incidence countries to increase, while in high incidence countries the incidence is stable (in Europe) or even decreasing (North America). Of note, among immigrant populations from low incidence countries to a high incidence country the incidence rate has shown a rapid increase towards that of the newly adopted country. This is an important argument in favor of environmental factors as etiological agents in colorectal cancer.

In the US, the burden of colorectal cancer has shown a decrease, both in terms of incidence and death rate (the latter from 28 deaths per 100,000 in 1975 to 14 per 100,000 in 2013). The incidence showed a similar reduction (by almost 50%). In European countries, in the same period colorectal cancer mortality increased for men (about 5%) but decreased (by 15%) for women. However, between European countries significant differences exist. Among men and in women in countries like Austria, Switzerland and the United Kingdom the reduction in mortality was even as high as 30% while in the Netherlands this was about 15%, but in central and eastern European countries mortality rates remained stable. Overall, reduction in mortality rate seems to be most convincing in high resource countries where specialized care and screening services such as endoscopy are available.

Screening for colorectal cancer is done through the fecal occult blood test, which has a reasonable sensitivity but low specificity. Colonoscopy is increasingly advocated, as colonoscopic polypectomy in principle would allow eradication of the disease. Molecular testing for disease-specific DNA abnormalities in feces or in the blood is under development, but not yet routinely applied. Likewise, hopes are high that screening might be feasible through "liquid biopsies," testing peripheral blood for the presence of abnormal (tumor) DNA or RNA or circulating tumor cells. Promising data have been obtained in an experimental setting but confirmation in clinical trials is still pending.

Risk Factors

Environmental Factors

Numerous epidemiological studies have documented risk factors for colorectal cancer. A high incidence is consistently observed in affluent "Western" populations with a diet rich in calories and animal fat and a sedentary lifestyle. This translates into obesity, meat consumption, smoking and alcohol consumption as important risk factors which are potentially amenable to improvement. Immigrants from low-incidence populations to a high incidence population have consistently shown an increase in incidence rate. Various factors, such as dietary consumption of fruits, vegetables and whole grains, dietary supplements such as calcium and vitamin D, prolonged use of nonsteroidal anti-inflammatory drugs, estrogen replacement therapy in women and physical activity are associated with lower incidence rates. Molecular pathways underlying these epidemiological associations have not been elucidated. This is a rather complex issue as in dietary factors macro- and micronutrient-composition of foodstuff and methods of food preparation are variables not easy to assess. In addition, hormonal effects and genetic characteristics differ between individuals. A noticeable exception is the effect of NSAID's. Large trials have been conducted to study the effect of these drugs on the incidence of colorectal adenoma, the precursor lesion of colorectal cancer, and effective chemoprevention using cyclooxygenase inhibitors has been documented. Other prevention strategies, such as increased fiber content of the diet and reduced intake of fat, have shown less convincing effects.

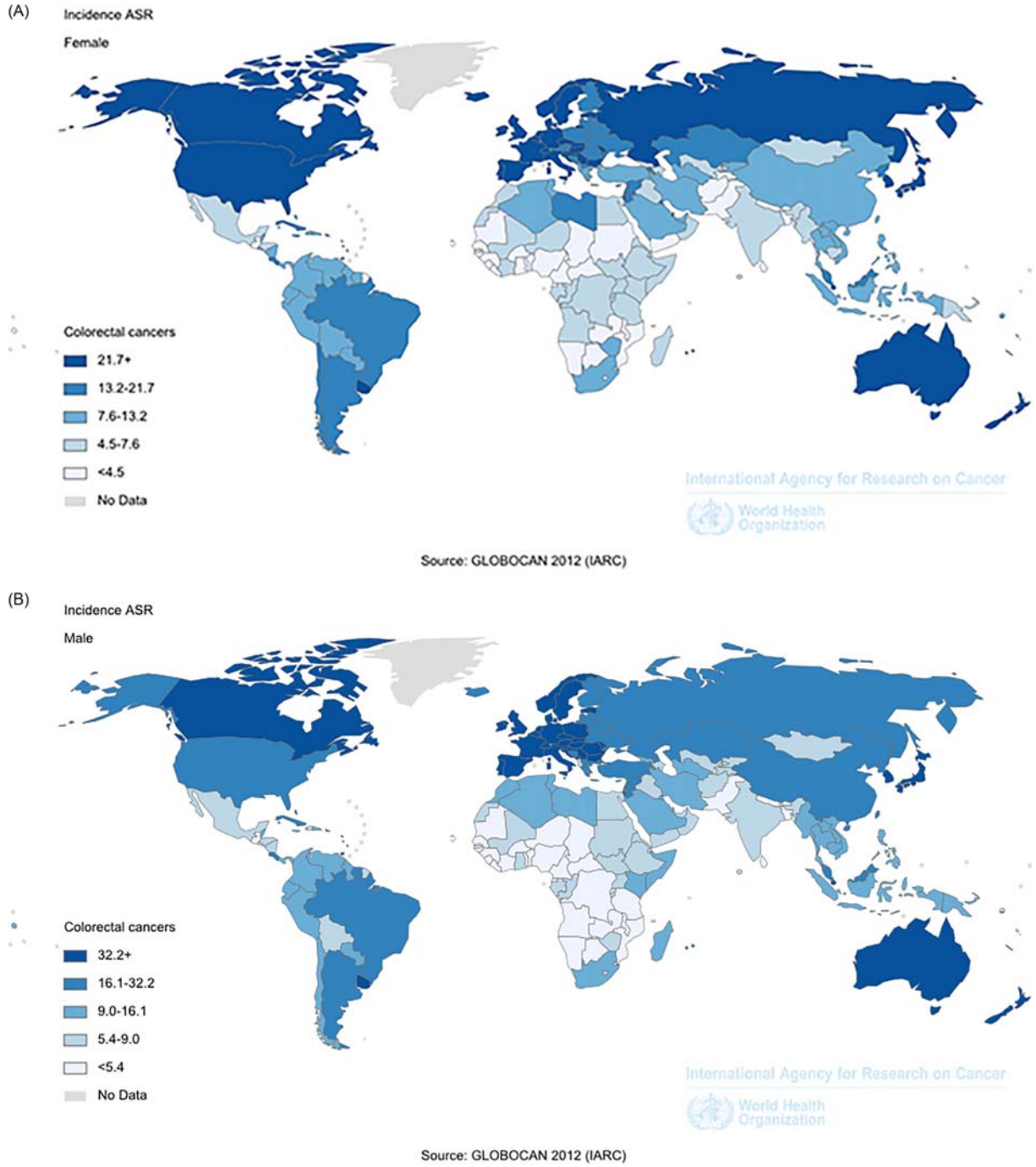

Fig. 1 Global incidence of colorectal cancer per gender (A) Females; (B) Males. Source: Globocan, 2012, http://globocan.iarc.fr.

Genetic Factors

More specific risk factors for colorectal cancer are genetic abnormalities, responsible for the different forms of familial colorectal cancer. These include familial adenomatous polyposis and its variants, Lynch syndrome, MUTYH-associated polyposis, DNA Polymerase-E or D1 deficiency related colorectal cancer and Peutz-Jeghers syndrome. Identification of the genetic abnormalities responsible for these syndromes has provided detailed insight into the molecular pathogenesis of colorectal cancer. Present understanding of the development of colorectal cancer indicates that colorectal cancer can develop along several distinct pathways: the chromosomal instability (CIN) pathway, the microsatellite instability (MIN) pathway and the CpG island methylator (CIMP) pathway. These will be discussed in detail in the paragraph on molecular pathology and genetics.

Chronic Inflammation

Chronic inflammatory bowel disease, notably when longstanding and active, has been recognized as a risk factor for colorectal cancer. This is more important for ulcerative colitis than for Crohn disease. It is assumed that DNA damage due to oxygen radicals in the context of active inflammation is involved. Chronic inflammation in the context of diverticular disease does not carry an increased risk for colorectal cancer.

Other Risk Factors

Although to some extent anecdotal, therapeutic pelvic irradiation and ureterosigmoidostomy have also been associated with colorectal cancer.

Pathology

Macroscopy

In general, early colorectal cancers will be found as a sign of progression in a precursor lesion, as a rule a pedunculated adenomatous polyp (Fig. 2). Size and architecture of these polyps can suggest malignancy: large adenomatous polyps of villous architecture (Fig. 3) are more frequently associated with malignancy and hence are called "high risk."

Advanced colorectal cancers most often present either as an exophytically growing mass or as a mass deeply infiltrating the bowel wall which tends to result in stenosis. The surface of both is often ulcerated. The exophytic growth pattern is most frequently encountered proximal to the splenic flexure (Fig. 4) while infiltrating stenosing cancers predominate in the distal colon and rectum. This is of significance notably in the rectum, as the proximity of urogenital organs carries a risk of invasion. These patterns often overlap as cancers which initially grow exophytically invariably will progress to infiltrate into the bowel wall.

Histology

Precursor Lesions

Carcinomas develop from adenomas, in what has become universally known as the "adenoma-carcinoma" sequence. Molecular studies have shown that in adenomas inactivation of the APC/Wnt pathway parallels that observed in colorectal carcinoma. In the adenoma-carcinoma sequence, genome abnormalities accumulate, responsible for the progression of the lesion to invasive cancer. Adenomas are defined as benign, premalignant epithelial proliferations of which the architecture reflects that of the normal colonic mucosa. They can be predominantly composed of glandular structures and then are called tubular (Fig. 2). Tubular adenomas are most frequently pedunculated, although when small may present as circumscript, slightly elevated patches of the mucosa, so-called flat adenomas. Villous adenomas have a multiple leaf-like architecture and are often sessile (Fig. 3). Tubulovillous adenomas have a mixture of both architectural patterns. Microscopically, adenomas contain glandular or crypt-like structures are composed of crowded hypercellular epithelium, the cells of which show varying degrees of hyperchromasia, pleomorphism and

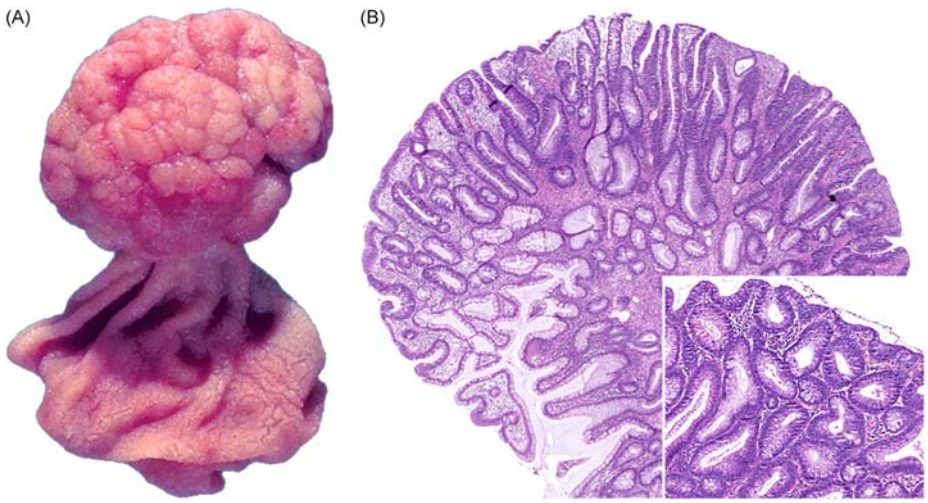

Fig. 2 Characteristic aspect of an adenomatous polyp (tubular adenoma) of the colon. (A) Gross image. Note the pedunculated architecture and slightly lobular surface. (B) Microscopic image. The basis of the stalk is covered with a relatively normal mucosa. In the head of the polyp the glandular architecture is disturbed. At higher magnification (insert) the dysplastic character of the epithelium can be discerned.

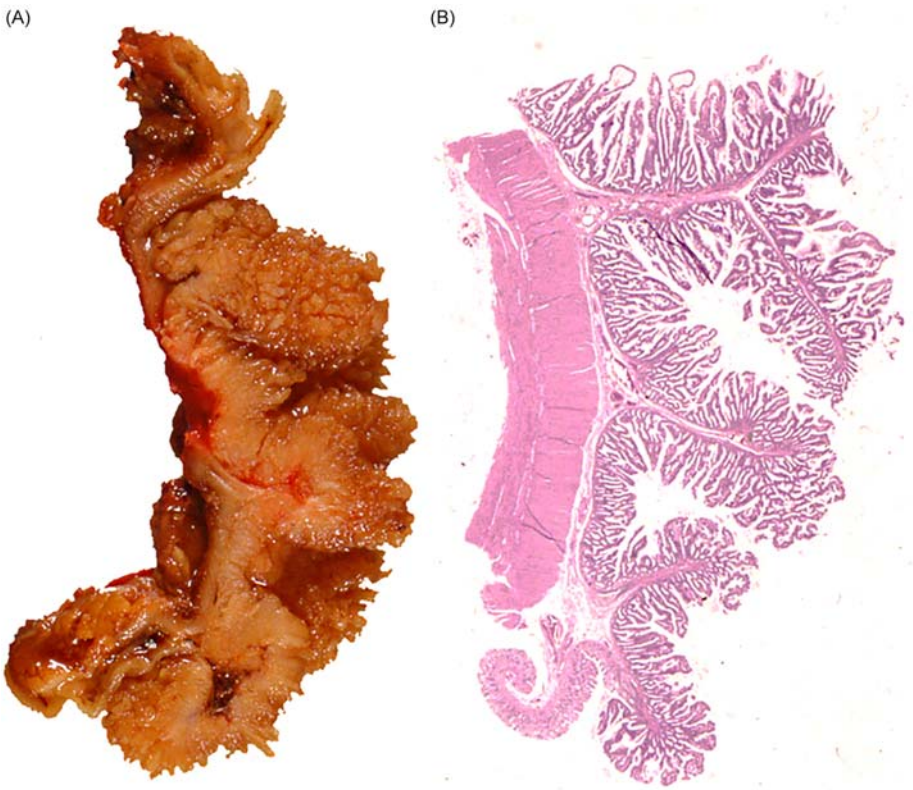

Fig. 3 Characteristic aspect of a villous adenoma. (A) Gross image. The adenoma has a broad ("sessile") implantation in the underlying mucosa. (B) Microscopic image. Note the villous (finger-like)architecture.

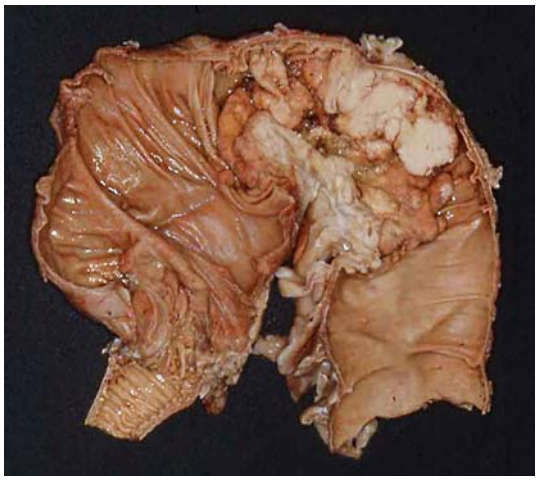

Fig. 4 Colon adenocarcinoma in the cecum of a patient with Lynch syndrome. Note voluminous exophytic growth into the lumen.

mitotic activity. The degree of epithelial cell crowding, nuclear hyperchromasia, mitotic activity and loss of glandular architecture determine dysplasia grade, which goes from low to high on a gradual scale. Adenomas larger than 1 cm and/or high grade and/or of villous architecture are called advanced as they are associated with a 3–5 fold increase in the risk of carcinoma. When in an advanced adenoma an area of high-grade dysplasia shows invasive growth that is limited to the lamina propria (e.g., no invasion of the submucosa) this is called intramucosal carcinoma. Risk of lymph node metastasis remains low as the lamina propria does not contain lymph vessels. Once invasion of the submucosa has developed the lesion is diagnosed as a colorectal carcinoma. It is important to underline that of all adenomas only about 10% would ever progress to a carcinoma.

A relatively recently defined adenomatous lesion is the so-called sessile serrated adenoma or polyp. As the term indicates, these lesions are sessile and occur more frequently in the right colon. The term serrated describes their microscopic appearance: composed

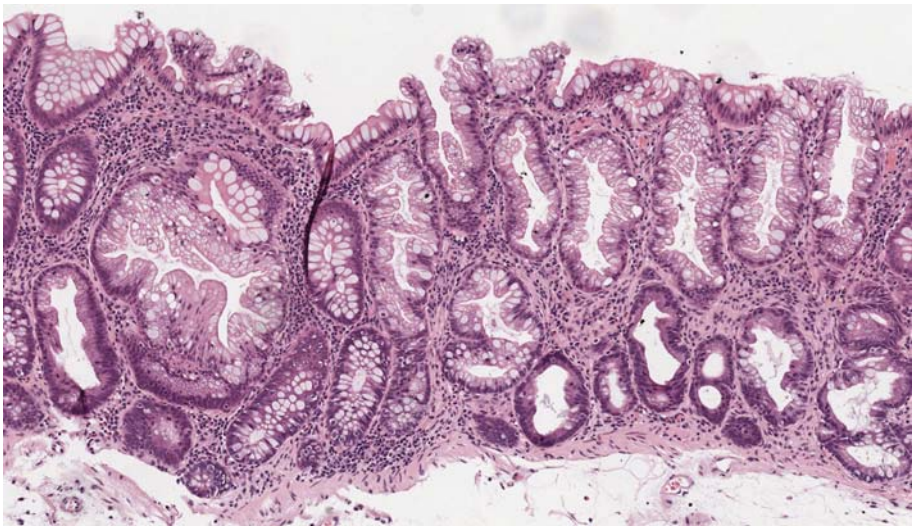

Fig. 5 Microscopic image of a sessile serrated adenoma. The basic architecture of this lesion is crypt-like, but the bases of the glands/crypts show architectural changes (dilation, lateral extension) as well as goblet cell differentiation deep down in the crypts.

of dilated crypts with a serrated (sawtooth-like) architecture of the epithelium and a tendency to branch towards the bottom of the crypt (Fig. 5). The term adenoma is justified by the clonal nature of the epithelial proliferation, as reflected in shared molecular abnormalities (high frequency of promoter methylation, loss of mismatch repair competency, *BRAF* mutation), even though the microscopic appearance of the epithelium is not dysplastic, which is why some insist that these lesions should not be called adenoma. It has become clear that sessile serrated adenomas do carry an increased risk of colorectal carcinoma, even though the risk is lower than that of conventional adenomas and time to progression is longer. For patients with (a) sessile serrated adenoma(s), surveillance as for conventional adenomas is advised. Sessile serrated adenomas rarely occur as a polyposis syndrome (sessile serrated polyposis). This goes along with a significantly higher risk of development of a colorectal carcinoma. Its molecular basis has not (yet) been clarified. Progression of a sessile serrated adenoma towards carcinoma passes through a phase of epithelial dysplasia, rather like that in conventional adenoma.

Another adenoma morphotype is that of traditional serrated adenoma. This is the least frequent of colorectal adenomas. Its architecture tends to be villous. The villous structures are covered with an epithelium that is usually mildly dysplastic with patches of epithelial cells with strikingly eosinophilic cytoplasm and thin elongated (penicillate) nuclei. A characteristic feature is an occurrence of "ectopic crypts": crypt-base like structure emerging from the epithelial lining of the villi.

Adenomas can be removed by endoscopic polypectomy. Theoretically, endoscopic surveillance with the removal of polyps would prevent the development of colorectal carcinoma. Large studies have shown that colonoscopic surveillance reduces colorectal cancer risk and mortality by as much as 70%.

Adenocarcinoma

Once a focus of high-grade dysplasia in any type of adenoma has invaded through the muscularis mucosae into the underlying submucosa, the lesion is classified as an adenocarcinoma. There is some discussion as to what exactly can be regarded as evidence of invasive growth in its initial phase. By definition, invasive growth starts with a breach in the epithelial basement membrane, but this is not a useful histological criterion as it is difficult to visualize and discontinuous basement membranes can also be seen in inflammatory conditions. Often, a "desmoplastic" stromal reaction is used as criterion. A more molecularly defined criterion is evidence of epithelial-mesenchymal transition, which characterizes most forms of invasive growth. Architectural criteria are also used, notably the existence of cribriform growth. Altogether, frank invasive growth is easy to identify for example, when tumor cell clusters are found in the submucosa. Early invasive growth, however, can be difficult to recognize. Frank invasive growth occurs in two patterns: "pushing" with sharp edges of tumor cell fields, and diffusely infiltrating which is frequently accompanied by tumor cell "budding," the occurrence of clusters of fewer than five tumor cells in the invasive front. The pattern of invasion has some prognostic value: diffuse infiltration with tumor cell budding is associated with poorer prognosis.

Several morphotypes of adenocarcinoma commonly are seen (Fig. 6). Most often the carcinoma has a glandular architecture, which is called well or moderately well differentiated. A solid growth pattern is called poorly differentiated. Some carcinomas show areas of mucinous differentiation, when this exceeds 50% of the tumor volume the carcinoma is called "mucinous." Occasionally a carcinoma can be composed (almost) entirely of signet ring cells, this is called a signet ring cell carcinoma. What has emerged recently is the importance of a high number of tumor-infiltrating lymphocytes (TIL), often in combination with clusters of dense follicle-like lymphocytic infiltrate around the tumor. Carcinomas with mucinous differentiation and poorly differentiated carcinomas with a solid growth pattern often contain a high number of TIL, tend to be more frequent in the right colon and mismatch repair

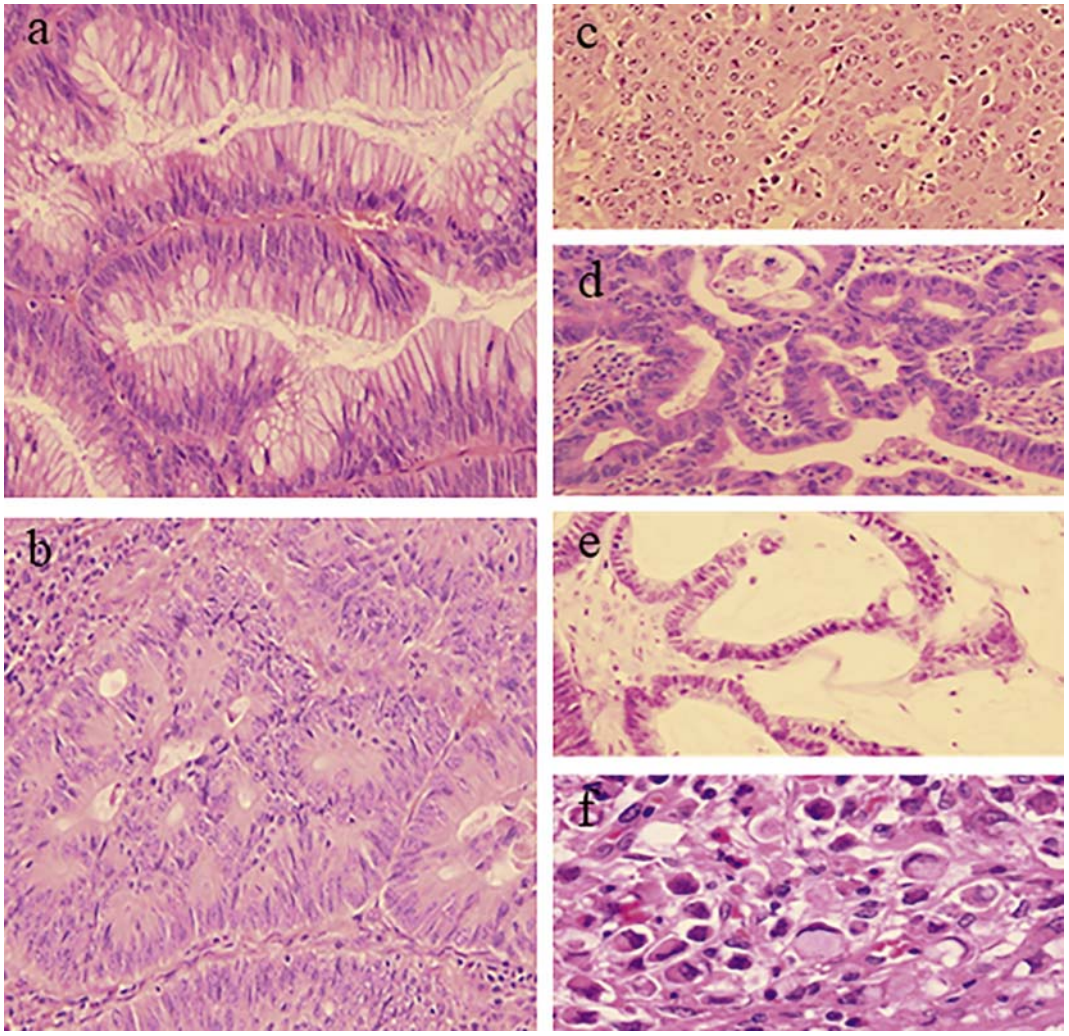

Fig. 6 Histological spectrum of colorectal cancer. Grading is as low grade (A) or high grade (B), the latter characterized by strong architectural and cytonuclear atypia. Solid tumors (C) tend to be infiltrated by lymphocytes and are associated with deficient mismatch repair, as are mucinous tumors (E). Other morphotypes are serrated (D) and signet ring-cell type (F).

deficient. Conversely, mismatch repair competent tumors tend to have a glandular growth pattern, often with areas of cell debris ("dirty necrosis"), and lack TIL. Colorectal carcinomas, however, display notoriously heterogeneous morphology and for a diagnosis of mismatch repair deficient (microsatellite instable) carcinoma, some form of molecular analysis needs to be undertaken.

Grading

Traditionally, colorectal adenocarcinomas are graded in three grades: well, moderately well and poorly differentiated. In grading, architectural as well as cytonuclear features are taken into account. Architectural features pointing towards high grade are the loss of epithelial polarity, loss of gland formation and cribriform growth pattern (Fig. 6A and B). Cytonuclear features include high nuclear pleomorphism and high mitotic activity, which is almost invariably accompanied by significant necrosis. Grading is somewhat subjective and relatively poorly reproducible. Nonetheless, grading is significantly associated with prognosis. Tumor grade is included in the list of parameters taken into consideration in deciding whether or not a stage 2 cancer will require adjuvant postoperative treatment.

Extension and Staging

Adenocarcinomas of the colorectum progress by invasion into the bowel wall. The depth of invasion determines the T-stage (Table 1). Metastasis will be initially to locoregional lymph nodes. Distant metastases occur most frequently in the liver. In

Table 1 TNM classification based staging of colorectal carcinoma

Stage grouping	*Tumor*	*Node*	*Metastasis*	*5y overall survival (%)*
Stage I	T1, T2	N0	M0	>90
Stage IIa	T3	N0	M0	85
Stage IIb	T4	N0	M0	65
Stage IIIa	T1, T2	N1, N2	M0	85
Stage IIIb	T3, T4	N1, N2	M0	60
Stage IV	any T	any N	M1	10

Overall survival data are approximations. T1 tumor limited to the submucosa; T2 tumor infiltrating into the muscularis propria; T3 tumor infiltrating into the subserosal fat; T4 tumor penetrating the serosal membrane and/or infiltrating an adjacent organ. N0 no lymph node metastasis; N1 metastasis in 1–3 regional lymph nodes; N2 metastases in four or more regional lymph nodes, M1 metastases in any distant organ.

advanced stages, metastases can be found in almost any organ. In a clinical context, stage grouping is practiced facilitating decisions on therapy. Stage grouping is explained in Table 1. Stage determines prognosis as well as the therapeutic approach, notably as regards the need for adjuvant therapy.

Molecular Pathology

Colorectal cancer is a classic example of stepwise progression, initially developing as an aberrant crypt focus, passing on to a still benign adenoma, and ultimately progressing to an invasive adenocarcinoma with the capacity to metastatic spread. The cell of origin most likely is a crypt base stem cell, from which a clone of aberrant transformed cells arises through the accumulation of multiple genetic abnormalities. The key signaling pathway affected in colorectal cancer is the Wnt pathway through inactivating mutation of *APC*, activating mutation of *KRAS* and subsequent mutation of *TP53* and genes in the TGF-β pathway (notably *SMAD4*) which confer invasive and metastatic capacity (Fig. 7). As discussed in the paragraph on pathology, many subtypes of colorectal cancer can be distinguished, differing in morphology, genetic background, molecular profile, clinical behavior and response to therapy. We will discuss the three main pathways involved, their relationship to familial colorectal cancer syndromes and how molecular pathology impacts on clinical decision making.

The Chromosomal Instability (CIN) Pathway

The prototypical pattern of the development of colorectal cancer is along the chromosomal instability (CIN) pathway, which represents about 85% of sporadic colorectal cancers. Chromosomal instability is reflected in a high number of allelic losses and gains, including recently discovered chromosomal translocations involving the *NTRK1* gene. CIN cancers have a relatively low gene point mutation rate, contrary to mismatch repair deficient microsatellite instable colorectal cancers. CIN colorectal cancers almost invariably carry a mutation in the Wnt pathway genes *APC* (70%) or *CTNNB1* (30%). KRAS mutations, usually in codons 12 and 13, occur in 45% of cases and constitutively activate the MAP-kinase/ERK signaling pathway which renders them insensitive to

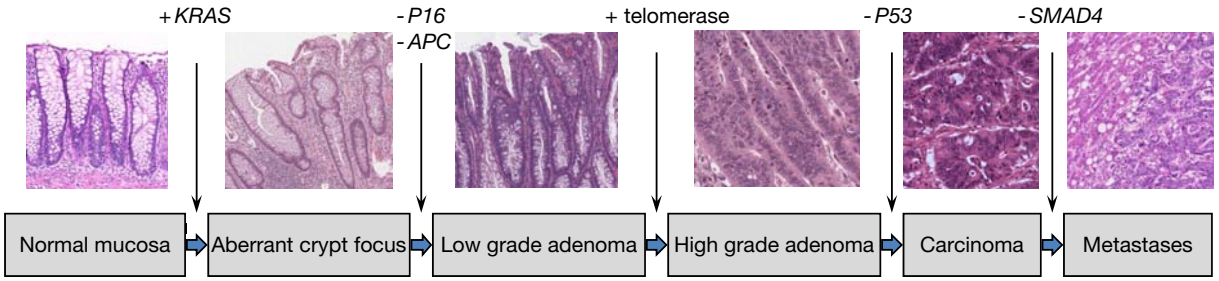

Fig. 7 The classical adenoma-carcinoma sequence of the development of colorectal cancer (after Vogelstein), which follows a CIN pattern of carcinogenesis. The initial lesion is called aberrant crypt focus and is characterized by a slight disturbance of crypt architecture and cytonuclear atypia of crypt cells. These often carry an activating (+) *KRAS* mutation. When an inactivating (−) *APC* mutation occurs epithelial dysplasia (as is shown in the second panel) ensues and an adenoma develops. Low grade adenomas are mostly small and tubular with little cytonuclear and architectural features of dysplasia. Upon telomerase activation (+) high grade features emerge: larger size, villous architecture and high grade cytonuclear features (loss of polarity, nuclear pleomorphism, mitotic activity). Progression towards invasive carcinoma is often accompanied by a *TP53* mutation and progression towards by further molecular events, such as inactivation of SMAD4 (−). Adapted from Bosman, F.T. (2014). World Cancer report 2014, In: Stewart, B.W., Wild, C.P. (eds.) ISBN 978–92–832-0429-9. Page 392. IARC publications. With permission from the publisher.

anti-EGFR therapy. *TP53* mutations are found in 70% of cases. In advanced colorectal cancer loss of SMAD4 (which functions downstream of the TGF-β2 receptor) occurs, which confers poor prognosis. Evidence emerging from The Cancer Genome Atlas (TCGA) studies has added to these classical molecular events >20 genes that are frequently mutated, including *ARID1A, SOX9 and FAM123B/WXT*.

In aberrant crypt foci, small patches of mucosa in which regular crypt architecture is disturbed, mutations in *KRAS* or *APC* gene are found. Aberrant crypt foci with an *APC* mutation are morphologically dysplastic, which signifies progression towards adenoma. Subsequent telomerase is activated, which confers unlimited lifespan and ultimately *TP53* mutation is acquired which is associated with progression from low grade to high-grade adenoma. The TGF-β pathway, including the SMAD family, is involved in the progression from a non-invasive adenoma to an invasive carcinoma. This is illustrated in **Fig. 7**.

The Microsatellite Instability (MIN) Pathway

Around 15% of colorectal carcinomas are characterized by microsatellite instability. This is a result of a deficient DNA mismatch repair system. This corrects mismatches (single base mismatches or short deletions or insertions) that have occurred during DNA replication. When this does not function, mutations accumulate and mismatch repair deficient cells effectively are hypermutated. This is reflected in the variable length of microsatellite repeats, hence the term microsatellite instability. Microsatellite instability can be visualized using a set of defined microsatellite sequences, which are almost invariably affected (the mononucleotides BAT25 and BAT26, and the dinucleotide repeats D2S123, D5S346, and D17S250). Mismatch repair deficiency is due to loss of function of one of the proteins that make up the effector complex of this system: MLH1, MSH2, MSH6, and PMS2. This can be due to a mutation of one of the responsible genes. Most cases of MSI colorectal carcinoma, however, are due to promoter methylation of MLH1 which silences expression of the gene. Patients with Lynch syndrome carry a germ-line mutation of one of the mismatch repair genes. Loss of function of these proteins can be visualized using immunohistochemical staining (**Fig. 8**). This is the most frequently used test approach for mismatch repair deficiency. Sporadic MSI cancers show a relatively high frequency of *BRAF* gene mutations, which are almost invariably of V600E type.

Morphologically, MSI cancers also follow an adenoma-carcinoma sequence. Precursor adenomas may be of the conventional type but more often of the sessile serrated type with dysplasia. The ensuing carcinomas are more often located in the right colon, show mucinous or medullary histology and a host response characterized by a high number of TIL. It is assumed that the hypermutated state of these cancers induces numerous cancer-cell related neoepitopes, which evoke a strong host immune response. MSI

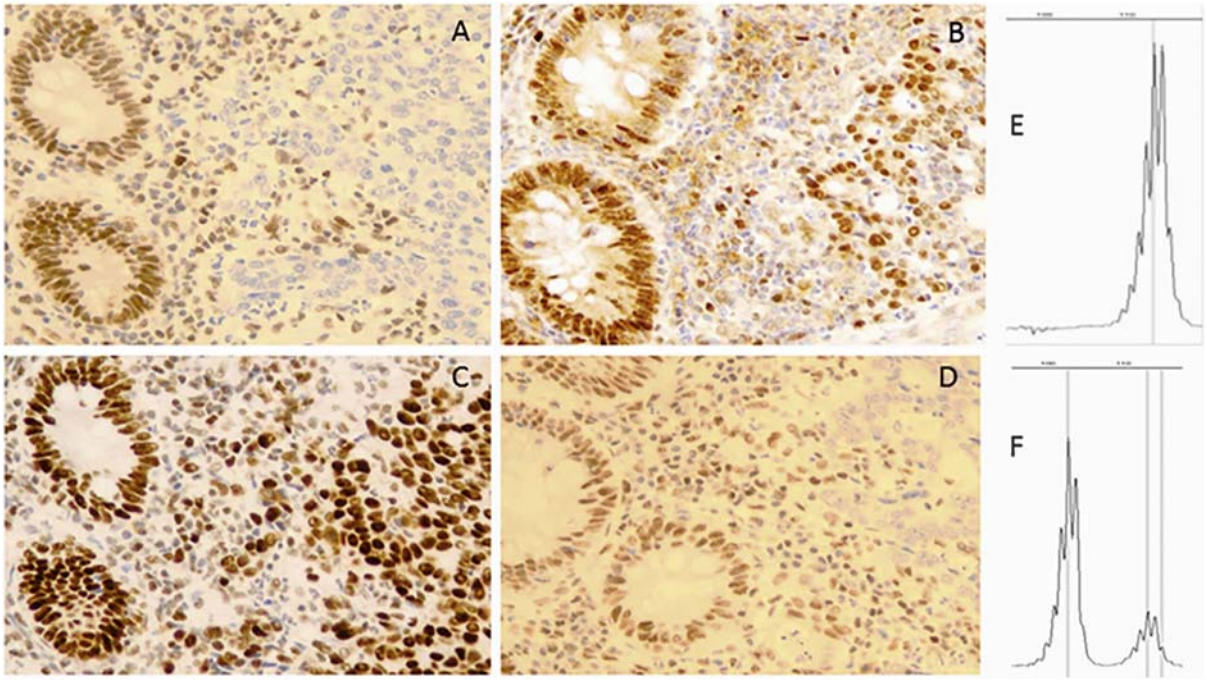

Fig. 8 Mismatch repair deficiency analysis using immunohistochemistry panels (A–C) and microsatellite instability. Immunohistochemical staining for MLH1 (A) and PMS2 (D) shows loss of expression (note nuclear staining of normal cells surrounding the cancer cells). MSH2 and MSH6 (panel B and C) show nuclear staining of cancer cells reflecting normal expression. Panels E and F show microsatellite analysis of a MS stable tissue sample (E) showing a single peak for the microsatellite marker (BAT26) whereas the existence of two peaks (F) indicates microsatellite instability. From Bosman, F. and Yan P. (2015). Molecular pathology of colorectal cancer. *Polish Journal of Pathology* **65**, 257–266. https://doi.org/10.5114/pjp.2014.48094.

cancers have a better prognosis than microsatellite stable (MSS) cancers but tend to be less responsive to 5-Fluorouracil, the standard chemotherapeutic drug used for colorectal cancer (adjuvant) treatment. Recent developments are quite exciting in that MSI cancers (not only colorectal cancers) appear to be highly sensitive to immune-checkpoint (PD1) blocking therapy, which potentially changes completely the treatment of advanced colorectal carcinoma.

The CpG Island Methylator Phenotype (CIMP) Pathway

This pathway is also known as the "serrated pathway," as it closely associated with the development and progression of sessile serrated adenomas (Fig. 9). It shows significant overlap with the microsatellite instability pathway. It is assumed that the earliest molecular event is promoter hypermethylation affecting a wide variety of genes (hence the term CpG island methylator phenotype) early on affecting MLH1 with microsatellite instability as a result, which then facilitates the accumulation of additional genetic abnormalities. In most cases, the *BRAF* gene is mutated (typically the V600E mutation). In more advanced stages of the pathway additionally affected genes correspond to those involved in the CIN pathway.

Colorectal Carcinoma in the Context of inflammatory Bowel Disease (IBD)

Inflammatory bowel disease is the generic term used to cover two specific entities: Crohn's disease and ulcerative colitis. Ulcerative colitis is a chronic inflammatory disease of the colon and/or rectum and in most cases restricted to the mucosa and submucosa. Crohn's disease is also a chronic inflammatory disease but can affect any part of the gastrointestinal tract although most commonly the terminal ileum. The pattern of the inflammatory reaction can be quite similar to that of ulcerative colitis but in a significant proportion of Crohn's disease cases, the inflammation is granulomatous. Inflammation extends through all layers of the bowel wall which may lead to stenosis through fibrosis but also perforation and formation of fistulae. Both conditions have a pattern of extra-intestinal manifestations. Important in this context is that in both conditions longstanding active disease is associated with increased risk of large bowel cancer or (in Crohn's disease) small bowel cancer. In this setting, carcinoma develops through a "dysplasia-carcinoma" sequence. The transcription factor NF-κB provides the link between inflammation and carcinogenesis, as it induces expression of the pro-inflammatory mediators COX-2 and TNF-α, which both play a role in colorectal carcinogenesis. Genome abnormalities in colorectal carcinoma in the context of IBD are similar to those in sporadic CRC but *TP53* mutations often occur early. *APC* mutations are less frequent.

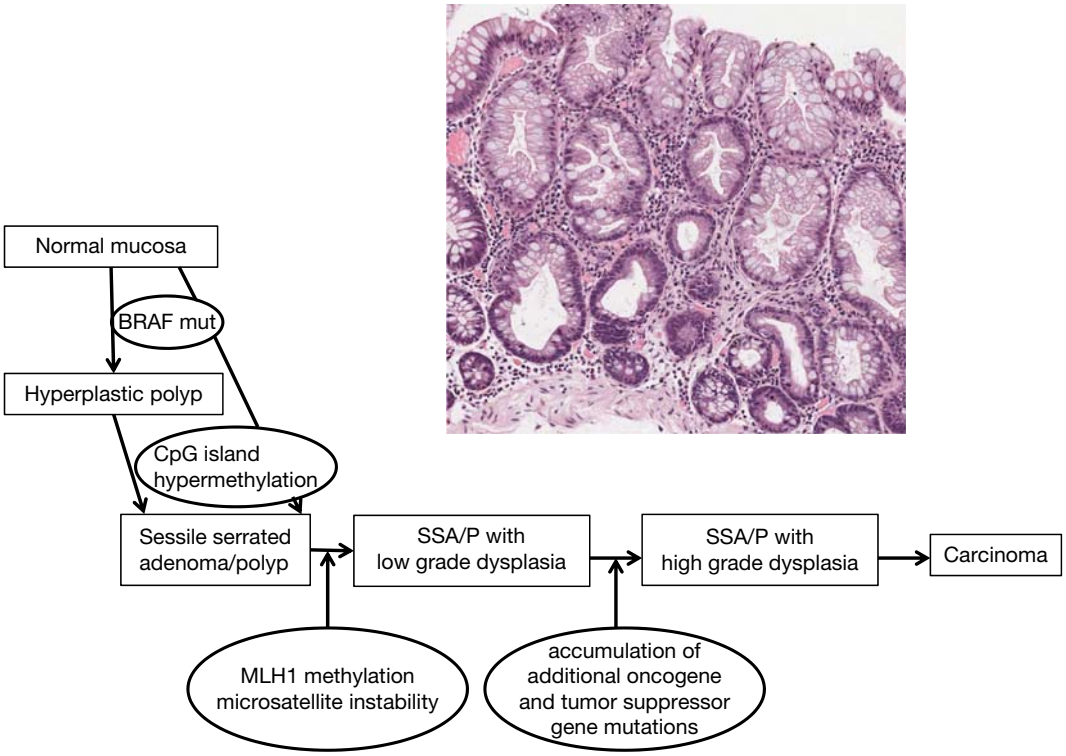

Fig. 9 Molecular pathway involved in the development of sessile serrated adenomas, which largely corresponds to the CIMP pathway. From Bosman, F.T. (2013). Molecular pathology of colorectal cancer. In: Chung, L., Eble, L.N. (eds.) Molecular surgical pathology. New York: Springer, pp. 1–16. ISBN 978–1–4614-4899-0.

Colorectal Cancer Genetics: Familial Colorectal Cancer Syndromes

About 20% of all colorectal carcinomas occur in a familial context: more family members are affected than would be expected by chance. An individual in a family with one or more cases of colorectal cancer has a two to sixfold chance to be affected by this cancer, depending on the number of affected family members. However, in only half of such families can a familial cancer syndrome be identified. Genome-wide association studies have pinpointed a variety of chromosomal loci and single nucleotide polymorphisms, associated with increased colorectal cancer risk. These might be less (if at all) penetrant or represent variants of which the effect depends on gene-environment interactions. For a substantial proportion of familial colorectal cancer cases, therefore, the genetic background still needs to be resolved.

Consequently, about 10% of all colorectal carcinomas arise in the context of a known familial cancer syndrome. Their number gradually increases. The first discovered syndrome is familial adenomatous polyposis coli. A later discovery was non-polyposis hereditary colon cancer or Lynch syndrome. More recent is the MutYH associated polyposis or MAP syndrome and a recently discovered syndrome is associated with DNA polymerase E/D1 deficiency. Other rare polyposis syndromes with increased colorectal cancer risk exist (e.g., Peutz-Jeghers syndrome, juvenile polyposis syndrome and Cowden syndrome).

Familial Adenomatous Polyposis

About 1% of all colorectal cancers occur in the context of familial adenomatous polyposis, of which the Gardner syndrome is a variant. The syndrome is characterized by the development early in life of an increasing number (between hundreds and thousands) of adenomatous polyps in the colon characteristically in the second decade of life (**Fig. 10**) If untreated these will progress to colorectal cancer; mean age of colorectal cancer diagnosis in FAP patients is 39 years. Extracolonic manifestations include adenomas in the stomach and the proximal duodenum, which can progress to carcinoma, and fundic gland polyps in the stomach. FAP-associated benign lesions are desmoids, lipomas, fibromas, sebaceous and epidermoid cysts in the skin, skeletal osteomas, dental abnormalities and congenital hyperplasia of the retinal pigment epithelium (known as CHRPE). Less common extracolonic malignancies are malignant neoplasms of the thyroid, brain, adrenal, hepatoblastoma in the liver and pancreaticobiliary tumors. The syndrome is caused by an autosomal dominantly inherited mutation of the adenomatous polyposis (*APC*) gene on chromosome 5. The APC protein plays a crucial role in the Wnt signaling pathway: in a normal cell it binds β-catenin (which is also integrated into the E-cadherin-catenin cell-adhesion complex) which will be then be degraded in the proteasome. Loss of APC results in accumulation of β-catenin, which migrates to the nucleus with transcription factor activity, promoting transcription of genes of which the protein products stimulate growth, such as MYC and cyclin D1 (**Fig. 11**).

In the context of FAP, >400 different mutations have been reported in the *APC* gene. Different mutations are associated with different phenotypes and distinct genotype-phenotype associations have emerged, as listed in **Table 2**. Most important are the mutations reported in exons 3, 4, 6, and 15 in association with attenuated FAP. In attenuated FAP fewer adenomatous polyps are found and colorectal cancer tends to develop at a more advanced age. Upper gastrointestinal lesions are associated with mutations in exon 15 and those beyond codon 1400. Mutations beyond codon 1444 are associated with the presence of desmoid tumors and those in codons 767–1513 with osteomas. Thyroid cancers are mostly seen with mutations in exons 1–15 and CHRPE's with mutations in exons 9–15 (**Table 3**).

The Gardner syndrome was first described before the discovery of the FAP syndrome or the *APC* gene. It refers to a FAP subtype with numerous gastrointestinal polyps in combination with other neoplasms including osteomas, desmoid tumors, epidermoid cysts and also dental anomalies. *APC* mutations in Gardner syndrome occur in a region encoding for the β-catenin-binding site of the protein. The use of the term Gardner syndrome is discouraged, as it clearly constitutes one of the subtypes of FAP.

The MUTYH-Associated Polyposis (MAP) Syndrome

Patients with the MUTYH-Associated Polyposis (MAP) syndrome have a FAP phenotype but lack a mutation in the *APC* gene. It is estimated that about 2% of colorectal cancers occur in the context of this syndrome. MAP is an autosomal recessive disorder caused

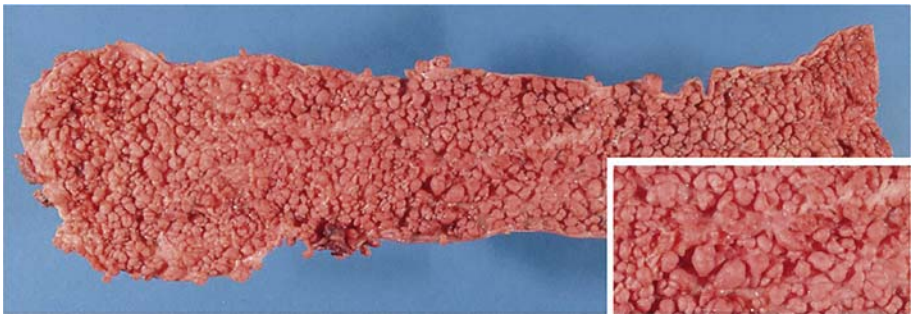

Fig. 10 Colon mucosa from a patient with familial adenomatous polyposis. The mucosa is diffusely seeded with polyps varying in size. Inset shows higher magnification.

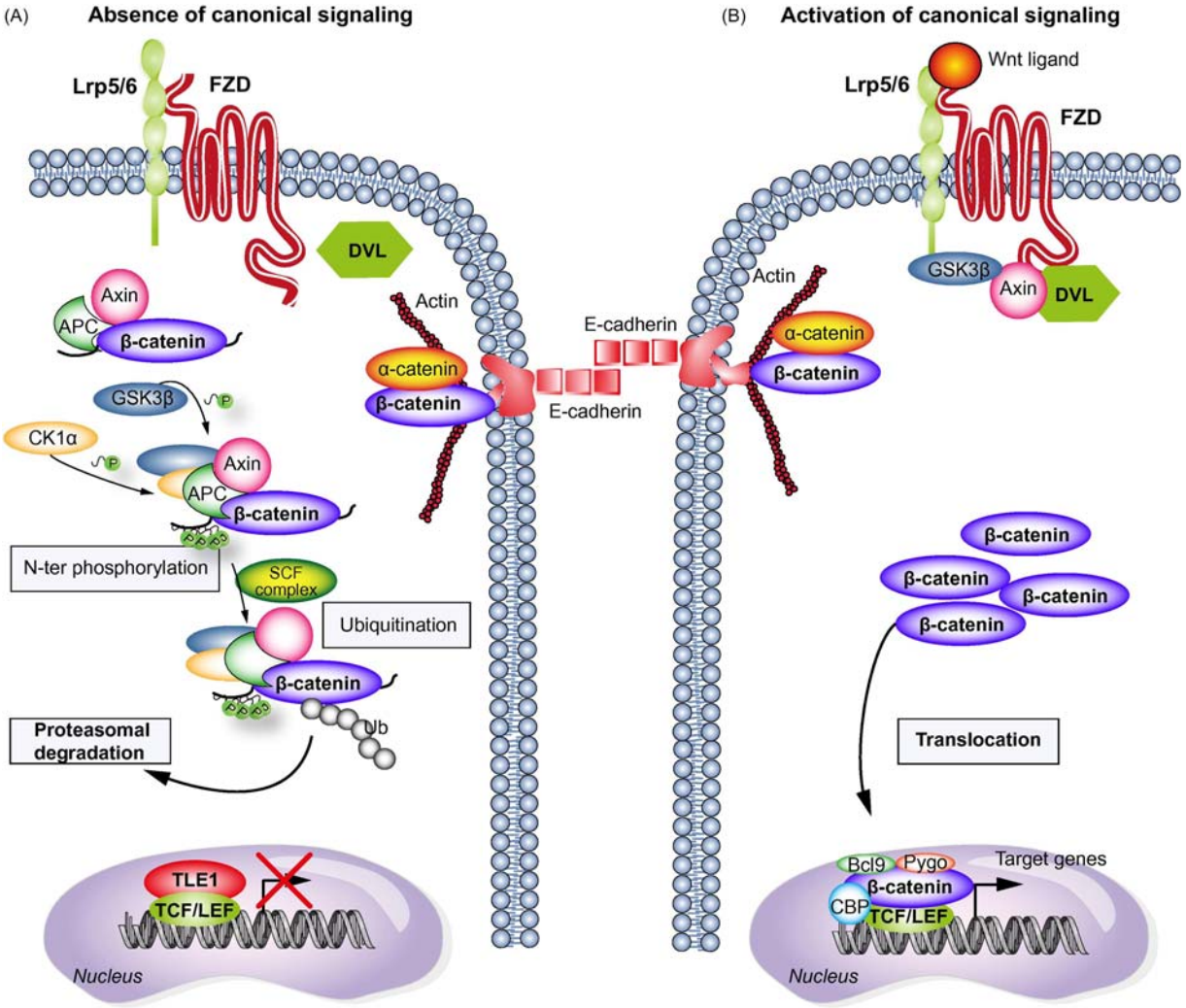

Fig. 11 Schematic representation of the canonical Wnt pathway in the absence (A) or presence (B) of a signaling event. In the absence of signaling β-catenin forms a complex initially with axin and APC, downstream completed with GSK3β and CK1α, which results in ubiquitination of β-catenin and ultimately its degradation. A proportion of β-catenin participates in the e-cadherin/catenin cell-adhesion complex. In the presence of a signaling event (B) β-catenin is not degraded, translocates to the nucleus where it forms a transcription factor in complex with, among others, TCF/LEF, pygopus and Bcl9, activating expression of target genes. From https://www.google.nl/search?q=wnt+pathway&source=lnms&tbm=isch&sa=X&ved=0ahUKEwjo0pX1sv_XAhVsC8AKHW8hCggQ_AUICigB&biw=1920&bih=925#imgrc=PUdZ-3WdhmeWRM.

Table 2 Genotype-phenotype associations for extracolonic manifestations in familial adenomatous polyposis (FAP) and attenuated familial addenomatosis (aFAP)

Syndrome phenotype	*Genotype*	*Characteristics*
FAP	Numerous different mutations reported (over 400)	Frequent extracolonic manifestations
FAP with		
Desmoid tumors	Mutations beyond codon 1444	
Duodenal polyposis	Mutations in exon 15, beyond codon 1400	
CHRPE	Mutations in exons 9–5	
Osteomas	Mutations in codons 767–1513	
Thyroid cancer	Mutations in exons 1–15	
aFAP	>35 mutations described (exons 3, 4, 6, 9, and 15; introns 3 and 9)	Other tumor types and desmoid tumors are less frequent Patterns of extracolonic manifestations not associated with specific mutations Often duodenal polyposis No CHRPE's

Table 3 Familial polyposis syndromes with polyp characteristics and responsible gene

Syndrome	*Polyp characteristics*	*Gene responsible*
Autosomal dominant		
Lynch syndrome	Few adenomatous polyps	*MLH1, MSH2, MSH6, PMS2*
Familial adenomatous polyposis	Numerous adenomatous polyps	*APC*
Polymerase proofreading-associated polyposis	Multiple mostly adenomatous or serrated polyps	*POLE, POLD1*
Peutz-Jeghers syndrome	Hamartomatous polyps also in upper GI tract	*LKB1/STK11*
Juvenile polyposis syndrome	Multiple hamartomatous polyps	*SMAD4/BMPR1A*
Cowden syndrome	Multiple adenomatous, hamartomatous or inflammatory polyps	*pTEN*
Autosomal recessive		
MUTYH associated polyposis syndrome	Multiple adenomatous or serrated polyps	*MUTYH*

by biallelic mutations in the Mut Y homolog (MUTYH) gene, located on chromosome 1p. The gene encodes a protein with an important function in the DNA base excision repair pathway. MAP syndrome resembles familial adenomatous polyposis in that multiple adenomatous polyps of the colorectum develop, with a very high lifetime risk of colorectal cancer. The number of adenomas ranges from very few to hundreds and therefore MAP resembles attenuated familial adenomatous polyposis. Extracolonic manifestations occur, including duodenal adenomatous polyps and adeno(carcino)mas, CHRPE's and breast cancer.

Hereditary Non-Polyposis Colon Cancer or Lynch Syndrome

About 3% of cases of colorectal cancers occur in the context of hereditary non-polyposis colon cancer or Lynch syndrome, the most common hereditary colorectal cancer predisposition syndrome. Lynch syndrome patients carry an 80% lifetime risk of developing colorectal cancer. Cancer occurs also in other organs, including (in decreasing order of frequency) in the endometrium, ovary, duodenum, urinary tract, stomach, pancreas, biliary tree, and brain. Lynch syndrome is autosomal dominant and is caused by mutations in one of the DNA mismatch repair genes mutL homolog 1 (*MLH1*) on chromosome 3p21, mutS homolog 2 (*MSH2*) on chromosome 2p16, mutS homolog 6 (*MSH6*) on chromosome 2p16 and postmeiotic segregation 2 (*PMS2*) on chromosome 7p22. The most frequent (about 80%) are *MLH1* and *MSH2* mutations. In the mismatch repair protein complex, which altogether is composed of seven DNA mismatch repair proteins (in addition to those previously mentioned *MLH3*, *MSH3* and *PMS1*), these work sequentially to initiate repair of DNA mismatches, base mismatches or small insertions or deletions that occur during DNA replication. MLH1 deficiency is relatively frequent in cancer, not through a mutation of the gene but through epigenetic silencing by methylation of the *MLH1* promoter. MSH2 deficiency can also occur through gene-silencing by transcriptional read-through of *TACSTD1*, a gene directly upstream of *MSH2* and encoding the Ep-CAM protein. Because of a germline deletion of the last exons of *TACSTD1*, its transcription is extended into *MSH2* with functional loss of MSH2 activity.

Around 15% of colorectal cancers are MisMatch Repair deficient (dMMR) and these characteristically show microsatellite instability (MSI), increased variability in length of the nucleotide repeats that occur throughout the genome. The large majority of these dMMR cancers show epigenetic silencing of MLH1 through promoter methylation. Only patients with a germline mutation in one of the MMR genes have the Lynch syndrome phenotype. Not all replication errors due to MMR are pathogenic. Mutations are significant when they occur in key genes in processes such as cell proliferation, apoptosis or DNA repair, with functional significance in the context of cancer development. MSI is detected through analysis of a consensus set of gene markers frequently affected by dMMR (the mononucleotides BAT25 and BAT26, and the dinucleotide repeats D2S123, D5S346, and D17S250) which show variations in length in MSI. When three or more markers are unstable this is designated as MSI-H(igh). dMMR can also be detected through immunohistochemical staining of the four MMR genes mentioned above. Loss of staining for one of the proteins qualifies the case as MSI (**Fig. 8**). In case of MLH1 loss, the *MLH1* gene might be mutated or, alternatively (and most frequently) silenced through promoter methylation. To diagnose such a case as sporadic or Lynch syndrome, the methylation status of the promoter needs to be assessed. When non-methylated the patient will be further screened for *MLH1* mutation. *BRAF* mutation status can be considered a surrogate marker, as in Lynch syndrome-associated colorectal cancer *BRAF* mutation is exceptionally rare. dMMR cancers are hypermutated: they show a very high frequency of gene point mutations and significantly fewer chromosomal rearrangements and allelic imbalances. While of colorectal cancers about 15% is MSI, this is much more frequent in gastric and esophageal cancer, non-small cell lung cancer and squamous cell carcinoma of head and neck. This has gained clinical significance recently as MSI cancers have been found to respond to immune checkpoint blocking therapy (PD1/PD-L1 inhibitors): the PD1 inhibitor Pembrolizumab was approved for recurrent solid tumors, regardless of the histological tumor type.

In Lynch syndrome, the dMMR colorectal cancers develop through an adenoma-carcinoma sequence with adenomatous polyps as precursor lesions. However, even though polyps may be multiple, they are never sufficiently numerous to call the condition polyposis. Common characteristics of (Lynch syndrome-associated and sporadic) dMMR colorectal cancers are typical localization in the right colon, mucinous or medullary histotype, and a striking lymphoid host reaction, partly as TIL's and partly as peritumoral

secondary lymphoid follicles. The prognosis is better than that of mismatch repair competent cancers, but mismatch repair-deficient cancers respond less well to adjuvant chemotherapy. Therapeutic options have changed significantly with the discovery of their response to immune-checkpoint blocking drugs.

DNA-Polymerase Epsilon/D1 Deficiency Syndrome

As with the three syndromes described above only a fraction of the familial cases of colorectal cancer could be explained, the search has been on to further identify inherited risk factors, through whole genome sequencing combined with linkage disequilibrium studies in families with multiple colorectal adenomas and early-onset colorectal cancer.

Such studies identified germline mutation of the DNA-polymerase epsilon and D1 gene (*POLE* and *POLD1*) as a new autosomal dominant syndrome with high-penetrance predisposition to colorectal cancer. The exonuclease domain of the protein has proofreading activity and removes wrongly incorporated nucleotides during DNA replication. Mutations in the part of the gene encoding for this domain result in lack of fidelity of DNA replication. The ensuing hypermutated phenotype contributes to tumorigenesis. The associated syndrome is called polymerase proofreading-associated polyposis. In families with germline *POLE* mutations, a predisposition for multiple adenomas and early-onset is characteristic but neoplasms in endometrium, ovary, brain, pancreas, small intestine and skin (melanoma) have also been reported. *POLD1* mutations also predispose carriers to multiple colorectal adenomas and carcinoma and in addition endometrial and breast cancer. The phenotype associated with polymerase proofreading-associated polyposis is not fully defined yet and will be further detailed as more families with this syndrome are identified.

Consensus Molecular Subtypes (CMS) of Colorectal Cancer, Tumor Microenvironment and the Immune Response

In recent years a series of independent publications proposed sub-classifications of colorectal cancer based upon molecular criteria, varying from expression profiles to genome aberrations. These classifications shared certain characteristics in approach and resulting classification but were far from identical. To resolve this conundrum an international consortium composed of the groups responsible for the original publications worked out a gene expression-based classification system, which is now known as the "consensus molecular subtypes" (CMS) of colorectal cancer. This classification distinguishes four CMSs of colorectal cancer. Characteristics of CMS1 are MSI, mutations of *BRAF*, infiltration with TIL composed of Th1 cells and cytotoxic T-lymphocytes and activation of immune evasion pathways. CMS2 tumors are characterized by high chromosomal instability (CIN) and activation of Wnt and MYC pathways. In the CMS3 category, *KRAS* mutations are frequent, and metabolic pathways are disrupted. Finally, CMS4 tumors show a strong desmoplastic reaction along with high expression of mesenchymal genes and angiogenesis in which activation of the transforming growth factor beta (TGF-β) plays a central role. The biological significance of molecular subtyping initially was supported by a difference in prognosis between the subtypes, notably with poor prognosis for CMS4. Between the four CMS's significant differences in the tumor microenvironment have been reported with high numbers of cytotoxic T-lymphocytes and macrophages in CMS1 and 4. In contrast, in CMS4 (myo)fibroblast proliferation and collagen deposition are higher than in the other CMS's. These differences go along with different patterns of expression of chemokines, mediators of inflammation, genes involved in regulation of the immune response and of angiogenesis. With the stellar increase of interest in immunotherapy, a strong focus has developed on quantifying infiltrating immune cells which has become known as the immunoscore. However, understanding the role of the tumor microenvironment in biology and clinical behavior of colorectal cancer will require the comprehensive study of other cellular and molecular players.

Prognostic and Predictive Biomarkers

Colorectal cancer patients are stratified according to prognostic parameters in view of eventual adjuvant chemotherapy. Central in this exercise remain the classical TNM stage parameters tumor extension (T) and lymph node metastasis (N). As these lack in precision (**Table 1**), more accurate parameters are proposed almost monthly. Most of these, however, do not hold up in subsequent validation studies and presently beyond TNM, (lymph)vascular invasion is reported as a rule and tumor budding as a sign of invasive activity might be added in the near future. In MSS cancers the small subgroup with the *BRAF* V600E mutation have a particularly poor prognosis. *KRAS* mutations status has no prognostic significance for overall survival of stage II/III patients, but does predict poor prognosis in advanced (stage IV) cases. MS status has gained in importance as MSI-H cancers have a better prognosis, even though they might respond less favorably to chemotherapy. Other prognostic factors have not made it into daily clinical practice. Recently published tests such as Oncotype DX (multiplex RT-PCR based) and Coloprint (array-based) might provide additional prognostic value, but this needs to be independently validated.

As predictive biomarker, *KRAS* mutation status has become essential, notably for advanced colorectal cancer as *KRAS* mutated carcinomas do not respond to anti-EGFR therapy. The responsible mechanism is the position of KRAS downstream of EGFR in the MAPK pathway. Constitutive activation of KRAS renders approaches, blocking the upstream activity of EGFR, ineffective (**Fig. 12**). Of note, no *KRAS* mutated but also only about 40% of *KRAS* wild-type colorectal cancers respond to anti-EGFR treatment. This indicates that *KRAS* mutation status does identify a subset of non-responders but does not predict who will respond. More recently, the status of other genes involved in the pathway, including *PIK3CA*, *BRAF*, *NRAS*, and *pTEN* has been found to improve predictive

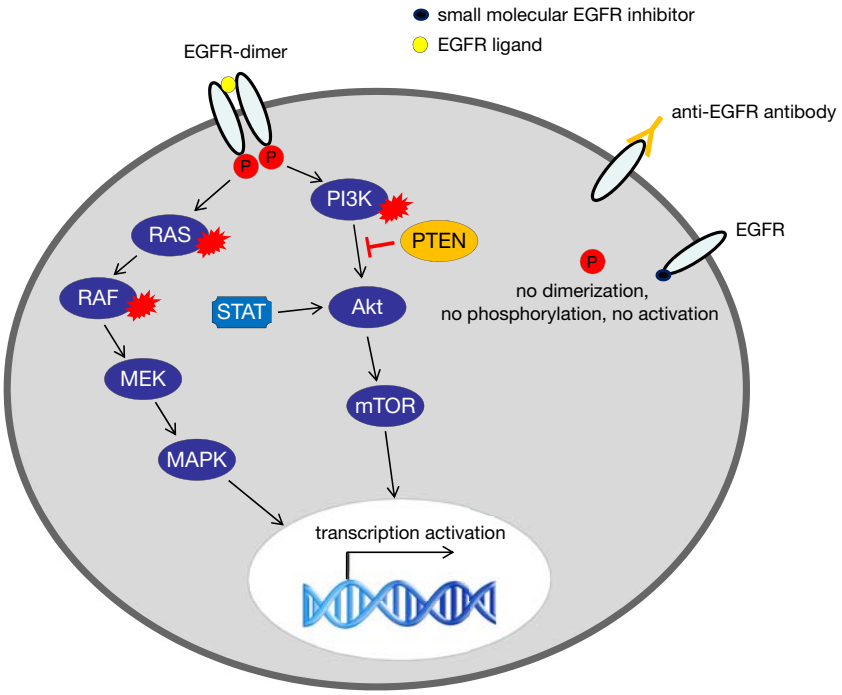

Fig. 12 Schematic representation of MAPK/mTOR signaling downstream of EGFR activation, and its role in insensitivity of KRAS mutated colorectal cancer for anti-EGFR treatment. Upon binding of an EGFR ligand to its receptor, EGFR dimerizes, phosphorylates and activates the signaling pathways. This can be inhibited by an anti-EGFR antibody or by a small molecular inhibitor. A mutation in *KRAS*, *RAF* or *PI3K* activates the pathway downstream of EGFR, thus circumventing EGFR inactivation. From Bosman, F. and Yan P. (2015). Molecular pathology of colorectal cancer. *Polish Journal of Pathology* **65**, 257–266. https://doi.org/10.5114/pjp.2014.48094.

power, but as yet unidentified genes are likely to be involved. MSI status identifies a subgroup of colorectal cancer patients with a high chance of favorable response to immunotherapy, notably PD1/PD-L1 blocking.

Prospects

For pathologists and for clinicians alike, heterogeneity of colorectal cancer remains a challenge. Which mechanisms are responsible for morphological heterogeneity? How can cancers that are histologically indistinguishable and in the same stage behave differently in terms of recurrence and response to chemotherapy? Some progress has been made with the introduction of molecular parameters and new molecular subtyping holds promise. However, this will have to be validated through molecular annotation of large series of colorectal cancers with detailed follow-up data. This will have to be repeated in connection with the emergence of new therapeutic targets and prognostic and predictive biomarkers.

An exciting field is the host response to the growing tumor cells, which creates the tumor micro-environment. It has become clear that the characteristics of the immune response of the host, in terms of the type, location and density of tumor infiltrating lymphocytes in combination with their functional molecular orientation, which together determine the immune contexture, have a significant impact on tumor progression and response to therapy. It will be essential to collect longitudinally samples from tumors before and after treatment, as they progress in recurrent or metastatic sites, as circulating tumor cells or circulating tumor DNA, to improve our understanding of the dynamics of tumor evolution and progression under therapy. Molecular analysis of such samples will clarify which (sub)clones exist in the primary tumor, which of these manage to gain access to the circulation and/or develop into a metastasis and how they respond to the selection pressure exerted by systemic therapy. Detailed knowledge of such tumor dynamics will ultimately allow the development of what has become known as "precision medicine," to improve outcome of patients with colorectal cancer.

See also: Colorectal Cancer: Diagnosis and Treatment.

Further Reading

Bijlsma, M.F., Sadanandam, A., Tan, P., Vermeulen, L., 2017. Molecular subtypes in cancers of the gastrointestinal tract. Nature Reviews. Gastroenterology & Hepatology 14, 333–342.

Bohanes, P., LaBonte, M.J., Winder, T., Lenz, H.J., 2011. Predictive molecular classifiers in colorectal cancer. Seminars in Oncology 38, 576–587.
Boland, C.R., Goel, A., 2010. Microsatellite instability in colorectal cancer. Gastroenterology 138, 2073–2087.
Fearon, E.R., 2011. Molecular genetics of colorectal cancer. Annual Review of Pathology 6, 479–507.
Fearon, E.R., Vogelstein, B., 1990. A genetic model for colorectal tumorigenesis. Cell 61, 759–767.
Gala, M., Chung, D.C., 2011. Hereditary colon cancer syndromes. Seminars in Oncology 38, 490–499.
Gibson, J.A., Odze, R.D., 2016. Pathology of premalignant colorectal neoplasia. Digestive Endoscopy 28, 312–323.
Guinney, J., Dienstmann, R., Wang, X., de Reyniès, A., Schlicker, A., Soneson, C., Marisa, L., Roepman, P., Nyamundanda, G., Angelino, P., Bot, B.M., Morris, J.S., Simon, I.M., Gerster, S., Fessler, E., De Sousa E Melo, F., Missiaglia, E., Ramay, H., Barras, D., Homicsko, K., Maru, D., Manyam, G.C., Broom, B., Boige, V., Perez-Villamil, B., Laderas, T., Salazar, R., Gray, J.W., Hanahan, D., Tabernero, J., Bernards, R., Friend, S.H., Laurent-Puig, P., Medema, J.P., Sadanandam, A., Wessels, L., Delorenzi, M., Kopetz, S., Vermeulen, L., Tejpar, S., 2015. The consensus molecular subtypes of colorectal cancer. Nature Medicine 21, 1350–1356.
Hamilton, S., Bosman, F., Boffetta, P., et al., 2010. Carcinoma of the colon and the rectum. In: Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), WHO Classification of Tumors of the Digestive. System, 4th edn. IARC press, Lyon, pp. 134–146.
Kinzler, K.W., Vogelstein, B., 1996. Lessons from hereditary colorectal cancer. Cell 87, 159–170.
Morley-Bunker, A., Walker, L.C., Currie, M.J., Pearson, J., Eglinton, T., 2016. Translating colorectal cancer genetics into clinically useful biomarkers. Colorectal Disease 18, 749–762.
Müller, M.F., Ibrahim, A.E., Arends, M.J., 2016. Molecular pathological classification of colorectal cancer. Virchows Archiv 469, 125–134.
Snover, D.C., 2011. Update on the serrated pathway to colorectal carcinoma. Human Pathology 42, 1–10.
The Cancer Genome Atlas Network, 2012. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337.
Xie, J., Itzkowitz, S.H., 2008. Cancer in inflammatory bowel disease. World Journal of Gastroenterology 14, 378–389.

Relevant Websites

https://www.cancer.gov/types/colorectal/hp - National Cancer Institute. Colorectal Cancer—Health Professional Version
http://www.nlm.nih.gov/medlineplus/colorectalcancer.html - MedlinePlus. Colorectal Cancer.
http://www.cancer.org/cancer/colonandrectumcancer/index - American Cancer Society. Colorectal Cancer.
http://seer.cancer.gov/statfacts/html/colorect.html - National Cancer Institute. Surveillance, Epidemiology, and End Results (SEER) Program. Cancer Stat Facts: Colorectal Cancer.
http://www.uptodate.com/contents/molecular-genetics-of-colorectal-cancer - UpToDate. Molecular genetics of colorectal cancer

Copy Number Variations in Tumors

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Glossary

Biomarker An easy-to-measure indicator of the disease status or subtype.
Copy number variation The number of copies within a genome region is different from the normal number of copies, 2.
Pan-cancer Diverse tumor types that share similar cancer features yet show different characteristics.
Targeted sequencing Resequencing of an isolated region of genome.
Whole genome sequencing The sequencing of the complete DNAs within an organism.

Human is a diploid organism with two copies of alleles for autosomal and pseudo autosomal genes. If there are no longer two copies, CNV (copy number variation) happens. When there are more than two copies, it is called gain or amplification. When there are less than two copies, it is called loss or deletion. CNVs are very common in diseases, especially in tumors when the biological systems dysfunction on multiple levels. CNV is usually the upstream change that triggers the cascade of signaling chaos and therefore can be the driver of disease. In tumors, CNV is found to be a very good biomarker for subtyping and survival prognosis. And it is helpful for understanding tumorgenesis and how mutations happen and in which order.

In this review, we will introduce the latest computational methods for CNV detections, especially the challenges of tumor CNV detection, and how to use the CNV data to investigate the mechanisms of several major types of cancers.

The Computational Methods for NGS Based CNV Detection

With the rapid development of next-generation sequencing (NGS) technology, CNV detection becomes easier and many computational methods have been proposed. The difficulties of NGS based CNV detection are filtering the noise, determining the region of CNV and estimating the copy numbers. The most widely used CNV software include PennCNV, CNVkit, and CNVtools, GISTIC and others. CNVkit is highly recommended for targeted sequencing.

To summary the principles underlying these software, there are several approaches: read-depth based methods, paired-end mapping based methods, split-read based methods and sequence assembly based methods. The four methods for CNV detection are shown in Fig. 1. The first three methods need to map the reads onto the reference genome while the last methods need to assemble the sequence from the beginning.

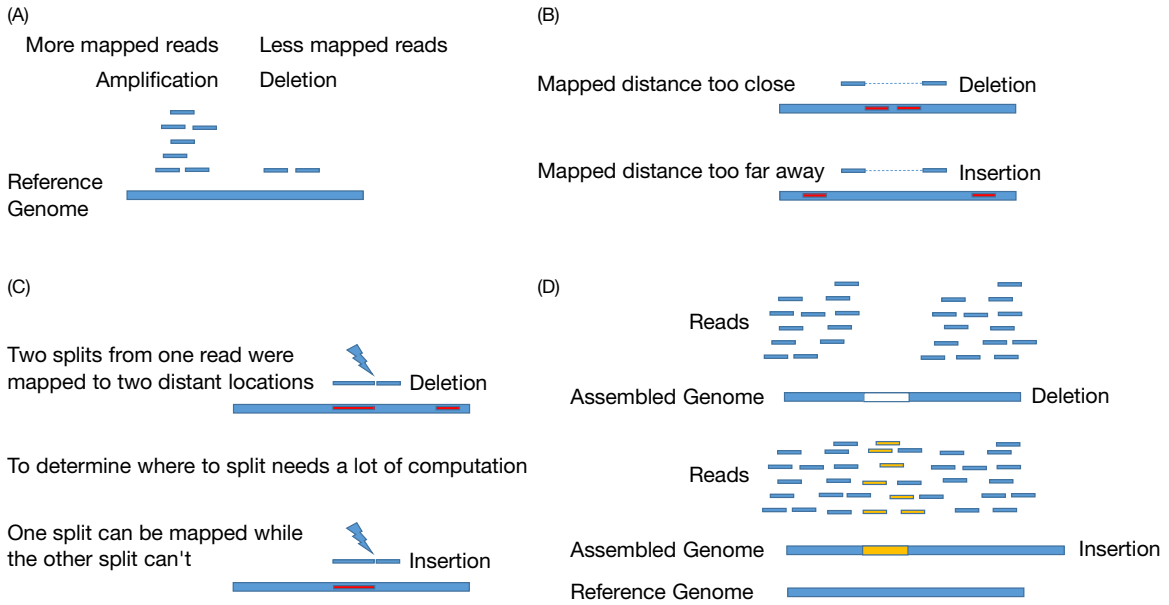

Fig. 1 The four methods for CNV detection. (A) Read-depth based method. (B) Paired-end mapping based method. (C) Split-read based method. (D) Sequence assembly based method.

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The Read-Depth Based Methods

The read-depth based methods are most intuitive and straightforward. These methods analyze the read depth at each position of the genome and find the differences across regions. It assumes that the read depth and copy number are positively correlated. This is generally correct, since the reads are randomly generated and should cover the genome without bias. Therefore, when a region is amplified or deleted, the reads within that region will change accordingly. But it is observed that the GC content can significantly affect the read depths. Usually, the region with extremely high or low GC content will have fewer reads. Another disadvantage of read depth methods is that the region with a lot of repeats tend to have more mapped reads since the mapping software could not assign the read onto the right region. This problem will become more serious when the length of reads is short. The simple but not perfect solution is to only consider the uniquely mapped reads or assign such reads onto a randomly chosen region. CNVnator is a representative CNV detection software that uses read-depth approaches.

The Paired-End Mapping Methods

The paired-end mapping methods need to break the DNA sequence into fragments with fixed length and then sequencing from both ends. Since the reads are paired, the mapped position of two reads within a pair can provide important information of the genome structure. It can not only detect CNV but also structural variation. When the distance between the mapped positions of two reads from a read pair is significantly short or long, this read pair is discordantly mapped and indicates CNVs between them. By clustering such discordantly mapped read pairs, the CNV regions and copy numbers can be detected. BreakDancer is one of the software that use paired-end mapping approaches.

The Split-Read Based Methods

The split-read based methods split the reads into smaller fragments and map the split fragments onto reference genome. To analyze the break point of the read with fragments that are mapped onto different regions, the CNV position can be accurately detected and the position can be as accurate as one base pair. If two fragments form one read are mapped onto two positions with a gap, it indicates a deletion between these two positions. If one fragment from a read can be mapped but the other fragment from this read can't be mapped, it indicates an insertion. How to split the reads is difficult to decide. To try more split methods means more computational time. And if the length of the original read is short, it will be difficult to split them and expect the even smaller fragments can be mapped onto the genome correctly. One of the split-read based software is Pindel. Generally speaking, the split-read based methods are slower.

The Sequence Assembly Based Methods

The sequence assembly based method does not require a reference genome and does not do mapping. It assembles the genome de novo and then detect the CNVs by analyzing the assembled genomes. Since the whole genome assembly is quite difficult, it usually does de novo assembly and local assembly to generate sequence clusters and compares with close reference genomes. Similar as read-depth based methods, the assembly based methods performs poorly at genome regions with many repeats. Velvet is a representative software that uses assembly based methods.

The Challenges of Tumor CNV Detection

Although there are already many software for CNV detections, it is still difficult to perform CNV detection for tumor samples. The main challenge is that the tumor samples usually are not pure tumor cells and include normal cells. This will contaminate the tumor sample and make the CNV detection from NGS data difficult. Even with careful selection to make sure the samples are from tumor, the heterogeneity of tumor cells is still troublesome. Single cell sequencing may be a good solution for this problem. But the small amount of DNA in single cell sequencing makes the sequencing procedure and analysis challenging. We need to distinguish the duplication during PCR, low coverage due to the small amount of DNA and actual CNVs.

Second challenge is that most methods assume that the genome is stable and most regions are diploid. But in tumor samples, it is quite common to have large structural variation and aneuploidy on a genome wide scale. Such systematic errors may cause great bias for CNV detection. It is better to detect structural variations before detecting CNVs for tumor samples. The contamination with normal cells, tumor heterogeneity, and tumor aneuploidy are very common in tumor samples and will make the CNV detection difficult.

The CNVs Contribute to Tumor Initiation and Progression

With the accumulation of more and more CNV data, several CNV databases have been built, such as CNVD and SCAN. And large tumor CNV datasets, such as TCGA (The Cancer Genome Atlas) and METABRIC (Molecular Taxonomy of Breast Cancer International Consortium), have been released.

Various pathological CNVs have been identified to be associated with tumor initiation and progression. For example, CNVs of well-known breast cancer genes *BRCA1* and *BRCA2*, are observed in breast cancer patients and suggest that the CNVs contribute to breast cancer. We will discuss the functions of CNVs in several major cancers.

CNVs in Breast Cancer

We downloaded the GISTIC processed TCGA breast cancer (BRAC) CNV data and METABRIC breast cancer data. The CNV status were quantified as "−2" for homozygous deletion, "−1" for heterozygous deletion, "0" for diploid, "1" for low-level gain, and "2" for high-level amplification. In TCGA and METABRIC breast cancer dataset, the most common deep deletion genes were *CSMD1*, *MYOM2*, *CDKN2A*, *DLGAP2*, *ERICH1*, *PTEN*, *RPL23AP53*, *ZNF596*, *ARHGEF10*, *CDKN2B*, and *KBTBD11*; the most frequent high-level amplification genes were *TRPS1*, *MYC*, *CASC8*, *LINC00536*, *PVT1*, *CCAT1*, *EIF3H*, *MIR1205*, *MIR1208*, and *TMEM75*. In breast cancer, gene amplification is more common than deletion. The frequencies of amplification and deletion in TCGA and METABRIC breast cancer datasets were shown in Fig. 2.

In Fig. 3, we plotted the top three deletion genes, *CSMD1*, *MYOM2*, *CDKN2A*, and the top three amplification genes, *TRPS1*, *MYC*, *CASC8*, in TCGA and METABRIC breast cancer dataset. It can be seen that the frequencies of the top amplification genes were much higher than the top deletion genes. The top amplification genes were consistently amplified in two datasets while the top deletion genes were deleted in most samples but amplified in a small proportion of other samples.

To investigate the CNV consistency in breast cancer, we plotted the amplification and deletion frequencies in both TCGA and METABRIC breast cancer dataset in Fig. 4. It can be seen that although the deletion frequencies of *CSMD1*, *MYOM2*, and *CDKN2A* were high in both TCGA and METABRIC, there were considerable large proportions of breast cancer samples from both TCGA and METABRIC with amplification of these genes. For top amplification genes *TRPS1*, *MYC* and *CASC8*, there were no deletions in 3253 TCGA and METABRIC breast cancer patients.

The CNV status of genes were reported to be associated with breast cancer stages, overall survival and disease-free survival. More specifically speaking, the breast cancer patients with deletions of *C20orf46* and *SCUBE2* had shorter overall survival time. And the

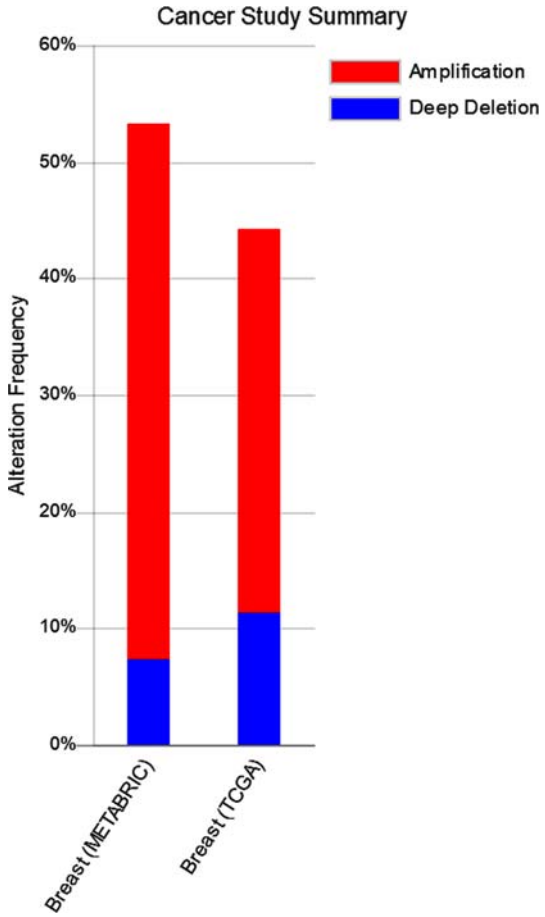

Fig. 2 The frequencies of amplification and deletion in TCGA and METABRIC breast cancer datasets. In breast cancer, the amplification is more common than deletion.

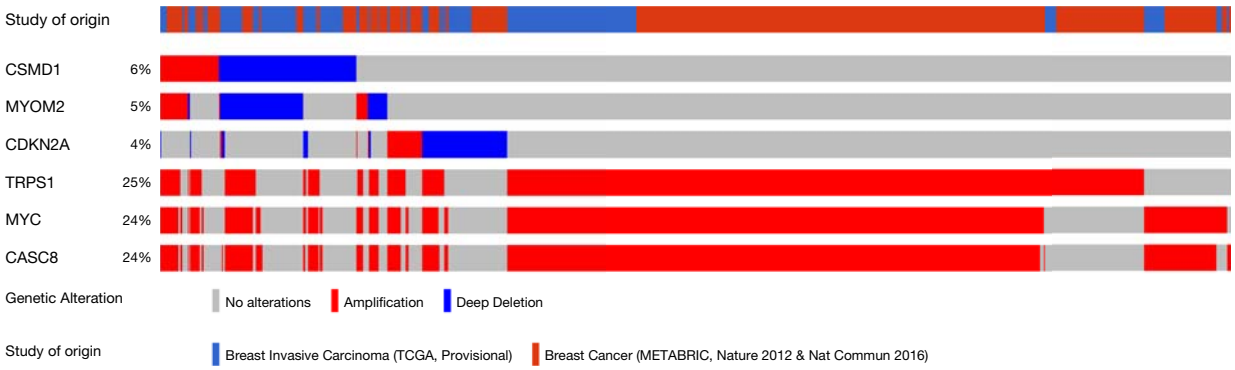

Fig. 3 The OncoPrint of the deletion genes, CSMD1, MYOM2, CDKN2A and the amplification genes, TRPS1, MYC, CASC8, in TCGA and METABRIC breast cancer dataset. CSMD1, MYOM2, CDKN2A were the top three deletion genes and TRPS1, MYC, CASC8 were the top three amplification genes. The patients without CNVs of these genes were not shown.

gain of *ZFP14*, *GSTM2*, and *JAK1* tended to have bad effect on overall survival while the loss of *PDGFRA* tended to have good effect on extend the overall survival. The loss of *PDGFRA* can not only extend survival time, but also postpone breast cancer recurrence. The gain of *ZFP14* and *LCE3C* will significantly accelerate breast cancer recurrence.

CNVs are not only biomarkers for breast cancer subtyping or prognosis prediction, but also useful for understanding how breast cancer begins and in which temporal order the mutations occur. With the oncogenetic tree model, the chronological CNV mutation timeline can be inferred. The general mutation orders of key driver genes in breast cancer were as follows: the *ERBB2* CNV mutation happened first, then the copy numbers of *AKT2*, *KRAS*, PTEN, and *CCND1* became abnormal and the mutation changes of *PIK3CA* happened after the changes of *KRAS*.

The CNV pattern of breast cancer varies a lot in different patients. Age is an important factor. For example, the prevalence of *PIK3CA* CNV in young breast cancer patients under 45 years old was much smaller than its prevalence in elderly patients over 70 years old. The prevalence of *TP53* mutations was the highest in patients between 46 and 69 years old. The mutation of *GATA3* only occurred in young patients under 45 years old but not in old patients while the mutations of *CDH1* were only found in patients over 45 years old. Another factor is ethnicity. For example, the amplification of 3q26.1 was detected in Chinese breast cancer patients but not in other populations.

CNVs in Lung Cancer

Non-small cell lung cancer (NSCLC) is the most common subtype of lung cancer and it includes adenocarcinoma and squamous cell carcinoma. In a CNV array dataset of over 200 lung adenocarcinoma patients and about 100 lung squamous cell carcinoma patients, 266 discriminative CNV probes were identified. They were able to classify the adenocarcinoma patients and squamous cell carcinoma patients nearly perfectly. They were from pathways, like hsa04310 Wnt signaling pathway, hsa04510 Focal adhesion, and hsa04512 ECM-receptor interaction. They located in the chromosome regions of 2q34, 10p15, 18q11, 3q26, 8p23, 3p21, 3q27, 22q12, Xq13, 2q36, 10p11, and 10p12. The CNV pattern of lung adenocarcinoma and squamous cell carcinoma are very significantly different.

There are many published array-based CNV signatures. Will these signatures still be useful if the NGS is used? To investigate this, we selected 20 CNV genes that can classify the adenocarcinoma patients and squamous cell carcinoma patients and mapped these genes on TCGA lung adenocarcinoma and TCGA lung squamous cell carcinoma dataset. The OncoPrint of the 20 CNV signatures derived from array data in the NGS based TCGA lung adenocarcinoma and TCGA lung squamous cell carcinoma dataset was shown in Fig. 5. It can be seen that the array-based CNV signatures can still classify the NGS lung adenocarcinoma and lung squamous cell carcinoma samples correctly. The genes *ESRRG* and *FZD8* were amplified more often in lung adenocarcinoma; *CD69* was deleted more frequently in lung adenocarcinoma. In lung squamous cell carcinoma, *TP63*, *NAALADL2*, *SOX2*, *BCL6*, *DGKG*, *DLG1*, *IL1RAP*, *LPP*, *LRRC15*, *MED12L*, *NLGN1*, *PAK2*, *PLSCR1*, *PLSCR2*, *TNIK*, *USP13*, and *ZIC4* were amplified more frequently. The array-based results and the NGS-based results were consistent. These results proved that the accumulated CNV array signatures can be adopted in the NGS-based methods.

The CNV is not just an adenocarcinoma or squamous cell carcinoma biomarker, but also indicates drivers for dysfunctions of multiple levels in lung cancer. By integrating CNV with gene expression, microRNA expression, DNA methylation in TCGA squamous cell lung carcinoma dataset, a whole genome wide integrative regulatory network can be constructed using regression model. The CNV of *FSCB*, *TCL6*, *MECOM*, *BCHE*, *ANO9*, *FBXO17*, *AGAP2*, and *C9orf53* were found to be the key drivers of the gene expression, microRNA expression, DNA methylation dysfunctions associated with lung squamous cell lung carcinoma. There have been many reports of how CNV affects gene expression through dosage effects. The regulatory roles of CNV in the integrative network of lung squamous cell lung carcinoma confirmed that CNV could regulate gene expression with dosage effect.

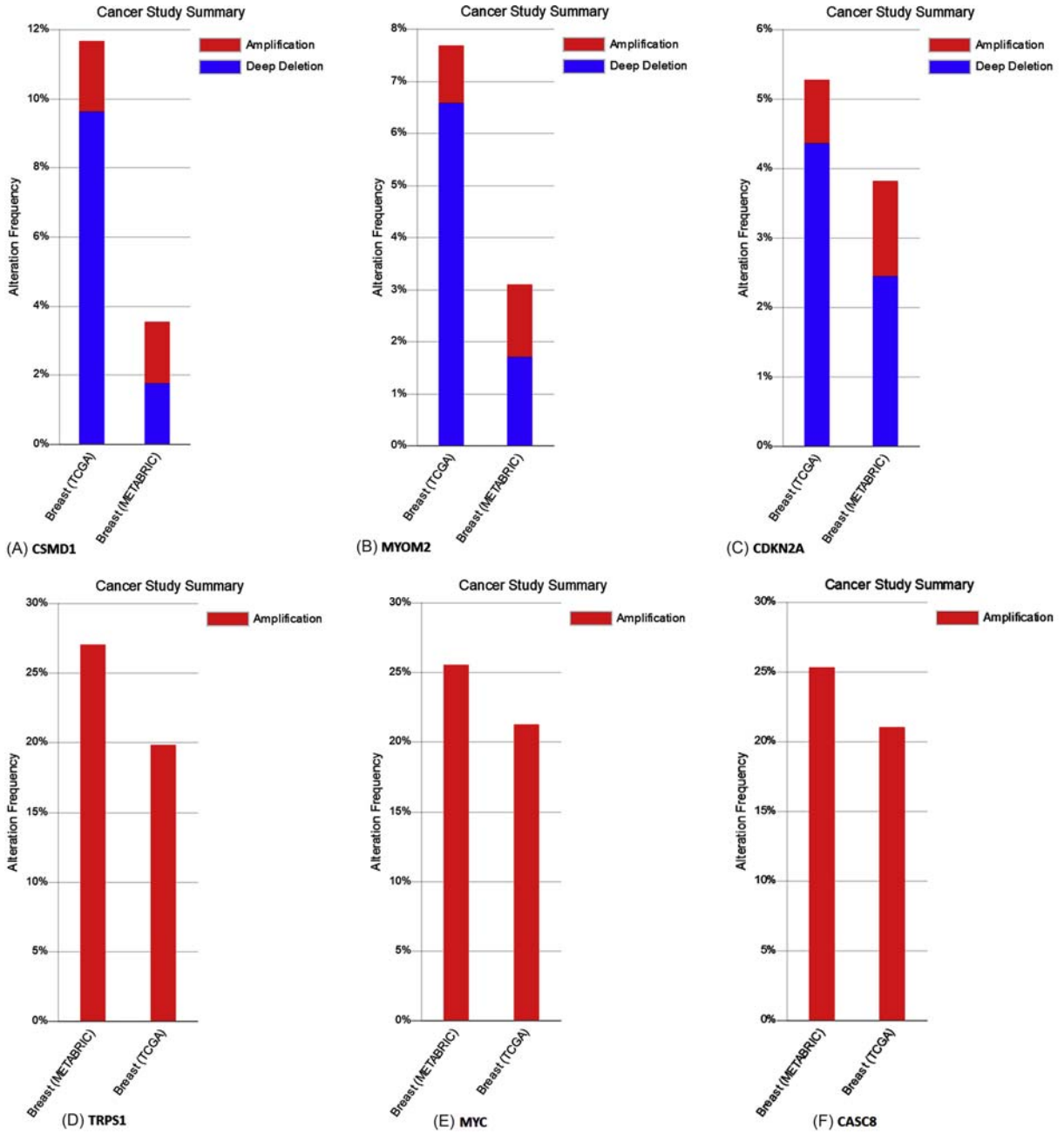

Fig. 4 The amplification and deletion frequencies of CSMD1, MYOM2, CDKN2A, TRPS1, MYC, and CASC8 in TCGA and METABRIC breast cancer dataset. The amplification and deletion frequencies of CSMD1 (A), MYOM2 (B), CDKN2A (C), TRPS1 (D), MYC (E), and CASC8 (F) in TCGA and METABRIC breast cancer dataset.

CNVs in Colorectal Cancer

In TCGA colon and rectal cancer dataset, the CNVs were measured using Affymetrix SNP 6.0 arrays. Meanwhile the methylation, microRNA and mRNA were quantified with Illumina HumanMethylation27 BeadChip, Agilent miRNA-Seq and Illumina RNA-Seq, respectively. The regulatory effect of CNV, methylation and microRNA on mRNA levels can be modeled as a linear regression. Based on how well the CNV, methylation and microRNA data can predict the gene expression level, the CNV-regulated genes, the methylation-regulated genes and the microRNA-regulated genes can be identified. The functions of these genes were investigated. It was found that the CNV-regulated genes have very different functions compared with the methylation-regulated genes and the microRNA-regulated genes. This finding indicates that the CNV is a unique regulator of gene expression.

Based on the TCGA colorectal cancer dataset, the CNVs prefer to regulate genes of cellular macromolecule metabolic process, gene expression, macromolecule modification, cellular protein modification process, protein modification process, transferase

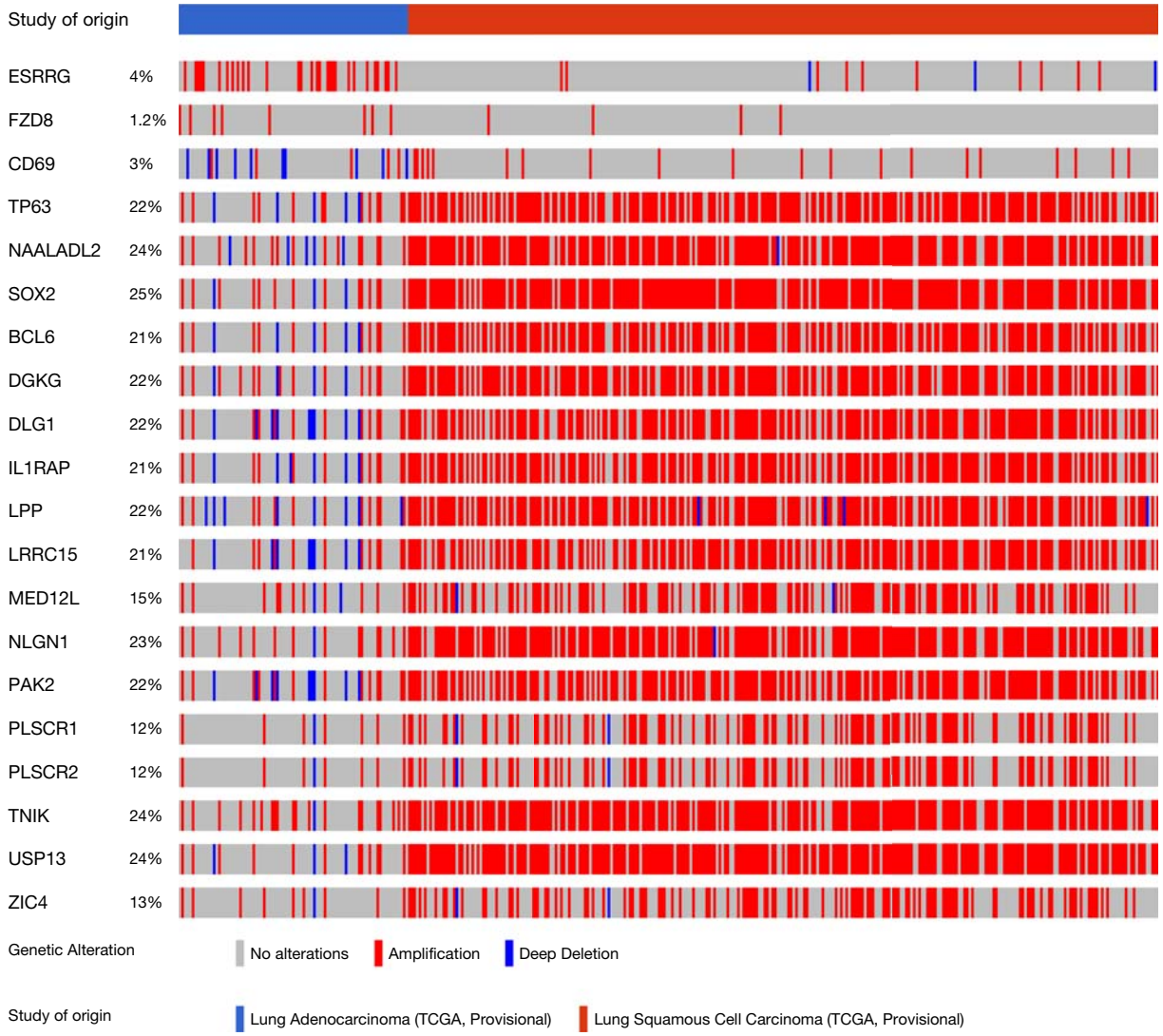

Fig. 5 The OncoPrint of the 20 CNV signature derived from array data in the NGS based TCGA lung adenocarcinoma and TCGA lung squamous cell carcinoma dataset. The array based CNV signature can still classify the NGS lung adenocarcinoma and lung squamous cell carcinoma samples correctly.

activity, transferring phosphorus-containing groups, protein kinase activity, phosphotransferase activity, and alcohol group as acceptor. The CNV-regulated genes are not like the methylation-regulated genes which are mostly from anatomical structure morphogenesis, immune system process, regulation of multicellular organismal process, response to external stimulus and calcium ion binding, or the microRNA-regulated genes that are enriched onto single-organism process, single-organism cellular process, biological regulation, protein binding, receptor activity, signal transducer activity, molecular transducer activity, and signaling receptor activity.

Through regulating key genes in colorectal cancer, CNV plays important roles for prognosis. For example, the CNV of *UQCRB* is a prognostic predictor for colorectal cancer. The receiver operative characteristic curve (AUC) of *UQCRB* CNV gain was 0.891. The CNVs of *CHD8*, *CD47*, and *RERG*, *ARHGDIB* are associated with colorectal cancer risk. The CNV of *TNFRSF10C* is associated with colorectal cancer metastasis. Similarly, there are also population specific CNVs in colorectal cancer. It was reported that the *ZNF217* and *CYP24A1* tend to be gained simultaneously in Chinese colorectal cancer patients.

CNVs in Pan-Cancer

The TCGA dataset is a large multi-omics dataset with about 30 types of cancers. The CNVs of 19 genes (*RPS15*, *IL17RC*, *CUL2*, *SMPD3*, *MIR4703*, *CDKN2A*, *RFFL*, *CTBP2*, *MMD2*, *SEMA6A*, *ZFPM1*, *CDC25A*, *ZMYND11*, *KBTBD6*, *CELF5*, *EGFR*, *PIGL*, *ZNF503-AS1*, *RBFOX1*) were found to be able to classify six major cancer types in TCGA: BRCA (breast invasive carcinoma),

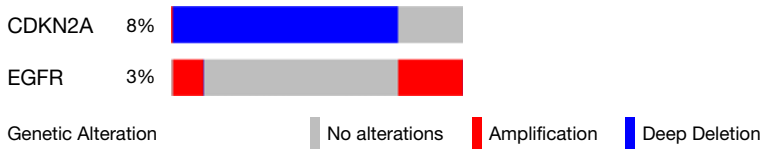

Fig. 6 The OncoPrint of CDKN2A and EGFR in MSK-IMPACT. Within the MSK-IMPACT cohort which included over 10,000 patients and over 360 types of cancers, 8% of them had CDKN2A deep deletion and 3% of them had EGFR amplification. The patients without CNVs of these two genes were not shown.

COAD/READ (colon adenocarcinoma/rectum adenocarcinoma), GBM (glioblastoma multiforme), KIRC (kidney renal clear cell carcinoma), OV (ovarian serous cystadenocarcinoma) and UCEC (uterine corpus endometrioid carcinoma). The accuracy of the 19-gene CNV signature was over 75%.

Another large pan-cancer CNV dataset is MSK-IMPACT in which over ten thousand cancer samples were included but only over four hundred genes were measured. The OncoPrint view of CNV will show how broadly CNV happens in cancer and how different their CNV pattern are. In Fig. 6, we plotted the OncoPrint of two genes from the 19 gene CNV signature discovered from TCGA dataset, *CDKN2A* and *EGFR*. Within the MSK-IMPACT cohort which included over 10,000 patients and over 360 types of cancers, 8% of them had *CDKN2A* deep deletion and 3% of them had *EGFR* amplification.

The Clinical Applications of CNVs in Tumors

As we discussed above, the CNV patterns were different in tumor and normal samples, different tumor subtype samples, tumor patients with different drug response, tumor patients with different survival time. CNV is an important biomarker. Unlike gene expression which can be easily disturbed and shows randomness, CNV is more robust and stable. Since it is known that the normal copy number for human is 2, it doesn't require complicated normalization baselines like gene expression based methods, such as RT-PCR, microarray or RNA-Seq. All these characteristics of CNV make it a perfect tumor signature.

In clinical practice, the targeted NGS-based CNV panels are widely used and highly reproducible. In a study with one cohort of 391 samples and another cohort of 2375 samples, 37 unique CNV events were detected by nine targeted NGS panels with 100% sensitivity. This assessment of CNV applications in clinical laboratory testing shows how reliable CNV biomarkers are.

There are many commercial CNV panels. For example, NanoString Technologies has an nCounter® v2 Cancer Copy Number Assay which includes 87 common tumor CNV genes, such as *AKT*, *BRCA*, *ERBB2*, *MYC*, *PIK3CA*, and *PTEN*. This panel can be used for formalin-fixed, paraffin-embedded (FFPE) samples. ArcherDX, Inc. has a comprehensive thyroid and lung (CTL) kit that can detect common oncogenic CNV drivers in lung cancers. NGeneBio Co., Ltd. also has a lung cancer kit called LUNGaccuTest™ that can easily detect lung cancer CNVs.

Unlike whole genome CNV analysis, the CNV panel analysis focus on a small set of genes but requires much higher accuracies since the results will directly affect the clinical practice, such as how to treat the cancer patients. Among the open source software, CNVkit is designed for targeted DNA sequencing and is a good choice for panel CNV data analysis. Of course, there are commercial software that are designed for specific platforms. For example, the NanoString platform has nCounter® Digital Analyzer.

Overall, CNV can be a great biomarker for tumors. It can be applied for tumor subtyping, drug response prediction, survival time prediction. If we can choose the right genes based on genome-wide CNV investigation, the targeted CNV panel can be easily designed and applied. We believe that more and more CNV applications in tumor diagnostics will be developed and applied.

Prospective Vision

The CNV analysis becomes more and more widely used. There are two big challenges. One is the CNV analysis in single-cell and ctDNA (circulating tumor DNA). When the amount of DNA is small or the genome is incomplete, the CNV measurements will become difficult and we must adjust these effects to distinguish actual copy number deletion and artificial low DNA fractions in a specific region. Another challenge is the integrative analysis of CNV with other multi-omics data. The CNV is only one level of data in the complex system of cancer which involves dysfunctions at almost all levels of regulation including SNP, CNV, methylation, mRNA, microRNA, lncRNA (Long non-coding RNA), circRNA (Circular RNA), protein, PTM (post-translational modification), and metabolites. We should not only study them on each level, but also integrate them and get a systems level picture of how tumors develop and progress.

See also: Chromosome Rearrangements and Translocations. Metastatic Signatures—The Tell-Tale Signs of Metastasis. Mutations: Driver Versus Passenger.

Further Reading

de Araujo Lima, L., Wang, K., 2017. PennCNV in whole-genome sequencing data. BMC Bioinformatics 18 (Suppl 11), 383.
Hehir-Kwa, J.Y., Pfundt, R., Veltman, J.A., 2015. Exome sequencing and whole genome sequencing for the detection of copy number variation. Expert Review of Molecular Diagnostics 15 (8), 1023–1032.
Huang, T., Yang, J., Cai, Y.-D., 2015. Novel candidate key drivers in the integrative network of genes, microRNAs, methylations, and copy number variations in squamous cell lung carcinoma. BioMed Research International 2015, 358125.
Huang, T., Li, B.-Q., Cai, Y.-D., 2016. The integrative network of gene expression, microRNA, methylation and copy number variation in colon and rectal cancer. Current Bioinformatics 11 (1), 59–65.
Li, B.Q., You, J., Huang, T., Cai, Y.D., 2014. Classification of non-small cell lung cancer based on copy number alterations. PLoS One 9 (2), e88300.
Li, X.C., Liu, C., Huang, T., Zhong, Y., 2016. The occurrence of genetic alterations during the progression of breast carcinoma. BioMed Research International 2016, 5237827.
Mermel, C.H., Schumacher, S.E., Hill, B., Meyerson, M.L., Beroukhim, R., Getz, G., 2011. GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. Genome Biology 12 (4), R41.
Pirooznia, M., Goes, F.S., Zandi, P.P., 2015. Whole-genome CNV analysis: Advances in computational approaches. Frontiers in Genetics 6, 138.
Qiu, F., Xu, Y., Li, K., Li, Z., Liu, Y., et al., 2012. CNVD: Text mining-based copy number variation in disease database. Human Mutation 33 (11), E2375–81.
Xu, L., Hou, Y., Bickhart, D.M., Song, J., Liu, G.E., 2013. Comparative analysis of CNV calling algorithms: Literature survey and a case study using bovine high-density SNP data. Microarrays (Basel, Switzerland) 2 (3), 171–185.
Zhang, N., Wang, M., Zhang, P., Huang, T., 2016. Classification of cancers based on copy number variation landscapes. Biochimica et Biophysica Acta (BBA) - General Subjects 1860 (11, Part B), 2750–2755.

Relevant Websites

CNVkit, Genome-wide copy number from high-throughput sequencing—http://cnvkit.readthedocs.io/en/stable/.
PennCNV—http://penncnv.openbioinformatics.org/en/latest/.
CNVtools, CNVtools—http://www.bioconductor.org/packages/release/bioc/html/CNVtools.html.
BreakDancer—http://breakdancer.sourceforge.net/.
SCAN, SNP and CNV Annotation Database—http://www.scandb.org/newinterface/.

Defective 5-Methylcytosine Oxidation in Tumorigenesis

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Glossary

DNA methylation The addition of methyl groups from the methyl group donor *S*-adenosyl-L-methionine onto carbon five of cytosine residues in DNA resulting in the formation of 5-methylcytosine. This enzymatic reaction is carried out by DNA methyltransferases and occurs preferentially at CpG sequences in most tissues.

DNA demethylation The loss of 5-methylcytosine from DNA resulting in replacement of 5-methylcytosine with cytosine. Demethylation can occur passively by continuing DNA replication in the absence of DNA methyltransferases, or actively, by TET protein-mediated oxidation of the methyl group of 5-methylcytosine.

Epigenetics The study of mechanisms that cause changes in gene expression based on chromatin-linked events that do not involve direct changes in the DNA sequence.

IDH Isocitrate dehydrogenase, an enzyme of the tricarboxylic acid (TCA) cycle that provides the TET enzyme cofactor alpha-ketoglutarate.

TET proteins Ten-Eleven-Translocation (TET) proteins are 5-methylcytosine oxidases that oxidize the methyl group of 5-methylcytosine leading to sequential formation of 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine.

TDG Thymine DNA glycosylase, an enzyme that was initially reported to remove thymine from T/G mispairs. TDG has strong activity to excise 5-formylcytosine and 5-carboxylcytosine from DNA. The resulting abasic site is processed by base excision repair. After TET-initiated 5-methylcytosine oxidation and base excision repair involving TDG, the outcome of these reactions is DNA demethylation.

Introduction

5-Methylcytosine (5mC) is an enzymatically produced modified cytosine base that has been known to exist in mammalian DNA for about 70 years. These decades of research have provided important clues as to its presumed functional role but there is no complete understanding yet. Numerous correlative studies have shown that the presence of 5mC at gene control elements such as promoters and enhancers is not compatible with gene expression. 5mC can directly interfere with binding of transcription factors. This modified base is often associated with inactive, repressed chromatin characterized by lack of histone acetylation. In some genomic regions, 5mC tends to co-exist with nucleosomes methylated at lysine 9 of histone H3 (H3K9me3), for example at repetitive DNA regions, or with nucleosomes methylated at lysine 36 of histone H3 (H3K36me3), when found in gene bodies. One major role of 5mC seems to be the suppression of unwanted gene activity, in particular its presence is thought to ensure the silenced state of retroviral and repetitive elements in the genome. This function could be described as aiming to reduce transcriptional noise genome-wide leaving unmethylated genomic regions as recognizable landmarks associated with active genes.

A surprising discovery in the field was made in 2009 when two research groups reported that 5mC is not the only modified base in mammalian genomes. An oxidized form of 5mC, 5-hydroxymethylcytosine (5hmC) was detected initially in embryonic stem cells and in certain types of neurons in the brain. The levels of 5hmC in brain tissue can be quite substantial and may represent up to 1% of all cytosines, being equivalent to a quarter to a third of all 5mC in neuronal cell types. In most other tissues, the levels of 5hmC range from about 005% to 0.2% of all cytosines, which is about 20–80 times lower than the levels of 5mC. Predominantly, 5hmC occurs at CpG dinucleotides. Like 5mC, which is a product of DNA methyltransferase activity, 5hmC is produced enzymatically. Mammalian genomes encode three evolutionary conserved proteins that can catalyze the conversion of 5mC to 5hmC by enzymatic oxidation. They are named TET1, TET2, and TET3. This name comes from the fact that TET1 was initially described as a gene involved in a translocation in leukemia involving chromosomes 10 and 11 (*T*en-*E*leven-*T*ranslocation 1, or TET1). The enzymatic reaction involves the transfer of molecular oxygen to the methyl group and requires the cofactors alpha-ketoglutarate (αKG), Fe^{2+} and ascorbate, the latter being important to preserve the 2+ state of iron (Fig. 1).

Although 5hmC is a modified DNA base that can be readily detected and quantitated by antibody-based assays or by mass spectrometry-based approaches in tissue samples, in vitro studies with purified TET proteins showed that the reaction does not stop at the 5hmC base, but can proceed to create 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (Fig. 2). However, in cultured cells or in tissues, the latter oxidation products are only barely detectable suggesting that they are either not effectively produced in vivo or that they are eliminated rapidly. One plausible pathway to process 5caC bases would be a decarboxylation reaction leading instantaneously back to unmodified cytosine (Fig. 2). Decarboxylation of 5caC does not occur spontaneously to a significant extent. So far, decarboxylase activity has not been demonstrated as an enzymatic function of TET proteins, and 5caC DNA decarboxylase enzymes have not been identified. Instead, it was shown that 5fC and 5caC can be removed from DNA by the base excision repair (BER) pathway initiated by thymine DNA glycosylase (TDG). This enzyme was previously known

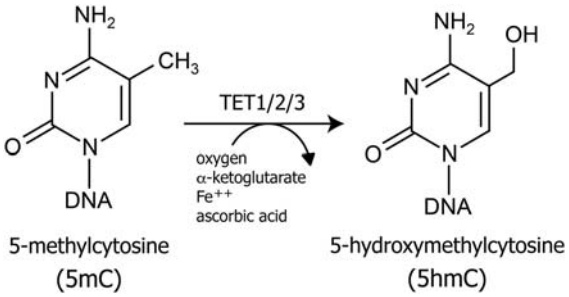

Fig. 1 Formation of 5-hydroxymethylcytosine by TET protein catalytic activity. The TET enzymes oxidize 5-methylcytosines at CpG sequences in DNA. They require oxygen, alpha-ketoglutarate, Fe^{2+} and ascorbic acid as cofactors.

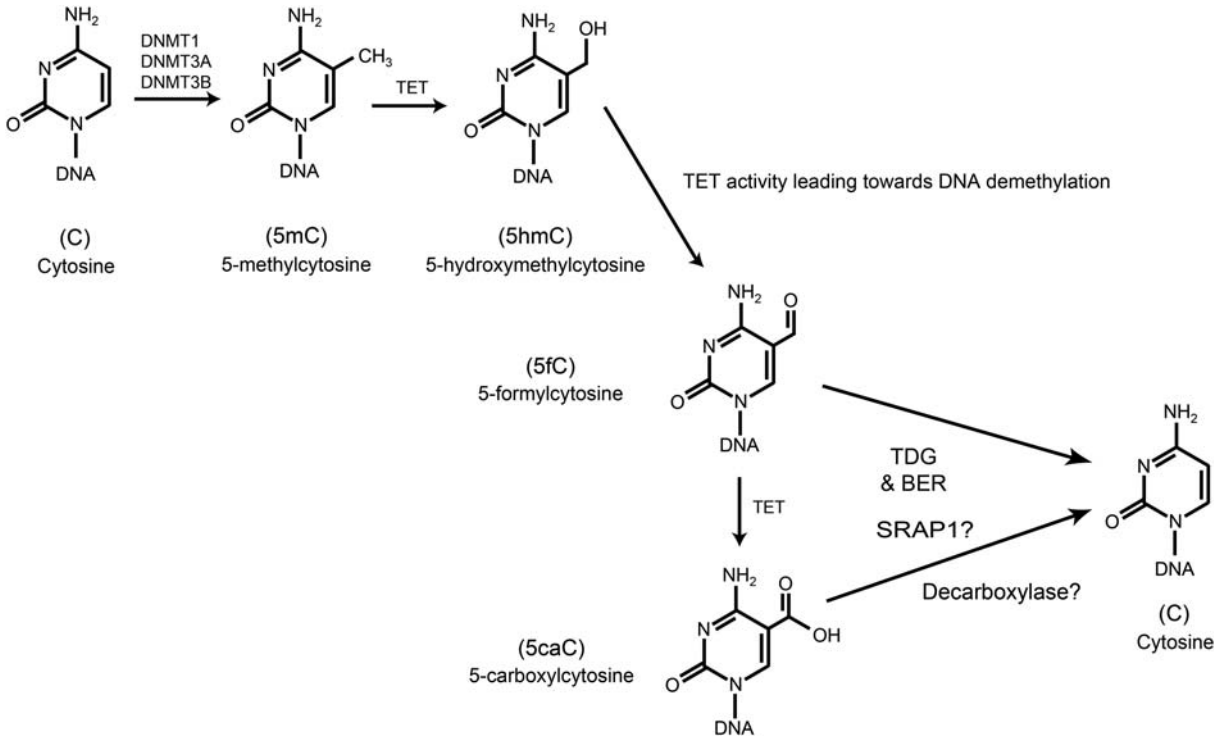

Fig. 2 Sequential enzymatic oxidation of 5-methylcytosine by TET proteins results in DNA demethylation. The TET enzymes oxidize 5mC to 5hmC, 5fC and 5caC at CpG sequences in DNA. 5hmC is observed as a relatively stable DNA base in cells. When 5hmC undergoes further oxidation, the pathway proceeds towards DNA demethylation via base excision repair that removes 5fC and 5caC from DNA. A 5caC decarboxylase is hypothetical and has not yet been identified.

to remove thymine from T/G mispairs in DNA, which are formed by spontaneous hydrolytic deamination of 5mC. This TDG-initiated DNA repair step seems to be important for cellular survival and genome integrity inasmuch as 5fC and 5caC are capable of arresting the progression of RNA and DNA polymerases on templates containing these oxidized bases. An alternative pathway for removal of 5caC bases that uses the SRAP1 autopeptidase-endonuclease has recently been described. It is still not well understood under what physiological conditions the TET-initiated 5mC oxidation pathway proceeds from 5hmC to the higher oxidized forms of 5mC resulting in complete elimination of the modified base (Fig. 2).

The biological role of 5hmC (and of 5fC and 5caC) as specific signaling marks rather than them simply being intermediates in an active DNA demethylation process has also been discussed. Several candidate proteins that may interact with oxidized 5mC bases have been identified using a mass spectrometry-based screening approach. However, the biological roles of these proteins when recognizing oxidized 5mC bases are not understood.

The first indication that the 5mC oxidation pathway may play an important role in tumorigenesis came from the discovery of common mutations in the *TET2* gene in hematological malignancies. We will discuss these mutations as well as other genetic and epigenetic changes in tumors that affect the balance between 5-methylcytosine formation and 5-methylcytosine removal (DNA demethylation).

TET2 Mutations

Around the same time when 5-hydroxymethylcytosine and the TET enzymes that produce this base modification were first reported, an analysis of hematological malignancies uncovered frequent mutations in the coding sequence of the *TET2* gene. This comprehensive analysis of *TET2* in over 400 hematopoietic tumor samples revealed *TET2* somatic mutations in 7.6% of myeloproliferative neoplasms, 42% of chronic myelomonocytic leukemia (CMML) and in 12% of acute myeloid leukemia (AML) cases analyzed. Many additional studies confirmed the prevalence of TET2 mutations in AML, myelodysplastic syndrome (MDS) and in CMML. However, TET2 mutations are uncommon in solid tumors as revealed by numerous cancer genome sequencing studies. The *TET2* mutations in hematological tumors are generally point mutations or deletions that lead to a lack of a functional protein, for example by truncation mutations or by point mutations in the catalytic domain. In many cases, one wildtype TET2 allele remains intact. The TET2 mutations are considered early events in the malignant transformation process. They also occur at low frequency during normal aging leading to clonal hematopoiesis.

Tet2-deficient mouse models also show an expansion of myeloid progenitor cells leading to the accumulation of pre-leukemic cell clones and the development of myeloid malignancies resembling CMML, MPD-like myeloid leukemia, and MDS, similar to the human disease. Somewhat surprisingly, the same human hematopoietic malignancy may harbor mutations in *TET2* and in the DNA methyltransferase gene *DNMT3A*. Such a finding suggests that TET2 and DNMT3A may have different genomic targets that are affected differentially by functional inactivation of either epigenetic enzyme. Mutation of both *Tet2* and *Dnmt3a* in mouse models leads to more severe phenotypes than single mutations of these genes. It is likely that TET2 loss of function, even when only partial, will lead to an imbalance of the methylation–demethylation cycle and one expectation is that CpG methylation at TET2-targeted loci should be increased. However, published studies have shown both a predominant loss and a predominant gain of methylation in tumors harboring TET2 mutations. One reason for the seemingly disparate finding may be that the tumors analyzed carry additional mutations in other epigenetic modifier genes (e.g., *DNMT3A*, *EZH2*) and it is difficult to judge the impact of a single mutation on genomic methylation patterns.

Ascorbic acid is an important co-factor for TET activities. Many standard cell culture media lack ascorbic acid, which could be one explanation for the extremely low levels of 5hmC generally found in cells propagated in tissue culture. Treatment of TET2-deficient cells with vitamin C enhances 5-hydroxymethylcytosine formation and suppresses human leukemic cell colony formation and leukemia progression. This effect is likely mediated by activation of TET3. TET2 and TET3, although they may also have unique gene targets, are believed to function redundantly in many tissues.

Mutations in TET1 and TET3

TET1 and *TET3* are rarely mutated in human tumors and no such mutations were identified in an initial screening of a variety of hematological malignancies. According to the COSMIC database, *TET1* is found mutated in a few percent, generally less than 5% of the cases of endometrial, stomach and intestinal tumors. *TET3* mutations are only found very rarely in solid or hematological tumors. Although *TET2* is expressed at high levels in cells of the hematopoietic system partially explaining its frequent mutation in hematological diseases, it is unknown why TET1 and TET3 are rarely affected by mutations in other tumor tissues.

IDH1 and IDH2 Mutations

Isocitrate dehydrogenases are NAD(+)- or NADP(+)-dependent enzymes that catalyze the conversion of isocitrate to alpha-ketoglutarate by oxidation and decarboxylation reactions. These enzymes are associated with the tricarboxylic acid (TCA) cycle. Mutations at specific amino acids of IDH1 were initially found in human brain cancers including astrocytomas, oligodendrogliomas and some glioblastomas. They are most common in lower grade gliomas (grade II or grade III). Mutations in IDH1 or IDH2 are also seen in acute myeloid leukemias, cholangiocarcinomas and in chondrosarcomas. The most common IDH1 mutations affect an amino acid within the active site of the protein and changes codon 132 from arginine to histidine. The change results in a gain of function producing a neomorphic enzyme that produces 2-hydroxyglutarate instead of alpha-ketoglutarate. Indeed, 2-hydroxyglutarate accumulates to high levels in IDH1-mutant cells. The theory proposes that 2-hydroxyglutarate is a competitive inhibitor of a number of alpha-ketoglutarate-dependent dioxygenase enzymes. In fact, the 2-hydroxyglutarate metabolite was shown to inhibit TET enzymatic activities leading to a defect in 5mC oxidation and thus to an accumulation of 5mC at genomic location that would normally be targeted by TET activities. As a result, IDH1-mutant gliomas are characterized by an overabundance of hypermethylated CpG islands (also called CpG island methylator phenotype or "CIMP"). In some instances, these hypermethylation events, for example when occurring at promoters of genes, will lead to silencing of the linked gene. One other class of dioxygenase enzymes that are effectively inhibited by 2-hydroxyglutarate are the large group of alpha-ketoglutarate-dependent histone lysine demethylases of the JmjC-domain family. The effect of IDH1 mutation will be an increase of the methylated state of different lysines on histone tails, for example, resulting in a potential dysregulation of gene expression. Since many lysine demethylases may be affected simultaneously, the disruption of the epigenetic state at specific gene loci is expected to be complex.

IDH1 or IDH2 mutations are relative early occurrences in glioma pathogenesis. They are a very common event in lower grade gliomas occurring in up to 80% of astrocytomas and oligodendrogliomas. Different molecular pathways seem to follow IDH1/2

mutations during tumor progression. These events include the loss of chromosomes 1p and 19q and the acquisition of *TERT* promoter mutations in oligodendrogliomas and mutations of *TP53* and *ATRX* in astrocytomas. On the other hand, mutation or amplification of the epidermal growth factor receptor gene (*EGFR*) occur rarely in IDH1-mutant brain tumors but are common in IDH1-wildtype primary glioblastomas. Mutations in IDH1/2 and TET2 do not co-occur in AML suggesting that the same downstream targets may be impacted by these mutations and that inhibition of TET2 activity by 2-hydroxyglutarate is indeed a key mechanism in disease pathogenesis.

Succinate and Fumarate

Two successively operating mitochondrial enzymes of the TCA cycle, succinate dehydrogenase (SDH) and fumarate hydratase (FH) show heterozygous mutations in their genes that cause predispositions to two types of inherited cancer syndromes. SDH mutations are linked to paraganglioma (PGL) and phaeochromocytoma and FH mutations predispose to leiomyomatosis and renal cell carcinoma. Mutation of these enzymes will lead to accumulation of their substrate metabolites, succinate and fumarate and dysregulation of metabolic pathways. Similar to 2-hydroxyglutarate, these metabolites are also competitive inhibitors of alpha-ketoglutarate-dependent dioxygenases, that is, lysine demethylases, 5-methylcytosine oxidases and prolyl hydroxylases. These events will likely lead to perturbations of epigenetic states of DNA and histones. However, these two genes are rarely mutated in other types of cancers.

General Loss of 5-Hydroxymethylcytosine in Human Solid Tumors

When viewed across the broad spectrum of multiple types of solid tumors, mutations in TET2, IDH1/2, FH, and SDH are still relatively rare and frequently occur in only a few tumor types such as gliomas and AML. The vast majority of human tumor specimens do not contain these aberrations. However, in 2011 it was discovered that the major product of 5mC oxidase activity, 5hmC, is strongly depleted in all solid tumors analyzed. These tumors included cancers of the lung, breast, colon, liver, pancreas, prostate, melanoma and several others. When quantitated with sensitive techniques such as liquid chromatography coupled with tandem mass spectrometry, the extent of 5hmC depletion in tumors can reach 80%–90%. This degree of loss of 5hmC is much greater than the loss of 5mC, which often is only in the range of 10%–30% in tumors when compared to corresponding normal tissue. Therefore, loss of 5hmC during tumorigenesis does not simply reflect the loss of its precursor, 5mC.

The loss of 5hmC may occur as an early event in carcinogenesis. For example, the loss is seen as a consequence of exposure of rodent liver to a nongenotoxic carcinogen, phenobarbital. Currently, it is unknown if other carcinogenic substances may also impair 5hmC maintenance in normal cells.

Loss of 5hmC, as monitored by immunocytochemistry or immunofluorescence staining, is characteristic of malignant lesions and may be useful as a biomarker for cancer. For example, when used for assessing the malignant potential of melanocytic lesions in the skin, benign nevi show strong staining for 5hmC but this staining is lost in primary or metastatic malignant melanomas. Interestingly, this modified base is also reduced in proliferating normal tissue including, for example intestinal crypt stem cell compartments.

The mechanisms that lead to a general depletion of 5hmC in tumors are not well understood and multiple pathways are probably involved. It has been known from earlier in vitro studies that the presence of 5hmC, in place of 5mC, on a parental DNA strand is incompatible with DNA methyltransferase 1 (DNMT1) activity that normally copies symmetrical methylation patterns at CpG sites shortly after DNA replication. This lack of DNMT1 activity on 5hmC-containing substrates leads to a loss of both 5mC and 5hmC in replicated daughter strand DNA molecules. However, in malignant tissue the global loss of 5hmC is much greater than the loss of 5mC so that other or additional mechanisms need to be invoked. A general reduction of *TET* gene expression, TET protein levels or TET enzymatic activity in tumor versus normal cells is one likely explanation. However, gene expression data from The Cancer Genome Atlas (TCGA) does not confirm a loss of *TET* gene expression across multiple tumor entities. To the contrary, *TET* gene expression levels are often increased in tumors. TET protein levels have rarely been measured in primary matched tumor-normal tissues and neither have their activities. There is also the possibility that there is reduced availability of co-factors in tumor tissues. For example, levels of ascorbic acid may be reduced in tumor patients or more specifically, in their tumor tissues. Metabolic reprogramming is a widespread phenomenon in tumor cells (e.g., the Warburg effect). As one possibility, the availability of the cofactor alpha-ketoglutarate may be limited in tumors. Equally important could be the maintenance of a correct balance between the dioxygenase enzyme cofactor alpha-ketoglutarate and their inhibitors such as succinate and fumarate. All three of these compounds are part of the TCA cycle and even subtle disturbances in the ratios of these metabolites may affect TET enzymatic activities. These parameters would need to be assessed systematically in normal and in tumor tissue.

One other unresolved issue is whether the depletion of 5hmC in cancer is perhaps only a consequence of tumor formation or enhanced cell proliferation or whether these changes have a causative role in tumorigenesis. There are not many studies that have addressed these issues other than those based on inactivation of *Tet* genes. *Tet2* inactivation in mice leads to an expansion of clonal hematopoiesis and emergence of premalignant clones as discussed earlier. Reintroduction of active TET2 into a melanoma cell line with low 5hmC levels led to suppression of melanoma growth and increased tumor-free survival in mice. Interestingly, combined loss of *Tet1* and *Tet2* in mice promotes B cell tumors, but not myeloid malignancies. However, germline deletion of *Tet2* in combination with acute loss of *Tet3* leads to a rapid, progressive leukocytosis, which developed within a few weeks into aggressive myeloid leukemia in 100% of the gene-targeted mice. These combinations of *Tet* genotypes are not usually found in human tumors;

however, the data leave open the possibility that other mechanisms of TET protein inactivation may occur and may be critically involved in human cancers as well.

Perturbation of DNA Methylation Patterns as a Consequence of Defective 5mC Oxidation

If one considers the state of methylation at a CpG-rich target to be the result of a balance between de novo methylation and DNA demethylation initiated by TET proteins, then the expectation is that loss of TET activity will shift that balance towards 5mC. These predictions have generally proven to be correct in experimental systems in which one or more *Tet* genes have been disabled. For example, genome-wide mapping of 5hmC has shown that enhancers are enriched for this modification. The results imply that certain TET proteins may be targeted to enhancers and that they are critically involved in regulating enhancer DNA methylation and thus the enhancer's accessibility and function. Indeed, when *Tet2* was deleted in embryonic stem cells, extensive loss of 5hmC occurred at enhancers and this loss was accompanied by DNA CpG hypermethylation at the same enhancer sequences. Combined loss of all three *Tet* genes in mouse embryonic stem cells inhibits their differentiation potential. These studies further revealed that promoters became hypermethylated upon loss of TET activities and this loss led to a deregulation of genes implicated in embryo development and differentiation. In mouse embryos, loss of all three Tet genes leads to a gastrulation defect also involving hypermethylation of normally demethylated critical gene control elements.

In patient samples, the contribution of TET2 mutations to DNA hypermethylation patterns is more difficult to appreciate because of the simultaneous occurrence of a number of other epigenetic modifier mutations in the same malignant cell populations. Cases of myeloproliferative neoplasms with TET2 mutations tend to show not only decreased levels of 5hmC but also distinct sets of hypermethylated genes. Hypermethylation of enhancers has also been reported for human AML cases with TET2 mutations. Therefore, similar as in mouse ES cells, loss of TET2 function in human hematopoietic and other somatic stem cells may be associated with a phenotype of enhancer hypermethylation and dysfunction that may contribute to the formation of leukemias and other malignancies (Fig. 3).

It is also conceivable that the global loss of 5hmC in solid tumors, which occurs by unknown mechanisms, will lead to DNA hypermethylation of regulatory gene sequences. At a global level, the extent of 5hmC loss does not seem to correlate with the number of DNA hypermethylation events observed in a tumor. Whether loss of 5hmC formation at specific sites (e.g., in regions flanking promoters or near enhancers) will lead to DNA hypermethylation of the same targets is more difficult to assess. It requires genome-wide high-resolution mapping of 5hmC in normal and tumor tissues, but single base mapping of 5hmC in mammalian genomes is still prohibitively expensive. Some of the initial studies using these approaches suggest that the presence of 5hmC is indeed negatively correlated with DNA methylation changes.

Prospective Vision

Perturbation of DNA methylation patterns in tumors, a phenomenon known for several decades, includes broad, genome-scale DNA hypomethylation and localized DNA hypermethylation at CpG-rich sequences. The loss of 5-hydroxymethylcytosine in

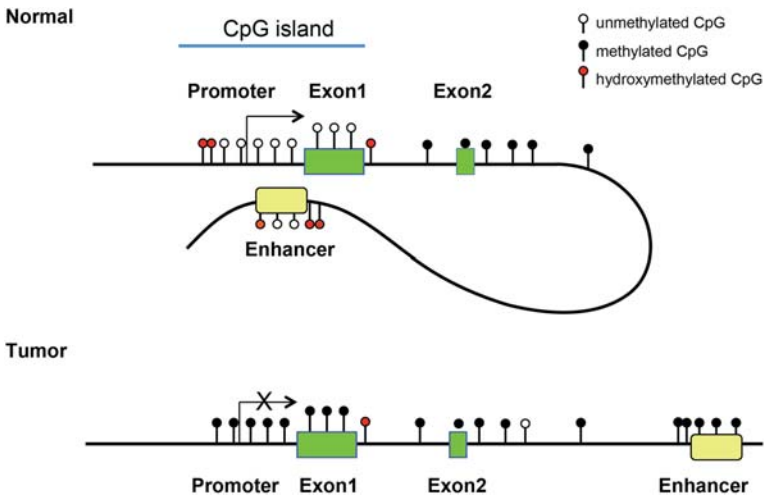

Fig. 3 Model for changes of 5hmC and 5mC in cancer. The diagram shows a hypothetical gene. Unmethylated CpG-rich sequences at enhancers and promoters in normal cells are protected from aberrant de novo methylation by TET-protein-catalyzed removal of erroneously introduced 5mC. In this scenario, TET-mediated 5mC oxidation leads to conversion of 5mC back to cytosine. Open circles, unmethylated CpG sequences; *black circles*, methylated CpG sequences (5mC), red circles, hydroxymethylated CpG sequences (5hmC). Loss of 5hmC near promoters and enhancers may be accompanied by de novo methylation of gene control elements in tumors.

tumors greatly exceeds the extent of the loss of 5-methylcytosine. This loss is not limited to tumors carrying mutations in *TET* genes or in *IDH* genes but occurs across a broad spectrum of malignancies. There is a need to understand the mechanisms of 5hmC loss since this process may be mechanistically linked to tumorigenesis. It is also tempting to speculate that there is an intrinsic connection between 5hmC perturbation and the changes in 5mC that are observed in tumors. Targeted genetic studies in mice will be needed to dissect the tissue-selective roles of specific *TET* gene inactivation in cancers other than those of the hematopoietic system. Manipulation of co-factor pathways relevant for TET protein function will also be required to understand the role of altered metabolism in the imbalance of DNA methylation and DNA demethylation pathways during tumor formation. Diagnostic assays assessing the DNA methylation status of a few specific genes are already in use for clinical tests with the goal of early cancer detection and patient management. Since widespread changes in 5hmC are also a ubiquitous characteristic of tumor genomes, similar assays analyzing 5hmC patterns in tissue or blood DNA samples could be developed for aiding cancer detection and diagnosis.

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See also: DNA Methylation Changes in Cancer: Cataloguing. DNA Methylation Changes in Cancer: Mechanisms. Environmental Exposures and Epigenetic Perturbations. Mutations in DNA Methyltransferases and Demethylases. TCA Cycle Aberrations and Cancer.

Further Reading

Abdel-Wahab, O., Mullally, A., Hedvat, C., Garcia-Manero, G., Patel, J., Wadleigh, M., Malinge, S., Yao, J., Kilpivaara, O., Bhat, R., Huberman, K., Thomas, S., Dolgalev, I., Heguy, A., Paietta, E., Le Beau, M.M., Beran, M., Tallman, M.S., Ebert, B.L., Kantarjian, H.M., Stone, R.M., Gilliland, D.G., Crispino, J.D., Levine, R.L., 2009. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 114, 144–147.

Bowman, R.L., Levine, R.L., 2017. TET2 in normal and malignant hematopoiesis. Cold Spring Harbor Perspectives in Medicine 7, a026518.

Dai, H.Q., Wang, B.A., Yang, L., Chen, J.J., Zhu, G.C., Sun, M.L., Ge, H., Wang, R., Chapman, D.L., Tang, F., Sun, X., Xu, G.L., 2016. TET-mediated DNA demethylation controls gastrulation by regulating lefty-nodal signaling. Nature 538, 528–532.

Dawlaty, M.M., Breiling, A., Le, T., Barrasa, M.I., Raddatz, G., Gao, Q., Powell, B.E., Cheng, A.W., Faull, K.F., Lyko, F., Jaenisch, R., 2014. Loss of Tet enzymes compromises proper differentiation of embryonic stem cells. Developmental Cell 29, 102–111.

Hashimoto, H., Liu, Y., Upadhyay, A.K., Chang, Y., Howerton, S.B., Vertino, P.M., Zhang, X., Cheng, X., 2012. Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. Nucleic Acids Research 40, 4841–4849.

He, Y.F., Li, B.Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L., Sun, Y., Li, X., Dai, Q., Song, C.X., Zhang, K., He, C., Xu, G.L., 2011. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333, 1303–1307.

Hon, G.C., Song, C.X., Du, T., Jin, F., Selvaraj, S., Lee, A.Y., Yen, C.A., Ye, Z., Mao, S.Q., Wang, B.A., Kuan, S., Edsall, L.E., Zhao, B.S., Xu, G.L., He, C., Ren, B., 2014. 5mC oxidation by Tet2 modulates enhancer activity and timing of transcriptome reprogramming during differentiation. Molecular Cell 56, 286–297.

Ito, S., Shen, L., Dai, Q., Wu, S.C., Collins, L.B., Swenberg, J.A., He, C., Zhang, Y., 2011. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333, 1300–1303.

Jin, S.G., Kadam, S., Pfeifer, G.P., 2010. Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic Acids Research 38, e125.

Jin, S.G., Jiang, Y., Qiu, R., Rauch, T.A., Wang, Y., Schackert, G., Krex, D., Lu, Q., Pfeifer, G.P., 2011. 5-Hydroxymethylcytosine is strongly depleted in human cancers but its levels do not correlate with IDH1 mutations. Cancer Research 71, 7360–7365.

Kohli, R.M., Zhang, Y., 2013. TET enzymes, TDG and the dynamics of DNA demethylation. Nature 502, 472–479.

Kriaucionis, S., Heintz, N., 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324, 929–930.

Lian, C.G., Xu, Y., Ceol, C., Wu, F., Larson, A., Dresser, K., Xu, W., Tan, L., Hu, Y., Zhan, Q., Lee, C.W., Hu, D., Lian, B.Q., Kleffel, S., Yang, Y., Neiswender, J., Khorasani, A.J., Fang, R., Lezcano, C., Duncan, L.M., Scolyer, R.A., Thompson, J.F., Kakavand, H., Houvras, Y., Zon, L.I., Mihm Jr., M.C., Kaiser, U.B., Schatton, T., Woda, B.A., Murphy, G.F., Shi, Y.G., 2012. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell 150, 1135–1146.

Lorsbach, R.B., Moore, J., Mathew, S., Raimondi, S.C., Mukatira, S.T., Downing, J.R., 2003. TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia 17, 637–641.

Moran-Crusio, K., Reavie, L., Shih, A., Abdel-Wahab, O., Ndiaye-Lobry, D., Lobry, C., Figueroa, M.E., Vasanthakumar, A., Patel, J., Zhao, X., Perna, F., Pandey, S., Madzo, J., Song, C., Dai, Q., He, C., Ibrahim, S., Beran, M., Zavadil, J., Nimer, S.D., Melnick, A., Godley, L.A., Aifantis, I., Levine, R.L., 2011. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell 20, 11–24.

Pfeifer, G.P., Kadam, S., Jin, S.G., 2013. 5-hydroxymethylcytosine and its potential roles in development and cancer. Epigenetics & Chromatin 6, 10.

Rasmussen, K.D., Jia, G., Johansen, J.V., Pedersen, M.T., Rapin, N., Bagger, F.O., Porse, B.T., Bernard, O.A., Christensen, J., Helin, K., 2015. Loss of TET2 in hematopoietic cells leads to DNA hypermethylation of active enhancers and induction of leukemogenesis. Genes & Development 29, 910–922.

Schuermann, D., Weber, A.R., Schar, P., 2016. Active DNA demethylation by DNA repair: Facts and uncertainties. DNA Repair (Amst) 44, 92–102.

Scourzic, L., Mouly, E., Bernard, O.A., 2015. TET proteins and the control of cytosine demethylation in cancer. Genome Medicine 7, 9.

Spruijt, C.G., Gnerlich, F., Smits, A.H., Pfaffeneder, T., Jansen, P.W., Bauer, C., Munzel, M., Wagner, M., Muller, M., Khan, F., Eberl, H.C., Mensinga, A., Brinkman, A.B., Lephikov, K., Muller, U., Walter, J., Boelens, R., van Ingen, H., Leonhardt, H., Carell, T., Vermeulen, M., 2013. Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized derivatives. Cell 152, 1146–1159.

Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., Rao, A., 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930–935.

Thomson, J.P., Lempiainen, H., Hackett, J.A., Nestor, C.E., Muller, A., Bolognani, F., Oakeley, E.J., Schubeler, D., Terranova, R., Reinhardt, D., Moggs, J.G., Meehan, R.R., 2012. Non-genotoxic carcinogen exposure induces defined changes in the 5-hydroxymethylome. Genome Biology 13, R93.

Tsagaratou, A., Lio, C.J., Yue, X., Rao, A., 2017. TET methylcytosine oxidases in T cell and B cell development and function. Frontiers in Immunology 8, 220.

Valinluck, V., Sowers, L.C., 2007. Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Research 67, 946–950.

Wu, X., Zhang, Y., 2017. TET-mediated active DNA demethylation: Mechanism, function and beyond. Nature Reviews. Genetics 18, 517–534.

Zhang, X., Su, J., Jeong, M., Ko, M., Huang, Y., Park, H.J., Guzman, A., Lei, Y., Huang, Y.H., Rao, A., Li, W., Goodell, M.A., 2016. DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. Nature Genetics 48, 1014–1023.

Diabetes and Cancer

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Introduction

Diabetes is a chronic progressive metabolic disease, defined by hyperglycemia. Diabetes is considered a worldwide epidemic. The world prevalence of diabetes for all ages is expected to double to 4.4% from the year 2000 to 2030. This means that by the year 2030 there will be 366 million people with diabetes living in the world. Older age is an important risk factor and the increase in the proportion of people >65 years of age across the world is an important reason for future projections on diabetes prevalence. Obesity is another important factor and given the increasing prevalence of obesity, it is likely that these figures provide an underestimate of future diabetes prevalence. Diabetes is more prevalent in men, but there are more women with diabetes in the world due to women's greater life-expectancy.

In his landmark article, published in Science in 1956, Otto Warburg linked metabolism with cancer. In the decades since, numerous epidemiological studies have demonstrated links between diabetes and cancer. Several publications during the year 2009 suggested that treatment with the insulin analogue, glargine, may increase the risk of cancer incidence. These studies, together with criticisms of them, raised interest in the relationship between diabetes and cancer, which went well beyond the use of glargine. In December 2009, representatives from American and European diabetes and cancer organizations met and produced a consensus report. This document supported associations of diabetes with increased risk of certain cancers, and corroborated cancer as a complication of diabetes. Hyperglycemia, hyperinsulinemia, adiposity, subclinical inflammation, and diabetes treatment were all identified for their involvement in the relationship between the two diseases. More research was called for to elucidate the mechanistic relationships between the diseases, as well as the effects of diabetes medications on carcinogenesis. Since the issuance of the consensus report, the publication of observational and clinical studies supporting associations between diabetes and cancer has soared. In this article we will first present the epidemiological evidence for associations between diabetes and all-site as well as site-specific cancers. Next, we will present possible mechanisms that may underlie associations of diabetes with cancer in general, as well as with a number of specific sites. Glucose lowering medications (the term used in this article for anti-diabetes drugs) will be discussed as possible confounders in the association of diabetes with cancer, as well as causative factors for carcinogenesis. We have tried to take a critical approach and urge our readers to do the same.

The Epidemiological Evidence

Epidemiological studies investigating associations of diabetes with cancer incidence or mortality are abundant. Diabetes is an exposure that cannot be imposed on study participants; thus, randomized controlled trials (RCTs) cannot be conducted to examine its association with cancer. Here, we present studies published since 2013, with an emphasis on systematic reviews and well-designed observational studies. Most of the meta-analyses conducted described considerable heterogeneity among included studies; and many lacked adjustments for potentially confounding factors. More recent studies more often adjusted for potentially confounding variables and some of them employed time-dependent approaches, which accounted for the date of diabetes diagnosis and assessed cancer incidence from only a certain period thereafter.

All-Site Cancer

Estimated population attributable fractions (PAFs) for diabetes, as well as for high body mass index (BMI, >25 kg/m^2) to cancer incidence in the year 2012 were published for 12 cancers, for 175 countries, with stratification by age and sex. Overall, the contribution of diabetes was estimated as 2%. However, higher PAFs were calculated for cancers of the liver (14.5%), pancreas (12.8%), gallbladder (7.8%), and colorectum (2.9%) in men; and for the liver (15.8%), pancreas (12.6%), endometrium (10.8%), gallbladder (7.4%), colorectum (2.8%), and breast (2.2%) in women. The overall estimated PAF for high BMI was calculated as 3.9%, almost twice that of diabetes. During the 10–32-year lag (starting in 1980–2002 and spanning to 2012) between exposure to diabetes or high BMI, a disproportionate number of those with high BMI evidently converted to diabetes, and this may have further contributed to their risk for cancer incidence, thus indicting BMI as a double whammy. Substantial differences were observed across geographical regions, supporting possible effects of factors other than diabetes and overweight. Though not investigated in that study, these could include differences in genetic susceptibility, in fetal and childhood nutrition and growth, in the detection and treatment of diabetes, and in lifestyle factors including nutrition and physical activity.

Most meta-analyses have reported positive associations of diabetes with cancer, yet some have only included a small number of cancer cases. A cohort of 2.3 million individuals followed for 11 years, using a time-dependent approach and a 2 year lag after diabetes diagnosis, reported hazard ratios (HRs) for all-site cancer of 1.42 (95% confidence interval (CI): 1.38, 1.46) in diabetic women and 1.33 (95%CI: 1.29, 1.36) in diabetic men, after adjusting for age, ethnic group, sociodemographic status (SES), and BMI. Several additional population-based studies have reported positive associations of diabetes with all-site cancer incidence. In studies

that stratified by sex, associations were generally stronger in women, but this is apparently due in large part to the lower incidence of prostate cancer reported for diabetic men, as well as to the increased risks of female-specific cancers. For non-sex-specific cancers, risks were generally similar between men and women. Potentially confounding factors were considered to varying degrees. The vast majority of studies that distinguished the type of diabetes, investigated type 2 (T2D). Higher risks of cancer were generally found for T2D than type 1 diabetes (T1D).

Site-Specific Cancers

In this section, we discuss meta-analyses of associations between diabetes and site-specific cancers, presented alphabetically, as well as a number of selected observational studies.

Bladder cancer

Differences between the sexes have been reported, with some studies finding a risk of bladder cancer in diabetic women and not men, and others finding the risk only in diabetic men and not women. Smoking may attenuate the risk. A number of studies have found an increased risk of bladder cancer, only or predominantly, during the first year following diabetes diagnosis. Such findings support the possibility that increased medical surveillance may explain observed associations of diabetes with bladder cancer. Nonetheless, a large historical population study, with a 2-year lagged analysis since diabetes diagnosis and 11 years follow up found a 35% increased risk in men and 70% in women, after adjusting for age, SES, and smoking.

Brain cancer

Brain cancer incidence was reported as 28% increased in diabetic women and 52% increased in diabetic men at 2–11 years after diabetes diagnosis.

Breast cancer

Several, but not all studies on the matter have shown an association of diabetes with an increased risk of breast cancer. However, these findings may not be relevant to Asian, Hispanic and African American populations, and this may be due, at least in part, to differences in mean body weight and fat distribution between populations. A large population study showed a 21% increased risk of breast cancer in postmenopausal, but not premenopausal women at 2–11 years after diabetes diagnosis and after adjustments for BMI, SES, ethnicity, and parity (HR = 1.21 95%CI 1.12, 1.30). A number of studies have shown that the association of diabetes with breast cancer may depend on the type of breast cancer. Triple-negative breast cancer tumors and estrogen receptor negative tumors have been associated with increased risk.

Cancer of unknown primary

Cancers of unknown primary (CUP) are metastatic malignant tumors for which the primary tumor is not identified; the prognosis of these cancers is generally very poor. Tobacco smoking is the only established risk factor for CUP. An association of diabetes with increased risk of CUP has been reported, particularly when the metastasis was to the liver and respiratory systems.

Colorectal cancer

Several studies have reported an association of diabetes with increased risk of colorectal cancer, in the range of 21%–26%. Some have found differences in risk between the sexes. A large population study with a time-dependent analysis reported 44% increased HR in women and 53% in men, for the 2–11 years after diabetes diagnosis, controlling for BMI. Another time-dependent analysis found an association of longer duration of obesity with increased risk of colorectal cancer, after adjusting for age, sex, smoking, alcohol consumption, and statin use in the previous 6 months. That study did not find an assocition with coloretal cancer of diabetes treatment stage; the latter was assessed as 90-day intervals of treatment with insulin or with one or more non-insulin glucose lowering medications alone or in combination. Some studies have shown differences in colon cancer risk, according to the location of the malignancy. However, the data are inconclusive, as both proximal and distal cancer have been found to be of greater risk.

Endometrial cancer

Several studies have found an association of diabetes with increased risk of endometrial cancer, which did not always remain statistically significant after adjusting for BMI.

Esophageal cancer

A number of studies have found a positive association of T2D with the risk of esophageal cancer; however, this association appears not to present in Asian populations. Male sex is a risk factor for esophageal cancer.

Gallbladder cancer

Several studies have found an increased risk of gallbladder cancer, similarly in men and women with diabetes, and independent of BMI, smoking, and a history of gallstones. Though, the possibility arises of confounding by alcohol consumption, the association was shown to persist in a study conducted in Israel, where alcohol consumption is not considered an important confounding factor.

Gastric/stomach cancer

Most, but not all studies on the matter have found an association of diabetes with gastric cancer. This risk may differ between the sexes, and may be particularly strong in East Asian populations.

Head and neck cancers

Associations of T2D with increased incidence of oral cancer, oropharyngeal cancer, and nasopharyngeal cancer have been reported, compared to non-diabetic individuals, matched by sex, age, obesity, and a number of comorbidities.

Liver cancer

Considerable evidence has accumulated for an association of diabetes with liver cancer, which appears to be stronger in non-Asian than Asian populations. Tobacco smoking and increased BMI, but not alcohol, have been identified as confounding factors. The association remains strong, even after excluding the first 2 years after diabetes diagnosis.

Lung cancer

Several studies have shown a robust association of diabetes with increased risk of lung cancer, particularly among women. However, the risk may be lower in Asian populations.

Ovarian cancer

Data seems conclusive for an association of diabetes with ovarian cancer, though the strength of the association varies between studies. Higher risks were found in studies published later and conducted outside of the United States or Europe, in studies that did not adjust for BMI or smoking status, and for T1D than T2D. An elevated risk of ovarian cancer was found to persist in diabetic women after adjustment for parity and the exclusion of women who had hysterectomies.

Pancreatic cancer

The pancreas, together with the liver, is directly responsible for glucose metabolism and insulin secretion. Thus, if diabetes is indeed associated with cancer via its metabolic pathways, the association would be expected to be the strongest in these organs. Indeed, the incidence of pancreatic cancer has consistently been shown to be elevated in the diabetic population. The population attributable fraction of diabetes to pancreatic cancer was found to be 13.5% and the BMI adjusted 2–11-year risk reportedly three times greater in the incident diabetic than in the non-diabetic population. The increased risk of pancreatic cancer is strong, even after excluding the first 2 years following diabetes diagnosis; and was shown to remain significant even after 20 years, and after adjusting for BMI and smoking.

Prostate cancer

Several studies have reported a lower risk for prostate cancer among men with than without diabetes, in both T1 and T2 diabetes. The risk appears to be lower in Europeans and Americans, and higher in Asians.

Thyroid cancer

The data are not conclusive regarding an association of diabetes with thyroid cancer, neither for men or women.

Site-specific cancers—summary

As the evidence presented here shows, considerable differences were found between studies for associations of diabetes with several types of cancer. While confounding factors and differences in study design likely account for some of the differences observed, real differences seem to exist between populations, and between the sexes.

Pathophysiology and Mechanisms Relevant to the Association of Diabetes With Cancer in General

Four main explanations are possible for the associations observed between diabetes and cancer; the relevance of these explanations differ among cancer types. Causality is the relationship by which the pathology of diabetes disease increases the risk of cancer. Since diabetes is diagnosed by hyperglycemia, we will consider direct cause as an effect of hyperglycemia on cancer risk. A second explanation for the associations observed between diabetes and cancer relates to shared risk factors. Insulin resistance and hyperinsulinemia, obesity, chronic inflammation, oxidative stress and alterations in sex hormones evidently comprise a metabolic milieu that predisposes to diabetes and that is conducive to cancer (Fig. 1). Shared lifestyle factors: physical inactivity, poor nutrition, smoking and excessive alcohol intake, as well as genetic susceptibility, may impact such metabolic milieu. A third possible explanation for the observed relationship between diabetes and cancer is reverse causality, in which the cancer pathology precedes the diabetes, and may even be the cause for hyperglycemia, though cancer is diagnosed only after diabetes. A fourth possibility is a spurious association, perceived due to biases or confounding factors, rather than a causal association between the diseases. We will discuss each of the four possible explanations for the observed associations between diabetes and cancer, considering cancer as a single entity. We will then present specific mechanisms by which diabetes may increase the risk of cancer, as pertains to specific cancers.

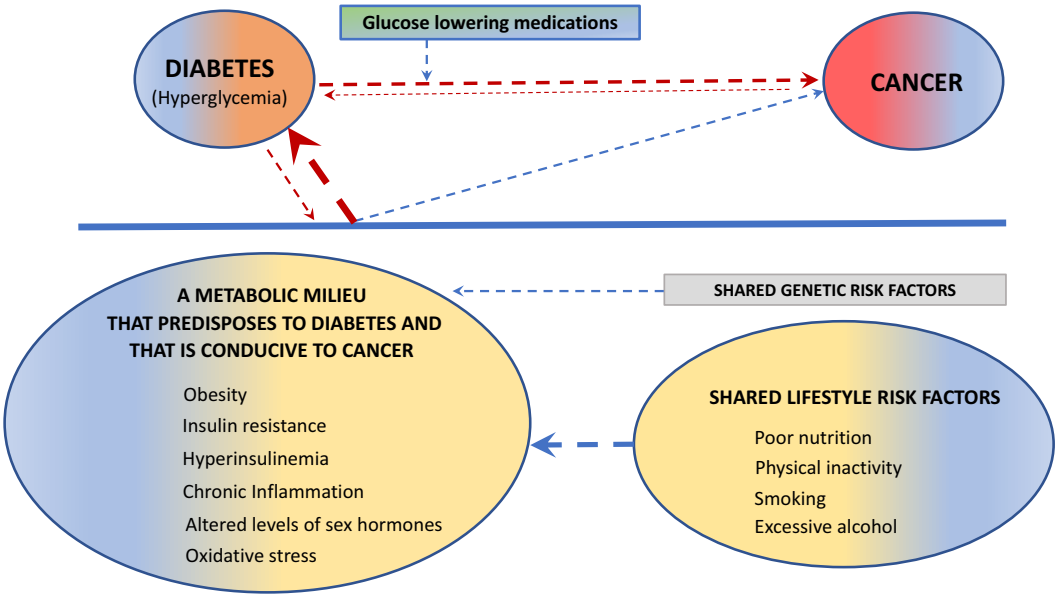

Fig. 1 The association of diabetes with cancer. The lines are *dotted* to indicate that the relationships are suspected and not verified, as they are based on observational studies. The thicker the line, the more evidence is currently available in support of the relationship.

Diabetes as a Risk Factor for Cancer

Distinguishing the impact on cancer of hyperglycemia from influences of pathophysiological conditions that accompany diabetes may be complex. The Warburg effect describes how irreversible damage to cell respiration leads some normal cells to become more proficient in using glucose for anaerobic glycolysis; these cells convert to highly proliferating undifferentiated cancer cells. However, this supposition does not infer that an increased supply of glucose, that is, hyperglycemia, promotes the development of cancer. Nonetheless, fatless hyperglycemic mice were shown to develop more aggressive tumors than normoglycemic mice. While this model neutralized the effect of obesity, it did not exclude hyperinsulinemia. Among diabetic mice that were insulin deficient, increased tumor growth was not observed. Taken together, while glucose is evidently essential for tumor growth, hyperglycemia in itself does not necessarily increase such growth. Still, knowledge accumulated since Warburg's publication has substantiated the perception of cancer as a metabolic disorder.

A number of publications have described direct effects of hyperglycemia on tumorigenesis. In nonmalignant human breast cancer cells, increased glucose uptake activated known oncogenic signaling pathways. Further, elevated glucose was shown to enhance the signaling of WNT/β-catenin in cancer cells, and this regulates cell proliferation and survival. In a mouse model, the glucose transporter GLUT1 was demonstrated as necessary for mammary tumorigenesis. Hyperglycemia was shown to promote features associated with cancer stem cells in premalignant and malignant pancreatic ductal epithelial cells, by activating signaling of the cytokine TGF-β.

Hyperglycemia promotes low-grade inflammation; and inflammatory processes are part of the etiology of cancer. Chronic hyperglycemia causes irreversible glycation and oxidation of proteins and lipids in the extracellular matrix; this results in the formation of advanced glycation end-products (AGE). Interactions of AGE and their receptors (RAGE) increase the generation of reactive oxygen species (ROS) in adipocytes, thus inhibiting glucose uptake and stimulating insulin resistance. Extracellular matrix glycation and RAGE activation may promote tumor growth by damaging proteins and DNA; this provides a link for the association between diabetes and cancer. Further evidence of an effect of elevated glucose on tumorigenesis by means of inflammation is the demonstration of increased phosphorylation of p38 MAPK, an essential kinase for inflammation, in pancreatic tumors from diabetic animals and in pancreatic cancer cells treated with high glucose.

The balance between oxidative and redox reactions in tumorigenesis is apparently complex. Along this line, James Watson, the Nobel prize winner, proposed that deficiencies rather than excesses in reactive oxygen species (ROS) may be the basis of diabetes and cancer, as well as other chronic diseases. He referred specifically to an insufficiency of the endoplasmic reticulum to generate the oxidative redox potential necessary to form the disulphide bonds that stabilize active proteins. Taken together, disruptions in oxidative or redox systems may link between diabetes and cancer.

Poorly controlled diabetes may perpetuate a pro-inflammatory state that may precede hyperglycemia, as will be detailed further below. The demonstration of higher levels of ROS among diabetic individuals with poorer glycemic control supports a role of hyperglycemia in pro-inflammation and oxidative stress. Further, poor glycemic control was found to be associated with serum gamma-glutamyltransferase and high sensitivity C-reactive protein levels, which are markers of inflammation. Nonetheless, individuals with poor glycemic control may tend to have more acute hyperinsulinemia, which is known to be associated with

pro-inflammatory effects and oxidative stress, as discussed below. This exemplifies the difficulty in distinguishing the effect of hyperglycemic from other pathophysiological components of diabetes.

The epidemiological evidence is inconclusive regarding a possible association of glucose control and the risk of cancer. While some studies have shown positive associations with any or site-specific cancers, others found no association. However, these studies generally did not consider factors that are relevant to the clinical manifestations and treatment of diabetes.

Studies on metabolic status in populations without known diabetes are not confounded by glucose lowering medications. Increased overall cancer risk has been reported in individuals without known diabetes yet with elevated HbA1c levels, as well as in diabetic patients. However, the great differences between cancer sites that have been reported for associations of HbA1c and cancer risk, highlight the importance of investigating cancer sites separately, in regard to associations with glycemia.

Shared Risk Factors Between Diabetes and Cancer

In individuals with diabetes, a number of factors interact with each other, generally before the diagnosis of diabetes; and separately or in combination, they may promote malignancy. While these factors are related to the development of diabetes, due to their precedence, we will consider them as entities shared by diabetes and cancer, rather than as the basis for a direct causal relationship between diabetes and cancer.

Obesity

Obesity is a major risk factor for both diabetes and cancer. Links between obesity and diabetes are complex and not fully understood; while most persons with T2D are obese, most obese persons do not have T2D. Certain cancers have long been associated with obesity. Already in 1980, endometrial cancer was found to be associated with excess body weight in postmenopausal women; and with increased levels of estradiol (E2) and decreased levels of sex hormone-binding globulin (SHBG). Among individuals with T2D, associations have been demonstrated of excess body weight with all cancer and with a number of site-specific cancers.

Adipocyte (fat) cells are central to energy storage, and particularly responsive to insulin. In addition to their metabolic role, adipocytes serve as endocrine cells that secrete a wide variety of hormones, among them cytokines, including tumor necrosis factor-alpha (TNF-α); steroid hormones, including estrogen; and adipokines, including leptin and adiponectin. Abnormal secretion of these hormones provides a link of obesity with diabetes and cancer. Obesity is associated with increased leptin and decreased adiponectin levels; these changes in endocrine function appear to have a role in cancer development. Insulin, estrogens, and TNF-α stimulate the release of leptin. Adiponectin regulates glucose metabolism, increases insulin sensitivity, and reduces the production of inflammatory cytokines.

Insulin resistance and hyperinsulinemia

Obesity, particularly abdominal obesity, leads to insulin resistance; insulin resistance can also contribute to obesity, and is a precursor of T2D. Insulin resistance is followed by compensatory hyperinsulinemia; this leads to beta cell compensation, which increases insulin production, resulting in hyperinsulinemia. The association between hyperinsulinemia and cancer was first hypothesized in relation to colon cancer; and was based on the recognition that similar factors are involved in hyperinsulinemia and colon cancer risk: namely, obesity, and abdominal obesity in particular; physical inactivity; and a low fiber, high sugar diet; the latter raises insulin levels by increasing the rate of blood glucose elevation and the blood glucose load. A number of epidemiological studies have substantiated the relationship between hyperinsulinemia and cancer. Moreover, factors related to hyperinsulinemia such as C-peptide, a biomarker of pancreatic insulin secretion and serum insulin growth factor (IGF)-1 were found to be associated with increased cancer risk.

A number of mechanisms have been proposed for the contribution of insulin resistance and hyperinsulinemia to cancer development. In addition to the metabolic effects of insulin and IGF, these hormones, together with their receptors and signal transduction networks, are recognized to have mitogenic effects. The binding of insulin receptor isoform A (IR-A) to IGF-II was described to result in mainly mitogenic effects; whereas, the binding of the same isoform to insulin results in mainly metabolic effects. Increased expression of IR-A has been detected in thyroid, colon, and breast tumors. Hyperinsulinemia impairs downregulation of insulin receptors in cancer cells; and affects signaling pathways of insulin and IGF-1, as well as plasma-free amino acid profiles; such activities may promote the development of both cancer and diabetes. Insulin and IGF-1, separately and in combination, increased cell proliferation and decreased apoptosis in colon cancer cells; and activated extracellular-signal regulated kinase 1/2 and c-Jun N-terminal kinase. All these activities are considered to contribute to the development of colon cancer.

Data of cancer incidence among individuals with T1D can help discriminate the roles of hyperglycemia and hyperinsulinemia, since only hyperglycemia and not hyperinsulinemia presents in T1D. A study of five nationwide diabetes registrars in European reported higher incidence of cancers of the stomach, liver, pancreas, endometrium and kidney in persons with T1D, compared to the general population; yet the rates were lower than for persons with T2D. These data support a role for hyperglycemia in cancer incidence, yet infer that other factors are evidently involved.

Effects of hyperglycemia and hyperinsulinemia may be additive or even synergistic. For example, the pro-inflammatory effect of hyperinsulinemia and hyperglycemia positively interact. Further, both hyperglycemia and hyperinsulinemia promote DNA

damage; and this has been proposed as a link in the relationship between diabetes and cancer: the mutations generated from DNA damage can lead to carcinogenesis. Disruptions in circadian rhythms that regulate metabolic processes has also been attributed a role in carcinogenesis.

Altered concentrations of sex hormones

Hyperinsulinemia affects concentrations of endogenous sexual hormones, and this may contribute to the development of both diabetes and cancer. High levels of insulin in the blood decrease the hepatic synthesis of SHBG, which leads to increased circulation of E2. Studies in both sexes have reported an inverse association of T2D with SHBG; and a positive association with E2. In women, elevated E2 has been shown to be associated with breast, endometrial, ovarian, and cervical cancers.

Oxidative stress and inflammation

While hyperglycemia induces oxidative stress, which causes insulin resistance that results in hyperinsulinemia, as described above, hyperinsulinemia also contributes to oxidative stress and low-grade inflammation. Associations are well established of pro-inflammatory agents with diabetes, as well as with several types of cancer. Apparently, in reciprocal relationships of chronic inflammation with both diabetes and cancer, inflammation may precede or be induced by diabetes and its associated conditions, as well as precede or be induced by oncogenic changes.

A number of mediators between insulin resistance and inflammation have received attention for their potential as links between diabetes and cancer. Elevated lactate promotes insulin resistance and inflammation, and has thus been coined "an interaction hub between diabetes and cancer." Similarly, the adipokine, chemerin, has been identified as a link between diabetes and cancer due to its involvement in inflammation and insulin resistance. Specifically, chemerin activates an inflammatory response, which increases oxidative stress in adipose tissue and thus contributes to insulin resistance. Further evidence for inflammation as a mediator in the link between diabetes and cancer is the reported association of increased concentration of growth differentiation factor 15 (GDF-15) with cancer, among individuals with type 2 diabetes. The stress responsive cytokine GDF-15 was found to be increased in individuals before type 2 diabetes diagnosis, yet not to be an independent predictor of diabetes. In addition, considerable evidence has accumulated for a role of the nuclear receptor transcription factor peroxisome proliferator activated receptor-γ (PPARγ) at the crossroad of obesity, diabetes, and several cancers. PPARγ is involved in the regulation of insulin and adipokine production and secretion; and of inflammatory pathways and differentiation. Thiazolidinediones, including rosiglitazone and pioglitazone, are synthetic ligands of PPARγ, which are used for the treatment of diabetes and whose pathways have been suggested to have potential benefit in the development of cancer therapies.

Genetically predisposing factors

Genetic factors may have both direct and indirect effects on the relationship between diabetes and cancer. Single nucleotide polymorphisms (SNPs) that were identified from genome-wide association studies as related to T2D, were associated with increased risk of a number of cancer types in diverse populations. Genetic factors may also affect the relationship between diabetes and cancer indirectly, by affecting shared predisposing factors. Along this line, the evidence does not support an independent relationship of the fat mass and obesity-associated (FTO) gene variant with cancer risk. Nonetheless, this variant may affect diabetes and cancer risk by its effect on obesity.

Lifestyle factors: diet, physical activity, alcohol consumption, smoking

A number of lifestyle factors have been identified as risk factors for both diabetes and cancer; and, as such, may contribute to obesity, as well as to other aspects of the metabolic milieu conducive to cancer, described above and depicted in **Fig. 1**.

Poor nutrition can contribute to imbalances in oxidation/redox systems, which can contribute to pro-inflammatory processes; and these, as discussed above, may increase risks of chronic illnesses including diabetes and some types of cancer. The role of diet in cancer development is particularly important to colorectal cancer, due to changes in the gut microbiota. Among the dietary factors that may be involved in the association between gut microbiota and the development of colorectal cancer are obesity, dietary patterns, and the consumption of red meat, sulfur, and fiber.

Evidence is robust for the benefit of physical activity to both primary and secondary prevention of chronic diseases, including diabetes and cancer. Among individuals with diabetes, cancer incidence was increased among those reporting low levels of physical activity (<2 h weekly), as well as among those who were overweight or obese, but not among those reporting high levels of physical activity (≥ 2 h weekly). According to James Watson's hypothesis, the benefit of physical activity to diabetes and cancer, as well as to other chronic diseases is in creating redox potential; specifically, the generation of ROS that oxidize free sulfhydryl groups of cysteine into the disulphide bonds that stabilize physiologically active proteins.

Elevated hazard ratios for diabetes and cancer were reported among women with a 20% increase in energy consumption; and decreased risks among women with a 20% increase in activity-related energy expenditure. Further, decreased risks of 25%, 14%, and 21% for diabetes, breast cancer, and colon cancer, respectively, were reported for individuals who were highly active (total weekly activity ≥ 8000 metabolic equivalents (METs)) compared with inactive individuals (<600 METs). Physical activity may affect breast cancer risk through biological pathways that involve adiposity, sex hormones, insulin resistance, adipokines, and chronic inflammation.

Several studies have reported an association of moderate alcohol consumption with a reduced risk for diabetes. However, some data have shown such association only for women. An association of occasional heavy drinking with an increased risk of diabetes

was also reported in women. Associations have been found of moderate alcohol consumption with increased risk of breast cancer and male colorectal cancer; and of light alcohol consumption with a reduced risk of lung cancer in both men and women.

Several studies have reported an association of cigarette smoking, including passive smoking with T2D. The cancer population attributable risk of smoking has been estimated as 20%, that is, the elimination of smoking could reduce one-fifth of all cancers. Up to one-third of cancer deaths has been estimated as preventable from smoking cessation; this includes the effect of passive smoking.

Reverse Causality

Reverse causality is a possible explanation for associations between diabetes and certain types of cancer. In a study of new users of diabetes medications, patients with upper gastrointestinal cancers (esophageal, stomach, pancreatic, liver cancers) were more likely than patients with other cancer diagnoses to have initiated insulin use during the 6 months prior to the cancer diagnosis. This supports the possibility of reverse causation, that is, that upper gastrointestinal cancers promoted diabetes, rather than the converse. To account for reverse causality, studies of the relationship between the two diseases have often excluded from analysis the early period, ranging from 3 months to 2 years, following the diabetes diagnosis.

Biases and Confounding Factors Relevant to the Exploration of the Relationship Between Diabetes and Cancer

A main difficulty in assessing the relationship between diabetes and cancer is the difficulty in identifying and mitigating the biases that may arise. In addition to biological mechanisms and shared risk factors, factors that are often not considered may influence the relationship between the diseases; these include: the duration and latency periods of both diseases, competing risks such as cardiovascular disease; glucose lowering medications, confounding by indication and cancer screening.

Since both diabetes and cancer may have long latency periods, their dates of diagnosis are somewhat arbitrary. Changes in glucose levels, and in insulin sensitivity and insulin secretion have been documented 3–6 years prior to the diagnosis of diabetes. For cancer subtypes, latency periods vary greatly, and should be considered in the determination of associations with diabetes. While not always a reflection of true biological disease onset, the dates of diagnosis of diabetes and cancer are important disease parameters, which generally reflect the initiation of treatment; the latter affects disease progression and possibly risks of other diseases.

Immortal time bias occurs from an inadequate definition of the follow up time of the exposed group, which ignores the period prior to diabetes diagnosis, thus implying that the study outcome, that is, cancer incidence, cannot occur during that period. The period before diabetes was diagnosed should not be ignored or attributed to the exposed arm, but rather to the unexposed group. This bias dilutes the strength of diabetes association with cancer risk towards the null hypothesis of no association.

Detection bias or ascertainment bias arises due to differences between persons with and without diabetes in cancer screening and surveillance; such differences may vary according to population. For example, United States and Israeli studies showed higher rates of screening among diabetic than non-diabetic men for colorectal cancer and for prostate cancer; however, in Korea, a lower rate of cancer screening was reported among people with than without diabetes; and a lower rate of screening for breast and cervical cancer was reported among Spanish women with than without diabetes. Several studies have shown particularly elevated cancer diagnoses during the first months, and up to 2 years following diabetes diagnosis, which could be due to increased cancer surveillance, as well as to reverse causality.

Detection/surveillance bias may be especially relevant to cancers of the thyroid and prostate, which have relatively long latency periods. For these cancers, particularly, symptoms of the diagnosed cancer may not present during a patient's lifetime; thus, the detection may be considered as an overdiagnosis, and the ensuing treatment may actually have negative consequences on physical and mental health, despite the cancer being asymptomatic. Detection bias can be mitigated in research by excluding from analysis an initial period, usually of up to 2 years following diabetes diagnosis, and adjusting for screening procedures.

Mechanisms Relevant to the Association of Diabetes With Certain Site-Specific Cancers

In addition to the mechanisms described above, which may increase cancer risk in any organ, several site-specific mechanisms affecting particular organs have been described; some of them are described below.

Colorectal Cancer

Disturbances in luminal factors may contribute to the increased risk of colorectal cancer in diabetic individuals; such factors include altered bile acid metabolism, delayed colonic transit, compositional change in gut microbiota, and reduction in the intestinal mucus barrier. Gut microbial dysbiosis has been reported in persons with T2D. NLRP6 inflammasomes have been reported to disrupt metabolism by altering gut microbiota and also to contribute to tumorigenesis. Various energy sensing pathways have been suggested to specifically link T2D with colorectal cancer. In addition, both diabetes and poor nutrition were shown to be independent and additive risk factors for colorectal cancer.

Endometrial Cancer

Estrogen and insulin have been shown to exhibit a synergistic effect in the development of type 1 endometrial carcinoma.

Liver Cancer

T2D is associated with increased incidences of a number of liver diseases that are associated with hepatocellular cancer (HCC); among them, hepatitis B virus infection, which accounts for more than half of HCC cases; hepatitis C virus infection, which contributes to 25% of HCC cases worldwide; and both alcohol-induced liver disease and nonalcoholic fatty liver disease, and their progression to HCC. In addition, excessive alcohol consumption is a risk factor for both T2D and HCC, and a synergistic interaction of alcohol, diabetes and hepatitis on HCC has been observed.

Prostate Cancer

While several studies have reported a lower risk for prostate cancer among diabetic men, a number of investigations have suggested that screening strategies may affect the association. In an Israeli study, the screening rate for the prostate-specific antigen (PSA) was 10% higher among diabetic than non-diabetic men, PSA positivity (>4 μg/L) was 20% lower, and only low-moderate grade, not high-grade tumors were decreased in diabetic men. These findings suggest that lower reported risks of prostate cancer in diabetic men may be due to lower PSA levels compared to non-diabetic men, rather than to a lower actual risk. In concurrence, a large Australian study identified a history of diabetes, as well as a number of socioeconomic and health system factors, such as frequency of visits to primary care physicians, as factors that affect rates of PSA testing. The possibility that a lower rate of prostate cancer screening among Asian than European men may explain, at least in part, the increased risk of prostate cancer in Asian diabetic men and decreased risk in European diabetic men needs investigation.

Thyroid Cancer

T2D as well as other metabolic diseases may increase the risk of thyroid cancer by affecting the level of thyroid stimulating hormone, iodine deficiency, chronic autoimmune thyroiditis and estrogen-dependent signaling.

Glucose Lowering Medications and Cancer Risk

Although diabetes is an exposure that cannot be imposed on study participants; glucose lowering medications are such an exposure and can be studied in RCTs with cancer as an outcome. Since mainly diabetic patients use these medications, separating effects of the disease from effects of the treatment is challenging. Phase-3 RCTs, as well as observational studies, provide evidence to this paradigm, each with its inherent methodological limitations. The pharmacologic treatment of T2D changes with increasing hyperglycemia, the indication of disease severity. Recommendations for the treatment of T2D consist of first-line treatment with metformin, followed by the successive addition of other oral glucose-lowering medications, or insulin, as needed to maintain glucose control, which is measured by the HbA1c level. T1D is treated by insulin, sometimes with the addition of oral glucose-lowering medications. Diabetes progression, together with its complications, the increased cancer risk over time, and the sequential nature of diabetes treatment and its combinations, makes it difficult to disentangle associations of diabetes pharmacotherapy and cancer risk.

The biases discussed above regarding all investigations of diabetes and cancer are also relevant to observational studies on glucose lowering medications. In addition, a number of parameters are particularly relevant to investigations of medications, among them: precise classification of drug exposure: dosages, duration and continuity. Factors such as increased hyperglycemia and the time of initiation of treatments are especially challenging to the investigation of associations of glucose lowering medications with cancer risk. Thus, disease severity may be a confounder between the glucose lowering medication investigated and cancer risk, a phenomenon called "confounding by indication." This bias is particularly relevant to non-randomized studies of diabetes therapies; whereby, for example, patients on metformin and insulin are compared, without considering that generally, metformin is a first-line treatment, and insulin is administered at a later stage when oral agents do not sufficiently reduce hyperglycemia. Another example arises in observational investigations of dose response relationships, when patients are stratified according to cumulative time under treatment or cumulative dose. However, such an approach may be confounded by disease severity, disease duration, and survival bias. Proper adjustments and using a time dependent statistical approach can attenuated these methodological limitations.

Adjustment for disease duration and the use of statistical methods like propensity scoring or instrumental variables can overcome, to some extent, lack of randomization and fundamental differences in the compared treatment groups, but they do not account for unmeasured confounders. In addition, while time varying variables: factors like HbA1c level, BMI and disease duration affect the selection of glucose lowering medications over the course of the disease, treatments also affect HbA1c levels and BMI. Associations of BMI and HbA1c with cancer risk may therefore confound the true association between glucose lowering medications and cancer. Not accounting for competing risks between diseases can result in a selection bias, as for example happens when

a diabetic patient dies due to another cause before treatment initiation. Excluding this patent from the analysis will result in immortal time bias. Time-dependent analysis, which accounts for the timing of changes in medications, can mitigate biases that arise in this context. In addition, such approach can overcome a differential misclassification bias, such as when patients are classified as using a medication of interest throughout the study period even though they may have used it only for a portion of the period. Referring unexposed person-years to the exposed group dilutes the magnitude of the association towards the null, and the investigated medication may even appear to show a protective effect with cancer, as often happens with metformin.

Glucose lowering medications are divided into classes according to their mechanism of action on various organs and tissues (Fig. 2). The diversity of these classes of medications reflects the complexity of diabetes as a multi-organ metabolic disease. The classes of medications can be categorized into: *insulins* (human and analogs); *insulin secretagogues* (including sulphonylureas, meglitinides, GLP-1 agonists, and DPP-4 inhibitors); *insulin sensitizers* (including biguanides and thiazolidinediones); and other medications, which at present include glucose absorption disrupters acting in the intestine (alpha-glucosidase inhibitors) and glucose reabsorption disrupters acting in the kidneys (SGLT2 inhibitors). Deciphering the association between glucose lowering medications and cancer risk can help elucidate the mechanisms underlying the diabetes cancer association. For example, findings that sulfonylureas and exogenous insulin are positively associated with cancer risk, and medications that decrease insulin resistance such as metformin and perhaps thiazolidinediones negatively associated with cancer risk, are congruent with a role of hyperinsulinemia in cancer risk.

Insulin

Insulin remains the most potent glucose-lowering agent. However, weight gain and risks of hypoglycemia may accompany improved glycemic control. In addition, all insulins to some extent act as a growth stimulating factor, raising concerns regarding their potential contribution to tumorogenicity. Since 2009, several studies have investigated cancer risks of insulin analogues (Aspart, Determir, Glargine, Glulisine, and Lispro), compared to human insulin. The insulin analogue glargine was associated with an increased risk of breast cancer risk in a number of observational studies that were later criticized for methodological flaws. RCTs have generally not found insulin treatment of any type to be associated with increased cancer risk, though some case-control and cohort studies showed increased risk. Further, the CARING five-country cohort study did not find consistently higher risk for 10 cancers following 5 or more years use of insulin glargine or insulin detemir compared to human insulin. A dose-response increased breast cancer risk was shown in women 40 years and older who used glargine compared to Hagedorn (NPH); this risk was evident only after 6 or more years of use. However, concerns that arise from the study's methodology are confounding by indication and differential misclassification of the outcome due to the screening policy for breast cancer in England. While there is no compelling

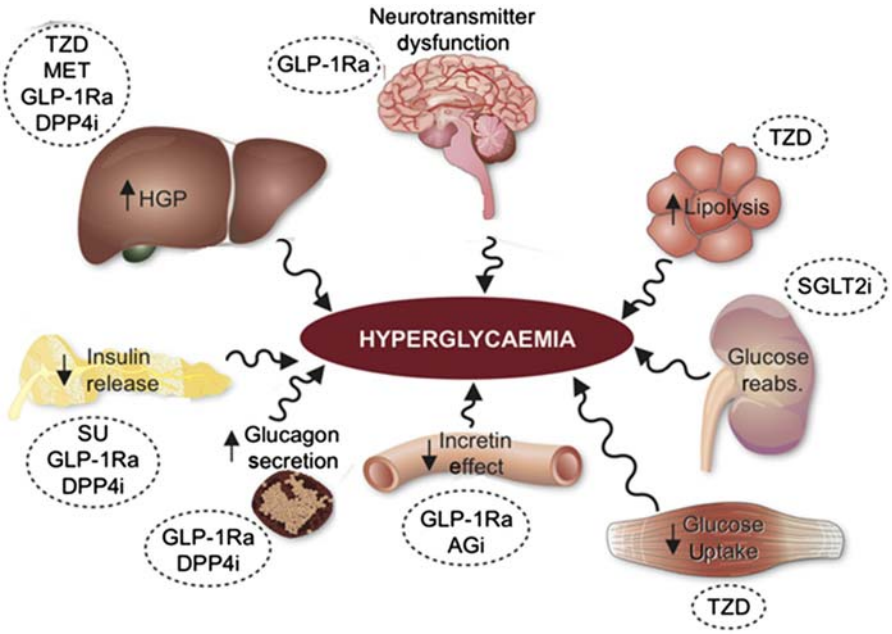

Fig. 2 Site of action of glucose-lowering medications on target organs and tissues contribution to hyperglycemia. Site of action of glucose-lowering medications on target organs are shown in the dotted circles. Contribution of different tissues to hyperglycemia is indicated by the direction of the small arrows indicating the direction of change in function of the organs in diabetes. HGP, hepatic glucose production; GLP-1Ra, GLP-1 receptor agonists; TZDs, thiazolidinediones; DPP4i, DPP4 inhibitors; SGLT2i, SGLT2 inhibitors; SU, sulfonylureas; AGi, α-glucosidase inhibitors. Adapted from Impact of glucose-lowering drugs on cardiovascular disease in type 2 diabetes. European Heart Journal 2015, **36**(*34*), 2288–2296. https://doi.org/10.1093/eurheartj/ehv239.

evidence that any clinically available insulin analogue or human insulin increases breast cancer risk, insulin might induce breast tumor progression by upregulating mitogenic signaling pathways.

Sulfonylureas

Sulfonylureas have been used to treat T2DM patients since the 1960s, although their use has declined with the emergence of new glucose lowering medications. Their main mechanism of action is to enhance insulin secretion by β-cells; the resultant hyperinsulinemia overcomes, in part, insulin resistance in liver and muscle, and leads to decreases in blood glucose and HBA1c. In vitro studies demonstrated that sulfonylureas accelerate β-cell failure, a process that was shown in an in vivo study to be reversible. Their main side effect is increasing the risk of hypoglycemia. Several observational studies of sulfonylureas have found an increased cancer risk, but such association is not evident in RCTs. Differences in cancer risk between types of sulfonylureas have been reported and warrant further research.

Metformin

Metformin, a biguanide, has been in use for over 50 years and its safety profile is well known. Metformin's glucose lowering effect is most probably confined to the liver and gut: reduction in hepatic glucose production and modest weight loss due to an anorectic effect and gastrointestinal side effects (diarrhea, abdominal discomfort, and flatulence). While not completely clear, the glucose lowering mechanism of metformin apparently involves inhibition of mitochondrial complex I, which increases the AMP/ATP ratio and leads to activation of the energy sensor Adenosine Monophosphate (AMP)-Activated Protein Kinase (AMPK). A direct anticancer mechanism of metformin may involve AMPK-independent and AMPK-dependent effects; while reductions in blood glucose, hyperinsulinemia, and IGF-1 level are possible indirect effects. Already in 2005, the first study to report an association between glucose lowering treatment and cancer suggested a protective effect of metformin. Numerous observational studies have reported a protective effect of metformin on all-site or specific cancer risk; however, those that accounted for biases were less likely to report such. Moreover, RCTs have generally not shown an association between metformin and cancer incidence. The metformin protective effect reported in observational studies has been claimed to result from time-related biases, as discussed above, and from overlooking aspects of the natural history of T2D. Several studies that accounted for time-related biases reported metformin not to be associated with a decreased risk of prostate, lung, colorectal, breast, or pancreas cancers. A number of clinical trials are in the process of investigating a protective effect of metformin on breast cancer risk in overweight or obese women. Metformin is thought to have antineoplastic activity directly via the modulation of cancer stem cell energy pathways, and indirectly by reducing hyperinsulinemia and glycemic levels. Clinical trials in prostate cancer patients are in process, with metformin as an additive to treatment arms, to examine a possible benefit in cancer survival.

Thiazolidinediones

The thiazolidinediones (TZDs, pioglitazone and rosiglitazone) are insulin sensitizers that activate peroxisome proliferator-activated receptors-γ (PPAR-γ). TZDs induce insulin secretion, preserve β-cell function, and improve insulin sensitivity. RCTs have generally shown no association of TZDs with all-site cancer; though some case-control and cohort studies have shown a protective effect. In December 2016, the U.S. Food and Drug Administration (FDA) re-issued a drug safety communication to the labelling information, having concluded that use of pioglitazone may be linked to an increased risk of bladder cancer. In France the drug has been removed from the market altogether. An analysis of close to 3 million individuals from several cohort and RCT studies showed higher risk of pioglitazone for bladder cancer for European populations, larger cumulative drug dose and longer use. Observational studies have generally not shown a significant association of bladder cancer with ever pioglitazone users compared with diabetic patients who had never used it, although an increased risk for bladder cancer may be possible after longer than 1-year exposure to pioglitazone.

Meglitinides

Meglitinides (repaglinide, mitiglinide and nataglinide) are insulin secretagogues that can cause weight gain and hypoglycemia. The evidence shows no association between Meglitinide use and all-site cancer risk.

Alpha-Glucosidase Inhibitors (AGIs)

AGIs (acarbose, miglitol, and voglibose) impede the breakdown of complex carbohydrates in the gastrointestinal tract, thus slowing carbohydrate absorption and reducing postprandial hyperglycemia. AGIs also increase plasma GLP-1 levels. Treatment with AGIs is usually associated with weight loss. RCTs have not shown an association between AGIs and cancers.

Dipeptidyl Peptidase-4 Inhibitors

Dipeptidyl peptidase-4 inhibitors (DPP4i) block the degradation of GLP-1, glucose-dependent insulinotropic polypeptide GIP, and other peptides, including brain natriuretic peptide. This class of drugs has a modest effect in reducing HbA1c and no effect on body

weight. Early concerns about an increased risk of acute pancreatitis and pancreatic cancer associated with DPP4i use have, for the most part, been refuted in more-recent studies. However, laboratory findings have raised concern that DPP4i can accelerate tumor metastasis. Numerous RCTs have shown no association between DPP-4is and cancer risk.

Incretins

The incretins, (GLP-1 receptor agonists—GLP-1RAs) mimic the action of endogenous glucagon-like peptide-1 (GLP-1). They enhance glucose homeostasis through: stimulation of insulin secretion; inhibition of glucagon secretion; direct and indirect suppression of endogenous glucose production; and delayed gastric emptying. The result is suppression of appetite, decreased post-prandial hyperglycemia, and enhanced insulin sensitivity secondary to weight loss. Laboratory studies suggest GLP-1RAs signaling promotes intestinal growth, and may promote colonic tumorigenesis. Several RCTs have reported no association of GLP-1RAs with cancer risk; a number of them specifically investigated the risk of pancreatic cancer. The possibility arises for a decreased risk of pancreatic cancer following long-term use of incretin therapies. GLP-1 analogues were demonstrated not to increase breast cancer incidence compared to DPP-inhibitors. The incretin therapies GLP-1 and Exendin-4 were shown to inhibit migration and promote apoptosis of human ovarian cancer cells, thus supporting the potential for a protective effect of these therapies on cancer risk.

Sodium-Glucose Co-Transporter-2 Inhibitors

Sodium-glucose co-transporter-2 inhibitors (SGLT2i) (dapagliflozin) represent the newest class of oral glucose lowering agents approved for the treatment of T2D in the United States and Europe. Inhibition of the SGLT2 transporter leads to increased urinary glucose excretion, resulting in a decline in plasma glucose concentrations. Due to their unique mechanism of action, the SGLT2i can be combined with all glucose-lowering medications, including insulin. The evidence shows no association of dapagliflozin with cancer risk. Long term RCTs are needed to rule out an association with bladder or urethral malignancies.

Glucose Lowering Medications—Summary of the Evidence

Meta-analyses from systematic reviews of observation studies (cohort and case control studies) showed increased risk for insulin and sulfonylureas, and a protective effect for metformin and TZDs. However, meta-analyses of RCTs showed no increased cancer risk conferred by any glucose lowering medication. We note that RCTs published to date on the association between glucose lowering medications and cancer risk were not designed for this purpose but were conducted to demonstrate cardiovascular safety and HbA1c reduction efficacy. These trials included diabetic patients with a specific risk profile to address their designated purposes; thus, some lacked statistical power to investigate cancer risk due to a short length of follow up or a small sample size. Further, since metformin, which is often used as the comparator or as background therapy, is the first-line oral therapy for type 2 diabetes, its comparison with other therapies that could be of lower clinical safety and effectiveness would be unethical. While meta-analyses overcome problems of small sample size, they are still subject to the limitations inherent in each of the included studies. Moreover, high heterogeneity between studies can reduce the validity of the findings.

To date, no study was published that accounts for the full complexity of diabetes disease, which exposes patients to sequentially changing treatments. Glucose lowering medications potentially act both as confounders and as causative factors in the association between diabetes and cancer. Thus, a research implication is that glucose lowering medications may confound associations of diabetes with cancer risk less than previously thought. A temporal association is consistently demonstrated between diabetes and cancer, which is not explained by glucose control or by glucose lowering medications. A clinical implication is that cancer risk need not be considered in the selection of diabetes treatments, in most circumstances. Still, the possibility that metformin, and perhaps also TZDs, may reduce cancer risk seems worthy of further investigation.

Concluding Remarks

Associations of diabetes with cancer risk are well established. Explanations are abundant and vary according to cancer type. Diabetes is evidently temporally associated with cancer risk directly, by means of hyperglycemia; as well as indirectly by means of shared risk and predisposing factors. Accounting for the numerous factors that can confound the association between the diseases and the potential biases is challenging and must be addressed. The evidence does not support causality of glucose lowering medications on cancer risk.

See also: Hormones and Cancer. Metformin.

Further Reading

Dang, C.V., 2012. Links between metabolism and cancer. Genes and Development 26, 877–890.

Dankner, R., Boffetta, P., Balicer, R.D., et al., 2016a. Time-dependent risk of cancer after a diabetes diagnosis in a cohort of 2.3 million adults. American Journal of Epidemiology 18, 1098–1106.

Dankner, R., Boffetta, P., Keinan-Boker, L., et al., 2016b. Diabetes, prostate cancer screening and risk of low- and high-grade prostate cancer: An 11 year historical population follow-up study of >1 million men. Diabetologia 59, 1683–1691.

Farmer, R.E., Ford, D., Forbes, H.J., Chaturvedi, N., Kaplan, R., Smeeth, L., Bhaskaran, K., 2017. Metformin and cancer in type 2 diabetes: A systematic review and comprehensive bias evaluation. International Journal of Epidemiology 46, 728–744.

Johnson, J.A., Bowker, S.L., 2011. Intensive glycaemic control and cancer risk in type 2 diabetes: A meta-analysis of major trials. Diabetologia 54, 25–31.

Johnson, J.A., Carstensen, B., Witte, D., et al., 2012. Diabetes and cancer (1): Evaluating the temporal relationship between type 2 diabetes and cancer incidence. Diabetologia 55, 1607–1618.

Kong, A.P.S., Xu, G., Brown, N., et al., 2013. Diabetes and its comorbidities—Where east meets west. Nature Reviews Endocrinology 9, 537–547.

Kyu, H.H., Bachman, V.F., Alexander, L.T., et al., 2016. Physical activity and risk of breast cancer, colon cancer, diabetes, ischemic heart disease, and ischemic stroke events: Systematic review and dose-response meta-analysis for the global burden of disease study 2013. BMJ 354, i3857.

Pearson-Stuttard, J., Zhou, B., Kontis, V., et al., 2017. Worldwide burden of cancer attributable to diabetes and high body-mass index: A comparative risk assessment. The Lancet Diabetes and Endocrinology 6, 95–104.

Shanik, M.H., Xu, Y., Skrha, J., et al., 2008. Insulin resistance and hyperinsulinemia: Is hyperinsulinemia the cart or the horse? Diabetes Care 31 (Suppl 2), S262–S268.

Suissa, S., Azoulay, L., 2012. Metformin and the risk of cancer: Time-related biases in observational studies. Diabetes Care 35, 2665–2673.

Tsilidis, K.K., Kasimis, J.C., Lopez, D.S., et al., 2015. Type 2 diabetes and cancer: Umbrella review of meta-analyses of observational studies. BMJ 350, 1–11.

Wu, L., Zhu, J., Prokop, L.J., Murad, M.H., 2015. Pharmacologic therapy of diabetes and overall cancer risk and mortality: A meta-analysis of 265 studies. Scientific Reports 5, 10147.

Zheng, C., Beresford, S.A., Van Horn, L., et al., 2014. Simultaneous association of total energy consumption and activity-related energy expenditure with risks of cardiovascular disease, cancer, and diabetes among postmenopausal women. American Journal of Epidemiology 180, 526–535.

Diet and Cancer

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Introduction

Diet plays a key role in the prevention of chronic diseases including cancer. Cancer is one of the leading cause of death accounting for 8.2 million deaths globally (International Agency for Research on Cancer, 2014). Cancer rates vary across geographical regions and population migration studies observed a 20-fold variation in rates (Rastogi et al., 2008; Ziegler et al., 1993; King et al., 1985) suggesting that environmental factors including diet play may be important contributors. Historically there is evidence supporting a role of diet in carcinogenesis from observations by Peyton Rous who, in Rous (1914) found that food restriction in mice reduced tumor metastasis (Rous, 1914), while in the 1920s obesity was found to be associated with greater cancer mortality and in the 1930s high plant food consumption was suggested to be protective in cancer development (Stocks et al., 2009). Doll and Peto (1981) estimated that approximately 35% of cancer deaths could be avoided by dietary modifications (Doll and Peto, 1981) and this seems to be confirmed by more recent epidemiological studies (McCullough and Giovannucci, 2004; Willett, 1995). Generally, high calorie diets as well as high consumption of red and processed meat are linked to increased cancer risk whereas high vegetable and fruit intake, particularly citrus fruit and vegetables from the brassica family, are consistently found to be protective against cancer development (Kushi et al., 2012; Pan et al., 2012; Santarelli et al., 2008; Willett, 1995; Bosetti et al., 2012; Turati et al., 2015b). There is a paucity of data from clinical trials of diet in cancer patients owing to the challenges and high costs of such trials. Below sections targeting nutrients, selected food groups and dietary patterns are discussed.

Dietary Carbohydrates and Glycemic Index

Amongst dietary compounds, carbohydrates have been implicated in the etiology of cancer at various sites (Franceschi et al., 1997; Giovannucci, 1995; Augustin et al., 2002) while sources of carbohydrates such as whole grains have been found to reduce cancer mortality in many studies and have been included in the guidelines for breast cancer recurrence of the American Cancer Society (Runowicz et al., 2016). In a recent meta-analysis published in 2016 of ten large international epidemiological studies showed that higher consumption of whole grains compared to low or no consumption reduced cancer mortality by 12% (Zong et al., 2016). This suggest that not all dietary carbohydrates are the same and some may give protection while others increase risk. Specifically it has been proposed that the extent of the blood glucose rise produced by carbohydrates and captured by the glycemic index (GI), may play a differential role in cancer development (Augustin et al., 2002). The GI is a ranking of carbohydrate foods based on the ability to raise blood glucose concentration by the food ingested (Jenkins et al., 1981). In equicarbohydrate amounts, low GI foods increase blood glucose levels mildly while high GI foods result in larger rises in blood glucose and insulin (Jenkins et al., 1981). Hyperglycemia and hyperinsulinemia have been suggested to increase cancer risk. McKeown-Eyssen (1994) and Giovannucci (1995) independently suggested that blood glucose and insulin may be important factors promoting malignant transformation and tumor growth. Indeed hyperglycemia, both fasting and postprandial, and even at concentrations below the diabetes threshold have been associated with a higher risk of cancer incidence and death independent of body weight (Jee et al., 2005; Stattin et al., 2007; Stocks et al., 2009) and cancer site (Crawley et al., 2014). A meta-analysis showed that blood glucose concentration above 110 mg/dL (6.11 mmol/L), increased risk of all cancers by 32% compared to glycemic concentrations below 110 mg/dL (6.11 mmol/L) (Crawley et al., 2014). Fasting blood glucose $\geq$140 mg/dL ($\geq$7.8 mmol/L) was associated with significantly higher (23%–29%) death rates from major cancer sites including colorectum, liver and pancreas, compared with blood glucose concentration $<$90 mg/dL ($<$5.0 mmol/L) (Jee et al., 2005). Furthermore, hyperglycemia can enhance cancer progression and promotion (Li et al., 2015), and in a dose–response meta-analysis it was shown that every 10 mg/dL (0.56 mmol/L) increase in blood glucose concentration corresponded to a 15% increased risk of pancreatic cancer (Liao et al., 2015). Reducing hyperglycemia pharmacologically by use of antihyperglycemic medications (e.g., metformin, acarbose) (Noto et al., 2012; Tseng et al., 2015) and consuming low GI diets instead of high GI diets has shown benefits in reducing the risk of major cancers especially colorectum, breast and endometrium (Augustin et al., 2001; Barclay et al., 2008; Turati et al., 2015a; Choi et al., 2012). Some evidence may also suggest reduced colorectal cancer recurrence (Meyerhardt et al., 2012). The mechanisms involved in increasing risk of cancer with high GI foods may include at least two pathways, hormonal and oxidative stress.

Potential Mechanisms

Regarding whole grains these carbohydrate foods are rich in dietary fiber, vitamins, minerals and phytochemicals which are protective against chronic diseases (Slavin, 2003). Dietary fiber down-regulates inflammation, probably through the production of short chain fatty acids, particularly butyrate and propionate, when fiber is fermented in the colon by gut microbiotia. There are however other mechanisms of action beyond fiber which have been suggested and include (Fardet, 2010). Furthermore, dietary fiber may reduce insulin resistance by decreasing the rate of carbohydrate absorption and increasing insulin-like growth factor binding protein 3 concentrations. These mechanisms are explained further below in relation to the dietary GI.

Insulin-like growth factor—1 (IGF-1)

High GI foods induce higher glycemic and insulinemic responses and insulin is known to have growth promoting and proliferating effects (Giovannucci, 2003; Kaaks and Lukanova, 2001) and to affect recurrence in breast cancer patients (Goodwin et al., 2002). Insulin is able to bind to the insulin-like growth factor (IGF)-1 receptor and produce the cascade of reactions typically seen with IGF-1 activation. IGF-1 is a peptide, similar in molecular structure to insulin, produced mainly by the liver in response to growth hormone, but can be synthesized by almost any tissue in the body (Olivecrona et al., 1999). IGF-1 is the primary circulating growth factor in the IGF system, comprising of other IGFs and their binding proteins, regulating cellular proliferation, differentiation and apoptosis. Meta-analyses of recent studies confirm previous reports regarding elevated serum levels of IGF-I to be associated with an increased risk of colorectal cancer (Chi et al., 2013; Rinaldi et al., 2010), breast cancer (Endogenous et al., 2010) and prostate cancer (Rowlands et al., 2009). Studies in healthy people of low versus high GI diets and changes in circulating IGF-1 and its binding proteins suggest that low GI diets beneficially affect the IGF-1 axis and therefore may lead to an environment that is less conducive to tumor growth compared to high GI foods (Brand-Miller et al., 2005; Runchey et al., 2012).

Oxidative stress

Damage to DNA by reactive metabolites has been widely accepted as a major cause of cancer (Ames and Gold, 1991; Beckman and Ames, 1997; Halliwell, 2007). Oxidative stress can activate a variety of transcription factors leading to the expression of over 500 different genes, including those for growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules (Reuter et al., 2010). Activation of these pathways via oxidative damage has been implicated in the initiation, promotion, and progression of a normal cell into a tumor cell (Reuter et al., 2010; Valko et al., 2007). Consumption of high glycemic index foods have been suggested to increase oxidative stress through formation of free radicals that are capable of damaging biological molecules and hence initiating abnormal cell growth through gene mutation. Hyperglycemia contributes to increased oxidative stress through an incremental generation of reactive oxygen species (ROS) during the mitochondrial oxidative metabolism of carbohydrates which depletes antioxidant defenses (Brownlee, 2005; Ceriello et al., 1998; Title et al., 2000). Reducing the GI of a meal using acarbose has been shown to significantly reduce markers of oxidative stress in individuals with impaired glucose tolerance (Inoue et al., 2006; Monnier et al., 2006; Quagliaro et al., 2005). Compared to a high GI diet, consumption of a low GI diet was associated with an increased plasma total antioxidant capacity in overweight and obese men (Botero et al., 2009) and with a lower marker of oxidative stress in overweight and obese women (Arikawa et al., 2015).

Conclusions and Future Directions

Despite population differences in dietary habits and carbohydrate intakes low GI diets seem to protect from cancer risk compared to high GI diets particularly for specific cancer sites: colorectum (WHS study) (Higginbotham et al., 2004), breast (ORDET and EPIC studies) (Sieri et al., 2007; Romieu et al., 2012) and endometrium (NBSS and NHS studies) (Silvera et al., 2005; Cui et al., 2011).

Populations who could benefit the most from consuming low GI diets may be those characterized by a high prevalence of overweight and/or obesity since the strongest associations have been found in people with higher body weight (Coleman et al., 2014; Franceschi et al., 2001) and this is possibly due to the underling insulin resistance in people who are overweight or obese which would result in higher insulin responses for the same dietary GI rank. In an environment characterized by sedentary behavior, overeating and obesity, higher circulating blood glucose as a result of high GI food consumption, could represent a greater burden for an already stressed metabolism caused by obesity and lack of physical activity. Lowering the GI of foods may therefore be particularly relevant in affluent societies and in countries with high consumption of dietary carbohydrates as in the Mediterranean region (Favero et al., 1997; Slimani et al., 2002; Wirfalt et al., 2002). Regarding prevention of cancer recurrence (secondary prevention), the guidelines for cancer survivors by the American Cancer Society include foremost maintenance of a healthy body weight, engaging in physical activity and consuming a healthy diet which are consistent with guidelines for cancer prevention and heart disease prevention (Kushi et al., 2012). There is no mention of carbohydrate quality beyond whole grains, with regards to longer survival. At present, there is a lack of clinical trial investigating low GI diets in secondary cancer prevention. Nevertheless, when summarizing the mechanistic evidence linking glycemia and insulinemia to carcinogenesis and the evidence from primary prevention studies, there is a general support for cancer protecting effects of lower fluctuations of blood glucose and insulin (Rock et al., 2012) and hence potentially for low GI diets. Considering the longer survival of people diagnosed with cancer and therefore their

potential risk of developing or worsening other chronic conditions such as diabetes, the inclusion in cancer guidelines for a preference of low GI whole grains and low GI carbohydrates in general would be a sensible decision.

Plant Based Proteins and Cancer

Plant based dietary patterns have been associated with reduced risk of developing several chronic diseases, including cancer. Consistent with these findings, guidelines support dietary patterns lower in animal-based foods and higher in plant-based foods, emphasizing beans/legumes including soy, whole grains, nuts/seeds, and vegetables, namely sources of plant protein (USDA, 2010; USDA, 2015; Kushi et al., 2012; Schüz et al., 2015). From a global standpoint, production of plant-based proteins is considered to have lower environmental burden, land and water requirements coupled with fewer greenhouse gas emissions, as well as having a lower financial cost compared with animal-based proteins (Pimentel and Pimentel, 2003; Reijnders and Soret, 2003). These reasons, along with concerns for animal welfare, likely explain the increasing interest in the potential of using plant-based protein sources. The following focuses on cancer morbidity and mortality related to plant compared with animal protein consumption, summarizing available evidence and considering implications for future research.

Plant Based Protein Sources

Plant-based proteins can be found in a variety of food sources, including soy beans and other legumes, nuts/seeds, whole grains containing gluten (e.g., seitan) as well as other vegetables although the amounts are usually small (USDA Food Composition Database, 2018). In Europe and the United States, cereals are the principal contributors to plant protein intake providing 40%–70% of plant protein. However, animal products are still the main source of dietary protein providing between 55% and 71%, depending on the country, with red meats contributing 16%–35% to animal protein intake (Halkjaer et al., 2009; Phillips et al., 2015; Camilleri et al., 2013).

Evidence for Plant Based Protein Compared to Animal Protein and Cancer Risk

Numerous studies have assessed the impact of animal protein consumption on various types of cancer, however, few have focused specifically on cancer and consumption of plant protein. Evidence regarding carcinogenic or anticarcinogenic effects of plant compared with animal protein is mixed. Yet, protein is not consumed in isolation, but as part of a food matrix. Thus, in epidemiological studies, it can be difficult to attribute any observed benefit solely to protein as the finding may be due to other nutrients found in the food source. As well, findings from observational studies may be confounded since individuals consuming a more plant-based diet may also tend to participate in "healthier" lifestyle behaviors, such as being a nonsmokers, consuming less alcohol, having a lower body weight, being more physically active, and/or consuming a generally healthier diet, all of which may contribute to decreased cancer risk (Tharrey et al., 2018; Davey et al., 2003). Due to these limitations the following will focus on research where protein was explicitly the nutrient of interest in the comparison of animal and plant based dietary exposures. Much of the available evidence for plant protein compared to animal protein in relation to specific cancers pertains to colorectal and breast cancers. In a meta-analysis of observational studies on colorectal cancer risk, comprising 21 studies and 8187 cases, showed no evidence of significance between dietary animal protein or vegetable protein intake (Lai et al., 2017). The association between dietary protein sources and breast cancer risk was assessed in a meta-analysis of 46 prospective cohort studies (60,615 cases and 2,749,307 participants, with a mean follow-up of 3.9–65 years). Findings indicated higher total red meat, fresh red meat, and processed meat intake were risk factors for breast cancer, whereas evaluation of plant protein sources showed soy protein exhibited a protective association, and nuts (peanuts and tree nuts) had no adverse association. In terms of cancer mortality, animal and plant protein intake has been prospectively examined in US health professionals (121,700 women and 51,529 men) followed for up to 32 years with repeated measures of dietary intake. Animal and plant protein consumption comprised 14% (9%–22%) and 4% (2%–6%) of total energy intake, respectively. Plant protein was inversely associated with cancer mortality when age was taken into account, however, when the model was further adjusted for dietary and lifestyle factors, such as smoking status, body mass index and physical activity, this relationship disappeared (Song et al., 2016).

Potential Mechanisms of Action: Plant vs. Animal Proteins

The mechanisms involved require further investigation. Proposed potential mechanisms relate to: (1) the reduction and/or displacement of animal protein intake with plant protein and (2) the food matrix within which plant protein tends to be contained. In reducing animal protein intake it is suggested there would be a subsequent reduction of the carcinogenic byproducts from consumption of red meats, such as heterocyclic amines and polycyclic aromatic hydrocarbons formed during high temperature cooking (Steck et al., 2007; Zheng and Lee, 2009). Animal protein is also a source of fat and heme iron, which have been implicated

in the production of oxidative stress and inflammation, where experimental data suggest oxidative stress and inflammation have a pro-carcinogenic role in cancer development (Farvid et al., 2014; Fonseca-Nunes et al., 2014; Hedlund et al., 2008; Negre-Salvayre et al., 2010; Samraj et al., 2015). Plant protein is found in food matrices that tend to include dietary fiber and antioxidants, thus possibly playing a role in cancer protection by lessening the negative effects of inflammation, insulin resistance and hyperinsulinemia by reducing IGF-1 concentrations, as well as oxidative damage. Additionally, plant foods are known to contain antioxidants, including vitamin C, vitamin E, and carotenoids, yet many plant proteins and peptides have also been identified as novel antioxidant agents, and are thought to contribute to carcinogenic protection by interfering with oxidative damage to DNA, lipids, and proteins (Lu et al., 2010; Zhang et al., 2010; Xue et al., 2009).

Conclusions and Recommendations and Future Directions

It is important for overall population health to continue to support dietary patterns with lower animal-based foods and higher plant-based foods (USDA, 2010; USDA, 2015; Kushi et al., 2012; Schüz et al., 2015). Cancer specific recommendations still require further research, yet, at this time it appears that consumption of plant-based proteins do not have adverse results thus supporting their intake. Higher quality research is recommended, differentiating cancer types and investigating cancer mortality rates for high versus low vegetable protein consumption.

Soy Foods and Cancer

Over the years epidemiological studies have consistently shown health benefits of soy foods related to the prevention of chronic diseases, including cancer (Messina, 2010). Of particular interest for human health are soy isoflavones, bioactive compounds in soybeans, that have shown anticancer properties due to their estrogen-like activity, as well as their ability to modulate cell cycle, apoptosis, differentiation, proliferation, and cell signaling (Rizzo and Baroni, 2018). Breast cancer incidence rates are lower in soy food consuming countries such as Asian populations (Pisani et al., 1999). Asian epidemiologic studies show that higher soy consumption is associated with an approximate one-third reduction in breast cancer risk (Chen et al., 2014). Opposite, in last decades, as Westernization of Asian cultures has occurred, breast cancer rates among Asian populations have steadily increased. In 1995 the "early intake" hypothesis emerged, in animal models, that exposure to soy isoflavones early in life reduce breast cancer risk (Lamartiniere et al., 1995a, b, 2000). Data from epidemiological studies indicate that higher soy intake early in life, such as during childhood and/or adolescence, is associated with 25%–60% reductions in risk (Wu et al., 2002; Shu et al., 2001; Lee et al., 2009). The "early-intake" hypothesis could also explain discrepancies in epidemiologic data from different countries. The estrogen-like activity of isoflavones by soybeans is at the base of data reporting soy isoflavones adversely affect the prognosis of breast cancer patients. Results from animal studies indicate that isoflavones activity stimulates the growth of existing mammary tumors (Ju et al., 2001; Allred et al., 2001; Helferich et al., 2008). However, results from in vitro and animal studies are controversial and not in line with human studies. The European Prospective Investigation into Cancer and Nutrition Study cohort (EPIC) reported no increase in cancer risk with higher soy isoflavone intake (Zamora-Ros et al., 2013). Regarding the association between soy food intake in breast cancer patients, clinical and prospective epidemiological data clearly show that soy consumption after breast cancer diagnosis was associated with reductions in breast cancer recurrence and beneficial effects were similar in Asian and non-Asian populations (Guha et al., 2009; Caan et al., 2011; Shu et al., 2009; Kang et al., 2010; Zhang et al., 2012). A meta-analysis of five cohort studies showed that soy isoflavones intake might be associated with lower recurrence in ER negative, ER+/PR+ breast cancer (Chi et al., 2013). Furthermore, it is reported that exposure to soy isoflavones was associated with reduced mortality and reduced cancer recurrence also in women with ER(+) and ER(−) breast cancer (Messina, 2016a, b; Shu et al., 2009). Results from in vivo studies shown that isolated isoflavones possibly interfere with the efficacy of tamoxifene, an antiestrogen prescribed to women with ER+ tumors (Ju et al., 2008; Jones et al., 2002), whereas other studies in humans report benefits of combined soy food intake and tamoxifen therapy use on the inhibition of breast cancer growth improving pharmacological efficacy (Kang et al., 2010; Nechuta et al., 2012).

As for breast cancer, prostate cancer incidence varies throughout the world and it appear higher in Western countries than in the Asian population. Specifically, the higher soy food intake in Asian population is associated with as much as a 50% reduction in prostate cancer risk (Zhang et al., 2016) even if prostate cancer has a lower incidence rate in Asian population and this phenomenon has been linked to difference in intestinal bacteria responsible for conversion of daidzein to equol in the gut (Akaza, 2012). Clinical studies indicate that isoflavone exposure has beneficial effects in prostate cancer patients (Messina et al., 2006; Kwan et al., 2010; Ide et al., 2010). A recent meta-analysis supports the existing evidence which indicates that the total soy food intake is associated with reduced prostate cancer risk (Applegate et al., 2018). Nevertheless, some studies reported controversial results on the effects of soy on prostate cancer (Bosland et al., 2010; Fleshner et al., 2011). Differences in study designs limit the implications of results.

The European Food Safety Agency (EFSA) commented on the possible association between the intake of isoflavones and adverse effect on sex-hormone responsive tissues (such as breast and endometrium), after reviewing 25 clinical studies and concluded that

35–150 mg per day of isoflavones from supplements do not adversely affect the endometrium (EFSA Panel on Food Additives and Nutrient Sources added to Food ANS, 2015). No effect of oral isoflavone supplementation on endometrial thickness overall was found in a meta-analysis of 23 randomized controlled trials in peri- and postmenopausal women (Liu et al., 2016). Moreover, data from a meta-analysis of 10 observational studies found that dietary soy isoflavones from soy beans and soy foods were inversely associated with endometrial cancer risk, and the protective effects were found for both Asian and non-Asian populations.

Evidence reported from a meta-analysis of 17 epidemiologic studies (including case-control and prospective cohort studies) revealed that soy isoflavone consumption, particularly with soy foods/products, was significantly associated also with reduced risk of colorectal cancer in Asian populations (Yu et al., 2016). Another meta-analysis of prospective studies showed higher isoflavone intakes significantly decreased the risk of stomach and lung cancer while nearly significantly decreased the risk of breast and colorectal cancer (Grosso et al., 2017).

Mechanisms of Action

Many biological explanations have been proposed for the cancer protective effects of soy foods (Grosso et al., 2017). Soy isoflavones play a competitive role with endogenous estrogens for binding of estrogen receptors (ERs) and stimulate cell proliferation (Trock et al., 2006; Taylor et al., 2009; Guha et al., 2009). Isoflavones act as weak ER agonists or antagonists, depending on the cell type and estrogen environment (Larkin et al., 2008). ER agonist/antagonist properties of isoflavones have been widely studied and because of their tissue-selective effects isoflavones are classified as selective ER modulators. Soy may act as an ER antagonist (Dorjgochoo et al., 2011) and soy isoflavones intake was associated with protective effects in both ER− and ER+ breast cancer patients (Chi et al., 2013).

The mechanisms by which soy intake may improve the prognosis of breast cancer patients is not obvious, taking into account also data showing a lack of effect of isoflavones on breast cancer markers. To date, neither soy protein nor isoflavones supplements seem to affect markers of breast cancer including mammographic density and breast cell proliferation (Hooper et al., 2010; Wu et al., 2015; Sartippour et al., 2004; Palomares et al., 2004; Cheng et al., 2007; Khan et al., 2012). Different mechanisms have been proposed to explain the role that isoflavones may play in prostate cancer prevention and modulation. Clinical and animal studies report that isoflavones are able to inhibit metastasis (Xu et al., 2009; Jin et al., 2013). Also, cell-based studies show that phytoestrogens exert a chemopreventive effect through binding ERβ, expressed in prostate epithelial cells, regulating genes that control cell cycle and apoptosis (Applegate et al., 2018; Mahmoud et al., 2014).

Several mechanisms for the protective role of early isoflavone exposure have been proposed (Messina and Hilakivi-Clarke, 2009). The protective effect of early isoflavone exposure may be related to isoflavones ability to impact mammary gland development, changing cells in ways that make them permanently less likely to be transformed into cancer cells. Early life exposure to phytoestrogens has been demonstrated to impact mammary gland development through accelerated terminal end bud differentiation. Furthermore, many similarities between the protective effect of early isoflavone exposure and early pregnancy have been observed (De Assis et al., 2011; Rahal and Simmen, 2011; Mishra et al., 2011).

Soy isoflavones also act independently of ER activity exhibiting antiproliferative, antioxidant and antiinflammatory properties in vitro and in vivo (Guha et al., 2009; Rizzo and Baroni, 2018; Zaheer and Humayoun Akhtar, 2017). Isoflavones act as antioxidants and reduce the long-term cancer risk by preventing DNA damage. Genistein in particular is a potent antioxidants and it seems to increase the production of SOD which removes free radicals from cells (Zaheer and Humayoun Akhtar, 2017).

Isoflavones interact with epigenetic modifications, such as hypermethylation of tumor suppressor genes. In vitro studies have demonstrated that phytoestrogens act on chromatin and modify transcription through the demetylation and acetylation of histones in breast cancer cell lines (Dagdemir et al., 2013; Zaheer and Humayoun Akhtar, 2017; Grosso et al., 2017).

Conclusions and Recommendations

Evidence indicates that soy foods, mainly by virtue of their isoflavones content, as having a number of health benefits. Despite the many benefits, soy foods have not been embraced by the medical community. Mainly the estrogen-like properties of isoflavones has led to concerns that soy may exert untoward effects in some subpopulation (e.g., postmenopausal women), increasing breast cancer risk and getting worse the prognosis of breast cancer patients. As a result, clinicians treating breast cancer patients frequently caution them to either avoid soy foods or use them in moderation. However, evidence published over the past decades indicates the safety of isoflavones exposure with respect to breast and endometrial cancer. The position of AICR and WFRC are that soy foods are safe and even beneficial if consumed by women with a breast cancer diagnosis (American Institute for Cancer Research (AICR); World Cancer Research Fund International (WCRF). In 2015, after a literature evaluation, EFSA concluded that isoflavones do not adversely affect the breast, uterus and thyroid (the three organs that were investigated) in *peri* and postmenopausal women (EFSA Panel on Food Additives and Nutrient Sources added to Food ANS, 2015). Clinical studies did not support the hypothesis of an increased risk of breast cancer and supplementation with 150 mg/day of soy isoflavones (up to 30 months) of had no adversely effect on endometrial thickness and in the uterus (EFSA Panel on Food Additives and Nutrient Sources added to Food ANS, 2015).

As suggested by the early hypothesis, it could be reasonable to encourage young women to consume one serving per day of soy foods because epidemiological data suggest it may be protective. However, the consumption of a variety of soy foods, such as soy flour and tofu, should be encouraged rather than purified forms of phytoestrogens. A moderate consumption of soy foods, such as one to two standard servings daily of soy foods (one serving averages approximately 25 mg isoflavones) is not links to increased breast cancer risk.

Future Directions

Although soy and isoflavones offer health benefits to humans, controversial results from different studies have raised doubts about the validity of such health claims. More studies are therefore needed to clarify the controversial results. Nutraceutical values of soybean, including isoflavone content of soy foods, can vary considerably due to the soybean type, cultivar and food processing.

Labeling of soy foods with isoflavones concentrations could be a helpful information for consumers and scientists. Dietary guidelines for soy foods and soy isoflavones consumption may improve cancer prevention and management.

Dietary Fats

Dietary fats include oils, butter and lard. They are composed of a 3-carbon backbone to which fatty acids are attached. Fatty acids are formed by long, short or medium hydrocarbon or aliphatic chain linked to a carboxylic acid end. The aliphatic chain of fatty acids can present double or single bonds between their carbon molecules, indicating unsaturated fats or saturated fats (SFA), respectively. Fatty acids with one double bound are called monounsaturated fats (MUFA), while polyunsaturated fats (PUFA) are fatty acids with more than one double bound. A type of unsaturated fat that occurs in small amounts in nature are trans-unsaturated fatty acids, with one or more of their double bonds in the "trans" rather than the common "cis" configuration. Cholesterol is not a fat but an organic molecule of the sterol family (similar to wax) and it is not used for calorie production hence it is not oxidized. Fats and oils are most abundant lipids in nature. They represent the major component of storage fat or adipose tissue in the human body and the major contributor to energy intake in diet. The fatty acids profile in serum and tissues reflects the composition of dietary fats. Fat-rich foods are formed by SFA (with no double-bond), MUFA (e.g., oleic acid) and PUFA ($n-3$, $n-6$ fatty acids, e.g., linolenic acid, omega 3 fatty acids). SFAs are found in dairy products such as butter, cream, cheese and milk, in meat and certain plants (e.g., palm oil, coconut oil). Dietary sources of MUFAs are olives, rapeseed, peanuts, sesame and safflower oil. Specific types of fish contain abundant levels of $n-3$ PUFAs, such as eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). Linoleic acid (LA, $n-6$ PUFAs) is present in oil of safflower, grape seed, hemp, corn, wheat germ, cotton-seed and soybean, while α-linoleic acid (ALA, $n-3$ PUFAs) is in flaxseed, walnuts, some vegetables and canola oils (Burdge, 2004). Trans-fatty acids are found especially in dairy products and meat, but in high amounts in baked foods (biscuits, crackers, cookies, etc.) and margarines. Cholesterol dietary sources are mainly egg yolk, organ meat, shrimp, squid and shell.

Dietary Fats and Cancer Prevention

Many studies have suggested that a high consumption of fat through diet is related to an increased incidence of breast, colon, pancreatic and prostate cancer (Othman, 2007). Independently from type of fat, excess dietary fat intake has been associated with cancer recurrence (Dal Maso et al., 2008; McEligot et al. 2006; Vernieri et al. 2018). However, when intervention studies with low fat diets were conducted in the USA no effect on primary (Prentice et al. 2006) or secondary prevention of breast cancer were observed (Pierce et al. 2007) unless the dietary change involved weight loss (Chlebowski et al. 2006).

Saturated Fatty Acids

Direct associations between SFAs intake and risk of breast cancer in postmenopausal breast cancer have been found but not in premenopausal women (Cao et al., 2016; Xia et al., 2015). Higher intakes of total fat and SFAs compared to lower intakes were associated with higher risk of breast cancer subtypes, in particular ER and PR positive breast cancer (Sieri et al., 2014). When investigating exposure to SFA consumption and mortality incidence, a recent meta-analysis of four studies found 50% higher risk of breast cancer mortality in the highest compared to the lowest SFA intakes.

In colorectal cancer total fat and SFAs did not show any significant associations in an Asian population (Zhong et al., 2013) but in a large European population SFA was directly and significantly associated with colorectal cancer risk (May-Wilson et al. 2017). A meta-analysis of endometrial cancer studies and dietary fat a 45% increased risk was found in case-control studies only (Zhao et al., 2016a, b). Evidence from observational studies (both case-control and prospective) indicate that there is no apparent relationship

between SFAs and pancreatic cancer risk. Case-controls study on prostate cancer and dietary fats shows that high consumption of SFAs was associated with a higher aggressiveness of prostate cancer (PC) which seemed to be related mainly to the SFA effects on serum cholesterol levels. Other studies confirmed a role of dietary cholesterol on PC aggressiveness (Allott et al., 2017; Mensink et al. 2003; Platz et al., 2006; Platz et al., 2009).

Monounsaturated Fat (MUFA)

Most recent epidemiologic evidence has shown that foods rich in MUFAs could reduce breast cancer risk (Pelucchi et al., 2011) but others do not support these results. High versus low consumption of MUFAs to SFAs ratio was associated with a significantly reduced risk of colorectal cancer in three case–control studies (Rosato et al., 2016). Inverse associations were observed between MUFAs and endometrial cancer risk in cohort studies, while nonsignificant associations were observed in case-control studies (Zhao et al., 2016b). No significant relationship between MUFAs and pancreatic cancer risk were observed in cohort studies (Yao and Saturated, 2015) and conflicting results were seen for prostate cancer.

MUFAs are found both in vegetable oils especially olive oil and canola oil but also in meat. This could explain the heterogeneity of results in international studies particularly between North American populations with high meat to olive oil intakes and Mediterranean populations who tend to consume larger amounts of olive oil and less meat. Indeed, when investigating vegetable oil and olive oil a protection is seen in breast cancer risk of 12% and 26%, respectively, in a meta-analysis of cohort and case-control studies (Xin et al. 2015). This protection was supported by results of the PREDIMED clinical trial conducted in Spain where the arm consuming higher extra virgin olive oil showed a 68% reduction in breast cancer risk after 4 years of intervention compared to the control group (Toledo et al. 2015).

Polyunsaturated Fat PUFA

Numerous studies (epidemiological, clinical, animal model and in vitro) confirm a preventive role of $n-3$ PUFAs in different types of cancer sites, including breast, colon and prostate (Berquin et al., 2008; Huerta-Yépez et al., 2016) while high dietary intake of $n-6$ PUFAs could increase risk of cancer development (Lawrence, 2013). In vitro and in vivo recent studies indicate positive association between $n-3$ PUFAs and cancer risk (Sawada et al., 2012), moreover data from epidemiologic studies confirm a protective role of this type of fats on cancer risk (Huerta-Yépez et al., 2016). Colorectal cancer is most widely developed in populations who consume a Western diet which is characterized by high fat and are especially rich in $n-6$ PUFAs particularly arachidonic acid (AA) which is associated with cancer promotion (Huerta-Yépez et al., 2016). Overall, $n-3$ PUFAs fats and fish may have positive effects on carcinogenesis and lower risk of colorectal cancer (Hall et al., 2008; Ronco et al., 2010). Results from meta-analysis indicate that high intakes of PUFAs was associated to reduced risk of pancreatic cancer, especially in case-control studies but not clearly in prospective studies while a high $n-6/n-3$ PUFA ratio was correlated with higher risk of high-grade prostate cancer (Williams et al., 2011). In recent meta-analysis, consumption of foods rich in $n-3$ PUFAs such as marine fish, EPA and DHA, was associated with lower risk of breast cancer (Yang et al., 2014). Meta-analysis on endometrial cancer risk indicate a relationship between intake of fish and lower risk of cancer in European studies (Hou et al., 2017). Results from prospective cohort studies confirm the benefit of $n-3$ PUFAs on breast cancer incidence (Zheng et al., 2013) which may be due to improvements in immune system for EPA/DHA in supplementation studies (Paixão et al., 2017).

Trans-Fatty Acid

Intakes of commercially produced trans-fatty acid are associated with several diseases, including several types of cancers (Thompson et al., 2008; Mozaffarian et al., 2006; Stender and Dyerberg, 2004). Data from correlational studies on trans-fatty acid intake and breast cancer indicate a positive correlation between intake and risk in premenopausal women (Hu et al., 2011) but studies on ruminant-derived trans-fatty acids do not confirm this association (Kolahdouz Mohammadi et al., 2017). However trans fatty acids in ruminants are different than those in industrial products, they are 10-fold lower in concentration and the position of the double bond is located on different carbons. Only few studies observed direct associations between intakes of trans-fatty acids and colon cancer or prostate cancer (Hu et al., 2010).

Cholesterol

Dietary cholesterol is found only in animal foods. There is an increased evidence of an important role of dietary cholesterol in increasing breast cancer risk (Li et al., 2016) and this may be due partly to the cholesterol metabolite, 27-hydroxycholesterol, which has estrogenic activity increasing proliferation of ER+ breast cancer cells (Nelson et al., 2013). High dietary cholesterol may also increase risk of pancreatic (Chen et al., 2015) and colorectal cancers in some studies (Järvinen et al., 2001; Lee et al., 2009), but not all (Nagata et al., 2001) and of endometrial and prostate cancers (Allott et al., 2017).

Mechanisms of Action

Potential mechanisms of dietary fats in the etiology of cancer include pathways linked to excess adiposity, reactive oxygen species (ROS), hyperinsulinemia, adipokine secretion and other key mechanism of inflammation which could induce an environment favorable to tumor growth (Austin et al., 2011; Sung et al., 2011). Dietary fats affect eicosanoids synthesis which are involved in the inflammatory cascade which in turn may stimulate cancer growth (Larsson et al., 2004; Escrich et al., 2011). ROS production is linked to higher consumption of dietary fat and induce oxidative stress that may promote carcinogenesis (Kang, 2002, Baracos et al., 2004). Fatty acids regulate transcription and expression of genes influencing cancer (Jump, 2004; Mandal et al., 2010). Different fatty acids can differently induce cell growth, proliferation, differentiation and motility of cancer cells (Zadra et al., 2013; Navarro-Tito et al., 2008). $N-3$ PUFAs for example reduce inflammation and seem to be protective against tumor growth (Schley et al., 2005).

Guidelines and Recommendations

The recommendations are to limit the intake of red meat and processed meats as much as possible since they are rich sources of SFA, AA, cholesterol and increase inflammation. It is important to avoid excess dietary fat which would lead of positive energy balance and therefore to overweight and obesity. The preferred sources of fats linked to better health and lower cancer risk are MUFA and PUFAs (ACS, American Cancer Society; WCRF, World Cancer Research Fund International).

Cruciferous Vegetables and Related Sulphoraphane

Cruciferous vegetables are part of the Brassica genus of plants and include arugula, broccoli, Brussels sprouts, cabbage, cauliflower, kale, turnip greens and mustard, among others. Cruciferous vegetables have been associated with protection against a range of cancers (AICR'S Foods That Fight Cancer—Broccoli and Cruciferous Vegetables; NIH—Cruciferous Vegetables and Cancer Prevention). They are a rich source of carotenoids (beta-carotene, lutein, zeaxanthin), vitamins C, dietary fiber and a major source of glucosinolate-derived bioactive compounds which have been shown to have anticarcinogenic properties. Glucosinolates are present in almost every member of Cruciferae family and these sulfur-containing compounds are responsible for the pungent aroma and bitter flavor that distinguish cruciferous vegetables from other vegetables (Verhoeven et al., 1996; Higdon et al., 2007). Chemically, glucosinolates are composed of thiohydroximate-*O*-sulfonate group linked to glucose, and an alkyl, aralkyl, or indolyl side chain (R) (Agerbirk and Olsen, 2012). Glucosinolates (Glucoraphanin) undergo hydrolysis upon contact with the enzyme myrosinase, which is present within plant tissues, and they form biologically active compounds such as indoles (indole-3-carbinol) and isothiocyanates (sulforaphane) that have well known anticarcinogenic properties in in vitro and animal studies (Bosetti et al., 2012; Zhang et al., 1992). Isothiocyanates (ITCs) in plants have antibacterial and antifungal activity and provide important protection from insect and herbivore attack (Rosa et al., 1997).

Glucoraphanin glucosinolate is found in the vacuoles of intact plant cells and it is spatially separated from myrosinase, which is in the cytoplasm cells. After cell rupture, induced by chewing during human consumption or by tissue damage during freezing, thawing, and chopping of edible plants, glucoraphanin comes into contact with myrosinase and is converted into sulforaphane and sulforaphane nitrile.

Raw vs Cooked

Storage, processing and cooking can change ITCs formation from glucosinolate and affect the cruciferous vegetables anticancer activity (Barba et al., 2016; Jones et al., 2010). Intake of raw cruciferous vegetables provides two to nine times the amount of ITCs in humans compared with similar intakes of their cooked counterparts (Rouzaud et al., 2004; Getahun and Chung, 1999; Conaway et al., 2000; Shapiro et al., 1998). Studies demonstrated that inverse association between cruciferous vegetable intake and cancer risk appear to be stronger with raw vegetable consumption than with their cooked counterparts. It is notable that ITCs may be largely reduced by cooking procedures due to heat-inactivating myrosinase, reducing the formation of sulforaphane (Rouzaud et al., 2004; Getahun and Chung, 1999; Conaway et al., 2000). Human intestinal microflora also possess myrosinase activity and is able to partially hydrolyze ingested glucosinolates, but studies have shown that isothiocyanate exposure after the consumption of cooked cruciferous vegetables is 60%–90% less than that after the ingestion of raw cruciferous vegetables (Rouzaud et al., 2004; Getahun and Chung, 1999; Conaway et al., 2000; Shapiro et al., 1998). Among thermal processing, steaming is preferred over boiling because glucosinolates can leach into the boiling water and because the action of myrosinase at internal temperatures exceeding 70°C is inhibited (Jones et al., 2010).

Freezing at −20°C is one way to preserve the content of nutrients and improve health benefits of cruciferous vegetables. Freezing at −20°C resulted in the formation of more sulforaphane of broccoli sprouts which was probably due to the enhancement of

myrosinase activity and to the damage caused by ice crystals that brings glucoraphanin into contact with myrosinase (Guo et al., 2015). However, freezing at −20°C and −40°C had a negative impact on the ascorbic acid content in broccoli leading to the enhancement of myrosinase activity (Guo et al., 2015).

Evidence That Cruciferous Vegetables Can Reduce Cancer Risk

There is substantial evidence from human studies that cruciferous vegetable consumption may reduce the risk of several common cancer (IARC 2004, WCRF/AICR 2007; Verhoeven et al., 1997; Turati et al., 2015).

Prostate Cancer

A meta-analysis of 13 studies found that high intakes of cruciferous vegetables were associated with the decreased risk of prostate cancer (Liu et al., 2012). Cohort studies have examined a wide range of daily cruciferous vegetable intakes and found little or no association with prostate cancer risk (Schuurman et al., 1998; Giovannucci et al., 2003; Key et al., 2004). However, some case-control studies have found that people who ate greater amounts of cruciferous vegetables had a lower risk of prostate cancer (Kolonel et al., 2000; Jain et al., 1999).

Colorectal Cancer

Results from cohort studies have generally found no association between cruciferous vegetable intake and colorectal cancer risk (McCullough et al., 2003; Flood et al., 2002; Michels et al., 2000). Two meta-analysis reported consistent findings on the protective effects of cruciferous vegetables for colorectal cancer suggesting that intake of cruciferous vegetables may reduce the risk of colorectal cancer in humans (Wu et al., 2013; Tse and Eslick, 2014). In an updated report from the World Cancer Research Fund and the American Institute for Cancer Research (WCRF/AICR), dietary fiber intake, including cruciferous vegetables, has been upgraded as a convincing protective dietary factor for CRC (WCRF Colorectal cancer).

Breast Cancer

Data from a meta-analysis of 13 studies suggest that cruciferous vegetables consumption may reduce the risk of breast cancer with a significantly reduced risk in postmenopausal but not in premenopausal women (Liu and Lv, 2013). Despite strong evidence of a protective role of cruciferous vegetables in breast cancer in animal and in in-vitro studies, data in human studies are often inconsistent. Some epidemiological studies reported no association (Smith-Warner et al., 2001) or a weak association with breast cancer risk (Zhang et al., 1999). No evidence of association between cruciferous vegetables consumption and breast cancer prognosis has been observed in breast cancer patients (Nechuta et al., 2013; Peng et al., 2017).

Overall

Significant reductions of colorectal and breast cancer risk in subjects consuming cruciferous vegetables at least once a week (compared to non-consumers) were reported from an analysis of a large network of case-control studies while no significant associations were seen in prostate cancer risk (Bosetti et al., 2012). Similar findings have been reported from a systematic review of Mediterranean countries (Turati et al., 2015).

Mechanisms

Similar to other plants, cruciferous vegetables contain various protective compounds including dietary fiber, several antioxidants and vitamins (i.e., carotenoids, polyphenols, vitamin C, and folate) (IARC 2004; Verhoeven et al., 1997; Higdon et al., 2007). In addition, cruciferae are rich unique sources of glucosinolates whose major breakdown products (indoles and ITCs) have shown anticarcinogenic properties. Indoles and ITCs may protect against cancer by modulating Phase I and Phase II enzymes, inhibiting carcinogenesis, stimulating cell cycle arrest and apoptosis, and by reducing inflammation and angiogenesis (Navarro et al., 2011; Lenzi et al., 2014).

Dietary Fiber

Cruciferous vegetables are good sources of dietary fiber which may play a protective role in colorectal cancer (Aune et al., 2011).

Mechanisms Modulating Estrogen Receptor-α Expression

There is strong evidence from cell and animal experiments of a preventive role of cruciferous vegetables in breast carcinogenesis by regulating the expression of estrogen receptor, altering the metabolism of estrogen, or suppressing cyclooxygenase 2 (COX-2) (Lin et al., 2017; Fowke et al., 2000; Kang et al., 2009). The chemopreventive effect of indole-3-carbinol on breast cancer may be linked to its ability of decreasing estrogen receptor-α expression (Bosetti et al., 2002; Fowke et al., 2000). Indeed indole-3-carbinol are able to bind to the estrogen receptor (ER), repressing ER signaling, downregulating the expression of estrogen-responsive genes (Meng et al., 2000) and thus preventing the development of estrogen-dependent cancers including breast, endometrial and cervical cancers. Besides indole-3-carbinol also ITCs can modulate expression of sex hormones and their receptors. ITCs function as ERα disruptors to abrogate mitogenic estrogen signaling in ER-positive breast cancer cells, which provides a molecular explanation for the growth inhibitory function of ITCs in breast cancer development, and a rational for further exploration of ITCs as chemopreventive agents for human mammary carcinogenesis. (Kang et al., 2009).

GST Polymorphism and Colorectal Cancer

Several studies have investigated the different capacity of individuals to metabolize and eliminate glucosinolate byproducts of hydrolysis and this theory may help explain the differential health benefits of cruciferous vegetables (Lampe and Peterson, 2002; Lin et al., 1998). Approximately half of the population lacks GSTM1 (a variant of glucosinolate enzymes) due to gene deletion (Lin et al., 1998) and the homozygous deletion of GSTM1 gives rise to the null phenotype in which there is no expression of GSTM1 protein. The hypothesis is that individuals with the null genotype of GSTM1 or GSTT1 polymorphisms would have less conjugation activity, and thus permitting ITCs to remain biologically active potentially providing greater protection against cancer (Cotton et al., 2000). Findings from a meta-analysis suggest a positive correlation between glucosinolate polymorphism and the protective effects of cruciferous vegetables consumption against colorectal cancer risk in individuals with a single null GSTT1 genotype (Tse and Eslick, 2014).

Epigenetic Modulation

Cruciferous vegetables may have epigenetic properties modulating cancer prevention. Most cancers are characterized by the overexpression of histone deacetylases and DNA methyltransferases, and the mis-expression of micro RNAs. Sulforaphane and indole-3-carbinol from cruciferous vegetables may regulate micro RNAs and inhibit histone deacetylases and DNA methyltransferases (Royston and Tollefsbol, 2015).

Recommendations

Dietary Guidelines generally recommend consuming a variety of vegetables each day because of their different content of bioactive compounds (USDA Choose my Plate). Usually cruciferous vegetables fall into the "dark-green vegetables" category. It is suggested to eat cruciferous vegetables as part of a healthy diet and to prefer eating them raw or after steaming and that sulforaphane appears to be an effective and safe chemopreventive molecule and a promising tool to fight cancer (Lenzi et al., 2014).

Pro-Inflammatory Vegetables: Is There Any Evidence?

High plasma levels of C reactive protein (CRP) are expression of chronic inflammatory status and are associated with poor prognosis of tumors. The inflammatory cytokines are released into the blood by the tumor and by the tumor-associated macrophages, their presence in the blood and the presence of high levels of CRP may indicate that the tumor is more aggressive.

It is a common belief that the Solanaceae plants (tomatoes, eggplants, peppers, and potatoes) may increase inflammation however there seems to be a lack of evidence (Berrino, 2016). In order to keep inflammation low, it is preferable to consume a diet rich in plant foods such as vegetables, fruit and whole grains with some omega-3 fats (flaxseeds, soybeans, wild herbs). Evidence of a possible association between the intake of tomatoes and tomato juice and inflammation has given mixed results: reduction in CRP levels in some studies (Jacob et al., 2008) or no significant change in CRP and other inflammatory mediators in others (Blum et al., 2007). Furthermore, isolated lycopene supplementation has not shown antiinflammatory effect (Markovitis

et al., 2009). Tomatoes, eggplants, peppers, and potatoes are rich the glycoalkaloids α- solanine and α—chaconine which are plants' natural defenses. These compounds however may stimulate the formation of polyamines, metabolites that induce cell proliferation, reduce immune defenses against tumors and promote metastatic migration of tumor cells and angiogenesis (Soda, 2011).

Supplements: Soy Isoflavones

Soy isoflavones are phytochemical compounds that are found in soybeans. These bioactive compounds are also known as phytoestrogen because of their chemical structure similar to animal endogenous estrogens which allows them to bind to the estrogen receptor (Setchell, 1998; Cabot, 2003). Mean isoflavone intake among adults range from 30 to 50 mg/day in Asian countries consuming traditional soy foods whereas is less than 3 mg/day in United States, Canada and Europe (Goodman-Gruen and Kritz-Silverstein, 2001; Bai et al., 2014; Van Erp-Baart et al., 2003; Van der Schouw et al., 2005).

Soy isoflavones occur mainly as glycosides form, bound to a sugar molecule (Murphy et al., 2002). After food fermentation during processing or gut digestion the sugar moiety is hydrolyzed, thereby allowing absorption to occur (Rowland et al., 2003). Breaking of this glycosidic bond leaves the isoflavones in their simple aglycone form (Murphy et al., 1999, 2002; Yamabe et al., 2007; Otieno et al., 2007). It is still not clear whether the effects are due only to the aglycone or its glucosidic portion or both. Soy isoflavones occur mostly in the form of genistein, daidzein and glycitein which account for approximately 50%, 40%, and 10%, respectively, of the total soy isoflavones content (Murphy et al., 2002).

Some human studies reported a wide inter-individual variability in isoflavones metabolism (Jackson et al., 2011) which depends primarily on intestinal flora polymorphisms (Moon et al., 2006; Nechuta et al., 2012a,b). Some individuals host intestinal bacteria that convert the isoflavone daidzein into the isoflavonoid equol (Jackson et al., 2011; Setchell, 1998; Setchell and Clerici, 2010) and this may explain some of the discrepancies in results seen for the health effects of soy foods in human studies (Bolca et al., 2007). It has been suggested that individuals with equol-producing intestinal bacteria may benefit from soy food consumption more than those without (Setchell et al., 2002; Setchell and Clerici, 2010; Setchell, 1998).

Sources

Plants synthetize isoflavones through environmental stresses, such as infections or paucity of nutrients (Lozovaya et al., 2005). Also, different climatic conditions and different cultivation practices lead to variable bean dimension as well as isoflavone content (Howitz and Sinclair, 2008). Isoflavones content can vary depending on temperature and soil moisture. During plant growth, the highest isoflavone concentrations occurring at low temperatures and high soil moisture (Rizzo and Baroni, 2018). Isoflavones are contained in different legumes, such as soy, kidney beans, navy beans, red clover and Japanese arrowroot called kudzu, but only soy beans and soy foods provide the main dietary sources of isoflavones in the human diet (Messina, 1999; Mazur et al., 1998). Soy foods include traditional Asian foods such as tofu, soy milk, tempeh and edamame.

Each gram of soy protein in soybeans and traditional soy foods is associated with approximately 3.5 mg of isoflavones (Messina et al., 2006a,b,c). One serving of a traditional soy food, such as 100 g of tofu or 250 mL soymilk, typically provides about 25 mg isoflavones. In 1999 USDA in conjunction with Iowa State University created a database providing data on the isoflavone (including daidzein, genistein, glycitein and total isoflavones) content of selected food items (USDA).

Health Effects

Beneficial effects have been associated with soy consumption, some of which may depend on phytoestrogen content (Rizzo and Baroni, 2018; Messina et al., 2006a,b,c). Epidemiological studies in Asian countries showed that a traditional diet rich in phytoestrogens was associated with a lower risk of chronic diseases and report on possible benefits in hormone-dependent prostate cancer (Applegate et al., 2018; Zuniga et al., 2013), colon cancer (Yu et al., 2016; Grosso et al., 2017a,b), breast and ovarian cancers (Loibl et al., 2011; Magee et al., 2004; Patisaul and Jefferson, 2010; Peeters et al., 2002; Grosso et al., 2017a), endometrial cancer (Zhong et al., 2018) and stomach cancer (Grosso et al., 2017b).

Some common potential mechanisms of action through which isoflavones could exert protective effects in carcinogenesis have been reported. Isoflavones are structurally similar to 17β-estradiol, the primary endogenous estrogen (Tham et al., 1998; Guha et al., 2009). These molecules play a competitive role with endogenous estrogens for binding to ERα and ERβ although they have lower estrogenic potency compared to estradiol (Breinholt and Larsen, 1998). Isoflavones possessed mixed ER agonist and antagonist properties, depending on the cell type and concentration of estrogen present in the surroundings (Larkin et al., 2008). It has been suggested that soy isoflavones may act as selective tissue estrogenic activity regulators and selective estrogen receptor modulator also with different mechanisms than direct receptor interactions (Nilsson and Gustafsson, 2002; Meegan and Lloyd, 2003; Riggs and Hartmann, 2003; Smith and O'Malley, 2004). ERα and ERβ display distinct expression patterns in men and women, thus phytoestrogens do not exert their activity as classical estrogen agonists (Matthews and Gustafsson, 2003; Hooper et al.,

2009). For example, the ability of genistein to bind to ERβ may be a key factor in the inhibition of prostatic carcinogenesis but other mechanisms include cellular proliferation, apoptosis and differentiation (Mahmoud et al., 2014). Indeed, in addition to ER-depending activity, anticancer effects of soy have been largely ascribed to isoflavones which can modulate cell cycle, apoptosis, differentiation, proliferation and cell signaling (Messina et al., 2006a,b,c; Zhou et al., 1999; Vitale et al., 2016). Soy isoflavones, as a polyphenol subclass, also act exhibiting antioxidant, anti-proliferative and anti-inflammatory properties in vitro and in vivo (Tham et al., 1998; Guha et al., 2009; Patel et al., 2001).

Newly discovered anticarcinogenic mechanisms of action of phytoestrogens include epigenetic modifications. In breast cancer cell lines phytoestrogens modulated chromatin transcription through epigenetic modifications such as methylation and acetylation of histones (Dagdemir et al., 2013; Grosso et al., 2017a,b).

A protective action of soy isoflavones during the cancer promotion phase may explain the reduced breast cancer risk and reduced recurrence in Asian women exposed to soy since infancy.

In 1995 the "early intake" hypothesis emerged showing that early isoflavone exposure may be protective against breast cancer in adulthood in animal studies. This effect may be related to isoflavones ability to impact on mammary gland development, changing cells in ways that make them permanently less likely to be transformed into cancer cells. Animal studies and epidemiological evidence support this hypothesis (Russo et al., 2005; Brown et al., 2010; De Assis et al., 2011; Rahal and Simmen, 2011; Mishra et al., 2011).

Concerns

The estrogen-like properties of isoflavones have been a concern suggesting it may increase estrogenic activities and thus cancer cell proliferation particularly in those who already had breast cancer. This concern stems mainly from animal studies where pharmacological doses of isoflavones were able to stimulate the growth of existing tumors in athymic ovariectomized mice implanted with estrogen-sensitive human breast cancer (Messina, 2016). However clinical and epidemiological studies in humans indicate that isoflavone exposure is safe for all women and it improves the prognosis of breast cancer patients (Grosso et al., 2017a,b; Rizzo and Baroni, 2018). This may be due partly to the selective nature of isoflavones in binding to estrogen receptors and to their other anticancer properties mentioned above. In 2015, after a comprehensive and multiyear evaluation of the literature, the European Food Safety Authority (EFSA) concluded that in postmenopausal women, intakes of 35–150 mg per day of isoflavones from food supplements do not adversely affect sex hormones-responsive tissues such as breast and utero after 2.5 years of treatment (EFSA Panel on Food Additives and Nutrient Sources added to Food ANS, 2015). Data from this systematic review does not support the hypothesis of an increased risk of breast cancer nor of an effect on mammographic density or proliferation marker Ki-67 expression (Wu et al., 2015). Moreover, higher isoflavone intakes in breast cancer patients were associated with reduced mortality and cancer recurrence in women with ER (+) and ER(-) breast cancer (Shu et al., 2009) and no contro-indications have been observed for women under treatment with tamoxifeen or anastrozole, two antihormonal drugs commonly used to treat breast cancer (Guha et al., 2009; Nechuta et al., 2012a,b; Kang et al., 2010).

Recommendations

Up to three servings/day (up to 100 mg/day of isoflavones) as consumed by Asian populations for thousands of years do not seem to be associated to increased breast cancer risk (American Institute for Cancer Research, AICR). Considering one serving averages about 7 g of protein and 25 mg isoflavones, a moderate soy isoflavone consumption is achieved with 1–2 standard servings daily of soybeans or soy foods.

Green Tea and Green Tea Extracts (GTEs)

Next to water, tea is the beverage most widely consumed worldwide, consisting of an infusion or decoction made from the leaves of a woody plant, *Camellia sinensis* (L.) Kuntze, belonging to the family Theaceae. The varieties of tea, derived from the leaves of the same plant, are created through different treatments and have different degrees of enzymatic oxidation of polyphenols by polyphenol oxidase (fermentation). Green tea is a nonoxidized nonfermented product which has similar phenolic compounds to unprocessed tea leaves. To preserve the polyphenols present in green tea, the leaves are arranged on bamboo surfaces and are exposed to the sun for a few hours, then they are steamed (Japanese Style Green Tea) or heated dried (Chinese Style Green Tea) to inactivate enzymes involved in the modification of polyphenols. Drying phases are alternated with folding or rolling allowing water to evaporate. When the leaves are well dried they are ready to be refined (dust and debris are eliminated) and packaged (Kosinska and Andlauer, 2014). The most common polyphenols present in green tea are catechins: (-)-epigallocatechin-3-gallate (EGCG) ≅ 59% of total catechins, (-)-epigallocatechin (EGC) (≅ 19%), (-)-epicatechin-3-gallate (ECG) (≅ 13.6%), (-)-epicatechin (EC), (+)-catechin (C), (-)-gallocatechin gallate (GCG) (≅ 6.4%) and glycosylated flavonols (Lin et al., 2008). They contribute to a higher antioxidant capacity of green tea and its sensory properties. Green tea products are also available as highly concentrated extracts (GTEs) for reconstituted tea drinks (in liquid and powdered forms) or supplements.

Recently, the EFSA ANS Panel provided a scientific opinion on the safety of green tea catechins from dietary sources including food supplements and infusions as a result of concerns about the possible hepatotoxicity, concluding that the catechins contained in green tea infusion and reconstituted drinks are risk-free. However, there is evidence from interventional clinical trials that intake of doses above 800 mg EGCG/day as a food supplement, may be a health concern due to increased serum transaminases (EFSA, Opinion 2018).

Previously peculiar to Asia, green tea is now gaining popularity in Western countries because its consumption seems to be linked to health benefits, especially for its potential anticancer effects in primary and secondary prevention (Cabrera et al., 2006) and in cancer therapy (Lecumberri et al. 2013). Several preclinical evidences have shown that EGCG is a multipotent chemopreventive and anticancer agent in vitro and in various animal models (Fujita et al., 1989; Fujiki and Suganuma, 2002; Gupta et al., 2001; Yamane et al., 1995; Yang et al., 2009; Yiannakopoulou, 2014). The mechanisms involved include inhibition of NF-kB signaling, protection from intracellular oxidation and DNA damage and apoptosis in tumor cells (Roy et al., 2010; Sadava et al., 2007). However, in contrast to the consistent results in in vitro and animal models, results from human studies are mixed (Johnson et al., 2012; Yuan, 2013). Two high quality systematic reviews assessed the link between green tea consumption and the risk of incidence, mortality and recurrence of all cancer types (Bohem et al., 2009; Sturgeon et al., 2009). The authors concluded that despite the associations between green tea consumption and decreased risk for some cancers, the overall evidence is limitated in quantity and quality. In intervention studies an inhibitory role of oral supplementation of green tea on a precancerous lesion of the oral cavity was shown (Li et al., 1999). A limited moderate to strong evidence has been investigated for lung cancer and seem inversely associated with green tea intake among never smokers but not among smokers, probably due to the potential confounding effect of smoking that cancel out the potential protective effect of green tea (Zhong et al., 2001). In prostate cancer, observational studies do not support a role of tea intake in reducing risk but some phase II clinical trials suggested that green tea catechins may have chemopreventive properties against prostate carcinogenesis (Bettuzzi et al., 2006).

The role of green tea infusion on cancer of the liver, colorectal and breast is not yet clear. However, two case control studies investigated the role of genotypes and suggested that differing angiotensin converting and folate metabolism activity genotypes in women probably play a role in the risk reduction of breast cancer with green tea (Inoue et al., 2008; Yoan et al., 2007).

A field to be investigate is the interaction between green tea/GTEs supplementation and cancer drug treatments. Studies showed an interference between green tea supplementation and the chemotherapy drug Bortezomib (Golden et al., 2009) and Sunitinib (Ge et al., 2011).

Until now, very little research has been done on the use of green tea or green tea extracts in treating cancer. Phase I and II trials of daily oral somministration of a green tea extract to patients with a chronic lymphocytic leukemia, who did not take any other treatment, showed that the number of leukemic cells and the size of their lymphones were reduced in a third of the participants (Shanafelt et al., 2009, 2013). More evidence is needed to confirm these preliminary results. At present there is no conclusive evidence that green tea helps to prevent or treat cancer in people because the number of studies especially randomized controlled trials are lacking and studies conducted thus far have utilized different amounts and types of green tea in different settings which makes it difficult to compare results.

Mediterranean Diet

The traditional Mediterranean diet represents the dietary pattern of the Mediterranean countries in 1960s (Kromhout et al., 1989; Sofi et al., 2008; Sofi et al., 2013). The traditional Mediterranean diet is characterized by a high intake of vegetables, legumes, fruits, nuts, unprocessed cereals and extra virgin olive oil as the main source of fat, it is also characterized by a low intake of saturated fats, meat and poultry, moderately high intake of fish, moderately low intake of dairy products and ethanol, mainly wine and generally during meals (Willett et al., 1995; Sofi et al., 2014). The Mediterranean diet contributes to the intake of important vitamins and phytochemicals including β-carotene, vitamins B, C, and E, folic acid, polyphenols and it contains on average: 55%–60% carbohydrates (80% complex carbohydrates and 20% simple sugars mainly from fruit), 10%–15% proteins (40% plant protein), and 25%–30% fats (mainly monousaturated fat from olive oil). The traditional Mediterranean diet received a great attention in the 1970s by the American physiologist Ancel Keys who lived for many years in Southern Italy and conducted the Seven Countries Study, the first major epidemiological study to investigate the relationship between diet and lifestyle in cardiovascular disease across countries and cultures (Menotti et al., 1989; Serra-Majem et al., 2006). This study suggested that the Mediterranean diet is protective against cardiovascular disease and cancer (Ferro-Luzzi and Branca, 1995). Numerous epidemiological studies showed that the Mediterranean diet is associated with a risk reduction of overall mortality, cardiovascular disease incidence and mortality, total cancer incidence and mortality, 25% reduction in colorectal cancer, 15% in breast cancer, and 10% in prostate, pancreas and endometrial cancers (Bloomfield et al., 2016).

Mediterranean Diet in Breast Cancer

High adherence to Med Diet has been shown to reduce incidence and recurrence of breast cancer (Bloomfield et al., 2016). A large European observational study supports a protective role of the Med Diet and its components in breast cancer risk (Buckland et al.,

2013). In international studies outside the Mediterranean basin, reductions in breast cancer risk have been observed with higher consumption of components of the Med Diet such as dietary fiber, low glycemic index carbohydrates, whole grains, fruit and vegetables and dietary marine $\omega - 3$ (Farvid et al., 2016; Kushi et al., 2012; Nicodemus et al., 2001; Zheng et al., 2013). The intervention study PREDIMED (Prevención con Dieta Mediterránea), showed that a Spanish Med Diet supplemented with at least 20% of calories as extra virgin olive oil reduced the incidence of breast cancer by 68% after 4.8 years (Toledo et al., 2015). Possible mechanisms seem to be linked to reduced inflammation, higher antioxidant, antiproliferative and apoptosis-inducing properties of specific compounds present in vegetables, fruit, nuts, extra virgin olive oil and fish, flavonoids such as polyphenols present in olive oil and red wine, dietary fiber and vitamin C present in vegetables and fruit, lignans in whole grains and seeds, monounsaturated fat and squalene in olive oil, and omega-3 fatty acids in fish (Dussaillant et al., 2016; Jing et al., 2013) improving insulin sensitivity and decreasing insulin-like growth factors (Barnard et al., 2006; Breneman and Tucker, 2013). In addition the antioxidant content in extra virgin olive oil (Carruba et al., 2006) may decrease endogenous estrogens, increase sex-hormone binding globulin levels (Wu et al., 2009), neutralize free radicals, prevent DNA damage and reduce oxidative stress (Mitjavila et al., 2013; Visioli et al., 2004). These results are confirmed also by the DIANA-5 study (Diet and Androgens) (Villarini et al., 2012).

Mediterranean Diet in Colorectal Cancer

The Med diet seems to be implicated also in a decreased risk of colorectal cancer although some studies are discordant. It seems that the positive effects of the Med Diet are more evident for the distal colon rather than the proximal colon (Donovan et al., 2017). Possible mechanisms are the antinflammatory effects as reported by preclinical studies. In vitro studies attributed this effect to the phenolic fraction and $\omega - 3$ content of olive oil and partly also to oleic acid, polyphenols from grapes, red wine, fruits and vegetables (Llor et al., 2003). Other studies assess that butyrate, produced from fiber by microorganisms in the gut, contribute to the oncoprotective effect. Future studies will focus on the role of the Med diet on epigenetic regulation and on changes of the microbiome as means of reducing colorectal cancer risk.

Mediterranean Diet in Prostate Cancer

Approximately 10% of the incidence of prostate cancer can be prevented following a traditional Mediterranean dietary pattern (Capurso and Vendemiale, 2017), reducing risk by 4% (Bloomfield et al., 2016). Possible protective compounds may be flavonoids in fruit and vegetables which exert antiinflammatory, antioxidant, antimutagenic and antiproliferative activities (Vance et al., 2013). Important is the antioxidation ativity of lycopene from tomatoes which modulate inflammatory mechanisms of carcinogenesis (Gann et al., 1999; Graff et al., 2016). Numerous studies are also investigating the antiflammatory action of $\omega - 3$ derived from fish in prostate cancer prevention and progression (Thompson et al., 2014; Aucoin et al., 2017).

Mediterranean Diet in Endometrial Cancer

Evidence associating endometrial cancer risk and specific dietary components are limitated. Howewer a 10% protection was found with the highest adherence to the Med Diet (Schwingshackl et al., 2017). Bioactive compounds and fiber of vegetables and fruit, as well as monounsatured fatty acid of extra virign olive oil, seem to modulate the estrogen levels and the inflammatory response. (Bravi et al., 2009; Filomeno et al., 2015; Lampe, 1999; Bandera et al., 2007; Fortner et al., 2017; Wang et al., 2011; Rose, 1990).

Mediterranean Diet in Pancreatic Cancer

Studies on the association between the Med Diet and pancreatic cancer are scanty and discordant. Howewer some studies suggest a protection of 10% for an high adherence to the Med Diet (Trichopoulou et al., 2000) possibly due to the high consumption of vegetables and fruit because of vitamin C, D, E, and K, folate and phenolic compounds (Larsson et al., 2006; Nothlings et al., 2007; Rossi et al., 2012; Trichopoulou et al., 2000; Buckland et al., 2013; Giacosa et al., 2013; Davis-Yadley and Malafa, 2015).

Guidelines and Future Perspectives

Scientific evidence supports healthy benefits of Mediterranean dietary pattern in primary and secondary prevention for the most common types of cancer. The World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR), based on scientific evidence, including the European code against cancer, provided lifestyle recommendations aimed to reduce the incidence and recurrence of the most common cancers worldwide. The recommendations are to avoid sugared beverages and processed

meat and to limit calorie-dense and salty food, alcoholic beverages and red meat. The central recommendation is to "Eat mostly food of plant origin, with a variety of nonstarchy vegetables and of fruit every day and with unprocessed cereals and/or pulses within every meal". these recommendations are at the base of the Med diet characteristics.(WCRF/AICR, 2007, www.iarc.it).

Fasting and Intermittent Fasting Diet

In the last decades a range of dietary interventions have gained considerable popularity for the prevention and treatment of age-related diseases, including cancer. Calorie restriction (CR) as well as fasting regimens are both dietary interventions of reduced caloric intake without malnutrition and have demonstrated a wide range of beneficial effects potentially able to prevent cancer and increase the efficacy of cancer therapies (Varady and Hellerstein, 2007; Mattson et al., 2017; Buono and Longo, 2018). Calorie restriction (CR) is a term commonly used to refer to a 20%–40% reduction in caloric intake without malnutrition (e.g., with an adequate micronutrient intake) (Mirzaei et al., 2016; Varady and Hellerstein 2007). As CR is not well tolerated by cancer patients and contraindicated for those at risk of weight loss, cachexia and sarcopenia, other caloric restricted approaches have been investigated, such as intermittent fasting (IF) which may be more suitable (Varady and Hellerstein, 2007; Rizza et al., 2014)

Fasting refers to a complete absence of food intake. The fasting period for the organism is a time for standby and repair in order to harvest energy when food becomes available (Longo and Panda, 2016). Alternate cycle of feeding and fasting are at the base of fasting physiology. Fasting can be administered at various time frames and can be repeated many times. Both reducing daily energy intake and restricting the time of food intake to a few hours may trigger the fasting physiology (Longo and Panda, 2016). IF comprises different forms of fasting including alternate day fasting (ADF), periodic fasting (PF), time-restricted feeding (TRF) and Ramadan fasting (Longo and Mattson, 2014; Varady, 2016). ADF regimens generally involve a 24-h fast followed by a 24-h non-fasting period (Horne et al., 2015). PF only requires participants to fast two or more consecutive days per week, every 2 or more weeks (Hutchison and Heilbronn, 2016). TRF requires individuals to confine the period of food intake to a set period of time during the day, for example an 8-h feeding window where all food is consumed between 10:00 am and 6:00 pm (Rothschild et al., 2014). The Islamic ritual of Ramadan fasting is a form of time-restricted feeding, where food consumption is only permitted during the night time (Mazidi et al., 2015).

Evidence in Cancer

Data from epidemiological and experimental studies suggest that calorie intake, the timing of food intake (e.g., fasting cycles), and dietary composition (e.g., protein, aminoacid, fat, mineral, vitamin and phytochemical intakes) are implicated in the pathogenesis of chronic diseases, including common types of cancer (Fontana et al., 2010; Mattson, 2005; Eyre et al., 2004).

Chronic CR provides both beneficial and detrimental effects as well as major compliance challenges, whereas different fasting regimens, without malnutrition, are emerging as interventions with the potential to be widely used to prevent and treat cancer (Patterson and Sears, 2017; Buono and Longo, 2018; Lee et al., 2012b; O'Flanagan et al., 2017).

First experiments in animal models demonstrated that fasting exerts a protective effect against DNA damage and promotes longevity (Longo and Fontana, 2010; Varady and Hellerstein, 2007). IF reduces the incidence of spontaneous tumors (Berrigan et al., 2002; Descamps et al., 2005), while PF can delay cancer progression as effectively as chemotherapy in animal models (Lee et al., 2012b). No data are available on the effects of IF on cancer rates in humans. Surrogate evidence that IF may reduce cancer risk can be derived from its effects on cancer risk factors. For example, most studies of ADF show benefits in weight reduction (Tinsley and La Bounty, 2015) and weight control is associated with a reduced risk of cancer (Renehan et al., 2008). IF has a beneficial effect on insulin resistance and diabetes which are cancer prognostic factors (Goodwin et al., 2015). Some studies have investigated the effect of fasting on the effectiveness of cancer therapy. Preliminary studies in 10 cancer patients fasted voluntarily during their chemotherapy and showed a decrease in the range of self-reported common side effects caused by chemotherapy compared to when they were on a standard diet (Safdie et al., 2009; Raffaghello et al., 2010b).

Mechanisms

CR and fasting have been shown to promote stress resistance as well as longevity in a wide variety of animal models. These dietary regimens affect cellular metabolism and growth by down regulating conserved nutrient-signaling proteins and by activating stress resistance transcription factors (Fontana et al., 2010). Metabolic and molecular antitumorigenic mechanisms of CR are well established. Accumulating data have showed that CR tumor suppressive effects are mediated by enhanced apoptosis, modulation of systemic signals such as IGF-1, insulin, metabolic and inflammatory pathways, and reduced angiogenesis (O'Flanagan et al., 2017; Fontana et al., 2010). Indirect evidence suggests that CR and fasting regimens may share common mechanisms. To date no data from human studies on the effect of IF or PF in cancer prevention are available. However their effect on reducing IGF-1, insulin and glucose levels, and increasing IGFBP1 and ketone body levels could generate a protective environment that reduces DNA damage and carcinogenesis, while at the same time creating hostile conditions for cancer and pre-cancerous cells (Longo and Mattson 2014). Elevated circulating IGF-1 is associated with an increased risk of developing several cancers (Chan et al.,

2000; Giovannucci et al., 2000) and individuals with severe IGF-1 deficiency caused by growth hormone receptor deficiency, rarely develop cancer (Guevara-Aguirre et al., 2011; Shevah and Laron, 2007). Findings in IGF-1 deficient subjects suggest that fasting may protect from cancer by reducing cellular and DNA damage but also by enhancing the programmed cell death of precancerous cells (Guevara-Aguirre et al., 2011). However, the effect of IF on IGF-1 levels in human studies has been variable. Some studies reported no changes in IGF-1 with weight loss due to intermittent fasting (Harvie et al., 2011), and others showed an increase in both IGFBP1 and IGFBP2 (Harvie et al., 2011; Fontana et al., 2010; Thissen et al., 1994). It is well established that a positive energy balance, as well as an increasing adiposity contributes to an increased risk of developing several types of cancer, including cancer of the colon, breast, prostate, endometrium, pancreas, liver and kidney, whereas weight loss lowers risk (Longo and Fontana, 2010; Calle and Kaaks, 2004).

Evidence demonstrates the important role of dysregulation in signal transduction and metabolic pathway in tumor initiation and progression. Cancer cells are characterized by an abnormally high level of glucose requirements necessary to produce the glycolytic ATP, as well as by accumulation of a variety of mutations (e.g., the IGF-1 receptor) (Buono and Longo, 2018) making them potentially sensitive to the changes in growth factors and circulating nutrients. Furthermore, a differential stress resistance (e.g., cell mechanism used during fasting to protect normal cells but not cancer cells from chemotherapy) and differential stress sensitization (e.g., cell mechanism used during fasting to preferentially kill cancer cells and other damaged cells in combination with chemotherapy or other cytotoxic agents) of fasting conditions in normal and cancer cells have been showed in in vitro and animal models (Buono and Longo, 2018; Raffaghello et al., 2008). Fasting conditions can be protective in normal cells because they are capable of adapting to starvation (Raffaghello et al., 2008) whereas cancer cells are characterized by inability to adapt to multi-stressed environments (Cheng et al., 2014; Lee et al., 2012b).

Future Directions

Several epidemiological studies clearly demonstrated that diet plays an important role in the initiation, promotion and progression of many common cancers (Kushi et al., 2006; Renehan et al., 2010). To date, there are no specific guidelines on the type of nutrition for each cancer type or cancer stage. According to the American Cancer Society, cancer patients receiving chemotherapy should increase calorie and protein intake (Doyle et al., 2006).

Findings from in vitro and animal studies have reported beneficial effects of combined fasting regimens with the standard-of-care pharmacotherapy. Dietary interventions mimicking CR can also be viewed as adjuvant anticancer strategies to be combined to standard cancer therapy (chemotherapeutic agents, ionizing radiation, and drugs with specific molecular targets). Clinical studies on the potential use of dietary treatments alongside conventional treatments are ongoing however the preliminary evidence suggests that these approaches may be a valid integrated therapy in cancer patients.

See also: Cancer Risk Reduction Through Lifestyle Changes. Dietary Factors and Cancer.

References

Agerbirk, N., Olsen, C.E., 2012. Glucosinolate structures in evolution. Phytochemistry 77, 16–45.

Akaza, H., 2012. Prostate cancer chemoprevention by soy isoflavones: Role of intestinal bacteria as the "second human genome." Cancer Science 103, 969–975.

Allott, E.H., Arab, L., Su, L.J., Farnan, L., Fontham, E.T., Mohler, J.L., Bensen, J.T., Steck, S.E., 2017. Saturated fat intake and prostate cancer aggressiveness: Results from the population-based North Carolina-Louisiana prostate Cancer project. Prostate Cancer and Prostatic Diseases 20 (1), 48–54. https://doi.org/10.1038/pcan.2016.39. Epub 2016 Sep 6.

Allred, C.D., Ju, Y.H., Allred, K.F., Chang, J., Helferich, W.G., 2001. Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. Carcinogenesis 22, 1667–1673.

Ames, B.N., Gold, L.S., 1991. Endogenous mutagens and the causes of aging and cancer. Mutation Research 250 (1–2), 3–16.

Applegate, C.C., Rowles, J.L., Ranard, K.M., Jeon, S., Erdman, J.W., 2018. Soy consumption and the risk of prostate Cancer: An updated systematic review and meta-analysis. Nutrients 10 (1).

Applegate, C.C., Rowles, J.L., Ranard, K.M., Jeon, S., Erdman, J.W., 2018. Soy consumption and the risk of prostate cancer: An updated systematic review and meta-analysis. Nutrients 10 (1).

Arikawa, A.Y., Jakits, H.E., Flood, A., Thomas, W., Gross, M., Schmitz, K.H., Kurzer, M.S., 2015. Consumption of a high glycemic load but not a high glycemic index diet is marginally associated with oxidative stress in young women. Nutrition Research 35 (1), 7–13. https://doi.org/10.1016/j.nutres.2014.10.005.

Aucoin, M., Cooley, K., Knee, C., Fritz, H., Balneaves, L.G., Breau, R., Fergusson, D., Skidmore, B., Wong, R., Seely, D., 2017. Fish-derived Omega-3 fatty acids and prostate Cancer: A systematic review. Integrative Cancer Therapies 16 (1), 32–62.

Augustin, L.S., Dal Maso, L., La Vecchia, C., Parpinel, M., Negri, E., Vaccarella, S., Kendall, C.W., Jenkins, D.J., Francesch, S., 2001. Dietary glycemic index and glycemic load, and breast cancer risk: A case-control study. Annals of Oncology 12 (11), 1533–1538.

Augustin, L.S., Franceschi, S., Jenkins, D.J., Kendall, C.W., La Vecchia, C., 2002. Glycemic index in chronic disease: A review. European Journal of Clinical Nutrition 56 (11), 1049–1071. https://doi.org/10.1038/sj.ejcn.1601454.

Aune, D., Chan, D.S., Lau, R., et al., 2011. Dietary fibre, whole grains, and risk of colorectal cancer: Systematic review and dose-response meta-analysis of prospective studies. BMJ 343, d6617.

Austin, G.L., Ogden, L.G., Hill, J.O., 2011. Trends in carbohydrate, fat, and protein intakes and association with energy intake in normal-weight, overweight, and obese individuals: 1971–2006. The American Journal of Clinical Nutrition 93 (4), 836–843.

Bai, W., Wang, C., Ren, C., 2014. Intakes of total and individual flavonoids by US adults. International Journal of Food Science 65, 9–14.
Bandera, E.V., Kushi, L.H., Moore, D.F., Gifkins, D.M., McCullough, M.L., 2007. Fruits and vegetables and endometrial cancer risk: A systematic literature review and meta-analysis. Nutrition and Cancer 58 (1), 6–21.
Baracos, V.E., Mazurak, V.C., Ma, D.W., 2004. N-3 polyunsaturated fatty acids throughout the cancer trajectory: Influence on disease incidence, progression, response to therapy and cancer-associated cachexia. Nutrition Research Reviews 17, 177–192.
Barba, F.J., Nikmaram, N., Roohinejad, S., Khelfa, A., Zhu, Z., Koubaa, M., 2016. Bioavailability of Glucosinolates and their breakdown products: Impact of processing. Frontiers in Nutrition 3, 24.
Barclay, A.W., Petocz, P., McMillan-Price, J., Flood, V.M., Prvan, T., Mitchell, P., Brand-Miller, J.C., 2008. Glycemic index, glycemic load, and chronic disease risk—A meta-analysis of observational studies. The American Journal of Clinical Nutrition 87 (3), 627–637.
Barnard, R.J., Gonzalez, J.H., Liva, M.E., Ngo, T.H., 2006. Effects of a low-fat, high-fiber diet and exercise program on breast cancer risk factors in vivo and tumor cell growth and apoptosis in vitro. Nutrition and Cancer 55 (1), 28–34.
Beckman, K.B., Ames, B.N., 1997. Oxidative decay of DNA. The Journal of Biological Chemistry 272 (32), 19633–19636.
Berquin, I.M., Edwards, I.J., Chen, Y.Q., 2008. Multi-targeted therapy of cancer by omega-3 fatty acids. Cancer Letters 269 (2), 363–377. https://doi.org/10.1016/j.canlet.2008.03.044. Epub 2008 May 13.
Berrigan, D., Perkins, S.N., Haines, D.C., Hursting, S.D., 2002. Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. Carcinogenesis 23, 817–822.
Berrino, F., 2016. I quattro pilastri alimentari della dieta adiuvante le terapie oncologiche. In: Berrino, F. (Ed.), Il cibo dell'uomo. La via della salute tra conoscenza scientifica e antiche saggezze, pp. 121–135. FrancoAngeli/Self-help.
Bettuzzi, S., Brausi, M., Rizzi, F., et al., 2006. Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: A preliminary report from a one-year proof-of-principle study. Cancer Research 66, 1234–1240.
Bloomfield, H.E., Koeller, E., Greer, N., MacDonald, R., Kane, R., Wilt, T.J., 2016. Effects on health outcomes of a Mediterranean diet with no restriction on fat intake: A systematic review and meta-analysis. Annals of Internal Medicine 165 (7), 491–500.
Blum, A., Monir, M., Khazim, K., Peleg, A., Blum, A., 2007. Tomato-rich (Mediterranean) diet does not modify inflammatory markers. Clinical and Investigative Medicine 30, E70–E74.
Bohem, K., Borrelli, F., Ernst, E., et al., 2009. Green tea (Camellia sinensis) for the prevention of cancer. Cochrane Database of Systematic Reviews 3, CD005004.
Bolca, S., Possemiers, S., Herregat, A., Huybrechts, I., Heyerick, A., De Vriese, S., Verbruggen, M., Depypere, H., De Keukeleire, D., Bracke, M., et al., 2007. Microbial and dietary factors are associated with the equol producer phenotype in healthy postmenopausal women. The Journal of Nutrition 137, 2242–2246.
Bosetti, C., Filomeno, M., Riso, P., Polesel, J., Levi, F., Talamini, R., Montella, M., Negri, E., Franceschi, S., La Vecchia, C., 2012. Cruciferous vegetables and cancer risk in a network of case-control studies. Annals of Oncology 23 (8), 2198–2203. https://doi.org/10.1093/annonc/mdr604.
Bosetti, C., Filomeno, M., Riso, P., Polesel, J., Levi, F., Talamini, R., Montella, M., Negri, E., Franceschi, S., La Vecchia, C., 2012. Cruciferous vegetables and cancer risk in a network of case-control studies. Annals of Oncology 23, 2198–2203.
Bosetti, C., Negri, E., Kolonel, L., et al., 2002. A pooled analysis of case-control studies of thyroid cancer. VII. Cruciferous and other vegetables (International). Cancer Causes & Control 13, 765–775.
Bosland, M.C., Kato, I., Zeleniuch-Jacquotte, A., Schmoll, J., Enk Rueter, E., Melamed, J., Kong, M.X., Brown, N.M., Belles, C.A., Lindley, S.L., Zimmer-Nechemias, L.D., Zhao, X., Witte, D.P., Kim, M.O., Setchell, K.D., 2010. The chemopreventive action of equol enantiomers in a chemically induced animal model of breast cancer. Carcinogenesis 31, 886–893.
Botero, D., Ebbeling, C.B., Blumberg, J.B., Ribaya-Mercado, J.D., Creager, M.A., Swain, J.F., Feldman, H.A., Ludwig, D.S., 2009. Acute effects of dietary glycemic index on antioxidant capacity in a nutrient-controlled feeding study. Obesity (Silver Spring) 17 (9), 1664–1670. https://doi.org/10.1038/oby.2009.203.
Brand-Miller, J.C., Liu, V., Petocz, P., Baxter, R.C., 2005. The glycemic index of foods influences postprandial insulin-like growth factor-binding protein responses in lean young subjects. The American Journal of Clinical Nutrition 82 (2), 350–354.
Bravi, F., Scotti, L., Bosetti, C., Zucchetto, A., Talamini, R., Montella, M., Greggi, S., Pelucchi, C., Negri, E., Franceschi, S., La Vecchia, C., 2009. Food groups and endometrial cancer risk: A case-control study from Italy. American Journal of Obstetrics and Gynecology 200 (3), 293. e1–7.
Breinholt, V., Larsen, J.C., 1998. Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. Chemical Research in Toxicology 11, 622–629.
Breneman, C.B., Tucker, L., 2013. Dietary fibre consumption and insulin resistance - the role of body fat and physical activity. The British Journal of Nutrition 110 (2), 375–383.
Brown, N.M., Belles, C.A., Lindley, S.L., Zimmer-Nechemias, L.D., Zhao, X., Witte, D.P., Kim, M.O., Setchell, K.D., 2010. The chemopreventive action of equol enantiomers in a chemically induced animal model of breast cancer. Carcinogenesis 31, 886–893.
Brownlee, M., 2005. The pathobiology of diabetic complications: A unifying mechanism. Diabetes 54 (6), 1615–1625.
Buckland, G., Travier, N., Cottet, V., et al., 2013. Adherence to the mediterranean diet and risk of breast cancer in the European prospective investigation into cancer and nutrition cohort study. International Journal of Cancer 132 (12), 2918–2927.
Buono, R., Longo, V.D., 2018. Starvation, stress resistance, and Cancer. Trends in Endocrinology and Metabolism 29, 271–280.
Burdge, G., 2004. Alpha-linolenic acid metabolism in men and women: Nutritional and biological implications. Current Opinion in Clinical Nutrition and Metabolic Care 7 (2), 137–144.
Caan, B.J., Natarajan, L., Parker, B., Gold, E.B., Thomson, C., Newman, V., Rock, C.L., Pu, M., Al-Delaimy, W., Pierce, J.P., 2011. Soy food consumption and breast cancer prognosis. Cancer Epidemiology, Biomarkers & Prevention 20, 854–858.
Cabot, W., 2003. Phytoestrogens. Journal of the American Academy of Orthopaedic Surgeons 11, 153–156.
Cabrera, C., Artacho, R., Giménez, R., 2006. Beneficial effects of green tea: A review. Journal of the American College of Nutrition 25, 79–99.
Calle, E.E., Kaaks, R., 2004. Overweight, obesity and cancer: Epidemiological evidenceand proposed mechanisms. Nature Reviews. Cancer 4, 579–591.
Camilleri, G.M., Verger, E.O., Huneau, J.F., Carpentier, F., Dubuisson, C., Mariotti, F., 2013. Plant and animal protein intakes are differently associated with nutrient adequacy of the diet of french adults. The Journal of Nutrition 143, 1466–1473.
Cao, Y., Hou, L., Wang, W., 2016. Dietary total fat and fatty acids intake, serum fatty acids and risk of breast cancer: A meta-analysis of prospective cohort studies. International Journal of Cancer 138 (8), 1894–1904. https://doi.org/10.1002/ijc.29938. Epub 2015 Dec 9.
Capurso, C., Vendemiale, G., 2017. The Mediterranean diet reduces the risk and mortality of the prostate Cancer: A narrative review. Front Nutr 4, 38.
Carruba, G., Granata, O.M., Pala, V., Campisi, I., Agostara, B., Cusimano, R., Ravazzolo, B., Traina, A., 2006. A traditional Mediterranean diet decreases endogenous estrogens in healthy postmenopausal women. Nutrition and Cancer 56 (2), 253–259.
Ceriello, A., Bortolotti, N., Crescentini, A., Motz, E., Lizzio, S., Russo, A., Ezsol, Z., Tonutti, L., Taboga, C., 1998. Antioxidant defences are reduced during the oral glucose tolerance test in normal and non-insulin-dependent diabetic subjects. European Journal of Clinical Investigation 28 (4), 329–333.
Chan, J.M., Stampfer, M.J., Giovannucci, E., Ma, J., Pollak, M., 2000. Insulin-like growth factor I (IGF-I), IGFbinding protein-3 and prostate cancer risk: Epidemiological studies. Growth Hormone & IGF Research 10 (Suppl A), S32–S33.
Chen, H., Qin, S., Wang, M., Zhang, T., Zhang, S., 2015. Association between cholesterol intake and pancreatic cancer risk: Evidence from a meta-analysis. Scientific Reports 5, 8243. https://doi.org/10.1038/srep08243.

Chen, M., Rao, Y., Zheng, Y., Wei, S., Li, Y., Guo, T., Yin, P., 2014. Association between soy isoflavone intake and breast cancer risk for pre- and post-menopausal women: A meta-analysis of epidemiological studies. PLoS One 9, e89288.
Cheng, C.W., et al., 2014. Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression. Cell Stem Cell 14, 810–823.
Cheng, G., Wilczek, B., Warner, M., Gustafsson, J.A., Landgren, B.M., 2007. Isoflavone treatment for acute menopausal symptoms. Menopause 14, 468–473.
Chi, F., Wu, R., Zeng, Y.C., Xing, R., Liu, Y., 2013. Circulation insulin-like growth factor peptides and colorectal cancer risk: An updated systematic review and meta-analysis. Molecular Biology Reports 40 (5), 3583–3590. https://doi.org/10.1007/s11033-012-2432-z.
Chi, F., Wu, R., Zeng, Y.C., Xing, R., Liu, Y., Xu, Z.G., 2013. Post-diagnosis soy food intake and breast cancer survival: A meta-analysis of cohort studies. Asian Pacific Journal of Cancer Prevention 14, 2407–2412.
Chlebowski, R.T., Blackburn, G.L., Thomson, C.A., Nixon, D.W., Shapiro, A., Hoy, M.K., Goodman, M.T., Giuliano, A.E., Karanja, N., McAndrew, P., Hudis, C., Butler, J., Merkel, D., Kristal, A., Caan, B., Michaelson, R., Vinciguerra, V., Del Prete, S., Winkler, M., Hall, R., Simon, M., Winters, B.L., Elashoff, R.M., 2006. Dietary fat reduction and breast cancer outcome: Interim efficacy results from the Women's intervention nutrition study. Journal of the National Cancer Institute 98 (24), 1767–1776.
Choi, Y., Giovannucci, E., Lee, J.E., 2012. Glycaemic index and glycaemic load in relation to risk of diabetes-related cancers: A meta-analysis. The British Journal of Nutrition 108 (11), 1934–1947. https://doi.org/10.1017/S0007114512003984.
Coleman, H.G., Kitahara, C.M., Murray, L.J., Dodd, K.W., Black, A., Stolzenberg-Solomon, R.Z., Cantwell, M.M., 2014. Dietary carbohydrate intake, glycemic index, and glycemic load and endometrial cancer risk: A prospective cohort study. American Journal of Epidemiology 179 (1), 75–84. https://doi.org/10.1093/aje/kwt222.
Conaway, C.C., Getahun, S.M., Liebes, L.L., et al., 2000. Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. Nutrition and Cancer 38, 168–178, 26.
Cotton, S.C., Sharp, L., Little, J., Brockton, N., 2000. Glutathione S-transferase polymorphisms and colorectal cancer: A HuGE review. American Journal of Epidemiology 151, 7–32.
Crawley, D.J., Holmberg, L., Melvin, J.C., Loda, M., Chowdhury, S., Rudman, S.M., Van Hemelrijck, M., 2014. Serum glucose and risk of cancer: A meta-analysis. BMC Cancer 14, 985. https://doi.org/10.1186/1471-2407-14-985.
Cui, X., Rosner, B., Willett, W.C., Hankinson, S.E., 2011. Dietary fat, fiber, and carbohydrate intake in relation to risk of endometrial cancer. Cancer Epidemiology, Biomarkers & Prevention 20 (5), 978–989. https://doi.org/10.1158/1055-9965.EPI-10-1089.
Dagdemir, A., Durif, J., Ngollo, M., Bignon, Y.J., Bernard-Gallon, D., 2013. Histone lysine trimethylation or acetylation can be modulated by phytoestrogen, estrogen or anti-HDAC in breast cancer cell lines. Epigenomics 5, 51–63.
Dagdemir, A., Durif, J., Ngollo, M., Bignon, Y.J., Bernard-Gallon, D., 2013. Histone lysine trimethylation or acetylation can be modulated by phytoestrogen, estrogen or anti-HDAC in breast cancer cell lines. Epigenomics 5, 51–63.
Dal Maso, L., Zucchetto, A., Talamini, R., Serraino, D., Stocco, C.F., Vercelli, M., Falcini, F., Franceschi, S., 2008. Prospective analysis of case-control studies on environmental factors and health (PACE) study group. Effect of obesity and other lifestyle factors on mortality in women with breast cancer. International Journal of Cancer 123 (9), 2188–2194. https://doi.org/10.1002/ijc.23747.
Davey, G.K., Spencer, E.A., Appleby, P.N., Allen, N.E., Knox, K.H., Key, T.J., 2003. EPIC-Oxford: Lifestyle characteristics and nutrient intakes in a cohort of 33 883 meat-eaters and 31 546 non meat-eaters in the UK. Public Health Nutrition 6 (3), 259–269.
Davis-Yadley, A.H., Malafa, M.P., 2015. Vitamins in pancreatic cancer: A review of underlying mechanisms and future applications. Advances in Nutrition 6 (6), 774–802.
De Assis, S., Warri, A., Benitez, C., Helferich, W., Hilakivi-Clarke, L., 2011. Protective effects of prepubertal genistein exposure on mammary tumorigenesis are dependent on BRCA1 expression. Cancer Prevention Research 4, 1436–1448.
De Assis, S., Warri, A., Benitez, C., Helferich, W., Hilakivi-Clarke, L., 2011. Protective effects of prepubertal genistein exposure on mammary tumorigenesis are dependent on BRCA1 expression. Cancer Prevention Research 4, 1436–1448.
Descamps, O., Riondel, J., Ducros, V., Roussel, A.M., 2005. Mitochondrial production of reactive oxygen species and incidence of age-associated lymphoma in OF1 mice: Effect of alternate-day fasting. Mechanisms of Ageing and Development 126, 1185–1191.
Doll, R., Peto, R., 1981. The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. Journal of the National Cancer Institute 66 (6), 1191–1308.
Donovan, M.G., Selmin, O.I., Doetschman, T.C., Romagnolo, D.F., 2017. Mediterranean diet: Prevention of colorectal Cancer. Frontiers in Nutrition 4, 59.
Dorjgochoo, T., Gu, K., Zheng, Y., Kallianpur, A., Chen, Z., et al., 2011. Soy intake in association with menopausal symptoms during the first 6 and 36 months after breast cancer diagnosis. Breast Cancer Research and Treatment 130, 879–889.
Doyle, C., Kushi, L.H., Byers, T., Courneya, K.S., Demark-Wahnefried, W., Grant, B., et al., 2006. Nutrition and physical activity during and after cancer treatment: An American Cancer Society guide for informed choices. CA: A Cancer Journal for Clinicians 56, 323–353.
Dussaillant, C., Echeverria, G., Urquiaga, I., Velasco, N., Rigotti, A., 2016. Revista Médica de Chile 144 (8), 1044–1052. Current evidence on health benefits of the mediterranean diet.
EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2015. Risk assessment for peri- and post-menopausal women taking food supplements containing isolated isoflavones: Safety of isoflavones from food supplements in menopausal women. EFSA Journal 13, 4246.
EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2015. Risk assessment for peri- and post-menopausal women taking food supplements containing isolated isoflavones: Safety of isoflavones from food supplements in menopausal women. EFSA Journal 13, 4246.
Endogenous, Hormones, Group Breast Cancer Collaborative, Key, T.J., Appleby, P.N., Reeves, G.K., Roddam, A.W., 2010. Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: Pooled individual data analysis of 17 prospective studies. The Lancet Oncology 11 (6), 530–542. https://doi.org/10.1016/S1470-2045(10)70095-4.
Escrich, E., Solanas, M., Moral, R., Escrich, R., 2011. Modulatory effects and molecular mechanisms of olive oil and other dietary lipids in breast cancer. Current Pharmaceutical Design 17 (8), 813–830.
European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to Food (ANS), 2018. Scientific opinion on the safety of green tea catechins. EFSA Journal 16, 5239.
Eyre, H., et al., 2004. Preventing cancer, cardiovascular disease, and diabetes: A common agenda for the American Cancer Society the American Diabetes Association, and the American Heart Association. CA: A Cancer Journal for Clinicians 54, 190–207.
Fardet, A., 2010. New hypotheses for the health-protective mechanisms of whole-grain cereals: What is beyond fibre? Nutrition Research Reviews 23 (1), 65–134. https://doi.org/10.1017/S0954422410000041.
Farvid, M.S., Cho, E., Chen, W.Y., Eliassen, A.H., Willett, W.C., 2014. Premenopausal dietary fat in relation to pre and post-menopausal breast cancer. Breast Cancer Research and Treatment 145, 255–265.
Farvid, M.S., Cho, E., Eliassen, A.H., Chen, W.Y., Willett, W.C., 2016. Lifetime grain consumption and breast cancer risk. Breast Cancer Research and Treatment 159 (2), 335–345.
Favero, A., Salvini, S., Russo, A., Parpinel, M., Negri, E., Decarli, A., La Vecchia, C., Giacosa, A., Franceschi, S., 1997. Sources of macro- and micronutrients in Italian women: Results from a food frequency questionnaire for cancer studies. European Journal of Cancer Prevention 6 (3), 277–287.
Ferro-Luzzi, A., Branca, F., 1995. Mediterranean diet, Italian-style: Prototype of a healthy diet. The American Journal of Clinical Nutrition 61 (6 Suppl), 1338S–1345S.
Filomeno, M., Bosetti, C., Bidoli, E., Levi, F., Serraino, D., Montella, M., La Vecchia, C., Tavani, A., 2015. Mediterranean diet and risk of endometrial cancer: A pooled analysis of three Italian case-control studies. British Journal of Cancer 112 (11), 1816–1821.

Fleshner, N.E., Kapusta, L., Donnelly, B., Tanguay, S., Chin, J., Hersey, K., Farley, A., Jansz, K., Siemens, D.R., Trpkov, K., et al., 2011. Progression from high-grade prostatic intraepithelial neoplasia to cancer: A randomized trial of combination vitamin-E, soy, and selenium. Journal of Clinical Oncology 29, 2386–2390.
Flood, A., Velie, E.M., Chaterjee, N., et al., 2002. Fruit and vegetable intakes and the risk of colorectal cancer in the breast Cancer detection demonstration project follow-up cohort. The American Journal of Clinical Nutrition 75, 936–943.
Fonseca-Nunes, A., Jakszyn, P., Agudo, A., 2014. Iron and cancer risk—A systematic review and meta-analysis of the epidemiological evidence. Cancer Epidemiology, Biomarkers & Prevention 23, 12–31.
Fontana, L., Partridge, L., Longo, V.D., 2010. Extending healthy life span–from yeast to humans. Science 328, 321–326.
Fortner, R.T., Husing, A., Kuhn, T., et al., 2017. Endometrial cancer risk prediction including serum-based biomarkers: Results from the EPIC cohort. International Journal of Cancer 140 (6), 1317–1323.
Fowke, J.H., Longcope, C., Hebert, J.R., 2000. Brassica vegetable consumption shifts estrogen metabolism in healthy postmenopausal women. Cancer Epidemiology, Biomarkers & Prevention 9, 773–779.
Franceschi, S., Dal Maso, L., Augustin, L., Negri, E., Parpinel, M., Boyle, P., Jenkins, D.J., La Vecchia, C., 2001. Dietary glycemic load and colorectal cancer risk. Annals of Oncology 12 (2), 173–178.
Franceschi, S., Favero, A., La Vecchia, C., Negri, E., Conti, E., Montella, M., Giacosa, A., Nanni, O., Decarli, A., 1997. Food groups and risk of colorectal cancer in Italy. International Journal of Cancer 72 (1), 56–61.
Fujiki, H., Suganuma, M., 2002. Green tea and cancer prevention. Proceedings of the Japan Academy 78, 263–270.
Fujita, Y., Yamane, T., Tanaka, M., et al., 1989. Inhibitory effect of (-)-epigallocatechin gallate on carcinogenesis with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine in mouse duodenum. Japanese Journal of Cancer Research 80, 503–505.
Gann, P.H., Ma, J., Giovannucci, E., Willett, W., Sacks, F.M., Hennekens, C.H., Stampfer, M.J., 1999. Lower prostate cancer risk in men with elevated plasma lycopene levels: Results of a prospective analysis. Cancer Research 59 (6), 1225–1230.
Ge, J., Tan, B.X., Chen, Y., et al., 2011. Interaction of green tea polyphenol epigallocatechin-3-gallate with sunitinib: Potential risk of dimin- ished sunitinib bioavailability. Journal of Molecular Medicine (Berl) 89 (6), 595e602.
Getahun, S.M., Chung, F.L., 1999. Conversion of glucosinolates to isothiocyanates in humans after ingestion of cooked watercress. Cancer Epidemiology, Biomarkers & Prevention 8, 447–451.
Giacosa, A., Barale, R., Bavaresco, L., Gatenby, P., Gerbi, V., Janssens, J., Johnston, B., Kas, K., La Vecchia, C., Mainguet, P., Morazzoni, P., Negri, E., Pelucchi, C., Pezzotti, M., Rondanelli, M., 2013. Cancer prevention in Europe: The Mediterranean diet as a protective choice. European Journal of Cancer Prevention 22 (1), 90–95.
Giovannucci, E., 1995. Insulin and colon cancer. Cancer Causes & Control 6 (2), 164–179.
Giovannucci, E., 2003. Nutrition, insulin, insulin-like growth factors and cancer. Hormone and Metabolic Research 35 (11 – 12), 694–704. https://doi.org/10.1055/s-2004-814147.
Giovannucci, E., Pollak, M., Platz, E.A., Willett, W.C., Stampfer, M.J., Majeed, N., Colditz, G.A., Speizer, F.E., Hankinson, S.E., 2000. Insulin-like growth factor I (IGF-I), IGF-binding protein-3 and the risk of colorectal adenoma and cancer in the nurses' health study. Growth Hormone & IGF Research 10 (Suppl A), S30–S31.
Giovannucci, E., Rimm, E.B., Liu, Y., Stampfer, M.J., 2003. Willett WC. A prospective study of cruciferous vegetables and prostate cancer. Cancer epidemiology. Biomarkers & Prevention 12, 1403–1409.
Golden, E.B., Lam, P.Y., Kardosh, A., et al., 2009. Green tea polyphenols block the anticancer effects of bortezomib and other boronic acid-based proteasome inhibitors. Blood 113 (23), 5927–5937.
Goodman-Gruen, D., Kritz-Silverstein, D., 2001. Usual dietary isoflavone intake is associated with cardiovascular disease risk factors in postmenopausal women. The Journal of Nutrition 131, 1202–1206.
Goodwin, P.J., Ambrosone, C.B., Hong, C.C., 2015. Modifiable lifestyle factors andbreast cancer outcomes: Current controversies and researchrecommendations. Adv. Exp. Med. Biol. 862, 177–192.
Goodwin, P.J., Ennis, M., Pritchard, K.I., Trudeau, M.E., Koo, J., Madarnas, Y., Hartwick, W., Hoffman, B., Hood, N., 2002. Fasting insulin and outcome in early-stage breast cancer: Results of a prospective cohort study. Journal of Clinical Oncology 20 (1), 42–51.
Graff, R.E., Pettersson, A., Lis, R.T., Ahearn, T.U., Markt, S.C., Wilson, K.M., Rider, J.R., Fiorentino, M., Finn, S., Kenfield, S.A., Loda, M., Giovannucci, E.L., Rosner, B., Mucci, L.A., 2016. Dietary lycopene intake and risk of prostate cancer defined by ERG protein expression. The American Journal of Clinical Nutrition 103 (3), 851–860.
Grosso, G., Bella, F., Godos, J., Sciacca, S., Del Rio, D., Ray, S., Galvano, F., Giovannucci, E.L., 2017a. Possible role of diet in cancer: Systematic review and multiple meta-analyses of dietary patterns, lifestyle factors, and cancer risk. Nutrition Reviews 75, 405–419.
Grosso, G., Godos, J., Lamuela-Raventos, R., Ray, S., Micek, A., Pajak, A., Sciacca, S., D'Orazio, N., Del Rio, D., Galvano, F., 2017b. A comprehensive meta-analysis on dietary flavonoid and lignan intake and cancer risk: Level of evidence and limitations. Molecular Nutrition & Food Research 61.
Grosso, G., Micek, A., Godos, J., Pajak, A., Sciacca, S., Galvano, F., Giovannucci, E.L., 2017. Dietary flavonoid and Lignan intake and mortality in prospective cohort studies: Systematic review and dose-response meta-analysis. American Journal of Epidemiology 1–13.
Guevara-Aguirre, J., Balasubramanian, P., Guevara-Aguirre, M., Wei, M., Madia, F., Cheng, C.W., Hwang, D., Martin-Montalvo, A., Saavedra, J., Ingles, S., de Cabo, R., Cohen, P., Longo, V.D., 2011. Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. Science Translational Medicine 3, 7–13.
Guha, N., Kwan, M.L., Quesenberry Jr., C.P., Weltzien, E.K., Castillo, A.L., Caan, B.J., 2009. Soy isoflavones and risk of cancer recurrence in a cohort of breast cancer survivors: The life after Cancer epidemiology study. Breast Cancer Research and Treatment 118, 395–405.
Guha, N., Kwan, M.L., Quesenberry, C.P., Weltzien, E.K., Castillo, A.L., Caan, B.J., 2009. Soy isoflavones and risk of cancer recurrence in a cohort of breast cancer survivors: The life after cancer epidemiology study. Breast Cancer Research and Treatment 118, 395–405.
Guo, L., Yang, R., Wanga, Z., Zhenxin, G., 2015. Effect of freezing methods on sulforaphane formation in broccoli sprouts. RSC Advances 5, 32290.
Gupta, S., Hastak, K., Ahmad, N., Lewin, J.S., Mukhtar, H., 2001. Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols. Proceedings of the National Academy of Sciences of the United States of America 98, 10350–10355.
Halkjaer, J., Olsen, A., Bjerregaard, L., Deharveng, G., Tjønneland, A., Welch, A., Crowe, F., Wirfält, E., Hellstrom, V., Niravong, M., 2009. Intake of total, animal and plant proteins, and their food sources in 10 countries in the European prospective investigation into cancer and nutrition. European Journal of Clinical Nutrition 63, S16–S36.
Hall, M.N., Chavarro, J.E., Lee, I.M., Willett, W.C., Ma, J., 2008. A 22-year prospective study of fish, n-3 fatty acid intake, and colorectal cancer risk in men. Cancer Epidemiology, Biomarkers & Prevention 17, 1136–1143.
Halliwell, B., 2007. Oxidative stress and cancer: Have we moved forward? The Biochemical Journal 401 (1), 1–11. https://doi.org/10.1042/BJ20061131.
Harvie, M.N., Pegington, M., Mattson, M.P., Frystyk, J., Dillon, B., Evans, G., et al., 2011. The effects of intermittent or continuous energy restriction on weight loss and metabolic disease risk markers: A randomized trial in young overweight women. International Journal of Obesity 35, 714–727.
Hedlund, M., Padler-Karvani, V., Varki, N.M., Varkia, A., 2008. Evidence for a human-specific mechanism for diet and antibody-mediated inflammation in carcinoma progression. Proceedings of the National Academy of Sciences of the United States of America 105, 18936–18941.
Helferich, W.G., Andrade, J.E., Hoagland, M.S., 2008. Phytoestrogens and breast cancer: A complex story. Inflammopharmacology 16, 219–226.
Higdon, J.V., Delage, B., Williams, D.E., Dashwood, R.H., 2007. Cruciferous vegetables and human cancer risk: Epidemiologic evidence and mechanistic basis. Pharmacological Research 55, 224–236.

Higginbotham, S., Zhang, Z.F., Lee, I.M., Cook, N.R., Giovannucci, E., Buring, J.E., Liu, S., Women's Health, S., 2004. Dietary glycemic load and risk of colorectal cancer in the Women's health study. Journal of the National Cancer Institute 96 (3), 229–233.
Hooper, L., Madhavan, G., Tice, J.A., Leinster, S.J., Cassidy, A., 2010. Effects of isoflavones on breast density in pre- and post-menopausal women: A systematic review and meta-analysis of randomized controlled trials. Human Reproduction Update 16, 745–760.
Hooper, L., Ryder, J.J., Kurzer, M.S., Lampe, J.W., Messina, M.J., Phipps, W.R., Cassidy, A., 2009. Effects of oy protein and isoflavones on circulating hormone concentrations in pre- and post-menopausal women: A systematic review and meta-analysis. Human Reproduction Update 15, 423–440.
Horne, B.D., Muhlestein, J.B., Anderson, J.L., 2015. Health effects of intermittent fasting: Hormesis or harm? A systematic review. The American Journal of Clinical Nutrition 102, 464–470.
Hou, R., Yao, S.S., Liu, J., Wang, L.L., Wu, L., Jiang, L., 2017. Dietary n-3 polyunsaturated fatty acids, fish consumption, and endometrial cancer risk: A meta-analysis of epidemiological studies. Oncotarget 8 (53), 91684–91693. https://doi.org/10.18632/oncotarget.18295 eCollection 2017 Oct 31.
Howitz, K.T., Sinclair, D.A., 2008. Xenohormesis: Sensing the chemical cues of other species. Cell 133, 387–391.
Hu, J., La Vecchia, C., de Groh, M., Negri, E., Morrison, H., Mery, L., 2011. Canadian Cancer registries epidemiology research group. Dietary transfatty acids and cancer risk. European Journal of Cancer Prevention 20 (6), 530–538. https://doi.org/10.1097/CEJ.0b013e328348fbfb.
Hu, J., La Vecchia, C., Negri, E., Mery, L., 2010. Nutrients and risk of colon cancer. Cancers (Basel) 2 (1), 51–67. https://doi.org/10.3390/cancers2010051.
Huerta-Yépez, S., Tirado-Rodriguez, A.B., Hankinson, O., 2016. Role of diets rich in omega-3 and omega-6 in the development of cancer. Boletín Médico del Hospital Infantil de México 73 (6), 446–456. https://doi.org/10.1016/j.bmhimx.2016.11.001. Epub 2016 Nov 30.
Hutchison, A.T., Heilbronn, L.K., 2016. Metabolic impacts of altering meal frequency and timing–does when we eat matter? Biochimie 124, 187–197.
IARC. Cruciferous vegetables, isothiocyanates and indoles. IARC Handbooks of Cancer Prevention Vol. 9. Lyon: IARC Press, 2004 (accessed on 14 April 2018).
Ide, H., Tokiwa, S., Sakamaki, K., Nishio, K., Isotani, S., Muto, S., Hama, T., Masuda, H., Horie, S., 2010. Combined inhibitory effects of soy isoflavones and curcumin on the production of prostate-specific antigen. Prostate 70, 1127–1133.
Inoue, M., Robien, K., Wang, R., et al., 2008. Green tea intake, MTHFR/TYMS genotype and breast cancer risk: The Singapore Chinese health study. Carcinogenesis 29, 1967–1972.
Inoue, I., Shinoda, Y., Nakano, T., Sassa, M., Goto, S., Awata, T., Komoda, T., Katayama, S., 2006. Acarbose ameliorates atherogenecity of low-density lipoprotein in patients with impaired glucose tolerance. Metabolism 55 (7), 946–952. https://doi.org/10.1016/j.metabol.2006.03.002.
International Agency for Research on Cancer, 2014. In: Stewart, B.W., Wild, C.P. (Eds.), World Cancer Report 2014. Lyon, France.
Jackson, R.L., Greiwe, J.S., Schwen, R.J., 2011. Emerging evidence of the health benefits of S-equol, an estrogen receptor beta agonist. Nutrition Reviews 69, 432–448.
Jacob, K., Periago, M.J., Bohm, V., Berruezo, G.R., 2008. Influence of lycopene and vitamin C from tomato juice on biomarkers of oxidative stress and inflammation. The British Journal of Nutrition 99, 137–146.
Jain, M.G., Hislop, G.T., Howe, G.R., Ghadirian, P., 1999. Plant foods, antioxidants, and prostate cancer risk: Findings from case-control studies in Canada. Nutrition and Cancer 34, 173–184.
Järvinen, R., Knekt, P., Hakulinen, T., Rissanen, H., Heliövaara, M., 2001. Dietary fat, cholesterol and colorectal cancer in a prospective study. British Journal of Cancer 85 (3), 357–361.
Jee, S.H., Ohrr, H., Sull, J.W., Yun, J.E., Ji, M., Samet, J.M., 2005. Fasting serum glucose level and cancer risk in Korean men and women. JAMA 293 (2), 194–202. https://doi.org/10.1001/jama.293.2.194.
Jenkins, D.J., Wolever, T.M., Taylor, R.H., Barker, H., Fielden, H., Baldwin, J.M., Bowling, A.C., Newman, H.C., Jenkins, A.L., Goff, D.V., 1981. Glycemic index of foods: A physiological basis for carbohydrate exchange. The American Journal of Clinical Nutrition 34 (3), 362–366.
Jin, Y., Qu, S., Tesikova, M., Wang, L., Kristian, A., Maelandsmo, G.M., Kong, H., Zhang, T., Jeronimo, C., Teixeira, M.R., et al., 2013. Molecular circuit involving KLK4 integrates androgen and mTOR signaling in prostate cancer. Proceedings of the National Academy of Sciences of the United States of America 110, E2572–E2581.
Jing, K., Wu, T., Lim, K., 2013. Omega-3 polyunsaturated fatty acids and cancer. Anti-Cancer Agents in Medicinal Chemistry 13 (8), 1162–1177.
Johnson, R., Bryant, S., Huntley, L.A., 2012. Green tea and gree tea catechin extracts: An overview of the clinical evidence. Maturitas 73, 280–287.
Jones, J.L., Daley, B.J., Enderson, B.L., et al., 2002. Genistein inhibits tamoxifen effects on cell proliferation and cell cycle arrest in T47D breast cancer cells. The American Surgeon 68, 575–577.
Jones, R.B., Frisina, C.L., Winkler, S., Imsic, M., Tomkins, R.B., 2010. Cooking method significantly effects glucosinolate content and sulforaphane production in broccoli florets. Food Chemistry (2), 237–242.
Ju, Y.H., Allred, C.D., Allred, K.F., Karko, K.L., Doerge, D.R., Helferich, W.G., 2001. Physiological concentrations of dietary genistein dose-dependently stimulate growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in athymic nude mice. The Journal of Nutrition 131, 2957–2962.
Ju, Y.H., Doerge, D.R., Woodling, K.A., et al., 2008. Dietary genistein negates the inhibitory effect of letrozole on the growth of aromatase-expressing estrogen-dependent human breast cancer cells (MCF-7Ca) in vivo. Carcinogenesis 29, 2162–2168.
Jump, D.B., 2004. Fatty acid regulation of gene transcription. Critical Reviews in Clinical Laboratory Sciences 41 (1), 41–78.
Kaaks, R., Lukanova, A., 2001. Energy balance and cancer: The role of insulin and insulin-like growth factor-I. The Proceedings of the Nutrition Society 60 (1), 91–106.
Kang, D.H., 2002. Oxidative stress, DNA damage, and breast cancer. AACN Advanced Critical Care 13 (4), 540–549.
Kang, L., Ding, L., Wang, Z.Y., 2009. Isothiocyanates repress estrogen receptor alpha expression in breast cancer cells. Oncology Reports 21, 185–192.
Kang, X., Zhang, Q., Wang, S., Huang, X., Jin, S., 2010. Effect of soy isoflavones on breast cancer recurrence and death for patients receiving adjuvant endocrine therapy. CMAJ 182, 1857–1862.
Kang, X., Zhang, Q., Wang, S., Huang, X., Jin, S., 2010. Effect of soy isoflavones on breast cancer recurrence and death for patients receiving adjuvant endocrine therapy. CMAJ 182, 1857–1862.
Key, T.J., Allen, N., Appleby, P., et al., 2004. Fruits and vegetables and prostate cancer: No association among 1104 cases in a prospective study of 130544 men in the European prospective investigation into Cancer and nutrition (EPIC). International Journal of Cancer 109, 119–124.
Khan, S.A., Chatterton, R.T., Michel, N., Bryk, M., Lee, O., Ivancic, D., Heinz, R., Zalles, C.M., Helenowski, I.B., Jovanovic, B.D., et al., 2012. Soy isoflavone supplementation for breast cancer risk reduction: A randomized phase II trial. Cancer Prevention Research 5, 309–319.
King, H., Li, J.Y., Locke, F.B., Pollack, E.S., Tu, J.T., 1985. Patterns of site-specific displacement in cancer mortality among migrants: The Chinese in the United States. American Journal of Public Health 75 (3), 237–242.
Kolahdouz Mohammadi, R., Bagheri, M., Kolahdouz Mohammadi, M., Shidfar, F., 2017. Ruminant trans-fatty acids and risk of breast cancer: A systematic review and meta-analysis of observational studies. Minerva Endocrinologica 42 (4), 385–396. https://doi.org/10.23736/S0391-1977.16.02514-1. Epub 2016 Sep 14.
Kolonel, L.N., Hankin, J.H., Whittemore, A.S., et al., 2000. Vegetables, fruits, legumes and prostate cancer: A multiethnic case-control study. Cancer epidemiology. Biomarkers & Prevention 9, 795–804.
Kosinska, A., Andlauer, W., 2014. Antioxidant capacity of tea: Effect of processing and storage. In: Preedy, V. (Ed.), Processing and impact on antioxidants in beverages. Elsevier, pp. 109–120.
Kromhout, D., Keys, A., Aravanis, C., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovic, S., Pekkarinen, M., et al., 1989. Food consumption patterns in the 1960s in seven countries. The American Journal of Clinical Nutrition 49 (5), 889–894.

Kushi, L.H., et al., 2006. American Cancer Society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. CA: A Cancer Journal for Clinicians 56, 254–281 quiz 313–314.
Kushi, L.H., Doyle, C., McCullough, M., Rock, C.L., Demark-Wahnefried, W., Bandera, E.V., Gapstur, S., Patel, A.V., Andrews, K., Gansler, T., 2012. The American Cancer Society 2010 nutrition and physical activity guidelines advisory committee. American Cancer Society Guidelines on Nutrition and Physical Activity for Cancer Prevention. CA: A Cancer Journal for Clinicians 62, 30–67.
Kushi, L.H., Doyle, C., McCullough, M., Rock, C.L., Demark-Wahnefried, W., Bandera, E.V., Gapstur, S., Patel, A.V., Andrews, K., Gansler, T., American Cancer Society N, Physical Activity Guidelines Advisory C, 2012. American Cancer Society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. Ca-A Cancer Journal for Clinicians, 62 (1), 30–67.
Kushi, L.H., Doyle, C., McCullough, M., Rock, C.L., Demark-Wahnefried, W., Bandera, E.V., Gapstur, S., Patel, A.V., Andrews, K., Gansler, T., Nutrition American Cancer Society, and Committee Physical Activity Guidelines Advisory, 2012. American Cancer Society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. CA: A Cancer Journal for Clinicians 62 (1), 30–67. https://doi.org/10.3322/caac.20140.
Kwan, W., Duncan, G., Van Patten, C., Liu, M., Lim, J., 2010. A phase II trial of a soy beverage for subjects without clinical disease with rising prostate-specific antigen after radical radiation for prostate cancer. Nutrition and Cancer 62, 198–207.
Lai, R., Bian, Z., Lin, H., Ren, J., Zhou, H., Guo, H., 2017. The association between dietary protein intake and colorectal cancer risk: A meta-analysis. World Journal of Surgical Oncology 15, 169.
Lamartiniere, C.A., Moore, J.B., Brown, N.M., Thompson, R., Hardin, M.J., Barnes, S., 1995b. Genistein suppresses mammary cancer in rats. Carcinogenesis 16, 2833–2840.
Lamartiniere, C.A., Moore, J., Holland, M., Barnes, S., 1995a. Neonatal genistein chemoprevents mammary cancer. Proceedings of the Society for Experimental Biology and Medicine 208, 120–123.
Lamartiniere, C.A., Zhao, Y.X., Fritz, W.A.G., 2000. Mammary cancer chemoprevention, in vivo mechanisms of action, potential for toxicity and bioavailability in rats. J. Women's. Cancer 2, 11–19.
Lampe JWand Peterson, S., 2002. Brassica, biotransformation and cancer risk: Genetic polymorphisms alter the preventive effects of cruciferous vegetables. The Journal of Nutrition 132, 2991–2994.
Lampe, J.W., 1999. Health effects of vegetables and fruit: Assessing mechanisms of action in human experimental studies. The American Journal of Clinical Nutrition 70 (3 Suppl), 475S–490S.
Larkin, T., Price, W.E., Astheimer, L., 2008. The key importance of soy isoflavone bioavailability to understanding health benefits. Critical Reviews in Food Science and Nutrition 48, 538–552.
Larkin, T., Price, W.E., Astheimer, L., 2008. The key importance of soy isoflavone bioavailability to understanding health benefits. Critical Reviews in Food Science and Nutrition 48, 538–552.
Larsson, S.C., Giovannucci, E., Wolk, A., 2006. Folate intake, MTHFR polymorphisms, and risk of esophageal, gastric, and pancreatic cancer: A meta-analysis. Gastroenterology 131 (4), 1271–1283.
Larsson, S.C., Kumlin, M., Ingelman-Sundberg, M., Wolk, A., 2004. Dietary long-chain n-3 fatty acids for the prevention of cancer: A review of potential mechanisms. The American Journal of Clinical Nutrition 79 (6), 935–945.
Lawrence, G.D., 2013. Dietary fats and health: Dietary recommendations in the context of scientific evidence. Advances in Nutrition 4 (3), 294–302. https://doi.org/10.3945/an.113.003657.
Lecumberri, E., Dupertuis, Y.M., Miralbell, R., Pichard, C., 2013. Green tea polyphenl epigallocatechin-3-gallate (EGCG) as adjuvant in cancer therapy. Clinical Nutrition 32, 894–903.
Lee, C., et al., 2012b. Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy. Science Translational Medicine 4, 124–127.
Lee, S.A., Shu, X.O., Li, H., Yang, G., Cai, H., Wen, W., Ji, B.T., Gao, J., Gao, Y.T., Zheng, W., 2009. Adolescent and adult soy food intake and breast cancer risk: Results from the ShanghaiWomen's health study. The American Journal of Clinical Nutrition 89, 1920–1926.
Lee, S.A., Shu, X.O., Yang, G., Li, H., Gao, Y.T., Zheng, W., 2009. Animal origin foods and colorectal cancer risk: A report from the shanghai Women's health study. Nutrition and Cancer 61 (2), 194–205. https://doi.org/10.1080/01635580802419780.
Lenzi, M., Fimognari, C., Hrelia, P., 2014. Sulforaphane as a promising molecule for fighting cancer. Cancer Treatment and Research 159, 207–223.
Li, J., Ma, J., Han, L., Xu, Q., Lei, J., Duan, W., Li, W., Wang, F., Wu, E., Ma, Q., Huo, X., 2015. Hyperglycemic tumor microenvironment induces perineural invasion in pancreatic cancer. Cancer Biology & Therapy 16 (6), 912–921. https://doi.org/10.1080/15384047.2015.1040952.
Li, N., Sun, Z., Han, C., Chen, J., 1999. The chemopreventive effects of tea on human oral precancerous mucosa lesions. Proceedings of the Society for Experimental Biology and Medicine 220, 218–224.
Li, C., Yang, L., Zhang, D., Jiang, W., 2016. Systematic review and meta-analysis suggest that dietary cholesterol intake increases risk of breast cancer. Nutrition Research 36 (7), 627–635. https://doi.org/10.1016/j.nutres.2016.04.009. Epub 2016 Apr 26.
Liao, W.C., Tu, Y.K., Wu, M.S., Lin, J.T., Wang, H.P., Chien, K.L., 2015. Blood glucose concentration and risk of pancreatic cancer: Systematic review and dose-response meta-analysis. BMJ 349, g7371. https://doi.org/10.1136/bmj.g7371.
Lin, L.Z., Chen, P., Harnly, J.M., 2008. New phenolic components and chromatographic profiles of green and fermented teas. Journal of Agricultural and Food Chemistry 56, 8130–8140.
Lin, H.J., Probst-Hensch, N.M., Louie, A., Kau, I.H., Witte, J.S., et al., 1998. Glutathione transferase null genotype, broccoli, and lower prevalence of colorectal adenomas. Cancer Epidem Biomar 7, 647–652.
Lin, T., Zirpoli, G.R., McCann, S.E., Moysich, K.B., Christine, B., Tang, A.L., 2017. Trends in cruciferous vegetable consumption and associations with breast Cancer risk: A case-control study. Current Developments in Nutrition 1, e000448.
Liu, X., Lv, K., 2013. Cruciferous vegetables intake is inversely associated with risk of breast cancer: A meta-analysis. Breast 22, 309–313.
Liu, B., Mao, Q., Cao, M., Xie, L., 2012. Cruciferous vegetables intake and risk of prostate cancer: A meta-analysis. International Journal of Urology 19, 134–141.
Liu, J., Yuan, F., Gao, J., Shan, B., Ren, Y., Wang, H., Gao, Y., 2016. Oral isoflavone supplementation on endometrial thickness: A ma-analysis of randomized placebo-controlled trials. Oncotarget 7, 17369–17379.
Llor, X., Pons, E., Roca, A., Alvarez, M., Mane, J., Fernandez-Banares, F., Gassull, M.A., 2003. The effects of fish oil, olive oil, oleic acid and linoleic acid on colorectal neoplastic processes. Clinical Nutrition 22 (1), 71–79.
Loibl, S., Lintermans, A., Dieudonné, A.S., Neven, P., 2011. Management of menopausal symptoms in breast cancer patients. Maturitas 68, 148–154.
Longo, V.D., Fontana, L., 2010. Calorie restriction and cancer prevention: Metabolic and molecular mechanisms. Trends in Pharmacological Sciences 31, 89–98.
Longo, V.D., Mattson, M.P., 2014. Fasting: Molecular mechanisms and clinical applications. Cell Metabolism 19 (2), 181–192.
Longo, V.D., Panda, S., 2016. Fasting, circadian rhythms, and time-restricted feeding in healthy lifespan. Cell Metabolism 14 (23), 1048–1059.
Lozovaya, V.V., Lygin, A.V., Ulanov, A.V., Nelson, R.L., Daydé, J., Widholm, J.M., 2005. Effect of temperature and soil moisture status during seed development on soybean seed Isoflavone concentration and composition. Crop Science 45, 1934–1940.
Lu, R.R., Qian, P., Sun, A., et al., 2010. Hempseed protein derived antioxidative peptides: Purification, identification and protection from hydrogen peroxide-induced apoptosis in PC12 cells. Food Chemistry 123 (4), 1210–1218.

Magee, P.J., McGlynn, H., Rowland, I.R., 2004. Differential effects of isoflavones and lignans on invasiveness of MDA-MB-231 breast cancer cells in vitro. Cancer Letters 208, 35–41.
Mahmoud, A.M., Yang, W., Bosland, M.C., 2014. Soy isoflavones and prostate cancer: A review of molecular mechanisms. The Journal of Steroid Biochemistry and Molecular Biology 140, 116–132.
Mahmoud, A.M., Yang, W., Bosland, M.C., 2014. Soy isoflavones and prostate cancer: A review of molecular mechanisms. The Journal of Steroid Biochemistry and Molecular Biology 140, 116–132.
Mandal, C.C., Ghosh-Choudhury, T., Yoneda, T., Choudhury, G.G., Ghosh-Choudhury, N., 2010. Fish oil prevents breast cancer cell metastasis to bone. Biochemical and Biophysical Research Communications 402 (4), 602–607.
Markovitis, N., Ben Amotz, A., Levy, Y., 2009. The effect of tomato-derived lycopene on low carotenoids and enhanced systemic inflammation and oxidation in severe obesity. The Israel Medical Association Journal 11, 598–601.
Matthews, J., Gustafsson, J.-A., 2003. Estrogen signaling: A subtle balance between ER alpha and ER beta. Molecular Interventions 3, 281–292.
Mattson, M.P., 2005. Energy intake, meal frequency, and health: A neurobiologicalperspective. Annual Review of Nutrition 25, 237–260.
Mattson, M.P., Longo, V.D., Harvie, M., 2017 Oct. Impact of intermittent fasting on health and disease processes. Ageing Research Reviews 39, 46–58.
May-Wilson, S., Sud, A., Law, P.J., et al., 2017. Pro-inflammatory fatty acid profile and colorectal cancer risk: A Mendelian randomisation analysis. European Journal of Cancer 84, 228–238.
Mazidi, M., Rezaie, P., Chaudhri, O., et al., 2015. The effect of Ramadan fasting on cardiometabolic risk factors and anthropometrics parameters: A systematic review. Pakistan Journal of Medical Sciences 31, 1250–1255.
Mazur, W.M., Duke, J.A., Wahala, K., Rasku, S., Adlercreutz, H., 1998. Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans. The Journal of Nutritional Biochemistry 9, 193–200.
McCullough, M.L., Giovannucci, E.L., 2004. Diet and cancer prevention. Oncogene 23 (38), 6349–6364. https://doi.org/10.1038/sj.onc.1207716.
McCullough, M.L., Robertson, A.S., Chao, A., et al., 2003. A prospective study of whole grains, fruits, vegetables and colon cancer risk. Cancer Causes & Control 14, 959–970.
McEligot, A.J., Largent, J., Ziogas, A., Peel, D., Anton-Culver, H., 2006. Dietary fat, fiber, vegetable, and micronutrients are associated with overall survival in postmenopausal women diagnosed with breast cancer. Nutrition and Cancer 55 (2), 132–140.
McKeown-Eyssen, G., 1994. Epidemiology of colorectal cancer revisited: Are serum triglycerides and/or plasma glucose associated with risk? Cancer Epidemiology, Biomarkers & Prevention 3 (8), 687–695.
Meegan, M.J., Lloyd, D.G., 2003. Advances in the science of estrogen receptor modulation. Current Medicinal Chemistry 10, 181–210.
Meng, Q., Yuan, F., Goldberg, I.D., Rosen, E.M., Auborn, K., Fan, S., 2000. Indole-3-carbinol is a negative regulator of estrogen receptor-alpha signaling in human tumor cells. The Journal of Nutrition 130, 2927–2931.
Menotti, A., Keys, A., Aravanis, C., Blackburn, H., Dontas, A., Fidanza, F., Karvonen, M.J., Kromhout, D., Nedeljkovic, S., Nissinen, A., et al., 1989. Seven countries study. First 20-year mortality data in 12 cohorts of six countries. Annals of Medicine 21 (3), 175–179.
Mensink, R.P., Zock, P.L., Kester, A.D., Katan, M.B., 2003. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. The American Journal of Clinical Nutrition 77, 1146–1155.
Messina, M.J., 1999. Legumes and soybeans: Overview of their nutritional profiles and health effects. The American Journal of Clinical Nutrition 70, 439S–450S. Review.
Messina, M., 2010. A brief historical overview of the past two decades of soy and isoflavone research. The Journal of Nutrition 140 (7), 1350S–4S. Review.
Messina, M., 2016a. Impact of soy foods on the development of breast Cancer and the prognosis of breast Cancer patients. Forschende Komplementärmedizin 23 (2), 75–80. Review.
Messina, M., 2016b. Soy and health update: Evaluation of the clinical and epidemiologic literature. Nutrients 8, 754.
Messina, M., 2016. Soy and health update: Evaluation of the clinical and epidemiologic literature. Nutrients 8, 754.
Messina, M., Hilakivi-Clarke, L., 2009. Early intake appears to be the key to the proposed protective effects of soy intake against breast cancer. Nutrition and Cancer 61 (6), 792–798.
Messina, M., Kucuk, O., Lampe, J.W., 2006. An overview of the health effects of isoflavones with an emphasis on prostate cancer risk and prostate-specific antigen levels. Journal of AOAC International 89, 1121–1134.
Messina, M., Kucuk, O., Lampe, J.W., 2006b. An overview of the health effects of isoflavones with an emphasis on prostate cancer risk and prostate-specific antigen levels. Journal of AOAC International 89, 1121–1134.
Messina, M., McCaskill-Stevens, W., Lampe, J.W., 2006a. Addressing the soy and breast cancer relationship: Review, commentary, and workshop proceedings. Journal of the National Cancer Institute 98 (18), 1275–1284. Review.
Messina, M., Nagata, C., Wu, A.H., 2006c. Estimated Asian adult soy protein and isoflavone intakes. Nutrition and Cancer 55, 1–12.
Meyerhardt, J.A., Sato, K., Niedzwiecki, D., Ye, C., Saltz, L.B., Mayer, R.J., Mowat, R.B., Whittom, R., Hantel, A., Benson, A., Wigler, D.S., Venook, A., Fuchs, C.S., 2012. Dietary glycemic load and cancer recurrence and survival in patients with stage III colon cancer: Findings from CALGB 89803. Journal of the National Cancer Institute 104 (22), 1702–1711. https://doi.org/10.1093/jnci/djs399.
Michels, K.B., Giovannucci, E., Joshipura, K.J., et al., 2000. Prospective study of fruit and vegetable consumption and incidence of colon and rectal cancers. Journal of the National Cancer Institute 92 (21), 1740–1752.
Mirzaei, H., Di Biase, S., Longo, V.D., 2016. Dietary interventions, cardiovascular aging, and disease: Animal models and human studies. Circulation Research 118 (10), 1612–1625.
Mishra, P., Kar, A., Kale, R.K., 2011. Prepubertal daidzein exposure enhances mammary gland differentiation and regulates the expression of estrogen receptor-alpha and apoptotic proteins. ISRN Oncology 2011, 896826.
Mishra, P., Kar, A., Kale, R.K., 2011. Prepubertal daidzein exposure enhances mammary gland differentiation and regulates the expression of estrogen receptor-alpha and apoptotic proteins. ISRN Oncology 2011, 896826.
Mitjavila, M.T., Fandos, M., Salas-Salvado, J., Covas, M.I., Borrego, S., Estruch, R., Lamuela-Raventos, R., Corella, D., Martinez-Gonzalez, M.A., Sanchez, J.M., Bullo, M., Fito, M., Tormos, C., Cerda, C., Casillas, R., Moreno, J.J., Iradi, A., Zaragoza, C., Chaves, J., Saez, G.T., 2013. The Mediterranean diet improves the systemic lipid and DNA oxidative damage in metabolic syndrome individuals. A randomized, controlled, trial. Clinical Nutrition 32 (2), 172–178.
Monnier, L., Mas, E., Ginet, C., Michel, F., Villon, L., Cristol, J.P., Colette, C., 2006. Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. JAMA 295 (14), 1681–1687. https://doi.org/10.1001/jama.295.14.1681.
Moon, Y.J., Wang, X., Morris, M.E., 2006. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. Toxicology In Vitro 20, 187–210.
Mozaffarian, D., Katan, M.B., Ascherio, A., Stampfer, M.J., Willett, W.C., 2006. Trans fatty acids and cardiovascular disease. The New England Journal of Medicine 354, 1601–1613.
Murphy, P.A., Barua, K., Hauck, C.C., 2002. Solvent extraction selection in the determination of isoflavones in soy foods. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences 777, 129–138.
Murphy, P.A., Song, T., Buseman, G., Barua, K., Beecher, G.R., Trainer, D., Holden, J., 1999. Isoflavones in retail and institutional soy foods. Journal of Agricultural and Food Chemistry 47, 2697–2704.
Nagata, C., Shimizu, H., Kametani, M., Takeyama, N., Ohnuma, T., Matsushita, S., 2001. Diet and colorectal adenoma in Japanese males and females. Diseases of the Colon and Rectum 44 (1), 105–111.

Navarro, S.L., Li, F., Lampe, J.W., 2011. Mechanisms of action of isothiocyanates in cancer chemoprevention: An update. Food & Function 2, 579–587.
Navarro-Tito, N., Robledo, T., Salazar, E.P., 2008. Arachidonic acid promotes FAK activation and migration in MDA-MB-231 breast cancer cells. Experimental Cell Research 314 (18), 3340–3355. https://doi.org/10.1016/j.yexcr.2008.08.018. Epub 2008 Sep 9.
Nechuta, S.J., Caan, B.J., Chen, W.Y., et al., 2012a. Soy food intake after diagnosis of breast cancer and survival: An in-depth analysis of combined evidence from cohort studies of US and Chinese women. The American Journal of Clinical Nutrition 96, 123–132.
Nechuta, S., Caan, B.J., Chen, W.Y., Kwan, M.L., Lu, W., Cai, H., Poole, E.M., Flatt, S.W., Zheng, W., Pierce, J.P., Shu, X.O., 2013. Postdiagnosis cruciferous vegetable consumption and breast cancer outcomes: A report from the after breast Cancer pooling project. Cancer Epidemiology, Biomarkers & Prevention 22, 1451–1456.
Nechuta, S.J., Caan, B.J., Chen, W.Y., Lu, W., Chen, Z., Kwan, M.L., Flatt, S.W., Zheng, Y., Zheng, W., Pierce, J.P., et al., 2012. Soy food intake after diagnosis of breast cancer and survival: An in-depth analysis of combined evidence from cohort studies of US and Chinese women. The American Journal of Clinical Nutrition 96, 123–132.
Nechuta, S.J., Caan, B.J., Chen, W.Y., Lu, W., Chen, Z., Kwan, M.L., Flatt, S.W., Zheng, Y., Zheng, W., Pierce, J.P., et al., 2012b. Soy food intake after diagnosis of breast cancer and survival: An in-depth analysis of combined evidence from cohort studies of US and Chinese women. The American Journal of Clinical Nutrition 96, 123–132.
Negre-Salvayre, A., Auge, N., Ayala, V., Basaga, H., Boada, J., Brenke, R., Chapple, S., Cohen, G., Feher, J., Grune, T., Lengyel, G., Mann, G.E., Pamplona, R., Poli, G., Portero-Otin, M., Riahi, Y., Salvayre, R., Sasson, S., Serrano, J., Shamni, O., Siems, W., Siow, R.C.M., Wiswedel, I., Zarkovic, K., Zarkovic, N., 2010. Pathological aspects of lipid peroxidation. Free Radical Research 44, 1125–1171.
Nelson, E.R., Wardell, S.E., Jasper, J.S., Park, S., Suchindran, S., Howe, M.K., Carver, N.J., Pillai, R.V., Sullivan, P.M., Sondhi, V., Umetani, M., Geradts, J., McDonnell, D.P., 2013. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. Science 342 (6162), 1094–1098. https://doi.org/10.1126/science.1241908.
Nicodemus, K.K., Jacobs Jr., D.R., Folsom, A.R., 2001. Whole and refined grain intake and risk of incident postmenopausal breast cancer (United States). Cancer Causes & Control 12 (10), 917–925.
Nilsson, S., Gustafsson, J.-A., 2002. Biological role of estrogen and estrogen receptors. Critical Reviews in Biochemistry and Molecular Biology 37, 1–28.
Nothlings, U., Murphy, S.P., Wilkens, L.R., Henderson, B.E., Kolonel, L.N., 2007. Flavonols and pancreatic cancer risk: The multiethnic cohort study. American Journal of Epidemiology 166 (8), 924–931.
Noto, H., Goto, A., Tsujimoto, T., Noda, M., 2012. Cancer risk in diabetic patients treated with metformin: A systematic review and meta-analysis. PLoS One 7 (3), e33411. https://doi.org/10.1371/journal.pone.0033411.
Olivecrona, H., Hilding, A., Ekstrom, C., Barle, H., Nyberg, B., Moller, C., Delhanty, P.J., Baxter, R.C., Angelin, B., Ekstrom, T.J., Tally, M., 1999. Acute and short-term effects of growth hormone on insulin-like growth factors and their binding proteins: Serum levels and hepatic messenger ribonucleic acid responses in humans. The Journal of Clinical Endocrinology and Metabolism 84 (2), 553–560. https://doi.org/10.1210/jcem.84.2.5466.
O'Flanagan, C.H., Smith, L.A., McDonell, S.B., Hursting, S.D., 2017. When less may be more: Calorie restriction and response to cancer therapy. BMC Medicine 15 (1), 106.
Othman, R.A., 2007. Dietary lipids and Cancer. The Libyan Journal of Medicine 2 (4), 180–184. Published online 2007 Dec 1. https://doi.org/10.4176/070831.
Otieno, D.O., Ashton, J.F., Shah, N.P., 2007. Isoflavone phytoestrogen degradation in fermented soymilk with selected beta-glucosidase producing *L. acidophilus* strains during storage at different temperatures. International Journal of Food Microbiology 115, 79–88.
Paixão, E.M.D.S., Oliveira, A.C.M., Pizato, N., Muniz-Junqueira, M.I., Magalhães, K.G., Nakano, E.Y., Ito, M.K., 2017. The effects of EPA and DHA enriched fish oil on nutritional and immunological markers of treatment naïve breast cancer patients: A randomized double-blind controlled trial. Nutrition Journal 16 (1), 71. https://doi.org/10.1186/s12937-017-0295-9.
Palomares, M.R., Hopper, L., Goldstein, L., Lehman, C.D., Storer, B.E., Gralow, J.R., 2004. Effect of soy isoflavones on breast proliferation in postmenopausal breast cancer survivors. Breast Cancer Research and Treatment 88, 4002.
Pan, A., Sun, Q., Bernstein, A.M., Schulze, M.B., Manson, J.E., Stampfer, M.J., Willett, W.C., Hu, F.B., 2012. Red meat consumption and mortality: Results from 2 prospective cohort studies. Archives of Internal Medicine 172 (7), 555–563. https://doi.org/10.1001/archinternmed.2011.2287.
Patel, R.P., Boersma, B.J., Crawford, J.H., Hogg, N., Kirk, M., Kalyanaraman, B., Parks, D.A., Barnes, S., Darley-Usmar, V., 2001. Antioxidant mechanisms of isoflavones in lipid systems: Paradoxical effects of peroxyl radical scavenging. Free Radical Biology & Medicine 31, 1570–1581.
Patisaul, H.B., Jefferson, W., 2010. The pros and cons of phytoestrogens. Frontiers in Neuroendocrinology 31, 400–419.
Patterson, R.E., Sears, D.D., 2017. Metabolic effects of intermittent fasting. Annual Review of Nutrition 37, 371–393.
Peeters, P.H.M., Keinan-Boker, L., van der Schouw, Y.T., Grobbee, D.E., 2002. Phytoestrogens and breast cancer risk: Review of the epidemiological evidence. IARC Scientific Publications 156, 331–336.
Pelucchi, C., Bosetti, C., Negri, E., Lipworth, L., La Vecchia, C., 2011. Olive oil and cancer risk: An update of epidemiological findings through 2010. Current Pharmaceutical Design 17 (8), 805–812.
Peng, C., WP1, L., Zhang, C.X., 2017. Fruit and vegetable intake and breast cancer prognosis: A meta-analysis of prospective cohort studies. The British Journal of Nutrition 117 (5), 737–749.
Phillips, S.M., Fulgoni, V.L., Heaney, R.P., Nicklas, T.A., Slavin, J.L., Weaver, C.M., 2015. Commonly consumed protein foods contribute to nutrient intake, diet quality, and nutrient adequacy. The American Journal of Clinical Nutrition 101, 1346S–1352S.
Pierce, J.P., Natarajan, L., Caan, B.J., et al., 2007. Influence of a diet very high in vegetables, fruit, and fiber and low in fat on prognosis following treatment for breast cancer: The Women's healthy eating and living (WHEL) randomized trial. Journal of the American Medical Association 298 (3), 289–298.
Pimentel, D., Pimentel, M., 2003. Sustainability of meat-based and plant-based diets and the environment. The American Journal of Clinical Nutrition 78, 660S–3S.
Pisani, P., Parkin, D.M., Bray, F., Ferlay, J., 1999. Estimates of the worldwide mortality from 25 cancers in 1990. International Journal of Cancer 83, 18–29.
Platz, E.A., Leitzmann, M.F., Visvanathan, K., Rimm, E.B., Stampfer, M.J., Willett, W.C., et al., 2006. Statin drugs and risk of advanced prostate cancer. Journal of the National Cancer Institute 98, 1819–1825.
Platz, E.A., Till, C., Goodman, P.J., Parnes, H.L., Figg, W.D., Albanes, D., et al., 2009. Men with low serum cholesterol have a lower risk of high-grade prostate cancer in the placebo arm of the prostate cancer prevention trial. Cancer Epidemiology, Biomarkers & Prevention 18, 2807–2813.
Prentice, R.L., Caan, B., Chlebowski, R.T., et al., 2006. Low-fat dietary pattern and risk of invasive breast cancer: The Women's Health Initiative randomized controlled dietary modification trial. Journal of the American Medical Association 295 (6), 629–642.
Quagliaro, L., Piconi, L., Assaloni, R., Da Ros, R., Maier, A., Zuodar, G., Ceriello, A., 2005. Intermittent high glucose enhances ICAM-1, VCAM-1 and E-selectin expression in human umbilical vein endothelial cells in culture: The distinct role of protein kinase C and mitochondrial superoxide production. Atherosclerosis 183 (2), 259–267. https://doi.org/10.1016/j.atherosclerosis.2005.03.015.
Raffaghello, L., et al., 2008. Starvation-dependent differentialstress resistance protects normal but not cancer cells against high-dose chemotherapy. Proceedings of the National Academy of Sciences of the United States of America 105, 8215–8220.
Raffaghello, L., et al., 2010b. Fasting and differential chemotherapy protection in patients. Cell Cycle 9, 4474–4476.
Rahal, O.M., Simmen, R.C., 2011. Paracrine-acting adiponectin promotes mammary epithelial differentiation and synergizes with genistein to enhance transcriptional response to estrogen receptor beta signaling. Endocrinology 152, 3409–3421.
Rahal, O.M., Simmen, R.C., 2011. Paracrine-acting adiponectin promotes mammary epithelial differentiation and synergizes with genistein to enhance transcriptional response to estrogen receptor beta signaling. Endocrinology 152, 3409–3421.
Rastogi, T., Devesa, S., Mangtani, P., Mathew, A., Cooper, N., Kao, R., Sinha, R., 2008. Cancer incidence rates among south Asians in four geographic regions: India, Singapore, UK and US. International Journal of Epidemiology 37 (1), 147–160. https://doi.org/10.1093/ije/dym219.
Reijnders, L., Soret, S., 2003. Quantification of the environmental impact of different dietary protein choices. The American Journal of Clinical Nutrition 78, 664S–8S.

Renehan, A.G., et al., 2010. Incident cancer burden attributable to excess body mass index in 30 European countries. International Journal of Cancer 126, 692–702.
Renehan, A.G., Tyson, M., Egger, M., Heller, R.F., Zwahlen, M., 2008. Body-massindex and incidence of cancer: A systematic review and meta-analysis ofprospective observational studies. Lancet 371, 569–578.
Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: How are they linked? Free Radical Biology & Medicine 49 (11), 1603–1616. https://doi.org/10.1016/j.freeradbiomed.2010.09.006.
Riggs, B.L., Hartmann, L.C., 2003. Selective estrogen-receptor modulators—Mechanisms of action and application to clinical practice. The New England Journal of Medicine 348, 618–629.
Rinaldi, S., Cleveland, R., Norat, T., Biessy, C., Rohrmann, S., Linseisen, J., Boeing, H., Pischon, T., Panico, S., Agnoli, C., Palli, D., Tumino, R., Vineis, P., Peeters, P.H., van Gils, C.H., Bueno-de-Mesquita, B.H., Vrieling, A., Allen, N.E., Roddam, A., Bingham, S., Khaw, K.T., Manjer, J., Borgquist, S., Dumeaux, V., Torhild Gram, I., Lund, E., Trichopoulou, A., Makrygiannis, G., Benetou, V., Molina, E., Donate Suarez, I., Barricarte Gurrea, A., Gonzalez, C.A., Tormo, M.J., Altzibar, J.M., Olsen, A., Tjonneland, A., Gronbaek, H., Overvad, K., Clavel-Chapelon, F., Boutron-Ruault, M.C., Morois, S., Slimani, N., Boffetta, P., Jenab, M., Riboli, E., Kaaks, R., 2010. Serum levels of IGF-I, IGFBP-3 and colorectal cancer risk: Results from the EPIC cohort, plus a meta-analysis of prospective studies. International Journal of Cancer 126 (7), 1702–1715. https://doi.org/10.1002/ijc.24927.
Rizza, W., Veronese, N., Fontana, L., 2014 Jan. What are the roles of calorie restriction and diet quality in promoting healthy longevity? Ageing Research Reviews 13, 38–45.
Rizzo, G., Baroni, L., 2018. Soy, soy foods and their role in vegetarian diets. Nutrients 5, 10. Review.
Rizzo, G., Baroni, L., 2018. Soy, soy foods and their role in vegetarian diets. Nutrients 5, 10. Review.
Rock, C.L., Doyle, C., Demark-Wahnefried, W., Meyerhardt, J., Courneya, K.S., Schwartz, A.L., Bandera, E.V., Hamilton, K.K., Grant, B., McCullough, M., Byers, T., Gansler, T., 2012. Nutrition and physical activity guidelines for cancer survivors. CA: A Cancer Journal for Clinicians 62 (4), 243–274. https://doi.org/10.3322/caac.21142.
Romieu, I., Ferrari, P., Rinaldi, S., Slimani, N., Jenab, M., Olsen, A., Tjonneland, A., Overvad, K., Boutron-Ruault, M.C., Lajous, M., Kaaks, R., Teucher, B., Boeing, H., Trichopoulou, A., Naska, A., Vasilopoulo, E., Sacerdote, C., Tumino, R., Masala, G., Sieri, S., Panico, S., Bueno-de-Mesquita, H.B., Van-der, A.D., van Gils, C.H., Peeters, P.H., Lund, E., Skeie, G., Asli, L.A., Rodriguez, L., Navarro, C., Amiano, P., Sanchez, M.J., Barricarte, A., Buckland, G., Sonestedt, E., Wirfalt, E., Hallmans, G., Johansson, I., Key, T.J., Allen, N.E., Khaw, K.T., Wareham, N.J., Norat, T., Riboli, E., Clavel-Chapelon, F., 2012. Dietary glycemic index and glycemic load and breast cancer risk in the European prospective investigation into Cancer and nutrition (EPIC). The American Journal of Clinical Nutrition 96 (2), 345–355. https://doi.org/10.3945/ajcn.111.026724.
Ronco, A.L., De Stéfani, E., Stoll, M., 2010. Hormonal and metabolic modulation through nutrition: Towards a primary prevention of breast cancer. Breast 19 (5), 322–332. https://doi.org/10.1016/j.breast.2010.05.005. Epub 2010 Jun 9.
Rosa, E.A.S., Heaney, R.K., Fenwick, G.R., Portas, C.A.M., 1997. Glucosinolates in crop plants. Horticultural Reviews 19, 99–215.
Rosato, V., Guercio, V., Bosetti, C., Negri, E., Serraino, D., Giacosa, A., Montella, M., La Vecchia, C., Tavani, A., 2016. Mediterranean diet and colorectal cancer risk: A pooled analysis of three Italian case-control studies. British Journal of Cancer 115 (7), 862–865. https://doi.org/10.1038/bjc.2016.245. Epub 2016 Aug 18.
Rose, D.P., 1990. Dietary fiber and breast cancer. Nutrition and Cancer 13 (1–2), 1–8.
Rossi, M., Lugo, A., Lagiou, P., Zucchetto, A., Polesel, J., Serraino, D., Negri, E., Trichopoulos, D., La Vecchia, C., 2012. Proanthocyanidins and other flavonoids in relation to pancreatic cancer: A case-control study in Italy. Annals of Oncology 23 (6), 1488–1493.
Rothschild, J., Hoddy, K.K., Jambazian, P., Varady, K.A., 2014. Time-restricted feeding and risk of metabolic disease: A review of human and animal studies. Nutrition Reviews 72, 308–318.
Rous, P., 1914. The influence of diet on transplanted and spontaneous mouse tumors. The Journal of Experimental Medicine 20 (5), 433–451.
Rouzaud, G., Young, S.A., Duncan, A.J., 2004. Hydrolysis of glucosinolates to isothiocyanates after ingestion of raw or microwaved cabbage by human volunteers. Cancer Epidemiology, Biomarkers & Prevention 13, 125–131.
Rowland, I., Faughnan, M., Hoey, L., Wahala, K., Williamson, G., Cassidy, A., 2003. Bioavailability of phyto-oestrogens. The British Journal of Nutrition 89, S45–S58.
Rowlands, M.A., Gunnell, D., Harris, R., Vatten, L.J., Holly, J.M., Martin, R.M., 2009. Circulating insulin-like growth factor peptides and prostate cancer risk: A systematic review and meta-analysis. International Journal of Cancer 124 (10), 2416–2429. https://doi.org/10.1002/ijc.24202.
Roy, P., Nigam, N., Singh, M., et al., 2010. Tea polyphenols inhibit cyclooxygenase-2 expression and block activation of nuclear factor-kappa B and Akt in diethylnitrosoamine induced lung tumors in Swiss mice. Investigational New Drugs 28 (4), 466–471.
Royston, K.J., Tollefsbol, T.O., 2015. The epigenetic impact of cruciferous vegetables on Cancer prevention. Current Pharmacology Reports 1, 46–51.
Runchey, S.S., Pollak, M.N., Valsta, L.M., Coronado, G.D., Schwarz, Y., Breymeyer, K.L., Wang, C., Wang, C.Y., Lampe, J.W., Neuhouser, M.L., 2012. Glycemic load effect on fasting and post-prandial serum glucose, insulin, IGF-1 and IGFBP-3 in a randomized, controlled feeding study. European Journal of Clinical Nutrition 66 (10), 1146–1152. https://doi.org/10.1038/ejcn.2012.107.
Runowicz, C.D., Leach, C.R., Henry, N.L., Henry, K.S., Mackey, H.T., Cowens-Alvarado, R.L., Cannady, R.S., Pratt-Chapman, M.L., Edge, S.B., Jacobs, L.A., Hurria, A., Marks, L.B., LaMonte, S.J., Warner, E., Lyman, G.H., Ganz, P.A., 2016. American Cancer Society/American Society of Clinical Oncology breast Cancer survivorship care guideline. CA: a Cancer Journal for Clinicians 66 (1), 43–73. https://doi.org/10.3322/caac.21319.
Russo, J., Mailo, D., Hu, Y.F., Balogh, G., Sheriff, F., Russo, I.H., 2005. Breast differentiation and its implication in cancer prevention. Clinical Cancer Research 11, 931s–936s.
Sadava, D., Whitlock, E., Kane, S.E., 2007. The green tea polyphenol, epigallocatechin 3- gallate inhibits telomerase and induces apoptosis in drug-resistant lung cancer cells. Biochemical and Biophysical Research Communications 360 (1), 233–237.
Safdie, F.M., et al., 2009. Fasting and cancer treatment in humans: A case series report. Aging (Albany NY) 1, 988–1007.
Samraj, A.N., Pearce, O.M., Laubli, H., Crittenden, A.N., Bergfeld, A.K., Banda, K., Gregg, C.J., Bingman, A.E., Secrest, P., Diaz, S.L., et al., 2015. A red meat-derived glycan promotes inflammation and cancer progression. Proceedings of the National Academy of Sciences of the United States of America 112, 542–547.
Santarelli, R.L., Pierre, F., Corpet, D.E., 2008. Processed meat and colorectal cancer: A review of epidemiologic and experimental evidence. Nutrition and Cancer 60 (2), 131–144. https://doi.org/10.1080/01635580701684872.
Sartippour, M.R., Rao, J.Y., Apple, S., Wu, D., Henning, S., Wang, H., Elashoff, R., Rubio, R., Heber, D., Brooks, M.N., 2004. A pilot clinical study of short-term isoflavone supplements in breast cancer patients. Nutrition and Cancer 49, 59–65.
Sawada, N., Inoue, M., Iwasaki, M., Sasazuki, S., Shimazu, T., Yamaji, T., Takachi, R., Tanaka, Y., Mizokami, M., Tsugane, S., Japan Public Health Center-Based Prospective Study Group, 2012. Consumption of n-3 fatty acids and fish reduces risk of hepatocellular carcinoma. Gastroenterology 142 (7), 1468–1475. https://doi.org/10.1053/j.gastro.2012.02.018. Epub 2012 Feb 16.
Schley, P.D., Jijon, H.B., Robinson, L.E., Field, C.J., 2005. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. Breast Cancer Research and Treatment 92 (2), 187–195.
Schuurman, A.G., Goldbohm, R.A., Dorant, E., van den Brandt, P.A., 1998. Vegetable and fruit consumption and prostate cancer risk: A cohort study in the Netherlands. Cancer epidemiology. Biomarkers & Prevention 7 (8), 673–680.
Schüz, J., Espina, C., Villain, P., Herrero, R., Leon, M.E., Minozzi, S., Romieu, I., Segnan, N., Wardle, J., Wiseman, M., Belardelli, F., Bettcher, D., Cavalli, F., Galea, G., Lenoir, G., Martin-Moreno, J.M., Nicula, F.A., Olsen, J.H., Patnick, J., Primic-Zakelj, M., Puska, P., van Leeuwen, F.E., Wiestler, O., Zatonski, W., 2015. Working groups of scientific experts. European code against cancer 4th edition: 12 ways to reduce your cancer risk. Cancer Epidemiology 39 (Suppl 1), S1–10.
Schwingshackl, L., Schwedhelm, C., Galbete, C., Hoffmann, G., 2017. Adherence to Mediterranean diet and risk of Cancer: An updated systematic review and meta-analysis. Nutrients 9 (10).
Serra-Majem, L., Roman, B., Estruch, R., 2006. Scientific evidence of interventions using the Mediterranean diet: A systematic review. Nutrition Reviews 64 (2 Pt 2), S27–S47.

Setchell, K.D., 1998. Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. The American Journal of Clinical Nutrition 68, 1333S–1346S.
Setchell, K.D., Brown, N.M., Lydeking-Olsen, E., 2002. The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. The Journal of Nutrition 132, 3577–3584.
Setchell, K.D., Clerici, C., 2010. Equol: Pharmacokinetics and biological actions. The Journal of Nutrition 140, 1363S–1368S.
Shanafelt, T.D., Call, T.G., Zent, C.S., et al., 2009. Phase I trial of daily, oral polyphenol E in patients with asymptomatic rai stage 0 to II chronic lymphocytic leukemia. Journal of Clinical Oncology 27, 3808–3814.
Shanafelt, T.D., Call, T.G., Zent, C.S., et al., 2013. Phase I trial of daily, oral polyphenol E in patients with asymptomatic rai stage 0 to II chronic lymphocytic leukemia. Cancer 119, 363–370.
Shapiro, T.A., Fahey, J.W., Wade, K.L., Stephenson, K.K., Talalay, P., 1998. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. Cancer Epidemiology, Biomarkers & Prevention 7, 1091–1100.
Shevah, O., Laron, Z., 2007. Patients with congenital deficiency of IGF-I seem protected from the development of malignancies: A preliminary report. Growth Hormone & IGF Research 17, 54–57.
Shu, X.O., Jin, F., Dai, Q., Wen, W., Potter, J.D., Kushi, L.H., Ruan, Z., Gao, Y.T., Zheng, W., 2001. Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. Cancer Epidemiology, Biomarkers & Prevention 10, 483–488.
Shu, X.O., Zheng, Y., Cai, H., Gu, K., Chen, Z., Zheng, W., Lu, W., 2009. Soy food intake and breast cancer survival. JAMA 302, 2437–2443.
Shu, X.O., Zheng, Y., Cai, H., Gu, K., Chen, Z., Zheng, W., Lu, W., 2009. Soy food intake and breast cancer survival. JAMA 302, 2437–2443.
Sieri, S., et al., 2014. Dietary fat intake and development of specific breast cancer subtypes. Journal of the National Cancer Institute 106 (5) pii: dju068. https://doi.org/10.1093/jnci/dju068.
Sieri, S., Pala, V., Brighenti, F., Pellegrini, N., Muti, P., Micheli, A., Evangelista, A., Grioni, S., Contiero, P., Berrino, F., Krogh, V., 2007. Dietary glycemic index, glycemic load, and the risk of breast cancer in an Italian prospective cohort study. The American Journal of Clinical Nutrition 86 (4), 1160–1166.
Silvera, S.A., Rohan, T.E., Jain, M., Terry, P.D., Howe, G.R., Miller, A.B., 2005. Glycaemic index, glycaemic load and risk of endometrial cancer: A prospective cohort study. Public Health Nutrition 8 (7), 912–919.
Slavin, J., 2003. Why whole grains are protective: Biological mechanisms. The Proceedings of the Nutrition Society 62 (1), 129–134. https://doi.org/10.1079/PNS2002221.
Slimani, N., Fahey, M., Welch, A.A., Wirfalt, E., Stripp, C., Bergstrom, E., Linseisen, J., Schulze, M.B., Bamia, C., Chloptsios, Y., Veglia, F., Panico, S., Bueno-de-Mesquita, H.B., Ocke, M.C., Brustad, M., Lund, E., Gonzalez, C.A., Barcos, A., Berglund, G., Winkvist, A., Mulligan, A., Appleby, P., Overvad, K., Tjonneland, A., Clavel-Chapelon, F., Kesse, E., Ferrari, P., Van Staveren, W.A., Riboli, E., 2002. Diversity of dietary patterns observed in the European prospective investigation into Cancer and nutrition (EPIC) project. Public Health Nutrition 5 (6B), 1311–1328. https://doi.org/10.1079/PHN2002407.
Smith, C.L., O'Malley, B.W., 2004. Coregulator function: A key to understanding tissue specificity of selective receptor modulators. Endocrine Reviews 25, 45–71.
Smith-Warner, S.A., Spiegelman, D., Yaun, S.S., et al., 2001. Intake of fruits and vegetables and risk of breast cancer: A pooled analysis of cohort studies. JAMA 285 (6), 769–776.
Soda, K., 2011. The mechanisms by which polyamines accelerate tumor spread. Journal of Experimental & Clinical Cancer Research 30, 95.
Sofi, F., Cesari, F., Abbate, R., Gensini, G.F., Casini, A., 2008. Adherence to Mediterranean diet and health status: Meta-analysis. BMJ 337, a1344.
Sofi, F., Macchi, C., Abbate, R., Gensini, G.F., Casini, A., 2013. Mediterranean diet and health. BioFactors 39 (4), 335–342.
Sofi, F., Macchi, C., Abbate, R., Gensini, G.F., Casini, A., 2014. Mediterranean diet and health status: An updated meta-analysis and a proposal for a literature-based adherence score. Public Health Nutrition 17 (12), 2769–2782.
Song, M., Fung, T.T., Hu, F.B., Willett, W.C., Longo, V., Chan, A.T., Giovannucci, E.L., 2016. Animal and plant protein intake and all-cause and cause-specific mortality: Results from two prospective US cohort studies. JAMA Internal Medicine 176 (10), 1453–1463.
Stattin, P., Bjor, O., Ferrari, P., Lukanova, A., Lenner, P., Lindahl, B., Hallmans, G., Kaaks, R., 2007. Prospective study of hyperglycemia and cancer risk. Diabetes Care 30 (3), 561–567. https://doi.org/10.2337/dc06-0922.
Steck, S.E., Gaudet, M.M., Eng, S.M., Britton, J.A., Teitelbaum, S.L., Neugut, A.I., Santella, R.M., Gammon, M.D., 2007. Cooked meat and risk of breast cancer—Lifetime versus recent dietary intake. Epidemiology 18, 373–382.
Stender, S., Dyerberg, J., 2004. Influence of trans fatty acids on health. Annals of Nutrition & Metabolism 48, 61–66.
Stocks, T., Rapp, K., Bjorge, T., Manjer, J., Ulmer, H., Selmer, R., Lukanova, A., Johansen, D., Concin, H., Tretli, S., Hallmans, G., Jonsson, H., Stattin, P., 2009. Blood glucose and risk of incident and fatal cancer in the metabolic syndrome and cancer project (me-can): Analysis of six prospective cohorts. PLoS Medicine 6 (12), e1000201. https://doi.org/10.1371/journal.pmed.1000201.
Sturgeon, J.L., Williams, M., van Servellen, G., 2009. Efficacy of green tea in the prevention of cancers. Nursing and Health Sciences 11, 436–446.
Sung, M.K., Yeon, J.Y., Park, S.Y., Park, J.H.Y., Choi, M.S., 2011. Obesity-induced metabolic stresses in breast and colon cancer. Annals of the New York Academy of Sciences 1229 (1), 61–68.
Taylor, C.K., Levy, R.M., Elliott, J.C., et al., 2009. The effect of genistein aglycone on cancer and cancer risk: A review of in vitro, preclinical, and clinical studies. Nutrition Reviews 67, 398–415.
Tham, D.M., Gardner, C.D., Haskell, W.L., 1998. Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. The Journal of Clinical Endocrinology and Metabolism 83, 2223–2235.
Tharrey, M., Mariotti, F., Mashchak, A., Barbillon, P., Delattre, M., Fraser, G.E., 2018. Patterns of plant and animal protein intake are strongly associated with cardiovascular mortality: The Adventist health Study-2 cohort. International Journal of Epidemiology 1–10.
Thissen, J.P., Ketelslegers, J.M., Underwood, L.E., 1994. Nutritional regulation of the insulin-like growth factors. Endocrine Reviews 15, 80–101.
Thompson Jr., I.M., Cabang, A.B., Wargovich, M.J., 2014. Future directions in the prevention of prostate cancer. Nature Reviews. Clinical Oncology 11 (1), 49–60.
Thompson, A.K., Shaw, D.I., Minihane, A.M., Williams, C.M., 2008. Trans-fatty acids and cancer: The evidence reviewed. Nutrition Research Reviews 21, 174–188.
Tinsley, G.M., La Bounty, P.M., 2015. Effects of intermittent fasting on body composition and clinical health markers in humans. Nutrition Reviews 73, 661–674.
Title, L.M., Cummings, P.M., Giddens, K., Nassar, B.A., 2000. Oral glucose loading acutely attenuates endothelium-dependent vasodilation in healthy adults without diabetes: An effect prevented by vitamins C and E. Journal of the American College of Cardiology 36 (7), 2185–2191.
Toledo, E., Salas-Salvado, J., Donat-Vargas, et al., 2015. Mediterranean diet and invasive breast cancer risk among women at high cardiovascular risk in the PREDIMED trial: A randomized clinical trial. JAMA Internal Medicine 175, 1752–1760.
Toledo, E., Salas-Salvado, J., Donat-Vargas, C., Buil-Cosiales, P., Estruch, R., Ros, E., Corella, D., Fito, M., Hu, F.B., Aros, F., Gomez-Gracia, E., Romaguera, D., Ortega-Calvo, M., Serra-Majem, L., Pinto, X., Schroder, H., Basora, J., Sorli, J.V., Bullo, M., Serra-Mir, M., Martinez-Gonzalez, M.A., 2015. Mediterranean diet and invasive breast Cancer risk among women at high cardiovascular risk in the PREDIMED trial: A randomized clinical trial. JAMA Internal Medicine 175 (11), 1752–1760.
Trichopoulou, A., Lagiou, P., Kuper, H., Trichopoulos, D., 2000. Cancer and Mediterranean dietary traditions. Cancer Epidemiology, Biomarkers & Prevention 9 (9), 869–873.
Trock, B.J., Hilakivi-Clarke, L., Clarke, R., 2006. Meta-analysis of soy intake and breast cancer risk. Journal of the National Cancer Institute 98, 459–471.
Tse, G., Eslick, G.D., 2014. Cruciferous vegetables and risk of colorectal neoplasms: A systematic review and meta-analysis. Nutrition and Cancer 66, 128–139.
Tseng, Y.H., Tsan, Y.T., Chan, W.C., Sheu, W.H., Chen, P.C., 2015. Use of an alpha-glucosidase inhibitor and the risk of colorectal Cancer in patients with diabetes: A Nationwide, population-based cohort study. Diabetes Care. https://doi.org/10.2337/dc15-0563.

Turati, F., Galeone, C., Gandini, S., Augustin, L.S., Jenkins, D.J., Pelucchi, C., La Vecchia, C., 2015a. High glycemic index and glycemic load are associated with moderately increased cancer risk. Molecular Nutrition & Food Research 59 (7), 1384–1394. https://doi.org/10.1002/mnfr.201400594.

Turati, F., Rossi, M., Pelucchi, C., Levi, F., La Vecchia, C., 2015. Fruit and vegetables and cancer risk: A review of southern European studies. The British Journal of Nutrition 113, S102–S110.

Turati, F., Rossi, M., Pelucchi, C., Levi, F., La Vecchia, C., 2015b. Fruit and vegetables and cancer risk: A review of southern European studies. The British Journal of Nutrition 113 (Suppl 2), S102–S110. https://doi.org/10.1017/S0007114515000148.

U.S. Department of Agriculture and Agriculture Research Service, 2018. USDA food composition databases. https://ndb.nal.usda.gov/ndb/nutrients/report/nutrientsfrm?max=25&offset=0&totCount=0&nutrient1=203&fg=20&fg=9&fg=16&fg=12&fg=11&subset=0&sort=c&measureby=g.

U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2010. Dietary guidelines for Americans, 7th edn. U.S. Government Printing Office., Washington, DC. December 2010.

U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015. 2015–2020 Dietary Guidelines for Americans, 8th edn. December. Available at: http://health.gov/dietaryguidelines/2015/guidelines/.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology 39 (1), 44–84. https://doi.org/10.1016/j.biocel.2006.07.001.

Van der Schouw, Y.T., Kreijkamp-Kaspers, S., Peeters, P.H., Keinan-Boker, L., Rimm, E.B., Grobbee, D.E., 2005. Prospective study on usual dietary phytoestrogen intake and cardiovascular disease risk in western women. Circulation 111, 465–471.

Van Erp-Baart, M.A., Brants, H.A., Kiely, M., Mulligan, A., Turrini, A., Sermoneta, C., Kilkkinen, A., Valsta, L.M., 2003. Isoflavone intake in four different European countries: The VENUS approach. The British Journal of Nutrition 89, S25–S30.

Vance, T.M., Su, J., Fontham, E.T., Koo, S.I., Chun, O.K., 2013. Dietary antioxidants and prostate cancer: A review. Nutrition and Cancer 65 (6), 793–801.

Varady, K.A., 2016. Impact of intermittent fasting on glucose homeostasis. Current Opinion in Clinical Nutrition and Metabolic Care 19 (4), 300–302.

Varady, K.A., Hellerstein, M.K., 2007 Jul. Alternate-day fasting and chronic disease prevention: A review of human and animal trials. The American Journal of Clinical Nutrition 86 (1), 7–13.

Verhoeven, D.T.H., Goldbohm, R.A., vanPoppel, G., Verhagen, H., vanden-Brandt, P.A., 1996. Epidemiological studies on brassica vegetables and cancer risk. Cancer Epidemiology, Biomarkers 5, 733–748.

Verhoeven, D.T., Verhagen, H., Goldbohm, R.A., van den Brandt, P.A., van Poppel, G., 1997. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. Chemico-Biological Interactions 103, 79–129.

Vernieri, C., Nichetti, R.A., Pusceddu, S., Platania, M., Berrino, F., de Braud, F., 2018. Diet and supplements in cancer prevention and treatment: Clinical evidences and future perspectives. Critical Reviews in Oncology/Hematology 123, 57–73. https://doi.org/10.1016/j.critrevonc.2018.01.002. Epub 2018 Feb 2.

Villarini, A., Pasanisi, P., Traina, A., Mano, M.P., Bonanni, B., Panico, S., Scipioni, C., Galasso, R., Paduos, A., Simeoni, M., Bellotti, E., Barbero, M., Macellari, G., Venturelli, E., Raimondi, M., Bruno, E., Gargano, G., Fornaciari, G., Morelli, D., Seregni, E., Krogh, V., Berrino, F., 2012. Lifestyle and breast cancer recurrences: The DIANA-5 trial. Tumori 98 (1), 1–18.

Visioli, F., Grande, S., Bogani, P., Galli, C., 2004. The role of antioxidants in the mediterranean diets: Focus on cancer. European Journal of Cancer Prevention 13 (4), 337–343.

Vitale, S.G., Laganà, A.S., Nigro, A., La Rosa, V.L., Rossetti, P., Rapisarda, A.M.C., La Vignera, S., Condorelli, R.A., Corrado, F., Buscema, M., et al., 2016. Peroxisome proliferator-activated receptor modulation during metabolic diseases and cancers: Master and minions. PPAR Research 2016, 6517313.

Wang, T., Rohan, T.E., Gunter, M.J., Xue, X., Wactawski-Wende, J., Rajpathak, S.N., Cushman, M., Strickler, H.D., Kaplan, R.C., Wassertheil-Smoller, S., Scherer, P.E., Ho, G.Y., 2011. A prospective study of inflammation markers and endometrial cancer risk in postmenopausal hormone nonusers. Cancer Epidemiology, Biomarkers & Prevention 20 (5), 971–977.

WCRF/AICR, 2007. Food, Nutrition, physical activity and the prevention of Cancer: A global perspective. Washington.

WCRF/AICR, 2007. Food, nutrition, physical activity and the prevention of Cancer: A global perspective.

Willett, W.C., 1995. Diet, nutrition, and avoidable cancer. Environmental Health Perspectives 103 (Suppl 8), 165–170.

Willett, W.C., Sacks, F., Trichopoulou, A., Drescher, G., Ferro-Luzzi, A., Helsing, E., Trichopoulos, D., 1995. Mediterranean diet pyramid: A cultural model for healthy eating. The American Journal of Clinical Nutrition 61 (6 Suppl), 1402S–1406S.

Williams, C.D., Whitley, B.M., Hoyo, C., Grant, D.J., Iraggi, J.D., Newman, K.A., Gerber, L., Taylor, L.A., McKeever, M.G., Freedland, S.J., 2011. A high ratio of dietary n-6/n-3 polyunsaturated fatty acids is associated with increased risk of prostate cancer. Nutrition Research 31 (1), 1–8. https://doi.org/10.1016/j.nutres.2011.01.002.

Wirfalt, E., McTaggart, A., Pala, V., Gullberg, B., Frasca, G., Panico, S., Bueno-de-Mesquita, H.B., Peeters, P.H., Engeset, D., Skeie, G., Chirlaque, M.D., Amiano, P., Lundin, E., Mulligan, A., Spencer, E.A., Overvad, K., Tjonneland, A., Clavel-Chapelon, F., Linseisen, J., Nothlings, U., Polychronopoulos, E., Georga, K., Charrondiere, U.R., Slimani, N., 2002. Food sources of carbohydrates in a European cohort of adults. Public Health Nutrition 5 (6B), 1197–1215. https://doi.org/10.1079/PHN2002399.

Wu, A.H., Spicer, D., Garcia, A., Tseng, C.C., Hovanessian-Larsen, L., Sheth, P., Martin, S.E., Hawes, D., Russell, C., MacDonald, H., et al., 2015. Double-blind randomized 12-month soy intervention had no effects on breast MRI fibroglandular tissue density or mammographic density. Cancer Prevention Research 8, 942–951.

Wu, A.H., Spicer, D., Garcia, A., Tseng, C.C., Hovanessian-Larsen, L., Sheth, P., Martin, S.E., Hawes, D., Russell, C., MacDonald, H., et al., 2015. Double-blind randomized 12-month soy intervention had no effects on breast MRI Fibroglandular tissue density or mammographic density. Cancer Prevention Research 8, 942–951.

Wu, A.H., Wan, P., Hankin, J., Tseng, C.C., Yu, M.C., Pike, M.C., 2002. Adolescent and adult soy intake and risk of breast cancer in Asian-Americans. Carcinogenesis 23, 1491–1496.

Wu, Q.J., Yang, Y., Vogtmann, E., Wang, J., Han, L.H., Li, H.L., Xiang, Y.B., 2013. Cruciferous vegetables intake and the risk of colorectal cancer: A meta-analysis of observational studies. Annals of Oncology 24, 1079–1087.

Wu, A.H., Yu, M.C., Tseng, C.C., Stanczyk, F.Z., Pike, M.C., 2009. Dietary patterns and breast cancer risk in Asian American women. The American Journal of Clinical Nutrition 89 (4), 1145–1154.

Xia, H., Ma, S., Wang, S., Sun, G., 2015. Meta-analysis of saturated fatty acid intake and breast Cancer risk. Medicine (Baltimore) 94 (52), e2391. https://doi.org/10.1097/MD.0000000000002391.

Xin, Y., Li, X.Y., Sun, S.R., Wang, L.X., Huang, T., 2015. Vegetable oil intake and breast Cancer risk: A meta-analysis. Asian Pacific Journal of Cancer Prevention 16 (12), 5125–5135.

Xu, L., Ding, Y., Catalona, W.J., Yang, X.J., Anderson, W.F., Jovanovic, B., Wellman, K., Killmer, J., Huang, X., Scheidt, K.A., et al., 2009. MEK4 function, genistein treatment, and invasion of human prostate cancer cells. Journal of the National Cancer Institute 101, 1141–1155.

Xue, Z., Yu, W., Liu, Z., Wu, M., Kou, X., Wang, J., 2009. Preparation and antioxidative properties of a rapeseed (Brassica napus) protein hydrolysate and three peptide fractions. Journal of Agricultural and Food Chemistry 57 (12), 5287–5293.

Yamabe, S., Kobayashi-Hattori, K., Kaneko, K., Endo, H., Takita, T., 2007. Effect of soybean varieties on the content and composition of isoflavone in rice-koji miso. Food Chemistry 100, 369–374.

Yamane, T., Takahashi, T., Kuwata, K., et al., 1995. Inhibition of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced carcinogenesis by (-)-epigallocatechin gallate in the rat glandular stomach. Cancer Research 55, 2081–2084.

Yang, B., Ren, X.L., Fu, Y.Q., Gao, J.L., Li, D., 2014. Ratio of n-3/n-6 PUFAs and risk of breast cancer: A meta-analysis of 274135 adult females from 11 independent prospective studies. BMC Cancer 14, 105. https://doi.org/10.1186/1471-2407-14-105.

Yang, C.S., Wang, X., Lu, G., Picinich, S.C., 2009. Cancer prevention by tea: Animal studies, molecular mechanisms and human relevance. Nature Reviews. Cancer 9, 429–439.
Yao X, Tian Z. Saturated, Monounsaturated and polyunsaturated fatty acids intake and risk of pancreatic Cancer: Evidence from observational studies. PLoS One 2015; 10(6):e0130870. doi: https://doi.org/10.1371/journal.pone.0130870. eCollection 2015.
Yiannakopoulou, E.C., 2014. Effect of green tea catechins on breast carcinogenesis: A systematic review of in-vitro and in-vivo experimental studies. European Journal of Cancer Prevention 23, 84–89.
Yoan, J.M., Koh, W.P., Sun, C.L., Lee, H.P., Yu, M.C., 2007. Green tea intake, ACE gene polymorphism and breast cancer risk among Chinese women in Singapore. Carcinogenesis 28, 1074–1078.
Yu, Y., Jing, X., Li, H., Zhao, X., Wang, D., 2016. Soy isoflavone consumption and colorectal cancer risk: A systematic review and meta-analysis. Scientific Reports 6, 25939.
Yu, Y., Jing, X., Li, H., Zhao, X., Wang, D., 2016. Soy isoflavone consumption and colorectal cancer risk: A systematic review and meta-analysis. Scientific Reports 6, 25939.
Yuan, J.M., 2013. Cancer prevention by green tea: Evidence from epidemiologic studies. The American Journal of Clinical Nutrition 98, 1676S–1681S.
Zadra, G., Photopoulos, C., Loda, M., 2013 Oct. The fat side of prostate cancer. Biochimica et Biophysica Acta 1831 (10), 1518–1532. https://doi.org/10.1016/j.bbalip.2013.03.010. Epub 2013 Apr 2.
Zaheer, K., Humayoun Akhtar, M., 2017. An updated review of dietary isoflavones: Nutrition, processing, bioavailability and impacts on human health. Critical Reviews in Food Science and Nutrition 57, 1280–1293.
Zamora-Ros, R., Ferrari, P., González, C.A., Tjønneland, A., Olsen, A., Bredsdorff, L., Overvad, K., Touillaud, M., Perquier, F., Fagherazzi, G., et al., 2013. Dietary flavonoid and lignan intake and breast cancer risk according to menopause and hormone receptor status in the European prospective investigation into Cancer and nutrition (EPIC) study. Breast Cancer Research and Treatment 139, 163–176.
Zhang, Q., Feng, H., Qluwakemi, B., Wang, J., Yao, S., Cheng, G., Xu, H., Qiu, H., Zhu, L., Yuan, M., 2016. Phytoestrogens and risk of prostate cancer: An updated meta-analysis of epidemiologic studies. International Journal of Food Sciences and Nutrition 1–15.
Zhang, S., Hunter, D.J., Forman, M.R., et al., 1999. Dietary carotenoids and vitamins A, C, and E and risk of breast cancer. Journal of the National Cancer Institute 91 (6), 547–556.
Zhang, Y.F., Kang, H.B., Li, B.L., et al., 2012. Positive effects of soy isoflavone food on survival of breast cancer patients in China. Asian Pacific Journal of Cancer Prevention 13, 479–482.
Zhang, Y., Talalay, P., Cho, C.-G., Posner, G., 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. Proceedings of the National Academy of Science USA 89, 2399–2403.
Zhang, J., Zhang, H., Wang, L., Guo, X., Wang, X., Yao, H., 2010. Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS/MS. Food Chemistry 119 (1), 226–234.
Zhao, J., Lyu, C., Gao, J., Du, L., Shan, B., Zhang, H., Wang, H.Y., Gao, Y., 2016b Jul. Dietary fat intake and endometrial cancer risk: A dose response meta-analysis. Medicine (Baltimore) 95 (27), e4121. https://doi.org/10.1097/MD.0000000000004121.
Zhao, H., Zhou, L., Shangguan, A.J., Bulun, S.E., 2016a. Aromatase expression and regulation in breast and endometrial cancer. Journal of Molecular Endocrinology 57 (1), R19–R33. https://doi.org/10.1530/JME-15-0310. Epub 2016 Apr 11.
Zheng, J.S., Hu, X.J., Zhao, Y.M., Yang, J., Li, D., 2013. Intake of fish and marine n-3 polyunsaturated fatty acids and risk of breast cancer: Meta-analysis of data from 21 independent prospective cohort studies. BMJ 346, f3706. https://doi.org/10.1136/bmj.f3706.
Zheng, J.S., Hu, X.J., Zhao, Y.M., Yang, J., Li, D., 2013. Intake of fish and marine n-3 polyunsaturated fatty acids and risk of breast cancer: Meta-analysis of data from 21 independent prospective cohort studies. BMJ 346, 346–f3706. f3706.
Zheng, W., Lee, S.A., 2009. Well-done meat intake, heterocyclic amine exposure, and cancer risk. Nutrition and Cancer 61, 437–446.
Zhong, X., Fang, Y.J., Pan, Z.Z., Li, B., Wang, L., Zheng, M.C., Chen, Y.M., Zhang, C.X., 2013. Dietary fat, fatty acid intakes and colorectal cancer risk in Chinese adults: a case-control study. European Journal of Cancer Prevention 22 (5), 438–447. https://doi.org/10.1097/CEJ.0b013e32835e88c4.
Zhong, X.S., Ge, J., Chen, S.W., Xiong, Y.Q., Ma, S.J., Chen, Q., 2018. Association between dietary Isoflavones in soy and legumes and endometrial cancer: A systematic review and meta-analysis. Journal of the Academy of Nutrition and Dietetics 118 (4), 637–651.
Zhong, L., Goldberg, M.S., Gao, Y.T., et al., 2001. Population-based case-control study of lung cancer and green tea consumption among women living in Shanghai, China. Epidemiology 12, 695–700.
Zhou, J.R., Gugger, E.T., Tanaka, T., Guo, Y., Blackburn, G.L., Clinton, S.K., 1999. Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. The Journal of Nutrition 129, 1628–1635.
Ziegler, R.G., Hoover, R.N., Pike, M.C., Hildesheim, A., Nomura, A.M., West, D.W., Wu-Williams, A.H., Kolonel, L.N., Horn-Ross, P.L., Rosenthal, J.F., Hyer, M.B., 1993. Migration patterns and breast cancer risk in Asian-American women. Journal of the National Cancer Institute 85 (22), 1819–1827.
Zong, G., Gao, A., Hu, F.B., Sun, Q., 2016. Whole grain intake and mortality from all causes, cardiovascular disease, and Cancer: A meta-analysis of prospective cohort studies. Circulation 133 (24), 2370–2380. https://doi.org/10.1161/CIRCULATIONAHA.115.021101.
Zuniga, K.E., Clinton, S.K., Erdman, J.W., 2013. The interactions of dietary tomato powder and soy germ on prostate carcinogenesis in the TRAMP model. Cancer Prevention Research 6, 548–557.

Further Reading

Adlercreutz, H., Markkanen, H., Watanabe, S., 1993. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet 342, 1209–1210.
Agnoli, C., Grioni, S., Sieri, S., Palli, D., Masala, G., Sacerdote, C., Vineis, P., Tumino, R., Giurdanella, M.C., Pala, V., Berrino, F., Mattiello, A., Panico, S., Krogh, V., 2013. Italian Mediterranean index and risk of colorectal cancer in the Italian section of the EPIC cohort. International Journal of Cancer 132 (6), 1404–1411.
AICR'S Foods That Fight Cancer—Broccoli & Cruciferous Vegetables. 2018. http://www.aicr.org/foods-that-fight-cancer/broccoli-cruciferous.html (accessed on 14 April 2018).
Alberti-Fidanza, A., Fidanza, F., Chiuchiu, M.P., Verducci, G., Fruttini, D., 1999. Dietary studies on two rural italian population groups of the seven countries study. 3. Trend of food and nutrient intake from 1960 to 1991. European Journal of Clinical Nutrition 53 (11), 854–860.
American Institute for Cancer Research (AICR). (2018) Soy is Safe for Breast Cancer Survivors. Available online: http://www.aicr.org/press/press-releases/soy-safe-breast-cancer-survivors.html (accessed on 14 April 2018).
American Institute for Cancer Research (AICR). 2018 Soybeans, soy nuts and edamame contain fiber. After a systematic review of the global scientific literature, AICR/WCRF weighed the strength of the evidence linking these factors to lower risk for several cancers. Available online: http://www.aicr.org/foods-that-fight-cancer/tab-content/soy-research-1.html. (accessed on 14 April 2018).
Aucoin, M., Cooley, K., Knee, C., Fritz, H., Balneaves, L.G., Breau, R., Fergusson, D., Skidmore, B., Wong, R., Seely, D., 2017. Fish-derived Omega-3 fatty acids and prostate Cancer: A systematic review. Integrative Cancer Therapies 16 (1), 32–62. https://doi.org/10.1177/1534735416656052.
Bach, A., Serra-Majem, L., Carrasco, J.L., Roman, B., Ngo, J., Bertomeu, I., Obrador, B., 2006. The use of indexes evaluating the adherence to the Mediterranean diet in epidemiological studies: A review. Public Health Nutrition 9 (1A), 132–146.

Balanza, R., Garcia-Lorda, P., Perez-Rodrigo, C., Aranceta, J., Bonet, M.B., Salas-Salvado, J., 2007. Trends in food availability determined by the food and agriculture Organization's food balance sheets in Mediterranean Europe in comparison with other European areas. Public Health Nutrition 10 (2), 168–176.

Bamia, C., Lagiou, P., Buckland, G., et al., 2013. Mediterranean diet and colorectal cancer risk: Results from a European cohort. European Journal of Epidemiology 28 (4), 317–328. https://doi.org/10.1007/s10654-013-9795-x. Epub 2013 Apr 12.

Bandera, E.V., Kushi, L.H., Moore, D.F., Gifkins, D.M., McCullough, M.L., 2007. Fruits and vegetables and endometrial cancer risk: A systematic literature review and meta-analysis. Nutrition and Cancer 58 (1), 6–21. https://doi.org/10.1080/01635580701307929.

Berrino, F., 2016. Mediterranean diet and its association with reduced invasive breast cancer risk. JAMA Oncology 2 (4), 535–536.

Bloomfield, H.E., Koeller, E., Greer, N., MacDonald, R., Kane, R., Wilt, T.J., 2016. Effects on health outcomes of a Mediterranean diet with no restriction on fat intake: A systematic review and meta-analysis. Annals of Internal Medicine 165 (7), 491–500. https://doi.org/10.7326/M16-0361.

Boker, L.K., Van der Schouw, Y.T., De Kleijn, M.J., Jacques, P.F., Grobbee, D.E., Peeters, P.H., 2002. Intake of dietary phytoestrogens by Dutch women. The Journal of Nutrition 132, 1319–1328.

Bravi, F., Scotti, L., Bosetti, C., Zucchetto, A., Talamini, R., Montella, M., Greggi, S., Pelucchi, C., Negri, E., Franceschi, S., La Vecchia, C., 2009. Food groups and endometrial cancer risk: A case-control study from Italy. American Journal of Obstetrics and Gynecology 200 (3), 293 e1–7. https://doi.org/10.1016/j.ajog.2008.09.015.

Brennan, S.F., Woodside, J.V., Lunny, P.M., Cardwell, C.R., Cantwell, M.M., 2017. Dietary fat and breast cancer mortality: A systematic review and meta-analysis. Critical Reviews in Food Science and Nutrition 57 (10), 1999–2008.

Buckland G et al. Adherence to the mediterranean diet and risk of breast cancer in the European prospective investigation into cancer and nutrition cohort study. International Journal of Cancer 132 (12), 2918–2927.

Buckland, G., Travier, N., Cottet, V., Gonzalez, C.A., Lujan-Barroso, L., Agudo, A., Trichopoulou, A., Lagiou, P., Trichopoulos, D., Peeters, P.H., May, A., Bueno-de-Mesquita, H.B., Bvan Duijnhoven, F.J., Key, T.J., Allen, N., Khaw, K.T., Wareham, N., Romieu, I., McCormack, V., Boutron-Ruault, M., Clavel-Chapelon, F., Panico, S., Agnoli, C., Palli, D., Tumino, R., Vineis, P., Amiano, P., Barricarte, A., Rodriguez, L., Sanchez, M.J., Chirlaque, M.D., Kaaks, R., Teucher, B., Boeing, H., Bergmann, M.M., Overvad, K., Dahm, C.C., Tjonneland, A., Olsen, A., Manjer, J., Wirfalt, E., Hallmans, G., Johansson, I., Lund, E., Hjartaker, A., Skeie, G., Vergnaud, A.C., Norat, T., Romaguera, D., Riboli, E., 2013. Adherence to the mediterranean diet and risk of breast cancer in the European prospective investigation into cancer and nutrition cohort study. International Journal of Cancer 132 (12), 2918–2927. https://doi.org/10.1002/ijc.27958.

Capurso, C., Vendemiale, G., 2017. The Mediterranean diet reduces the risk and mortality of the prostate Cancer: A narrative review. Frontiers in Nutrition 4, 38. https://doi.org/10.3389/fnut.2017.00038.

Chao, A., Thun, M.J., Connell, C.J., McCullough, M.L., Jacobs, E.J., Flanders, W.D., Rodriguez, C., Sinha, R., Calle, E.E., 2005. Meat consumption and risk of colorectal cancer. Journal of the American Medical Association 293 (2), 172–182.

da Silva, R., Bach-Faig, A., Raido Quintana, B., Buckland, G., Vaz de Almeida, M.D., Serra-Majem, L., 2009. Worldwide variation of adherence to the Mediterranean diet, in 1961-1965 and 2000-2003. Public Health Nutrition 12 (9A), 1676–1684.

Davis-Yadley, A.H., Malafa, M.P., 2015. Vitamins in pancreatic cancer: A review of underlying mechanisms and future applications. Advances in Nutrition 6 (6), 774–802. https://doi.org/10.3945/an.115.009456.

De Kleijn, M.J., van der Schouw, Y.T., Wilson, P.W., Adlercreutz, H., Mazur, W., Grobbee, D.E., Jacques, P.F., 2001. Intake of dietary phytoestrogens is low in postmenopausal women in the United States: The Framingham study (1–4). The Journal of Nutrition 131, 1826–1832.

Di Sebastiano, K.M., Mourtzakis, M., 2014. The role of dietary fat throughout the prostate Cancer trajectory. Nutrients 6 (12), 6095–6109. Published online 2014 Dec 22. https://doi.org/10.3390/nu6126095.

Dinu, M., Pagliai, G., Casini, A., Sofi, F., 2018. Mediterranean diet and multiple health outcomes: An umbrella review of meta-analyses of observational studies and randomised trials. European Journal of Clinical Nutrition 72 (1), 30–43. https://doi.org/10.1038/ejcn.2017.58.

Economopoulos, K.P., Sergentanis, T.N., 2010. GSTM1, GSTT1, GSTP1, GSTA1 and colorectal cancer risk: A comprehensive meta-analysis. European Journal of Cancer 31, 1617–1631.

Farnetti, S., Malandrino, N., Luciani, D., Gasbarrini, G., Capristo, E., 2011. Food fried in extra-virgin olive oil improves postprandial insulin response in obese, insulin-resistant women. Journal of Medicinal Food 14, 316–321.

Faulkner-Hogg, K.B., Selby, W.S., Loblay, R.H., 1999. Dietary analysis in symptomatic patients with coeliac disease on a gluten-free diet: The role of trace amounts of gluten and non-gluten food intolerances. Scandinavian Journal of Gastroenterology 34, 784–789.

Ferro-Luzzi, A., Branca, F., 1995. Mediterranean diet, Italian-style: Prototype of a healthy diet. The American Journal of Clinical Nutrition 61 (6 Suppl), 1338S–1345S.

Fidanza, F., Alberti, A., Lanti, M., Menotti, A., 2004. Mediterranean adequacy index: Correlation with 25-year mortality from coronary heart disease in the seven countries study. Nutrition, Metabolism, and Cardiovascular Diseases 14 (5), 254–258.

Filomeno, M., Bosetti, C., Bidoli, E., Levi, F., Serraino, D., Montella, M., La Vecchia, C., Tavani, A., 2015. Mediterranean diet and risk of endometrial cancer: A pooled analysis of three Italian case-control studies. British Journal of Cancer 112 (11), 1816–1821. https://doi.org/10.1038/bjc.2015.153.

Filomeno, M., Bosetti, C., Bidoli, E., Levi, F., Serraino, D., Montella, M., La Vecchia, C., Tavani, A., 2015. Mediterranean diet and risk of endometrial cancer: A pooled analysis of three Italian case-control studies. British Journal of Cancer 112 (11), 1816–1821. https://doi.org/10.1038/bjc.2015.153. Epub 2015 May 12.

Fortner, R.T., Husing, A., Kuhn, T., Konar, M., Overvad, K., Tjonneland, A., Hansen, L., Boutron-Ruault, M.C., Severi, G., Fournier, A., Boeing, H., Trichopoulou, A., Benetou, V., Orfanos, P., Masala, G., Agnoli, C., Mattiello, A., Tumino, R., Sacerdote, C., Bueno-de-Mesquita, H.B., Peeters, P.H., Weiderpass, E., Gram, I.T., Gavrilyuk, O., Quiros, J.R., Maria Huerta, J., Ardanaz, E., Larranaga, N., Lujan-Barroso, L., Sanchez-Cantalejo, E., Butt, S.T., Borgquist, S., Idahl, A., Lundin, E., Khaw, K.T., Allen, N.E., Rinaldi, S., Dossus, L., Gunter, M., Merritt, M.A., Tzoulaki, I., Riboli, E., Kaaks, R., 2017. Endometrial cancer risk prediction including serum-based biomarkers: Results from the EPIC cohort. International Journal of Cancer 140 (6), 1317–1323. https://doi.org/10.1002/ijc.30560.

Gann, P.H., Ma, J., Giovannucci, E., Willett, W., Sacks, F.M., Hennekens, C.H., Stampfer, M.J., 1999. Lower prostate cancer risk in men with elevated plasma lycopene levels: Results of a prospective analysis. Cancer Research 59 (6), 1225–1230.

Garcia-Closas, R., Berenguer, A., Gonzalez, C.A., 2006. Changes in food supply in Mediterranean countries from 1961 to 2001. Public Health Nutrition 9 (1), 53–60.

Giacosa, A., Barale, R., Bavaresco, L., Gatenby, P., Gerbi, V., Janssens, J., Johnston, B., Kas, K., La Vecchia, C., Mainguet, P., Morazzoni, P., Negri, E., Pelucchi, C., Pezzotti, M., Rondanelli, M., 2013. Cancer prevention in Europe: The Mediterranean diet as a protective choice. European Journal of Cancer Prevention 22 (1), 90–95. https://doi.org/10.1097/CEJ.0b013e328354d2d7.

Giampaoli, S., Krogh, V., Grioni, S., Palmieri, L., Gulizia, M.M., Stamler, J., Vanuzzo, D., Gruppo di ricerca dell Osservatorio epidemiologico cardiovascolare/health examination S, 2015. Eating behaviours of italian adults: Results of the Osservatorio epidemiologico cardiovascolare/health examination survey. Epidemiologia e Prevenzione 39 (5–6), 373–379.

Gibney, M.J., Forde, C.G., Mullally, D., Gibney, E.R., 2017. Ultra-processed foods in human health: A critical appraisal. The American Journal of Clinical Nutrition 106 (3), 717–724.

Gong, T.T., Li, D., Wu, Q.J., Wang, Y.Z., 2016. Cholesterol consumption and risk of endometrial cancer: A systematic review and dose-response meta-analysis of observational studies. Oncotarget 7 (13), 16996–17008. https://doi.org/10.18632/oncotarget.7913.

Graff, R.E., Pettersson, A., Lis, R.T., Ahearn, T.U., Markt, S.C., Wilson, K.M., Rider, J.R., Fiorentino, M., Finn, S., Kenfield, S.A., Loda, M., Giovannucci, E.L., Rosner, B., Mucci, L.A., 2016. Dietary lycopene intake and risk of prostate cancer defined by ERG protein expression. The American Journal of Clinical Nutrition 103 (3), 851–860. https://doi.org/10.3945/ajcn.115.118703.

Gupta, K., Kshirsagar, S., Chang, L., Schwartz, R., Law, P.Y., Yee, D., Hebbel, R.P., 2002. Morphine stimulates angiogenesis by activating proangiogenic and survival-promoting signaling and promotes breast tumor growth. Cancer Research 62 (15), 4491–4498.
Kapiotis, S., Hermann, M., Held, I., Seelos, C., Ehringer, H., Gmeiner, B.M., 1997. Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. Arteriosclerosis, Thrombosis, and Vascular Biology 17, 2868–2874.
Khodarahmi, M., Azadbakht, L., 2014. The association between different kinds of fat intake and breast cancer risk in women. International Journal of Preventive Medicine 5, 6–15.
King, D.E., Egan, B.M., Woolson, R.F., et al., 2007. Effect of a high-fiber diet vs a fiber-supplemented diet on C-reactive protein level. Archives of Internal Medicine 167, 502–506.
Kromhout, D., Keys, A., Aravanis, C., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovic, S., Pekkarinen, M., et al., 1989. Food consumption patterns in the 1960s in seven countries. American Journal of Clinical Nutrition 49 (5), 889–894. https://doi.org/10.1093/ajcn/49.5.889.
Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139, 4252–4263.
Kwan, H.Y., Chao, X., Su, T., Fu, X.Q., Liu, B., Tse, A.K., Fong, W.F., Yu, Z.L., 2015. Dietary lipids and adipocytes: Potential therapeutic targets in cancers. The Journal of Nutritional Biochemistry 26 (4), 303–311. https://doi.org/10.1016/j.jnutbio.2014.11.001. Epub 2014 Dec 3.
Lampe, J.W., 1999. Health effects of vegetables and fruit: Assessing mechanisms of action in human experimental studies. The American Journal of Clinical Nutrition 70 (3 Suppl), 475S–490S.
Larsson, S.C., Giovannucci, E., Wolk, A., 2006. Folate intake, MTHFR polymorphisms, and risk of esophageal, gastric, and pancreatic cancer: A meta-analysis. Gastroenterology 131 (4), 1271–1283. https://doi.org/10.1053/j.gastro.2006.08.010.
Lee, C., Longo, V.D., 2011. Fasting vs dietary restriction in cellular protection and cancer treatment: From model organisms to patients. Oncogene 30 (30), 3305–3316.
Lee, C., Raffaghello, L., Brandhorst, S., Safdie, F.M., Bianchi, G., Martin-Montalvo, A., et al., 2012a. Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy. Science Translational Medicine 4, 124–127.
Levy, R.M., D'Anna, R., et al., 2008. Breast safety and efficacy of genistein aglycone for postmenopausal bone loss: A follow-up study. The Journal of Clinical Endocrinology and Metabolism 93, 4787–4796.
Li, Y., Li, S., Meng, X., Gan, R.Y., Zhang, J.J., Li, H.B., 2017 Jul 8. Dietary natural products for prevention and treatment of breast Cancer. Nutrients 9, E728.
Macias, V., Kajdacsy-Balla, A., Lumey, L.H., et al., 2013. Effect of soy protein isolate supplementation on biochemical recurrence of prostate cancer after radical prostatectomy: A randomized trial. JAMA 310, 170–178.
Menotti, A., Keys, A., Aravanis, C., Blackburn, H., Dontas, A., Fidanza, F., Karvonen, M.J., Kromhout, D., Nedeljkovic, S., Nissinen, A., et al., 1989. Seven countries study. First 20-year mortality data in 12 cohorts of six countries. Annals of Medicine 21 (3), 175–179.
Messina, M.J., Wood, C.E., 2008. Soy isoflavones, estrogen therapy, and breast cancer risk: Analysis and commentary. Nutrition Journal 7, 17. Review.
NIH-Cruciferous Vegetables and Cancer Prevention. https://www.cancer.gov/about-cancer/causes-prevention/risk/diet/cruciferous-vegetables-fact-sheet. (Accessed 14 April 2018).
Nothlings, U., Murphy, S.P., Wilkens, L.R., Henderson, B.E., Kolonel, L.N., 2007. Flavonols and pancreatic cancer risk: The multiethnic cohort study. American Journal of Epidemiology 166 (8), 924–931. https://doi.org/10.1093/aje/kwm172.
Parekh, N., Chandran, U., Bandera, E.V., 2012. Obesity in cancer survival. Annual Review of Nutrition 32, 311–342.
Raffaghello, L., Longo, V., 2017. Metabolic alterations at the crossroad of aging and oncogenesis. International Review of Cell and Molecular Biology 332, 1–42.
Raffaghello, L., Safdie, F., Bianchi, G., Dorff, T., Fontana, L., Longo, V.D., 2010a. Fasting and differential chemotherapy protection in patients. Cell Cycle 9 (22), 4474–4476.
Reddy, B.S., 2002. Types and amount of dietary fat and colon cancer risk: Prevention by omega-3 fatty acid-rich diets. Environmental Health and Preventive Medicine 7 (3), 95–102. https://doi.org/10.1265/ehpm.2002.95.
Rose, D.P., 1990. Dietary fiber and breast cancer. Nutrition and Cancer 13 (1–2), 1–8. https://doi.org/10.1080/01635589009514040.
Rossi, M., Lugo, A., Lagiou, P., Zucchetto, A., Polesel, J., Serraino, D., Negri, E., Trichopoulos, D., La Vecchia, C., 2012. Proanthocyanidins and other flavonoids in relation to pancreatic cancer: A case-control study in Italy. Annals of Oncology 23 (6), 1488–1493. https://doi.org/10.1093/annonc/mdr475.
Schwingshackl, L., Schwedhelm, C., Galbete, C., Hoffmann, G., 2017. Adherence to Mediterranean diet and risk of Cancer: An updated systematic review and meta-analysis. Nutrients 9 (10). https://doi.org/10.3390/nu9101063.
Serra-Majem, L., Roman, B., Estruch, R., 2006. Scientific evidence of interventions using the Mediterranean diet: A systematic review. Nutrition Reviews 64 (2 Pt 2), S27–S47.
Serra-Majem, L., Trichopoulou, A., Ngo de la Cruz, J., Cervera, P., Garcia Alvarez, A., La Vecchia, C., Lemtouni, A., Trichopoulos, D., International Task Force on the Mediterranean D, 2004. Does the definition of the Mediterranean diet need to be updated? Public Health Nutrition 7 (7), 927–929.
Sofi, F., Cesari, F., Abbate, R., Gensini, G.F., Casini, A., 2008. Adherence to Mediterranean diet and health status: Meta-analysis. BMJ 337, a1344. https://doi.org/10.1136/bmj.a1344.
Sofi, F., Macchi, C., Abbate, R., Gensini, G.F., Casini, A., 2013. Mediterranean diet and health. BioFactors 39 (4), 335–342. https://doi.org/10.1002/biof.1096.
Sofi, F., Macchi, C., Abbate, R., Gensini, G.F., Casini, A., 2014. Mediterranean diet and health status: An updated meta-analysis and a proposal for a literature-based adherence score. Public Health Nutrition 17 (12), 2769–2782. https://doi.org/10.1017/S1368980013003169.
Thompson Jr., I.M., Cabang, A.B., Wargovich, M.J., 2014. Future directions in the prevention of prostate cancer. Nature Reviews. Clinical Oncology 11 (1), 49–60. https://doi.org/10.1038/nrclinonc.2013.211.
Trichopoulou, A., Lagiou, P., Kuper, H., Trichopoulos, D., 2000. Cancer and Mediterranean dietary traditions. Cancer Epidemiology, Biomarkers & Prevention 9 (9), 869–873.
Tucker, K.L., 2010. Dietary patterns, approaches, and multicultural perspective. Applied Physiology, Nutrition, and Metabolism 35 (2), 211–218.
UNESCO, 2010. http://www.unesco.it/it/PatrimonioImmateriale/Detail/384.
USDA Database for the Isoflavone Content of Selected Food, Release 2.1 (November 2015). https://data.nal.usda.gov/dataset/usda-database-isoflavone-content-selected-foods-release-21-november-2015 (accessed on 14 April 2018).
USDA 2018 Choose my plate. https://www.choosemyplate.gov/vegetables (accessed on 14 April 2018).
Vance, T.M., Su, J., Fontham, E.T., Koo, S.I., Chun, O.K., 2013. Dietary antioxidants and prostate cancer: A review. Nutrition and Cancer 65 (6), 793–801. https://doi.org/10.1080/01635581.2013.806672.
Varady, K.A., 2011. Intermittent versus daily calorie restriction: Which diet regimen is more effective for weight loss? Obesity Reviews 12 (7), e593–e601.
Vareiro, D., Bach-Faig, A., Raido Quintana, B., Bertomeu, I., Buckland, G., Vaz de Almeida, M.D., Serra-Majem, L., 2009. Availability of Mediterranean and non-Mediterranean foods during the last four decades: Comparison of several geographical areas. Public Health Nutrition 12 (9A), 1667–1675.
Wang, T., Rohan, T.E., Gunter, M.J., Xue, X., Wactawski-Wende, J., Rajpathak, S.N., Cushman, M., Strickler, H.D., Kaplan, R.C., Wassertheil-Smoller, S., Scherer, P.E., Ho, G.Y., 2011. A prospective study of inflammation markers and endometrial cancer risk in postmenopausal hormone nonusers. Cancer Epidemiology, Biomarkers & Prevention 20 (5), 971–977. https://doi.org/10.1158/1055-9965.EPI-10-1222.
WCRF/AICR, 2007. Food, nutrition, physical activity and the prevention of Cancer: A global perspective.
Willett, W.C., Sacks, F., Trichopoulou, A., Drescher, G., Ferro-Luzzi, A., Helsing, E., Trichopoulos, D., 1995. Mediterranean diet pyramid: A cultural model for healthy eating. The American Journal of Clinical Nutrition 61 (6 Suppl), 1402S–1406S.
World Cancer Research Fund International (WCRF). Breast cancer survivors. Available online: http://www.wcrf.org/int/research-we-fund/continuous-update-project-findings-reports/breast-cancer-survivors (accessed on 14 April 2018).

World Cancer Research Fund/American Institute for Cancer Research. 2018 Colorectal cancer. https://wcrf.org/int/continuous-update-project/cup-findings-reports/colorectal-cancer (accessed on 15 April 2018).
www.iarc.it. https://cancer-code-europe.iarc.fr/index.php/it.
www.iarc.it. https://cancer-code-europe.iarc.fr/index.php/it/.

Relevant Websites

http://www.heart.org/HEARTORG/—American Heart Association.
https://www.dietitians.ca/—Dietitians of Canada.

Dietary Factors and Cancer*

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Glossary

Case control study An epidemiological study in which the participants are chosen based on their disease or condition (cases) or lack of it (controls) to test whether past or recent history of an exposure is associated with the risk of disease.

Cohort study An epidemiological study of a group of people whose characteristics are recorded at recruitment, followed for a period of time during which the outcomes of interest are noted. Differences in the likelihood of a particular outcome are presented as the relative risk comparing one level of exposure to another.

Confidence interval A measure of uncertainly in an estimate usually reported as 95% confidence interval.

Dietary fiber Constituents of plant cell walls that are not digested in the small intestine. Non-starch polysaccharides are a consistent feature and are fermented by colonic bacteria to produce energy and short chain fatty acids including butyrate.

Fatty acids A carboxylic acid with a carbon chain of varying length, which may be either saturated (no double bonds) or unsaturated (one or more double bonds).

Meta-analysis The process of using statistical methods to combine the results of different studies.

Pooling In Epidemiology, a type of study where original individual-level data from two or more original studies are obtained, combined and reanalyzed.

Randomized controlled trial (RCT) A study in which a comparison is made between one intervention (often a treatment or prevention strategy) and another (control). Groups are randomized to one intervention or the other, so that any difference in outcome between the two groups can be ascribed with confidence to the intervention.

Relative risk (RR) The ratio of the rate of the disease or death among people exposed to a factor, compared to the rate among the unexposed.

Introduction

Interest in the cause and prevention of cancer arose from the observation that cancer rates vary greatly between countries and are correlated to dietary factors, and that nutrition can modify cancer incidence in animals. Furthermore, ecological studies showing correlation between dietary habits and cancer incidence and mortality, and migrant studies on changing rates with change in diet and life style, have stimulated research on the role of diet in cancer prevention. To provide more specific information on human cancer, many retrospectives case-control studies, some prospective cohort studies and a few randomized controlled trials (RCT) have been conducted. The most extensive review of the existing evidence on diet and cancer has been conducted by the World Cancer Research Funds/American Institute for Cancer Research (WCRF/AICR) (2007) and the continuous update project (CUP/WCRF). Lifestyle recommendations for cancer prevention were drawn up on the basis of nutrition-related factors judged to be convincingly or probably causally related to cancer, according to predefined criteria for judging the strength of the evidence regarding causality. Results from the most recent evaluations on the association between dietary factors and alcohol consumption and major cancers are presented in Table 1. According to the recommendations, a healthy diet for cancer prevention is a diet (1) that allows a person to be as lean as possible without being underweight; (2) is rich in fruit, vegetables, whole grains and pulses; (3) contains low amounts of red meat; (4) does not contain processed meats; (5) limits salt intake; (6) avoid sugary drinks, and limits intake of calorie-rich foods. A healthy diet also limits consumption of alcoholic drinks. In this article we address the mechanisms involving nutritional factors in cancer development, and the challenges in studying these associations, then we present and discuss the most recent evidence on the link between dietary factors and cancer development.

Main Mechanisms Involving Nutritional Factors in Cancer Development

Excessive caloric intake results in weight gain and ultimately obesity, which in turn is associated with an increased risk of several cancers (colorectal, endometrium, kidney, esophagus, postmenopausal breast, gallbladder, pancreas, gastric cardia, liver, ovary, thyroid, meningioma, multiple myeloma, and advanced prostate cancers). Many experimental studies have shown that calorie restriction suppresses the carcinogenic process. The interactions between cellular energetics in cancer cells and the systemic metabolic changes associated with obesity are emerging as critical drivers of obesity-related cancer. In obesity, alterations occur in

*This work was undertaken during the tenure of Senior Visiting Scientist at the International Agency for Research on Cancer, Lyon, France.

Table 1 World Research Cancer Funds/American Institute for Cancer Research, summary of evidence on diet and cancer prevention[a] (updated CUP 2017)

	Oropharynxnx 2007	*Osesopahgus 2014*	*Lung 2007*	*Stomach 2016*	*Pancreas 2012*	*Gallbladder 2015*	*Liver 2015*	*Colorectum 2017*	*Breast Premenopausal 2017*	*Breast Postmenopausal 2017*	*Ovary 2014*	*Endometrium 2013*	*Prostate 2014*	*Kidney 2015*	*Skin 2007*
Fat[b]			↑		↑										
Red meat			↑		↑			↑↑							
Processed meat[c]		↑	↑	↑↑	↑			↑↑↑							
Salt, salted foods				↑↑											
Dairy products[d]								↓↓	↓				↑		
Fruits[e]	↓↓	↓		↓				↓							
Vegetables (non-starchy)[f]	↓↓	↓						↓	↓	↓					
Dietary fiber								↓↓							
Glycemic load												↑↑			
Coffee							↓↓					↓↓			
Soy															
Diet high in calcium								↓↓	↓	↓			↑		
Carotenoids[g]	↓↓	↓↓	↓↓						↓	↓					
Alcoholic beverage[h]	↑↑↑	↑↑↑		↑↑	↑		↑↑↑	↑↑↑	↑↑	↑↑↑				↓↓	

↓↓↓, convincing decreased risk; ↓↓, probable decreased risk; ↓, limited-suggestive evidence decreased risk.
↑↑↑, convincing increased risk; ↑↑, probable increased risk; ↑, limited-suggestive evidence increased risk.
AICR/WCRF evidence stratification.
Convincing: Evidence strong enough to support causal relationship. Evidence from more than one study type, data from at least two cohort studies, no unexplained heterogeneity between studies with regard to the presence or absence of an association, good quality studies where random or systematic error are unlikely, presence of a dose–response relationship, and strong and plausible experimental evidence.
Probable: Evidence from at least two cohort studies or at least five case-control studies, no unexplained heterogeneity between studies with regard to the presence or absence of an association, good quality studies where random or systematic error are unlikely, evidence of biological plausibility.
Limited-suggestive: Evidence is too limited to permit a probable convincing judgment but there is evidence of a direction of effect. Evidence from at least two cohort studies or at least five case-control studies, some evidence of biological plausibility, and the direction of the effect is generally consistent.
Limited-no conclusion: evidence limited in number of studies or yielding inconsistent results.
[a]For unmarked items there is limited evidence and no conclusion could be drawn.
[b]Include evidence for saturated fat for pancreas.
[c]Includes evidence for stomach (noncardia).
[d]Includes evidence from total dairy, milk, cheese and dietary calcium intakes for colorectum.
[e]Includes evidence on foods containing carotenoids for oropharynx (mouth, pharynx, larynx), foods containing beta-carotene for esophagus; foods containing vitamin C for esophagus and stomach (cardia).
[f]Includes evidence on foods containing carotenoids for oropharynx (mouth, pharynx, larynx) and breast (pre and postmenopausal); foods containing beta-carotene for esophagus; foods containing vitamin C for esophagus; stronger effect on ER− breast tumors.
[g]Includes evidence for foods containing carotenoids for oropharynx (mouth, pharynx, larynx) and breast (stronger effect for ER− tumors). Includes evidence on foods containing beta-carotene for esophagus and lung. Increased risk for lung derived from studies using beta-carotene supplements.
[h]Based on evidence for alcohol intake up to 30 g/day (about two drinks a day). There is insufficient evidence for intake >30 g/day.

circulating levels of insulin and insulin-like growth factor-1 (IGF-1); adipokines, such as leptin and adiponectin; inflammatory factors; several chemokines; lipid mediators, and vascular-associated factors. Each of these factors has a putative role in the development and progression of cancer as well as several other chronic diseases including cardiovascular disease and type 2 diabetes.

In addition, nutrition influences inflammatory and immune responses, and this has led to the development of the "dietary inflammatory index" and the use of the term immune-nutrition. Obesity is strongly linked to chronic inflammation and to immunologic abnormalities with fewer cytotoxic CD8 + T cells and natural killer cells, while fasting has been associated with antiinflammatory effects. These factors may influence cellular processes and lead to the accumulation of the well-established hallmarks of cancer cells: reprogrammed energy metabolism, sustained proliferative signaling, increased chronic inflammation, increased genome instability, enabled replicative immortality, enhanced angiogenesis, activated processes related to invasion and metastasis, and resistance to growth suppressors, cell death inducers, and immunoregulators. A growing body of evidence indicates that lowering the energy density (the amount of energy in a particular weight of food) of diet can reduce caloric intake. Energy dense diets contain less fiber-rich foods, and are usually high in fats, processed starch, and added sugars. Sugar-sweetened soft drinks play an important role in the development of obesity.

Nutrition can also cause oxidative stress, augment a cascade of molecular reactions in cells and alter the metabolism of tissues. Overnutrition may generate free radicals and subsequently elevated oxidative stress and ROS-mediated modulation of various molecular pathways and chronic inflammation. Some nutrients have been linked to an increase in oxidative stress response. Carbohydrates quality can influence the plasma level of glucose and insulin resistance, leading to the formation of free radicals by an imbalance in the ratio of NADH to NAD and by non-enzymatic glycation increased by glucose in cells. Fatty acids appear to affect differentially the cancer risk, in particular through inflammatory pathways. The cyclo'oxygenase-2 (COX-2) enzyme can convert omega-6 FAs into prostaglandin E2, a proinflammatory cytokine, which enables angiogenesis and cell proliferation, whereas prostaglandin E3 is produced from omega-3 FAs with the help of COX-2 which does not facilitate mitogenic angiogenesis. The ratio between omega-6 and omega-3 polyunsaturated fatty acids (n-6 PUFAs and n-3 PUFAs) could therefore play an important role.

Fatty acids could also act through other mechanisms. In breast cancer, "de novo" lipogenesis through the action of the stearoyl-CoA desaturase-1 (SCD-1) has been suggested to increase cancer risk. Trans fatty acids are used in industrial processed sweet and salty foods and have been associated to alterations in metabolic and signaling pathways, higher circulating levels of lipids, systemic inflammation, endothelium dysfunction and possibly increased visceral adiposity, body weight and insulin resistance.

Vitamins and minerals such as carotenoids, folate, vitamin C, D, E, and B6 and selenium might reduce cancer through preventive oxidative damage, affecting immune response, inhibiting cell proliferation, inducing cell-cycle arrest, and maintaining DNA methylation. Similarly, phytochemicals such as polyphenols are natural antioxidants modulating intracellular ROS levels resulting into epigenetic alterations of essential genes in tumorigenesis. Flavonoids compounds have been linked to an anticancer activity. Isoflavone possesses a well-characterized anti-estrogenic activity; acts in intracellular steroid metabolism and has antiangiogenic, antiproliferative and proapoptotic activities in tumor cells. Lignans (enterolactone) are strong antioxidant and antiinflammatory substances.

Some food compounds can act as *direct mutagens and carcinogens*. In processed red meat, haem iron is nitrosylated, because curing salt contains nitrate or nitrite leading to the synthesis of nitroso compounds that are mutagenic and potential carcinogens. In red meat, haem iron may act by catalyzing the formation of nitroso compounds and of lipid oxidation end products such as 4-hydroxynonenal. Heterocyclic amines and polycyclic aromatic hydrocarbons are formed when cooking food at high temperatures for a long time, or exposed to a direct flame such as a barbecue. In addition, various single-nucleotide polymorphisms involved in the metabolism of these potential carcinogens may modulate the association of carcinogens formed in meat with cancer risk.

Sodium chloride is a food preservative used in processed foods. In animal experiments, salt intake facilitates gastric Helicobacter pylori colonization, one of the main predisposing factors for stomach cancer development, and induces mucosal damage. It may also promote or enhance the effect of nitroso-compounds and other carcinogens.

Dietary fiber from plant foods can reduce the risk of cancer by several mechanisms. Dietary fiber stimulates bacterial anaerobic fermentation in the large bowel with production of short-chain fatty acids, acetate, propionate, and butyrate, shown to reduce cell proliferation and induce apoptosis. Dietary fiber may also protect against colorectal cancer by reducing the transit time of the intestinal content. Dietary fiber interferes with the enterohepatic circulation of oestrogens and experimental studies in mice have shown that soluble fiber can reduce mammary tumor growth, angiogenesis and metastasis. In addition, fiber-rich foods are important sources of phytoestrogens, estrogen-like plant compounds that may modulate the risk of hormone-dependent cancers, especially breast cancer.

Carbohydrates and carbohydrate quality could influence carcinogenesis by affecting insulin resistance and plasma levels of insulin and glucose and level of insulin-like growth factor-1. In addition to increased oxidative stress, elevated circulating insulin level could promote carcinogenesis either directly by stimulating the production of insulin receptors, or indirectly by suppressing insulin growth factor binding protein 1 and 3 affecting the bioavailability of insulin growth factor-1.

Folate, originating mainly from green leafy vegetables and fruits, is an important B vitamin. Folate is a critical factor in the network of biochemical reaction referred to as "one-carbon metabolism" together with other B vitamins (B2, B6, B12) that plays critical role in DNA methylation and DNA synthesis, and in turn, facilitates the cross-talk between genetic and epigenetic processes. Thus, the 1-carbon metabolism can impact both genetic and epigenetic pro-carcinogenic processes, and these biologic roles potentially make folate and other related B vitamins important in cancer prevention. However, serum folate has been inconsistently associated with DNA methylation. The effect of diet on epigenetics could be indirect by modulating chronic inflammation, an important factor associated to epigenetic changes and cancer risk.

Despite its central function in maintaining DNA integrity, the role of folate in cancer prevention seems to be affected by folate status and the timing of folate administration. In particular, data from a supplementation study suggest that folate administrated prior to the existence of preneoplastic lesions can prevent tumor development, whereas provision of folate once early lesions are established appears to increase tumorigenesis. In addition, other factors may impact the relationship between folate intake and cancer risk, such as alcohol intake, and polymorphisms in methylenetetrahydrofolate reductase (*MTHFR*), which codes for a key 1-carbon metabolizing protein. Further, the impact of folate may be related to the tumor type, and other B vitamin namely vitamin B2, B6, B12 that serve critical cofactors for several pivotal enzymes in 1-carbon metabolism.

Activation of the vitamin D pathway with calcitriol, the active component of vitamin D, or its analogues reduced tumor development and growth in numerous animal models. Calcitriol acts by binding to the vitamin D receptor (VDR) and by functioning via both genomic and nongenomic pathways to regulate target gene expression. Calcitriol has been shown to have antiproliferative, prodifferentiation and antiinflammatory and immunomodulating activities.

Alcohol dinking has been classified as carcinogenic to humans (Group 1) by the International Agency for Cancer Research. Ethanol in alcoholic beverages and acetaldehyde associated with the consumption of alcoholic beverages have also been classified as carcinogenic to humans (Group 1). Overall, there is no consistent difference in cancer risk between different types of alcoholic beverages. While the mechanisms of alcohol carcinogenesis are not fully understood, several mechanisms have been suggested: the direct carcinogenicity of ethanol and its metabolites; the interplay with folate metabolism regulated in part by methylenetetrahydrofolate reductase (MTHFR); impact on endocrine and growth factor pathway leading to an increased levels of androgen and estrogen; impacts on bioactive lipids metabolism including lipoperoxidation and generation of free-radical oxygen species, all of which would be further modulated by use patterns and genetic and environmental factors.

The human microbiota is a very diverse ecosystem composed of a large number of microorganisms (close to 100 trillion). Nutrition plays a pivotal role in the regulation of the bacterial community in the gut. Excess intake or imbalance in micro or macronutrients have deleterious effects. Microbiota interacts with the host via direct contact through surface antigens or via soluble molecules which are produced by the microbial metabolism. Most of the data on the link between microbiota and cancer is derived from intestinal tumor models in mice that suggest a causal role for bacterial diversity and community changes. Patients with colorectal cancer and polyposis show dysbiosis, or altered diversity of microbiota supporting the search for tumor-promoting or tumor-protectives species. Bacterial species such as enterogenic *Bacteroides fragilis* could confer protumorigenic properties. Microbiota can also affect the risk of cancer through its metabolic activity and functions. For example, the metabolism of fiber is a critical one for colorectal cancer. A reduction in butyrate levels has been associated with tumorigenesis in mouse models with oncogenic K-ras activation. Metagenomic analyses have consistently shown a reduction of butyrate producers in colorectal cancer patients. In addition, the host genotype may interact with diet to alter the diversity and function of the microbiota in the gut.

Challenge in Diet–Cancer Research

Foods are naturally complex and provide numerous bioactive substances that can act individually or synergically to influence processes such as cell differentiation, apoptosis and hormonal regulation of cellular functions. However, demonstrating relationship between diet and cancer risk in epidemiological studies and intervention, has been challenging because of the potential for bias and random misclassification in both exposure and disease.

Study Design

Prospective cohorts are least susceptible to bias, yet require very large sample size, long term follow-up and good ascertainment of cases. Randomized controlled trials (RCTs) of dietary change or supplements provide the highest level of evidence on diet–cancer relationship. However, adherence to the intervention and sufficient change in nutritional intake are needed to detect an effect. In addition, dietary effects are likely to differ based on baseline nutritional status, and volunteers who participate in dietary intervention are likely to be more health-conscious. Dietary or nutritional supplement interventions are usually conducted over a few years which is a brief period relative to the long latency of most cancers. Therefore, depending on the proposed mechanism of action, the timing of the intervention might affect the outcome. Finally, it is becoming more evident that early life exposure and exposure during infancy and adolescence might impact the risk of cancer later in life. Greater growth/stature is a risk factor common to many cancers, including breast, ovary, endometrium, kidney, testicular, colon, and rectal cancer, and malignant melanoma, non-Hodgkin's lymphoma, and leukemia. Studying the relevant period of exposure becomes an important challenge, and focusing only on exposure in adulthood may obscure an association.

Dietary Assessment

Food frequency questionnaire (FFQs) have been mostly used to assess dietary intake in cancer studies. Being self-administered, FFQs are considered easy to use with practical application in cohort studies. Accuracy of dietary assessment is obtained through validation studies using different methods of assessment (e.g., 24-h report or 24-h recall) and assessment of biomarkers in blood and urine samples measuring recovery biomarkers such as urinary nitrogen, potassium and sodium in order to calibrate FFQ data. In addition, concentrations of biomarkers correlated to intake of food or food group are available such as carotenoids, vitamin E or fatty acids and

can be used to validate a FFQ. Direct measurement of blood biomarkers has the advantage of integrating the bioavailability of the nutrients and not relying on reported food intake and food composition tables so improving dietary assessment. Recently, high-throughput technologies developments have provided tools to improve dietary assessment. Metabolomics, by measuring the full profile of small molecules metabolites in biological samples such as saliva, blood and urine, can improve our knowledge of metabolic pathways relevant to diet–cancer relationship. Moreover, because nutritional epidemiological studies rely on self-reported dietary assessment methods subject to recall bias and measurement error, and because objective biomarkers do not exist for all nutrients and foods, metabolomics can be a promising technique for objectively identifying dietary biomarkers and dietary patterns.

Cancer Phenotype

The biology of cancer has shown heterogeneity within and across different organ sites and a number of carcinogenic pathways are involved through various genetic mutations; therefore, tumors originating within the same organ site are often etiologically different and are likely to be associated with different risk factors. For example, in the pooling project of prospective studies on diet and cancer, the intake of fruit and vegetables had a consistent protective effect on estrogen receptor negative (ER−) tumors, but not on estrogen receptor positive (ER+) breast cancer across 20 cohorts. It is therefore important to classify adequately tumor subtypes.

Early Life Exposure

Diet in early life can affect markers of adolescent growth and development such as age at menarche, age at peak height growth velocity, and peak height growth velocity. This association has implications for chronic diseases such as breast cancer which has some risk factors stemming from adolescent growth and development, in particular because the breast has not yet completed cell differentiation. Several longitudinal studies have shown that the intake of animal protein in young girls has been associated to earlier age at menarche, while vegetable protein has been associated to a later onset of menarche. Animal protein intake has also been related to faster growth and higher attained height. Higher peak height growth velocity and early age at menarche each increase the risk of breast cancer.

Data on the contribution to breast cancer risk of childhood and adolescent dietary exposure is little documented. Soy intake particularly in childhood has been related to lower risk of breast cancer. Data from the ongoing Nurses 'Health study II (NHS II), a large cohort study of young US nurses 24 to 44 years of age, suggest that meat intake and fatty diet during adolescence are related to an increase of premenopausal breast cancer. A significant linear association was observed with every additional 100 g of red meat consumed per day during high school (20% increase in risk; Relative risk (RR) = 1.10 95% CI = 1.00–1.43). Fat intake in the highest quintile (142 g/day) was associated with a 35% increase in risk when compared to fat intake in the lowest quintile (105 g/day) (RR = 1.35 95% CI = 1.00–1.81). Fiber intake during adolescence was also related to the risk of breast cancer; women in the highest quintiles (27.8 g/day) had 24% less risk of premenopausal breast cancer than women in the first quintile (18.2 g/day) (RR = 0.76 95% CI = 0.58% 1.00%). Higher total fruit intake during adolescent independently of fruit intake in adulthood was also associated to a reduced risk of premenopausal breast cancer. In addition, consumption of fish, fruit and vegetables during adolescence has been associated with a reduced risk of colorectal adenomas, a precursor lesion of colorectal cancer.

"Prudent" dietary pattern, characterized by high intake of vegetables, fruits, legumes, fish and poultry, during adolescence was inversely associated with premenopausal breast cancer. Similar results were observed by scoring higher on the Alternative Healthy Eating Index (AHEI) and the association appeared to be stronger for ER−/progesterone receptor-negative (PR−) tumors. High consumption of fish, fruit and vegetables during adolescence was associated with a reduced risk of colorectal adenomas. This association is plausible since dietary fiber can modify the composition of the gut microbiota to metabolize and reduce circulating estrogen. In addition to reducing the risk of early cancers, prevention efforts that begin in early life may also provide important benefits much later in life by shifting the long-term trajectory of risk accumulation.

The importance of the timing of exposure during adolescence is further emphasized by the adverse effect of alcohol consumption. Adolescent alcohol consumption is directly related to the risk of premalignant and invasive breast cancer in prospective cohort studies. In NHS II, alcohol consumption between menarche and first full-time pregnancy (FFTP) was associated with an 11% increased risk per 10 g/day (1 drink) in breast cancer (RR = 1.11 95% CI = 1.00–1.23) and a 16% increase in benign breast disease (RR = 1.16 95% CI = 1.02–1.32). In addition, a stronger effect was observed with binge drinking pattern. In the European prospective investigation into cancer and nutrition (EPIC), a large multicenter European cohort, alcohol intake was associated with both pre- and postmenopausal breast cancer. However, breast cancer risk was stronger among women who started drinking prior to FFTP. An increase of 10 g of alcohol/day was related to an 8% (RR = 1.08 95% CI = 1.02–1.14) increased risk of ER− tumors in women who start drinking prior to FFTP, while no association could be detected among women who start drinking after FFTP.

Association of Adult Diet and Cancer in Population Studies

Fat

Among dietary factors, fat has received the greatest attention due to strong international correlations with rates of several cancers common in developed countries. However, prospective studies have consistently shown little relationship of fat intake with breast

cancer risk. Some epidemiological studies indicate that, rather than total fat intake, subtypes of fatty acids could be more determinant and diversely affect breast cancer risk; although, overall findings to date are conflicting. Epidemiological data on biomarkers of exposure to fatty acids and breast cancer risk are also limited. A meta-analysis of prospective studies has suggested a protective effect of n-3 PUFAs on breast cancer risk. One prospective study showed a significant association between high blood levels of industrial trans-fatty acids (ITFA) and increased risk of breast cancer. However, in general prospective studies have not shown clear associations between patterns of fatty acids and risk of breast cancer, overall and by hormonal receptor status. In two large RCT of low-fat diet, there was no significant effect on risk of breast cancer.

Prospective studies of colon cancer have also not supported the positive relationship with fat intake suggested by international comparison of cancer rates. Prospective studies of dietary fat and prostate cancer are fewer but do not generally support the relation with total or specific types of dietary fats. Intake of saturated fatty acids has been related to an increase in pancreatic cancer. In a recent meta-analysis including 5 cohort studies, an 11% increase in pancreatic cancer incidence per 10 g saturated fatty acid per day (RR = 1.11 95% CI = 1.01–1.21) was observed while no consistent results were observed for total fat intake. Studies of lung cancer have found inconsistent evidence of an association with total fat.

Red and Processed Meat

Numerous studies have shown an association between high intake of processed meat (such as ham, bacon, sausage and hot dogs), red meat (mainly beef, pork and lamb) and colorectal cancer. Processed meat has been classified as carcinogenic to human by the International Agency for Research on Cancer (IARC) (group 1) while red meat has been classified as probably carcinogenic to human (group 2A). A meta-analysis including 10 cohort studies has shown significant dose–response relationship and concluded to an18% increase (RR = 1.18 95% CI = 1.10–1.28) per 50 g/day of processed meat intake and a 17% increase (RR = 1.17 95% CI = 1.05–1.31) per 100 g/day of red meat. Processed meat cured with nitrite contains high content of nitroso compounds (NOCs), potent carcinogenic agent in animal experiment, and nitrosylated haem iron potential carcinogenic to human. Carcinogens formed during the cooking of meat heterocyclic aromatic amines (HAAs), polycyclic aromatic hydrocarbon, and benzopyrene have provided conflicting results, in part because of the difficulty in assessing dietary carcinogen intake. However, interaction between specific phenotypes of *N*-acetyltransferase 2 (NAT2) (rapid, intermediate or slow acetylation), and enzyme requires for the bioactivation of HAAs, and the risk of colorectal cancer has been observed. Red and processed meat intake has also been linked to stomach, pancreatic cancer and overall cancer mortality. Although consumption of meat during midlife or later has not been associated with risk of breast cancer, a positive association has been seen with intake in adolescent and early adult life. Studies on red meat and the risk of prostate cancer have been limited and results are inconsistent.

Salt Intake

Animal and human studies supported the cocarcinogenic effect of salt on gastric cancer through synergic action with Helicobacter pylori infection, in addition to some independent effects such as increase in the rate of cell proliferation and of endogenous mutations. Salt intake may also promote or enhance the carcinogenic effect of nitroso compounds. The epidemiologic evidence is consistent across studies investigating the intakes of salt, pickle vegetables, dry fish and other salty foods.

Dairy Products

Higher consumption of milk and dairy products have been associated with lower risk of colon cancer with a decreased risk of 13% (RR = 0.87; 95% CI = 0.83–0.90) per 400 g/day. Dairy products were not associated with rectal cancer. This protective effect is probably due to the calcium content, lactic-acid producing bacteria and potentially lactoferrin, vitamin D and short chain fatty acid butyrate. In contrast, higher consumption of dairy products has been associated with increased risk of total prostate cancer in many studies (RR = 1.07 95% CI = 1.02–1.12 per 400 g/day). The fat component of milk does not appear to account for these associations.

Fruits, Vegetables, Pulses and Phytochemicals

Higher intake of essential nutrients and other biological active constituents of plants have reduced cancer incidence in laboratory animals. However, the potential protective effect of fruit and vegetables against cancer observed in case control epidemiological studies has not been strongly supported in cohort studies and is limited to some cancers. Fruit and vegetable intakes have been inversely associated to cancer of the mouth, pharynx and larynx in a large consortium of case-control studies. Fruit and vegetable intake have also been inversely associated with oesophageal squamous cell carcinoma in a large meta-analysis (31 studies). The evidence for fruit intake and stomach cancer has been confirmed in a meta-analysis of 22 cohort studies showing that individual with lower fruit intake were at higher risk of stomach cancer and an association with citrus fruit was confirmed in a further meta-analysis of prospective cohort studies. A 13% reduction of gastric cancer according to the intake of citrus fruit when comparing highest to lowest intake was observed (RR = 0.87, 95% CI = 0.76–0.99). The effect was stronger for cardia gastric cancer than noncardia gastric cancer. This association was later confirmed in cohort studies based on plasma vitamin C. For vegetables, no association with stomach cancer was observed, but allium vegetables (garlic, onions and shallots) showed a protective effect in a large meta-analysis.

Lung cancer is the most common cancer death and fruits and vegetables containing carotenoids and other antioxidants have been hypothesized to decrease lung cancer risk. Data from large meta-analysis of cohort studies report a protective effect of fruits and vegetables on lung cancer risk. When comparing highest versus lowest intake, a 18% decrease risk was observed for fruit intake (RR = 0.82 95% CI = 0.76–0.89) and 8% decrease for vegetable intake (RR = 0.92 95% CI = 0.87–0.97). The effect was stronger in current smokers. For each increase of 100 g/day an inverse dose response was observed for vegetables (RR = 0.94 95%CI 0.89–0.98 and fruits (RR = 0.92, 95% CI = 0.89–0.95). Vegetable intake has been associated to a small decreased risk of colorectal cancer (2% decrease; RR = 0.98, 95% CI = 0.96–0.99 per100g/day). No effect was observed for rectal cancer. Intake of fruits was not related to the risk of colorectal cancer. For breast cancer, results from the pooling of large cohort studies have shown an inverse association between vegetable intake and ER− breast cancer (18% decrease, RR = 0.82 95% CI = 0.74–0.90 comparing highest vs. lowest vegetable intake). No protective effect was observed in ER+ tumors. Recent data from the EPIC study suggest a protective effect from vegetable fiber on breast cancer risk.

Whole Grain and Cereal Fiber

Whole grain is rich in fiber, phytochemicals, B vitamins and other active substances, while refined cereals retain almost only the starchy endosperm when the germ and bran are removed. The majority of studies focused on gastrointestinal cancers. Most of the evidence is related to the association of whole grain intake and colorectal cancer. Whole grain intake has been associated with a 7% decreased risk of colon cancer (RR = 0.83 95% CI = 0.78–0.89) per 90 g/day; no effect was observed for rectal cancer in a meta-analysis (4 studies). Association with other gastrointestinal cancer, genitourinary cancers (prostate and renal cancers), breast and endometrium and neck cancers are inconsistent.

Cereal fiber intake has been associated to a decreased risk of colorectal cancer with a decreased risk of 9% (RR = 0.91 95% CI = 0.88–0.94) per 10 g/day intake. This was confirmed by results from the large prospective EPIC study (RR = 0.89 95% CI = 0.82–0.97 per 10 g/day). For other gastrointestinal cancers data are sparse and inconclusive. No effect has been observed on genitourinary cancers. For head and neck cancer there is suggestion of a protective effect. For breast cancer a borderline protective effect has been observed cereal fiber.

Carbohydrate, Glycaemic Index, and Glycaemic Load

Cancer with potential links to diabetes leading to an increased risk include bladder cancer, breast cancer, colorectal cancer, endometrial cancer, liver cancer and pancreatic cancer while prostate cancer could possibly be lower among diabetic individuals. Many factors influence how rapidly carbohydrates are digested and absorbed and hence what their glycaemic and insulinemic effects will be. Refined carbohydrate such as pure sugar is rapidly absorbed. This physiologic response of carbohydrates can be quantified by the glycaemic index (GI), which compares the plasma glucose response to specific foods with that induced by the same amount of a standard carbohydrate source, usually white bread or pure glucose. The GI is therefore a measure of carbohydrate quality. However, both the quality and quantity of dietary carbohydrates need to be considered in relation to metabolic effects; the glycaemic load (GL) of a specific food, calculated as the product of GI and the amount of dietary carbohydrates in a food item, has been proposed as a global indicator of the glucose response and insulin demand induced by a serving of food. A recent meta-analysis of prospective studies reported a significant positive association between GI and GL and total diabetes-induced cancers risk; the pooled multivariable-adjusted RR of the overall diabetes related cancer risk when comparing the highest versus the lowest levels of GI and GL were 1.07 (95% CI = 1.04–1.11) for GI and 1.02 (95% CI = 0.96–1.08) for GL. For colorectal cancer a borderline positive association between GI and colorectal cancer risk was observed when comparing the highest with the lowest category of intake (RR = 1.08; 95% CI = 1.00–117). No significant association was observed between GL intake; for endometrial cancer risk no significant association was observed with GI intake while GL intake was significantly associated with a 21% greater risk of developing endometrial cancer compared with the lowest category of intake (RR = 1.21 95% CI = 1.07–1.37); for breast cancer RR comparing the highest with the lowest category were 1.06 (95% CI = 1.01–1.10) for GI and 1.04 (95% CI = 0.97–1.11) for GL. No association with GI or GL were observed for pancreatic and prostate cancers while data were very sparse for liver and bladder cancers (only 1 study for each) and no conclusion could be made.

Polyphenols and Food Containing Polyphenols

Polyphenols/flavonoids

Polyphenols are a large and diverse family of phytochemicals widely consumed by humans. Food sources for major classes of polyphenol include berries, citrus fruits, leafy vegetables, tea and chocolate, soybean and legumes for flavonoids; tea, berries, wine, fruits, vegetables and coffee for phenolic acid; and sesame seeds, whole grains, vegetables and olive oil for lignans.

Flavonoids are the most studied subgroup of polyphenols with regard to cancer risk. In recent epidemiological studies, total flavonoid intake has rarely been associated with a reduction in cancer risk. However, isoflavones, whose main dietary source is soy foods, plausibly reduce the risk of colorectal, breast, and prostate cancers, especially in Asian countries. Soy consumption during childhood and adolescence might be the most relevant period and only studies in Asian populations have evaluated this association. In Western countries, the greatest potential is from the intake of tea catechins, although evidence is limited and still controversial for colorectal, breast, and prostate cancers. Other flavonoids require much more extensive study in relation to major cancers,

especially in prospective studies. In brief, epidemiological evidence for a protective effect of polyphenol intake upon cancer risk remains weak. It should be kept in mind that many in-vitro studies have administered pharmacological doses of polyphenols in nonglycosylated form rather than as metabolites. Lignans are converted to the estrogen-like compounds enterolactone and enterodiol during digestion and could affect breast cancer.

Coffee and caffeine

Coffee is among the most commonly consumed beverage worldwide and has been associated to human health effects. Several beneficial compounds are present in coffee including antioxidants polyphenols (phenolic acid). Meta-analysis of coffee consumption have shown reduced risk in several cancers including endometrial, colon, liver, prostate, pancreatic and gallstone when comparing highest versus lowest consumption. However, to date, evidence of a probable protective effect is only observed for liver and endometrial cancers.

Vitamins and Minerals

Folate and B vitamins

Among epidemiological studies that explore the relation between folate intake and the risk of developing cancer, the evidence has been most compelling for colorectal cancer. In a meta-analysis of cohort studies, the resulting summary risk estimate for high versus low dietary folate intake has shown a marginal decreased risk of 8% when comparing the highest to the lowest intake (RR = 0.92, 95% CI = 0.81–1.05). The protective effect was larger for colon cancer (RR = 0.75, 95% CI = 0.57–0.99). This result was confirmed by a large prospective study with a 30% lower risk of developing colorectal cancer among subjects with the highest versus lowest dietary folate intake (RR = 0.81, 95% CI = 0.52–0.97). The protective effect is more pronounced in moderate to heavy consumers of alcohol, a known inhibitor of folate metabolism. A role of folate in colorectal carcinogenesis is supported by the association between a genetic polymorphism in MTHFR, and enzyme involved in folate metabolism and risk of colorectal cancer.

Epidemiological studies on the association between folate intake and breast cancer risk have provided mixed results. Results from a meta-analysis showed no significant association between dietary folate intake and breast cancer risk (pooled RR = 0.95, 95% CI = 0.87–1.03); however higher folate intake may reduce breast cancer risk for those with high alcohol intake. Results from prospective studies also suggest a U-shaped relationship for the dietary folate intake and breast cancer risk. Women with daily dietary folate intake between 153 and 400 mcg showed a significant reduced breast cancer risk compared with those <153 mcg, but not for those >400 mg. These results were further confirmed in the EPIC study in which, a 14% reduction in breast cancer risk was observed when comparing the highest with the lowest dietary folate tertiles in women having a high (>12 alcoholic drinks/week) alcohol intake (RR = 0.86, 95% CI = 0.75 to 0.98). There is no clear evidence for a significant association between blood folate levels and breast cancer risk.

A protective role for adequate folate status in other cancers is not compelling but there are some observations implying a protective role for adequate folate intake in oropharynx, esophagus, stomach, pancreas lung, cervix neuroblastoma and leukemia.

Vitamin D

Vitamin D is mostly synthesized in the skin by ultraviolet B radiation while dietary intake and supplements also contribute to overall vitamin D status. Several cancers have been associated with low sun exposure, in particular colorectal and breast cancers. An inverse association has also been observed between prediagnostic circulating serum 25-hydroxyvitamin D (25(OH)D) levels and risk for colorectal cancer. In a meta-analysis an inverse association of colorectal cancer risk was observed with dietary vitamin D (RR per 100 IU/day = 0.95, 95% CI = I 0.93–0.98; range of intake (midpoints) 39–719 IU/day) and serum/plasma 25-(OH)D (RR = 0.96, 95% CI = 0.94–0.97 per 100 IU/L; range 200–1800 IU/L). However, the association has been less consistent for other cancer types. In a recent large meta-analysis, the pooled RR of breast cancer for the highest (>500 IU/day, mean) versus lowest categories of vitamin D intake (<148 IU/day, mean) was 0.95 (95% CI = 0.88–1.01). The pooled RR of breast cancer for the highest (>31 ng/ml, mean) versus lowest categories of 25(OH)D blood levels (<18 ng/ml, mean) provided similar results. Several lines of evidence suggest that effects of vitamin D may be stronger for cancer mortality than for incidence. Cancer patients with higher prediagnostic 25(OH)D have lower risks of dying from prostate, colorectal and breast cancer.

Carotenoids

Carotenoids and retinol are considered biomarkers of fruits and vegetables intake, and have important antiinflammatory and antioxidant properties. In a meta-analysis of prospective studies of blood concentrations of carotenoids and retinol, and lung cancer risk, blood concentrations of α-carotene, β-carotene, total carotenoids, and retinol were significantly inversely associated with lung cancer risk or mortality. The summary RR was 0.66 (95% CI = 0.55–0.80) per 5 μg/100 mL of α-carotene, 0.84 (95% CI = 0.76–0.94) per 20 μg/100 mL of β-carotene, 0.66 (95% CI = 0.54–0.81) per 100 μg/100 mL of total carotenoids, and 0.81 (95% CI = 0.73–0.90) per 70 μg/100 mL of retinol. In stratified analysis by sex, the significant inverse associations for β-carotene and retinol were observed only in men and not in women. Carotenoids have also been linked to a decreased risk in cancer of the oropharynx. A systematic review and meta-analysis of cohort studies of dietary intake and blood concentrations of carotenoids and breast cancer risk reported stronger associations with blood concentrations than with dietary estimates, probably because the measurement error in the dietary assessment of carotenoid intake from fruits (such as mango, orange, tangerine, melon, papaya) and vegetables (such as carrots, pumpkin, tomato, kale, sweet potato) may have attenuated associations with breast cancer risk. Of

the six dietary carotenoids assessed, only intake of β-carotene was significantly associated with a reduced breast cancer risk (RR = 0.95 95% CI = 0.91–0.99; per 5000 mg/day). In contrast, blood concentrations of total carotenoids, β-carotene, α-carotene, and lutein were inversely associated with breast cancer risk. In a recent report, an inverse association between plasma carotenoids and risk of premalignant breast diseases was found in younger women, consistent with inverse associations reported for invasive breast cancer suggesting that carotenoids may play a role in breast cancer early development. The relative levels of the different types of carotenoids are important to take into account as well as a potential influence from other lifestyle factors.

Alcohol

The association between alcohol drinking and the risk of various cancers has been evaluated in a large number of case controls and cohort studies. Alcohol consumption has been convincingly associated to the cancer of the oral cavity, pharynx, larynx, esophagus, colorectal, liver (hepatocellular carcinoma) and female breast cancers. For the majority of these cancers, the association appears mostly linear. Among nonsmokers, reported RRs for oesophageal squamous cell carcinoma and laryngeal cancer range from no effect for light intake (1 drink per day or 10–12 g of ethanol) to a threefold increase (RR = 3.09, 95% CI = 1.75–5.46) for high intakes (4 or more drinks/day). Risks for oropharyngeal cancer are as high as fivefold increase (RR = 5.04, 95% CI = 4.49–6.50) in high intake. The combined exposure to alcohol drinking and tobacco smoking results in a synergistic effect which enhances the risk of these neoplasms up to 14-fold, among heavy-smokers and heavy drinkers (4 or more drinks/day).

Summary effect estimates for colorectal cancer are about 11% (RR = 1.11, 95% CI = 0.90–1.38) at one drink per day (corresponding to 10–12 g of ethanol) and 40% (RR = 1.41, 95% CI = 1.16–1.72) at more than four to five drinks per day. Associations of alcohol consumption with hepatocellular carcinoma are generally found at high intakes with significant increased risks of about 30%–40% at consumption levels >40 g/day. For breast cancer, the risk increases by 5% for premenopausal breast cancer (RR = 1.05, 95% CI = 1.02–1.08) and by 9% for postmenopausal breast cancer (RR = 1.09, 95% CI = 1.07–1.12) per 10 g/day of ethanol. While studies on binge drinking and cancer risk are still sparse and a standardized measure of exposure is lacking, the available evidence shows that the risk is increased between 33% (RR = 1.33, 95% CI = 1.11–1.59, for monthly binge drinking) and 55% (RR = 1.55, 95% CI = 1.07–2.26, for weekly binge drinking). For other cancers sites, kidney, bladder, stomach, ovary, small intestine, Hodgkin and non-Hodgkin lymphoma, ampulla of Vater, pancreas and thyroid, the evidence is considered "not conclusive".

Vitamin and Mineral Supplementation

The role of vitamin and mineral supplements in cancer prevention has been examined in both prospective and RCTs. Trials of beta-carotene and other single supplements, including vitamin E and selenium have not shown benefits. In some studies, adverse effects were observed: an increased risk of lung cancer with beta-carotene in heavy smokers and an increased risk of prostate cancer with high doses of vitamin E. In trials using combinations of multiple vitamins and mineral at lower doses than those in single supplements, a reduction in cancer rates has been seen in a Chinese population with multiple nutrient deficiencies as well as a decrease in overall mortality after 10 years follow up. However, no effect on overall and specific mortality was observed after 20 years follow up. Recent reports from the Physicians' Health Study (PHS) II, a RCT of daily multivitamins among US physicians, found fewer total cancers in multivitamin recipients, but no effect on overall mortality. Overall supplements with multivitamins and minerals are likely to be beneficial in populations with multiple nutrients deficiency but not in well-nourished individuals.

Randomized controlled trials of folic acid conducted among individuals with previously resected colonic adenomas provide conflicting results. Supplements of folic acid (with dose varying from 0.5 to 5 mg/day) did not reduce recurrent adenomas as a whole, while among subjects with lowest plasma folate a marginal reduction was observed. In one trial an increased risk of recurrent adenoma was seen. These results suggest that an adequate amount of folate intake is protective compared to an inadequate intake, but supplementation of those who are already folate-replete conveys no additional protection against colorectal cancer.

Total calcium intake is consistently associated with a reduction of colorectal cancer in observational studies. Recent results of large prospective studies (NHS and PHS) report a decreased risk of colon cancer when comparing high versus low intake ($\geq$1400 vs. <600 mg) (RR = 0.78, 95%CI = 0.65–0.95). In subjects reporting the use of calcium supplementation, a protective effect against colorectal cancer has been observed. However, trials have shown a protective effect of calcium supplementation on colorectal adenomas, a precursor of colorectal cancer, but not against colorectal cancer itself.

Several lines of evidence suggest that effects of vitamin D may act on cancer incidence and mortality. A meta-analysis of RCTs conducted over 2–7 years of duration, showed that vitamin D supplementations had little effect on total cancer incidence (400–1100 IU/day) (RR = 1.00, 95% CI = 0.94–1.06, comparing the intervention vs. the control groups) but significantly reduced total cancer mortality (400–833 IU/day, summary RR = 0.88, 95% CI = 0.78–0.98). In addition recent review and meta-analysis of RCTs suggested that vitamin D supplementation is not associated with a reduced risk of breast cancer.

Dietary Patterns

Dietary patterns are defined as the quantities, proportions, variety or combinations of different foods and beverages in diets, and the frequency with which they are habitually consumed.

The dietary pattern has gained considerable attention because nutrients and foods are eaten in combination and may interact; therefore, the evaluation of intake pattern provides a better understanding and is more readily available for dietary recommendations. The impact of dietary pattern on health outcomes including cancer has been reviewed recently to define the dietary guideline for American 2015–2020.

Approaches to derive dietary patterns

Several methods have been used to define dietary patterns. The first method is based on an "a priori index" based on a set of dietary recommendations for a healthy dietary pattern. An individual index/score is derived by comparing and quantifying their adherence to the criterion food/nutrient component of the index. Examples of dietary quality scores include: The Healthy Eating Index (HEI)—2005 and 2010, the Alternate HEI (AHEI) and updated AHEI—2010, the Recommended Food Score (RFS), the Dietary Approaches to Stop Hypertension (DASH) score, the Mediterranean Diet Score (MDS), and the Alternate Mediterranean Diet Score (aMed) (for a review see **USDA, 2015**). The second method of dietary pattern assessment is through data-driven approaches "some posteriori", such as cluster, factor analyses of other statistical methods to define specific patterns and link these patterns to a health outcome such as cancer. Generally, the dietary patterns investigated are a prudent or healthy dietary pattern (high in vegetables and/or fruits, poultry, fish, low-fat dairy and/or whole grains) as compared to the Western diet or so-called unhealthy diet (red or processed meats, refined grains, sweets and/or high-fat dairy).

The cancer sites that have been most investigated include colorectal, breast, lung and prostate. The scientific evidence has been classified as convincing, moderate and limited. For colon/rectal cancer, moderate evidence indicates an inverse association between dietary patterns that are higher in vegetables, fruits, legumes, whole grains, lean meats/seafood, and low-fat dairy, and moderate in alcohol; and low in red and/or processed meats, saturated fat, and sodas/sweets relative to other dietary patterns. Conversely, diets that are higher in red/processed meats, French fries/potatoes, and sources of sugars (i.e., sodas, sweets, and dessert foods) are associated with a greater colon/rectal cancer risk.

For breast cancer, moderate evidence indicates that dietary patterns rich in vegetables, fruit, and whole grains, and lower in animal products and refined carbohydrates, are associated with a reduced risk of postmenopausal breast cancer. The data regarding this dietary pattern and premenopausal breast cancer risk point in the same direction, but the evidence is limited due to fewer studies.

For lung cancer, limited evidence from a small number of studies suggests a lower risk of lung cancer associated with dietary patterns containing more frequent servings of vegetables, fruits, seafood, grains/cereals, and legumes, and lean versus higher fat meats and lower fat or nonfat dairy products. However, for lung cancer as well as prostate cancer no conclusions can be drawn due to the small number of studies and the wide variation in study design, dietary assessment methodology and prostate cancer outcome ascertainment.

Inflammatory dietary index

Chronic inflammation has been linked to alteration of cellular processes and carcinogenesis. Evidence also shows that diet plays a central role in the regulation of chronic inflammation. The dietary inflammatory index DII has been developed to categorized the inflammatory (anti or pro) potential of individual foods and nutrients based on an extensive review of literature. Higher score indicates a more proinflammatory diet while a lower score indicates a more antiinflammatory diet. For example, the Mediterranean diet characterized by high consumption of plant foods, whole grain products, vegetables, fruits, nuts and legumes and regular intake of fish and seafood, low intake of red and processed meat and high-fat dairy products, olive oil and moderate alcohol consumption (preferably red wine taken with meals) has been linked to antiinflammatory proprieties. A recent meta-analysis including 24 studies reports that highest versus lowest DII categories are associated to a 25% overall increase of cancer incidence (RR = 1.25 95% CI = 1.16–1.35) and a 67% higher cancer mortality (RR = 1.67 95% CI = 1.13–2.48). For each of the cancer studies (colorectal, breast and lung) higher DII was related to a high incidence of cancer. The DII provides a useful summary measure of total inflammatory potential of multiple food items and can inform cancer prevention efforts.

Future in the Field

Cancers are increasingly seen as metabolic diseases; however, the contribution of metabolic dysfunction to the onset or development of cancer remains poorly understood. Recently developed technologies including metabolomics and deep DNA sequencing have become major tools to explore the metabolism of cancer cells. Hundreds to thousands of metabolites can now be measured simultaneously in bio specimens and give a highly detailed picture of metabolites profiles in tumor tissues and biofluids. The metabolome—the sum of low molecular-weight metabolites at a given time—is the most fundamental biochemical indicator of biological systems and an indicator of both genetic and environmental conditions. Identification of metabolites and metabolic pathways can improve our understanding on the mechanisms of carcinogenesis and related nutritional factors. In addition, both the modulating effect of diet on epigenetics and the connection between diet and the microbiota are fields of intense research as potential mechanisms of action on cancer development.

See also: Cancer-Related Inflammation in Tumor Progression. Prevention and Control: Nutrition, Obesity, and Metabolism.

Further Reading

Arkan, M.C., 2017. The intricate connection between diet, microbiota, and cancer: A jigsaw puzzle. Seminars in Immunology 32, 35–42.

Baumann, J., Sevinsky, C., Conklin, D.S., 2013. Lipid biology of breast cancer. Biochimica et Biophysica Acta 1831, 1509–1517.

O'flanagan, C.H., Bowers, L.W., Allott, E.H., Hursting, S.D., 2017. Molecular and metabolic mechanisms underlying the obesity-cancer link. In: Romieu, I., Dossus, L., Willett, W.C. (Eds.), Energy balance and obesity. International Agency for Research on Cancer/World Health Organization, Lyon.

Guasch-Ferre, M., Bhupathiraju, S.N., Hu, F.B., 2018. Use of Metabolomics in Improving Assessment of Dietary Intake. Clinical Chemistry 64 (1), 82–98. https://doi.org/10.1373/clinchem.2017.272344.

International Agency for Research on Cancer, 2010. Alcohol consumption and ethyl carbamate, vol. 96. International Agency for Research on Cancer/World Health Organization, Lyon.

International Agency for Research on Cancer, 2015. Red meat and processed meat, vol. 114. Lyon, International Agency for Research on Cancer/World Health Organization.

Mason, J.B., Tang, S.Y., 2017. Folate status and colorectal cancer risk: A 2016 update. Molecular Aspects of Medicine 53, 73–79.

Moukayed, M., Grant, W.B., 2013. Molecular link between vitamin D and cancer prevention. Nutrients 5, 3993–4021.

Norat, T., Scoccianti, C., Boutron-Ruault, M.C., Anderson, A., Berrino, F., Cecchini, M., Espina, C., Key, T., Leitzmann, M., Powers, H., Wiseman, M., Romieu, I., 2015. European code against cancer 4th edition: Diet and cancer. Cancer Epidemiology 39 (Suppl 1), S56–66.

Rothwell, J.A., Knaze, V., Zamora-Ros, R., 2017. Polyphenols: Dietary assessment and role in the prevention of cancers. Current Opinion in Clinical Nutrition and Metabolic Care 20, 512–521.

Saha, S.K., Lee, S.B., Won, J., Choi, H.Y., Kim, K., Yang, G.M., Dayem, A.A., Cho, S.G., 2017. Correlation between oxidative stress, nutrition, and cancer initiation. International Journal of Molecular Sciences 18.

Sapienza, C., Issa, J.P., 2016. Diet, nutrition, and cancer epigenetics. Annual Review of Nutrition 36, 665–681.

Scoccianti, C., Cecchini, M., Anderson, A.S., Berrino, F., Boutron-Ruault, M.C., Espina, C., Key, T.J., Leitzmann, M., Norat, T., Powers, H., Wiseman, M., Romieu, I., 2016. European code against cancer 4th edition: Alcohol drinking and cancer. Cancer Epidemiology 45, 181–188.

USDA Department of Agriculture, 2015. Scientific report of the 2015 dietary guidelines advisory committee. Department of Human Services, Washington, DC.

Willett, W.C., Key, T., Romieu, I., 2014. Diet, obesity and physical activity. In: Stewart, B.W., Wild, C.P. (Eds.), World Cancer Report. International Agency for Research on Cancer/World Health Organization, Lyon, p. 2014.

Willett, W.C., 2013. Nutritional epidemiology. Oxford University Press, New York.

Zitvogel, L., Pietrocola, F., Kroemer, G., 2017. Nutrition, inflammation and cancer. Nature Immunology 18, 843–850.

World Research Cancer Funds International/American Institute for Cancer Research (WCRF/AICR) Continuous Update Project (CUP), 2017. *http://www.wcrf.org/int/research-we-fund/continuous-update-project-cup/cup-expert-panel* [Online], [Accessed].

DNA Methylation Changes in Cancer: Cataloguing

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Glossary

Bisulfite sequencing Bisulfite treatment of DNA converts cytosine residues to uracil, but 5-methylcytosine residues are unaffected, which reveals the methylation status of the region of interest at the single nucleotide level.

CpG island (CGI) Genomic regions with a high frequency of CpG dinucleotides, usually over 200 base pairs in length.

DNA methylation Addition of methyl-groups to cytosines, predominantly in the context of CpG dinucleotides.

Genome-wide DNA methylation profiling Analysis of the methylation status of CpGs in the regions of interest or in the whole genome.

High-throughput (or next-generation) sequencing Millions of DNA strands are sequenced in parallel, eliminating the need for fragment-cloning before nucleotide sequence determination. It provides single-nucleotide resolution.

Microarray (DNA chip) A laboratory tool (a microscope slide) that carries thousands of regions of the investigated genome, including promoters, CGIs and any nonrepetitive loci of the genome.

Restriction endonucleases Specific enzymes that cleave DNA into fragments at specific recognition sites. DNA cleavages can be affected by the methylation status at the recognition site.

Introduction

DNA methylation is an epigenetic process that converts cytosines, chiefly at CpG dinucleotide sequences, into their methylated state. The reaction is carried out by three catalytically active mammalian DNA methyltransferases, DNMT1, DNMT3A and DNMT3B. All of these enzymes are required for survival in mice pointing to a critical role of DNA methylation in development and cellular physiology. 5-methylcytosine, when occurring in gene regulatory sequences is generally associated with gene inactivity and loss of methylation can occur when genes become activated.

The first carcinogenesis-associated DNA methylation (i.e., 5-methylcytosine [5mC]) changes were described in the early 1980s when global DNA hypomethylation in tumors was observed. Several years later, the first gene-specific hypermethylation events were detected in tumor samples. Hypermethylation commonly affects CpG island sequences, which in some cases, may be associated with silencing of well-studied tumor suppressor genes such as CDKN2A or MLH1. Since then, a number of differentially methylated regions have been described in the context of all types of cancer. Some types of tumors, even different malignancies originating in the same tissue or organ, can be distinguished by their unique DNA methylation profiles. Tumor classification based on the methylation profile of numerous sequences within the tumor when compared to the corresponding normal tissue has only become possible after sufficiently sensitive genome-scale approaches for methylation analysis had been developed. This field has moved from conducting single gene analysis or scanning of a few selected genes to obtaining overviews of the changes occurring genome-wide (i.e., the "methylome").

To identify altered DNA methylation at the genome scale, a large number of approaches have been developed. The currently employed DNA methylome mapping methodologies can be traced back to three major principles, including DNA methylation-sensitive restriction endonucleases, sodium bisulfite modification of genomic DNA and DNA methylation-specific antibodies or proteins (Table 1). In the last 15 years, significant developments have been made in genome-wide DNA methylation profile analysis, whereby the methods are based on coupling the aforementioned three principle-based approaches with various microarray or next-generation sequencing (NGS) platforms.

Table 1 Commonly used methods for genome-scale analysis of DNA methylation and DNA hydroxymethylation profiles

Genome-wide DNA methylation profiling approaches		
DNA methylation sensitive endonuclease-based methods	Biological affinity-based methods	Sodium bisulfite treatment-based methods
Differential methylation hybridization	Methylated DNA immuno-precipitation (MeDIP)	BS-sequencing (targeted and whole genome)
HELP assay	MDB-Seq	RRBS
MCAM	MIRA	Infinium assay
	hMeDIP (5hmC)	TAB-Seq (5hmC)
	T4-BGT-assisted (5hmC)	Ox-BS-Seq (5hmC)

The methods discussed in this article are subdivided into restriction enzyme-based, biological affinity-based approaches and sodium bisulfite-dependent techniques.

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The recent discovery of 5-hydroxymethylcytosine (5hmC), the first in a series of oxidative products leading to active demethylation of 5mC, in mammalian DNA challenged almost all previously used DNA methylation (i.e., 5mC) mapping methods because they could not distinguish between 5mC and 5hmC bases. Thus, by employing "classical" 5mC-mapping technology, one needs to be careful if the goal is to distinguish between these two bases. Indeed, 5mC and 5hmC have completely different biological functions with 5hmC being often associated with active genes or their control elements and 5mC being linked to repressed or silent chromatin. This can be particularly critical in cases of investigating tissues where high levels of 5hmC are present, for example, in neurons of the brain. Accordingly, new techniques were developed for selective detection of the two closely related cytosine residues. In this article, we will present the most frequently used genome-wide DNA methylation profiling methods that can be effectively employed for cataloguing DNA methylation changes in tumors.

Methylation-Sensitive Restriction Endonuclease-Based Methods

This family of methylome mapping techniques relies on restriction endonucleases that have a different sensitivity towards the methylation status of cytosine residues at the cleavage site. Accordingly, the methylation status of cytosine determines the actual restriction endonuclease digestion pattern, which reflects the level of DNA methylation of the investigated genomic region. A number of the originally applied methods, including methylation-specific digital karyotyping (MSDK), methylation-sensitive representational difference analysis (MS-RDA), methylation-sensitive restriction fingerprinting (MSRF) and restriction landmark genomic scanning (RLGS), are not used very widely anymore. However, at least two of these techniques are still being used; the HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR) and the methylated CpG island amplification coupled to microarray (MCAM) method. Both methods employ adapter ligation and PCR amplification steps after the digestion of chromosomal DNA with methylation-specific enzymes. The PCR amplified and labeled fragment pools are hybridized to microarray platforms or the corresponding amplicons are sequenced by one of the available NGS platforms. Here, we present MCAM, which employs SmaI (methylation sensitive) and XmaI (methylation insensitive) restriction endonuclease pairs (Fig. 1). The unmethylated cleavage sites can be cut with the SmaI enzyme, generating blunt ended fragments. A second digestion with the XmaI enzyme can cut the methylated cleavage sites as well and generate sticky ends for the subsequent adapter ligation. PCR amplification and fluorescent labelling of amplicons is followed by hybridization of labeled fragments onto microarray platforms carrying CpG-rich regions, including promoters and CpG islands.

The main drawback of the methylation sensitive enzyme-based profiling methods is the uneven occurrence of SmaI/XmaI cleavage sites in the genomes. Consequently, a significant number of potentially interesting genomic regions, such as promoters and CpG islands, cannot be investigated because of the lack of this particular cleavage site. Numerous enzyme pair combinations have been used to overcome the described limitations, but all of them have reduced genomic coverage, which partly explains why the methylation sensitive enzyme-based approach has been less popular recently.

Recent studies have revealed that the DNA base N6-methyladenine (6 mA), previously considered to be a prokaryote-specific epigenetic modification, also occurs in higher organisms including mammals. This base can be mapped using restriction enzymes that are inhibited by 6 mA within the cleavage site, affinity-based approaches using 6 mA-specific antibodies, or by single molecule sequencing that distinguishes between 6 mA and other bases.

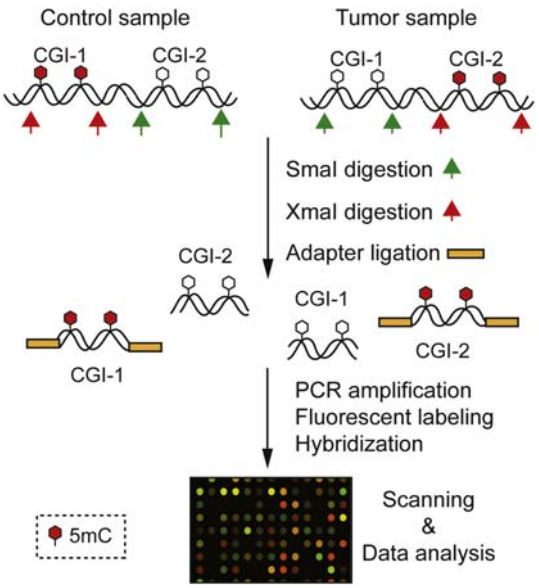

Fig. 1 The main steps of methylated CpG island amplification coupled to microarrays (MCAM). A detailed description of the method is provided in the text. *Arrows* point to *Sma*I and *Xma*I cleavage sites.

Biological Affinity-Based Methods

High affinity towards methylated DNA is the principle of these genome-wide DNA methylation-profiling techniques. Methylated DNA fragments can be affinity-purified by using either 5mC-specific antibodies or methyl-CpG-binding domain (MBD) proteins that specifically bind to methylated DNA sequences. Consequently, DNA fragments containing methylated CpG sites are effectively captured by these proteins (i.e., antibodies or MBDs) and can be selectively extracted and enriched. Using these approaches, methylation profiles can be generated with a resolution of approximately 100 base pairs, similar to chromatin immunoprecipitation (ChIP)-sequencing.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP first utilizes a 5mC-specific antibody to capture methylated DNA fragments after nonspecific fragmentation of genomic DNA (i.e., by sonication) (Fig. 2). Next, the immunoprecipitated DNA fraction and the input (nonenriched) genomic DNA can be labeled with different fluorescent dyes (e.g., Cy5 and Cy3) and cohybridized onto microarray platforms. The ratio of the fluorescent intensity of the two dyes reflects the methylation status of the region of interest. The main limitations of the MeDIP method are that single-stranded (denatured) DNA is needed for analysis and the quality of anti-5mC antibodies can be variable. MeDIP is compatible with NGS (MeDIP-seq), which provides much higher coverage than microarray-coupled technologies.

MBD Protein-Based Affinity Pull-Down Methods

A relatively small family of proteins including MBD1, MBD2, MBD3, MBD4 and MeCP2, possess a very similar methyl-CpG-binding domain. With the exception of human MBD3, each of these proteins is capable of binding specifically to methylated 5mC containing DNA. An affinity column-based method was initially developed, which employed the methyl-CpG binding domain of MeCP. However, it was recently demonstrated that MeCP2 could also bind to 5hmC with good affinity and therefore may not effectively discriminate between 5mC and 5hmC bases. This indiscriminative nature of MeCP's MBD can compromise the accuracy of methylation profile mapping. However, the other MBD proteins' recombinant MBD fragments can be fused to affinity tags (e.g., His- or GST-tag) and be used for capturing the methylated DNA fraction from genomic DNA fragment pools. These fragments can then be analyzed, as done earlier using microarrays or, more recently, by employing NGS.

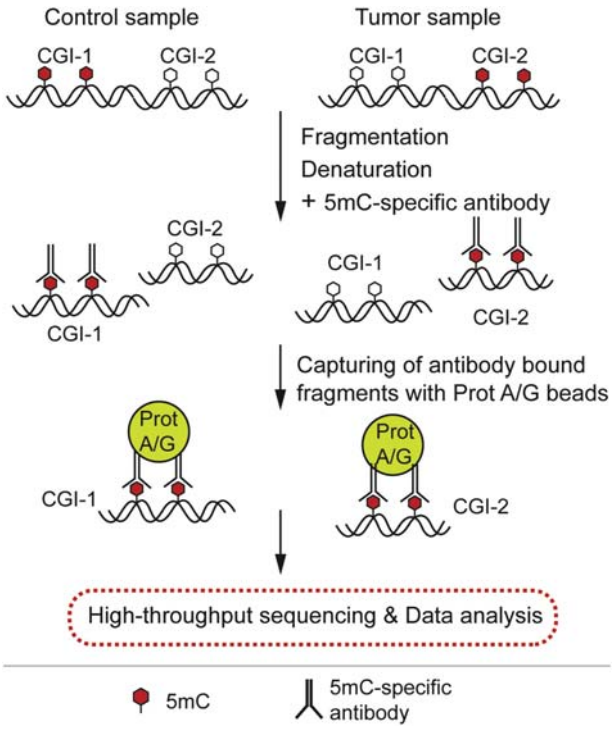

Fig. 2 Methylated DNA immunoprecipitation sequencing (MeDIP-seq). The detailed description is provided in the text.

Methylated-CpG Island Recovery Assay (MIRA)

MBD2b, the shorter isoform of MBD2, has the highest affinity to methylated DNA and can form heterodimers with other MBD proteins via its C-terminal coiled-coil domain. One of its interacting partners is MBD3L1. MBD3L1 has no DNA binding domain itself, but can act as a hetero-dimerization partner and then bind to methylated DNA. There is no dependence on DNA sequences other than that it requires a minimum of two methylated CpGs in the captured fragment. The MBD2b/MBD3L1 heterodimer complex has a higher affinity to methylated DNA templates than MBD2b alone, a feature that is harnessed in the methylated-CpG island recovery assay (MIRA) (Fig. 3). Bacterially expressed recombinant GST-MBD2b and His-MBD3L1 proteins are mixed and incubated with the fragmented genomic DNA. The heterodimer complex captures methylated DNA fragments, which are then purified, PCR amplified, labeled and analyzed on microarray platforms (MIRA-chip). The MIRA-enriched methylated fraction can be also coupled with NGS platforms (MIRA-seq). MIRA can reliably be performed on a few hundred nanograms of genomic DNA. The MIRA technique was used to profile DNA methylation patterns at a resolution of 100 base pairs in the entire genome of human B lymphocytes, providing one of the first mammalian "methylome" data sets.

Affinity-Based 5hmC Mapping Methodologies

Discovery of 5hmC in mammalian genomic DNA demanded new technologies that could distinguish between 5hmC and 5mC bases. For this purpose, two biological affinity-based methods were developed.

The hMeDIP approach is based on a 5hmC-specific antibody that has been used for immunoprecipitation of DNA sequences harboring 5hmC residues. This method is similar to the detailed MeDIP technique, except that the employed high affinity antibody is specific for 5hmC in single-stranded DNA (Fig. 2).

Another method(s) requires the specific modification of 5hmC using T4 bacteriophage-derived β-glucosyltransferase (T4-BGT), which can transfer a modified glucose moiety to the hydroxyl group of 5hmC. Glucosylated 5hmC residue carrying fragments can be enriched by using a specific protein (e.g., Jbp1) or can be further modified to be suitable for biotin-streptavidin pull-down (Fig. 4). NGS is conducted on the enriched fractions to annotate the genomic locations of 5hmCs. The best resolution that can be achieved by these methods is about 100 bp, which is determined by the size fragmentation and by the employed NGS platform.

Sodium Bisulfite Treatment-Based Methods

The previously discussed genome-wide DNA methylation mapping techniques provide valuable information regarding the locations of 5mC and 5hmC residues, but they also have several obvious drawbacks, including the relatively low resolution of mapping,

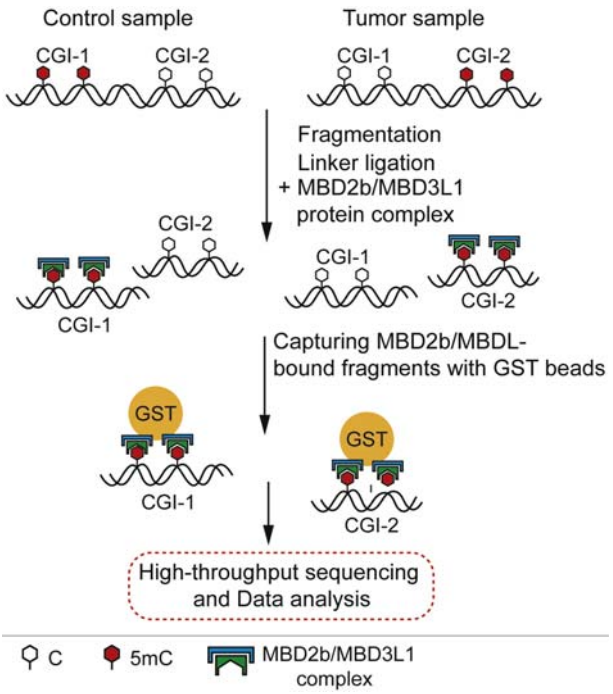

Fig. 3 Methylated-CpG island recovery assay (MIRA-seq). Please see text for detailed description of this methodology.

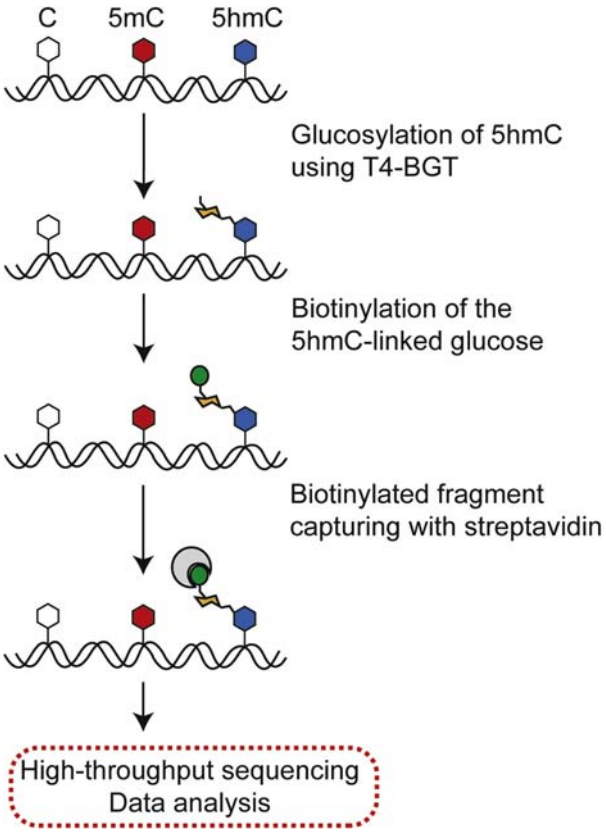

Fig. 4 Mapping of 5-hydroxymethyl cytosine (5hmC) in the genome. Detailed description is provided in the text.

and that the number of the modified nucleotides cannot be identified at the single nucleotide level. Bisulfite treatment-based profiling approaches overcome these issues inasmuch as they provide single base resolution and modified cytosine residues are countable within single molecules. Bisulfite sequencing is based on the premise that unmodified cytosine and 5mC showing different sensitivity towards sodium bisulfite treatment. Sodium bisulfite provokes deamination of cytosine and turns it into uracil, but 5mC is resistant to bisulfite-induced deamination. Thus, bisulfite treatment introduces specific changes in the DNA sequence, which depends on the methylation status of cytosine residues. Whole-genome bisulfite sequencing (WGBS) consists of several main steps: NGS library preparation, high-throughput sequencing of NGS libraries, and data analysis. NGS library preparation is a multi-step process that involves the fragmentation of genomic DNA followed by adapter ligation, bisulfite conversion and amplification using adapter-specific PCR primers. The actual nucleotide sequence analysis is conducted by using Roche 454, Illumina, or ABI/SOLiD platforms. Finally, the collected sequencing reads are aligned to "computationally bisulfite-converted" model genomes and the actual methylation status of all cytosines is revealed. Although WGBS is a powerful method that provides the most complete genome-wide DNA methylation profiling data, the associated high cost of sequencing still limits its widespread application (about $ 2000–4000 per sample). Therefore, a related method, the reduced representation bisulfite sequencing (RRBS) (Fig. 5), was developed, which is a cost-effective alternative to WGBS, although only 85% of CpG islands and 60% of promoters can be investigated. In RRBS, genomic DNA is digested with MspI, a methylation-insensitive endonuclease, which is followed by adapter ligation and fragment size selection. Adapter-ligated fragments in the several hundred bp size range are isolated and subjected to bisulfite conversion. Bisulfite-treated samples are PCR amplified and sequenced. RRBS is frequently employed when high-throughput and low-cost DNA methylation profiling is desired, such as in clinical applications. The method is very sensitive and requires low input allowing the analysis of limited numbers of cells.

Infinium Methylation Bead Arrays

The Illumina Infinium methylation assay is a company-developed platform that provides quantitative array-based measurement at the single-CpG-site level (Fig. 6). The assumption behind this experimental design (i.e., focusing on a single CpG in a particular region of interest) is that neighboring CpGs are usually (but not always) at similar methylation states. The monitored CpG sequences are selected from promoter regions, first exons, gene bodies and 3'UTRs. The flagship of this methylation platform has been the Infinium 450 k methylation assay, which could be used for detecting the methylation status of ~480,000 CpG of

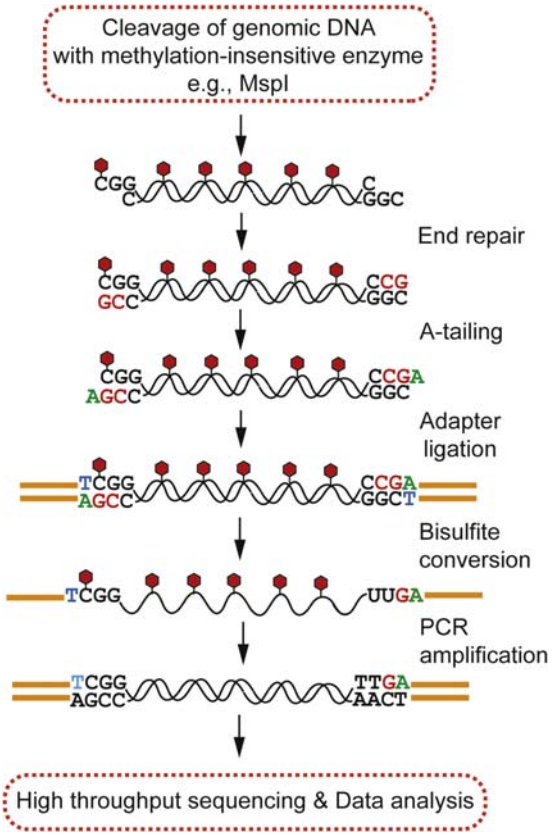

Fig. 5 Reduced representation bisulfite sequencing (RRBS). *MspI* cleavage enriches genomic fragments from CpG islands and other CpG-rich regions. See text for details.

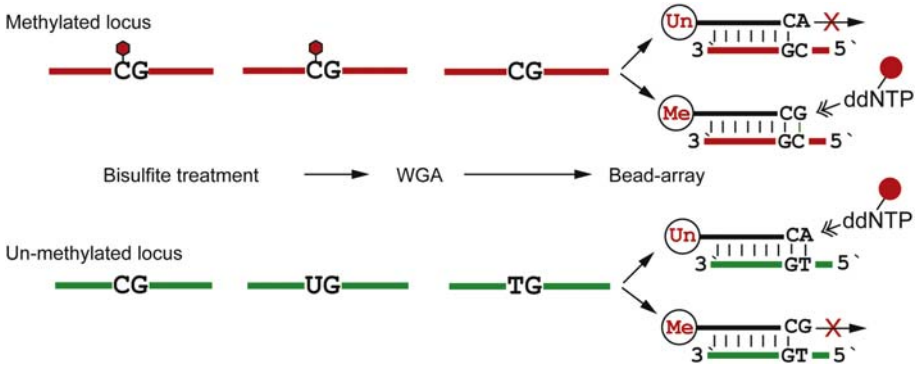

Fig. 6 Infinium 450 k methylation bead-array.The detailed description is provided in the text.

the human genome. An improved version of this platform, the EPIC array, is suitable to methylation profiling of ~850,000 CpG sites. The EPIC array now covers 99% of RefSeq gene promoters, 95% of CpG islands, many distant enhancers and miRNA promoters. However, the coverage of the large number of enhancers in mammalian genomes is still limited. Genomic DNA is bisulfite-treated and amplified, employing a special DNA polymerase (Phi29) with very low error rate. Whole genome amplification is carried out and the product of this reaction is enzymatically fragmented and applied to the Infinium chip (bead array). On the chip, there are two bead types for each CpG site per locus. Each bead type is attached to a single stranded 50-mer DNA oligonucleotide that differs in sequence only at the 3′ end, which corresponds to the investigated cytosine at CpG sequences. The bisulfite converted and denatured DNA can hybridize to either the methylation-specific probe or the nonmethylation probe, and in a single-base extension reaction, labeled dideoxynucleotides can be incorporated. Through conducting appropriate labelling and

scanning, the methylation status of the particular CpG site can be determined. This methylation bead array technology is now used very commonly in basic research and clinical settings.

5hmC Mapping at Single Nucleotide Level

Soon after the discovery of 5hmC in mammalian genome, it was realized that the conventional bisulfite treatment cannot be used directly for identifying 5hmC, since both bases are resistant to this treatment. Thus, such methodologies have been developed that can distinguish between these two closely related nucleotides. Currently, there are two available methods that can be used for unambiguous identification of 5hmC at single-base resolution.

TET-Assisted Bisulfite Sequencing (TAB-Seq)

The TET enzymes catalyze the oxidative demethylation of 5mC in a stepwise manner. The TETs convert 5mC to 5hmC, then 5-formylcytosine (5fC), and then 5-carboxylcytosine (5caC). It is thought that 5fC and 5caC are then converted to unmodified C by base excision repair initiated by thymine DNA glycosylase (TDG). In TAB-seq (Fig. 7. left panel), 5hmC is first protected from further oxidation by using T4-BGT enzyme that transfers a glucose moiety to the hydroxyl group of 5hmC. Next, DNA is treated with recombinant TET1 enzyme or its catalytic domain that oxidizes the 5mC to 5caC, while leaving the glucosylation-protected 5hmC intact. Subsequent bisulfite treatment converts unmodified C and 5caC residues to uracils that can be read as T after sequencing. Accordingly, any C-containing reads at CpG sequences in the resulting sequence can be interpreted as a direct readout of 5hmC.

Oxidative Bisulfite Sequencing (OxBS-Seq)

In OxBS-Seq (Fig. 7. Right panel), first, 5hmC is oxidized to 5fC by employing a strong oxidizing agent (KRuO4). KRuO4 treatment of DNA leaves 5mC and unmodified C residues intact. Unlike 5mC and 5hmC, 5fC is sensitive to bisulfite-induced deamination; thus, subsequent bisulfite-treatment converts the 5fC to uracil. Next, the KRuO4 and bisulfite treated DNA is subjected to NGS. In OxBS-Seq, T reads in the resulting sequence can be either originating from 5hmC or from unmodified C. Therefore, OxBS-Seq and conventional BS-Seq must be run parallel and, by comparing the two sequences, 5hmC can be identified. The

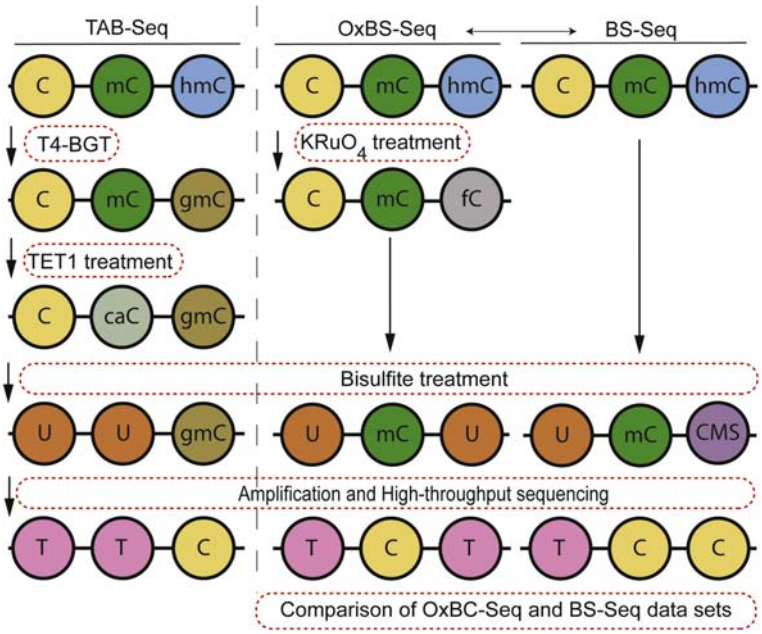

Fig. 7 TET-assisted bisulfite sequencing (TAB-seq) and oxidative bisulfite sequencing (OxBS-Seq). *Left panel* depicts TAB-seq, *right panel* describes the main steps of OxBS-Seq. OxBS-Seq displays 5mC only and requires a comparison to standard BS-seq, which shows the sum of 5mC and 5hmC. A detailed description of these approaches is provided in the text. *caC*, 5-Carboxylcytosine; *CMS*, cytosine-5-methylsulfonate; *gmC*, glucosylated 5-hydroxymethylcytosine; *hmC*, 5-hydroxymethylcytosine; *mC*, 5-methylcytosine.

disadvantage of OxBs-Seq that it displays 5hmC only indirectly, but the advantage is that this methodology does not require recombinant TET protein.

Prospective Vision

Epigenome-focused studies have already made remarkable progress towards exploring carcinogenesis-involved genes, pathways and mechanisms. It is quite predictable that DNA profiling techniques will receive more and more applicability and credit in clinical diagnostics. Application of high-throughput technologies can contribute to better cancer management in multiple ways: (i) by exploring cancer-specific epigenetic biomarkers, these methods can help in early tumor detection and prognosis, (ii) by monitoring and evaluating the efficacy of therapies, and (iii) by revealing novel "druggable" targets. The method of choice depends on multiple factors, including the number and complexity of the investigated regions, the depth of the required information (i.e., single base resolution is needed, or lower resolution is sufficient). Cost may have a decisive role as well. It is expected that the technological development of high-throughput approaches will continue, which might allow for more cost-effective analysis. The currently employed DNA profiling techniques are multistep and still time-consuming methodologies. New technologies, such as nanopore sequencing, are on the horizon; they allow eliminating PCR amplification and fluorescent labelling from the protocol. Nanopore sequencing has the potential to discriminate between the four canonical DNA bases and their oxidized derivatives.

In summary, efficient cataloguing of cancer-associated DNA methylation changes has more than academic significance inasmuch as it can provide novel inroads to diagnosis and choice of treatment options.

See also: Chromatin Dynamics in Cancer: Epigenetic Parameters and Cellular Fate. Defective 5-Methylcytosine Oxidation in Tumorigenesis. DNA Methylation Changes in Cancer: Mechanisms. Mutations in DNA Methyltransferases and Demethylases.

Further Reading

Booth, M.J., Ost, T.W., Beraldi, D., Bell, N.M., Branco, M.R., Reik, W., et al., 2013. Oxidative bisulfite sequencing of 5-methylcytosine and 5-hydroxymethylcytosine. Nature Protocols 8 (10), 1841–1851.

Brinkman, A.B., Simmer, F., Ma, K., Kaan, A., Zhu, J., Stunnenberg, H.G., 2010. Whole-genome DNA methylation profiling using MethylCap-seq. Methods 52 (3), 232–236.

Cross, S.H., Charlton, J.A., Nan, X., Bird, A.P., 1994. Purification of CpG islands using a methylated DNA binding column. Nature Genetics 6 (3), 236–244.

Dedeurwaerder, S., Defrance, M., Calonne, E., Denis, H., Sotiriou, C., Fuks, F., 2011. Evaluation of the Infinium methylation 450K technology. Epigenomics 3 (6), 771–784.

Estecio, M.R., Yan, P.S., Ibrahim, A.E., Tellez, C.S., Shen, L., Huang, T.H., et al., 2007. High-throughput methylation profiling by MCA coupled to CpG island microarray. Genome Research 17 (10), 1529–1536.

Hahn, M.A., Szabo, P.E., Pfeifer, G.P., 2014. 5-Hydroxymethylcytosine: A stable or transient DNA modification? Genomics 104, 314–323.

Huang, Y., Pastor, W.A., Shen, Y., Tahiliani, M., Liu, D.R., Rao, A., 2010. The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One 5, e8888.

Jin, S.G., Kadam, S., Pfeifer, G.P., 2010. Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic Acids Research 38, e125.

Jung, M., Kadam, S., Xiong, W., Rauch, T.A., Jin, S.G., Pfeifer, G.P., 2015. MIRA-seq for DNA methylation analysis of CpG islands. Epigenomics 7 (5), 695–706.

Khulan, B., Thompson, R.F., Ye, K., Fazzari, M.J., Suzuki, M., Stasiek, E., et al., 2006. Comparative isoschizomer profiling of cytosine methylation: The HELP assay. Genome Research 16 (8), 1046–1055.

Kriaucionis, S., Heintz, N., 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324 (5929), 929–930.

Rauch, T., Li, H., Wu, X., Pfeifer, G.P., 2006. MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. Cancer Research 66 (16), 7939–7947.

Rauch, T.A., Wu, X., Zhong, X., Riggs, A.D., Pfeifer, G.P., 2009. A human B cell methylome at 100-base pair resolution. Proceedings of the National Academy of Sciences of the United States of America 106 (3), 671–678.

Sun, Q., Huang, S., Wang, X., Zhu, Y., Chen, Z., Chen, D., 2015. N6-methyladenine functions as a potential epigenetic mark in eukaryotes. BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology 37 (11), 1155–1162.

Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., et al., 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324 (5929), 930–935.

Weber, M., Davies, J.J., Wittig, D., Oakeley, E.J., Haase, M., Lam, W.L., et al., 2005. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nature Genetics 37 (8), 853–862.

Yu, M., Hon, G.C., Szulwach, K.E., Song, C.X., Jin, P., Ren, B., et al., 2012. Tet-assisted bisulfite sequencing of 5-hydroxymethylcytosine. Nature Protocols 7 (12), 2159–2170.

DNA Methylation Changes in Cancer: Mechanisms

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Glossary

CpG island CpG sites are observed less often than expected in the genome. However, there are clusters of CpG sites, and such a cluster is named as a CpG island. If a CpG island is located at a promoter region, its DNA methylation can strongly repress transcription of its downstream gene.

CpG island methylator phenotype (CIMP) In some cancers, a large number of CpG islands are simultaneously methylated, and such a phenotype is designated as the CpG island methylator phenotype. CIMP can be associated with specific cancer pathophysiology, such as patient prognosis and drug sensitivity.

DNA methylation DNA modification that occurs at the 5th position of the cytosine residue (5-methylcytosine, 5-mC) within a CpG dinucleotide (CpG site).

DNMT Enzymes that can catalyze DNA methylation are collectively designated as DNA methyltransferases (DNMTs), and DNMTs can be classified into two classes, de novo type (DNMT3A and DNMT3B) and maintenance type (DNMT1). DNMT3A and DNMT3B are mainly involved in the establishment of DNA methylation status during early embryogenesis. DNMT1 is mainly involved in the maintenance of DNA methylation status at DNA replication.

TET An enzyme that can catalyze the conversion of 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carbocylcytosine (5-caC). 5-hmC, 5-fC, and 5-caC are removed by base excision repair. Therefore, TET enzymes are critical for the DNA demethylation process.

Introduction

DNA methylation is involved in various biological processes, such as embryogenesis, tissue differentiation, genomic imprinting, and inactivation of the X chromosome. At the same time, DNA methylation status can be altered by exposure to various environmental stimuli, and alterations of DNA methylation status are deeply involved in development and progression of human cancers.

Regulation Mechanism of DNA Methylation

DNA methylation is present at the 5th position of the cytosine residue (5-methylcytosine, 5-mC) within a CpG dinucleotide (CpG site) (Fig. 1). DNA methylation status is established during early embryogenesis by de novo type DNA methyltransferases (DNMTs), DNMT3A and DNMT3B. Once DNA methylation status is established, it is accurately transmitted upon somatic cell division (DNA replication) by maintenance DNMT (DNMT1) (Fig. 2).

At the same time, DNA demethylation can take place at specific biological periods in preimplantation embryos and primordial germ cells. Biochemically, 5-mC can be converted to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and finally 5-carboxylcytosine (5-caC) by tet methylcytosine dioxygenase (TET). These modified bases are removed by the action of a DNA repair pathway, namely base excision repair (BER), and unmethylated cytosine is incorporated (Fig. 1).

Changes of DNA Methylation in Cancers

Regional Hypermethylation

DNA methylation status is altered in cancer cells, and the alteration is generally classified into two types; regional hypermethylation and global hypomethylation (Fig. 3). Regional hypermethylation, also called aberrant DNA methylation, represents increased DNA methylation of a CpG island (a cluster of CpG sites) unmethylated in normal cells. Aberrant DNA methylation of a promoter CpG island completely represses transcription of its downstream gene (methylation-silencing) (Fig. 4A), and can be involved in repression of various tumor-suppressor genes, depending upon cancer types. *RB* gene is repressed in many types of cancers, including retinoblastomas and pituitary adenomas, *CDKN2A* also in a variety of cancers, *BRCA1* gene predominantly in breast and ovarian cancers, *CDH1* gene in gastric and breast cancers, *MLH1* gene in colorectal, gastric, and lung cancers, and *RASSF1A* gene in various cancer types, such as breast and lung cancers.

In addition to tumor-suppressor genes, aberrant DNA methylation can be present at promoter CpG islands of thousands of genes. Most of such genes are not transcribed in corresponding normal tissues (as described in section "Aberrant DNA Methylation Induction by Environmental Stimuli"). Since these genes are considered not to be functional in normal tissues, their aberrant DNA methylation is not considered to be "driver methylation" but "passenger methylation".

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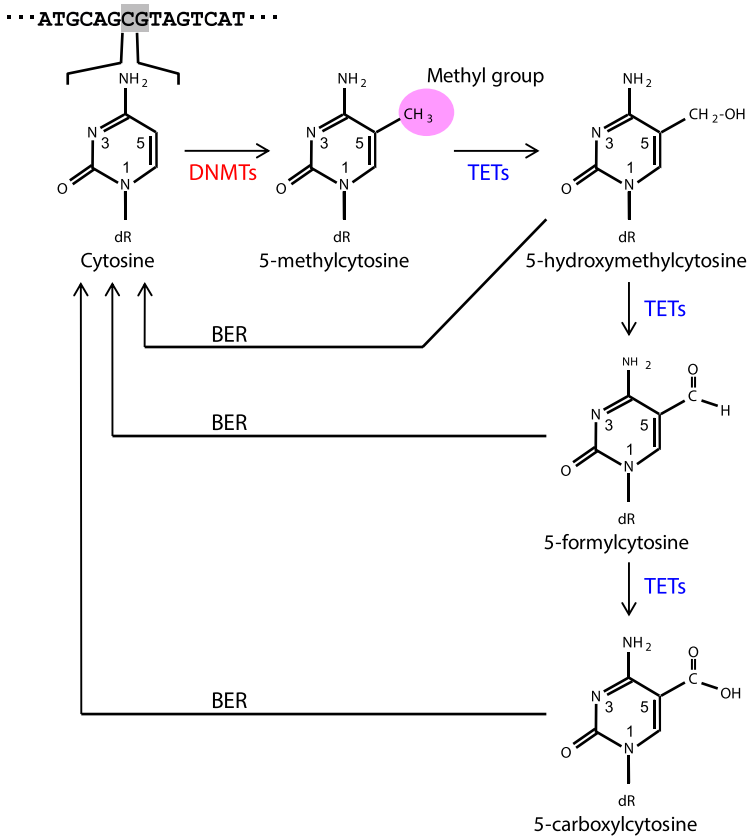

Fig. 1 DNA methylation and demethylation. DNA methylation is present at the 5th position of the cytosine residue (5-methylcytosine, 5-mC) within a CpG dinucleotide (CpG site). 5-mC can be converted to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and finally 5-carboxylcytosine (5-caC) by tet methylcytosine dioxygenase (TET). These modified bases are finally removed by base excision repair (BER).

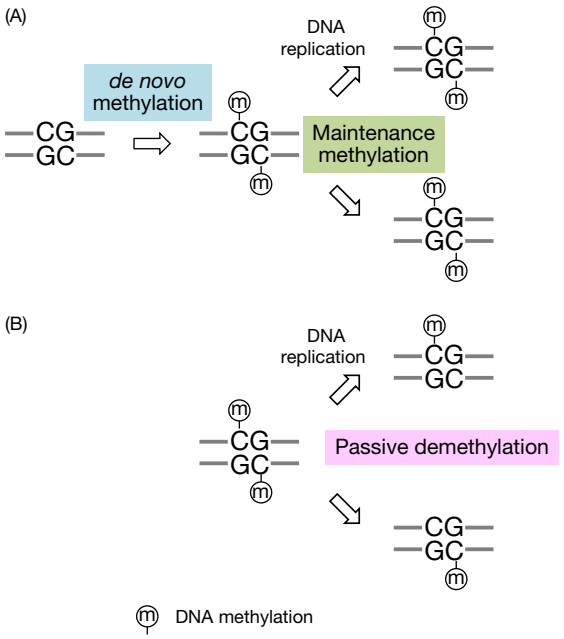

Fig. 2 Maintenance of DNA methylation. Once DNA methylation status is established, it can be accurately transmitted, even after cell division by maintenance DNMT (DNMT1) (A). When maintenance methylation is inhibited, passive DNA demethylation takes place (B).

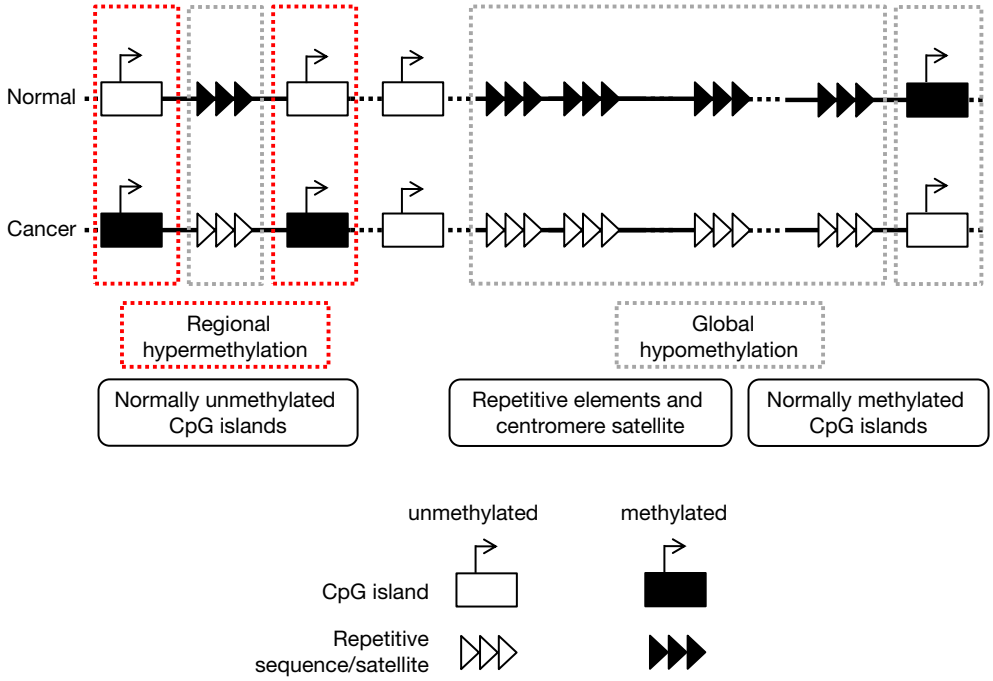

Fig. 3 Alterations of DNA methylation in cancer cells. They are generally classified into two types, namely regional hypermethylation and global hypomethylation. Regional hypermethylation represents increased DNA methylation of a CpG island unmethylated in normal cells. Global hypomethylation represents decreased 5-mC content in genomic DNA, due to mainly demethylation of repetitive sequences highly methylated in normal cells.

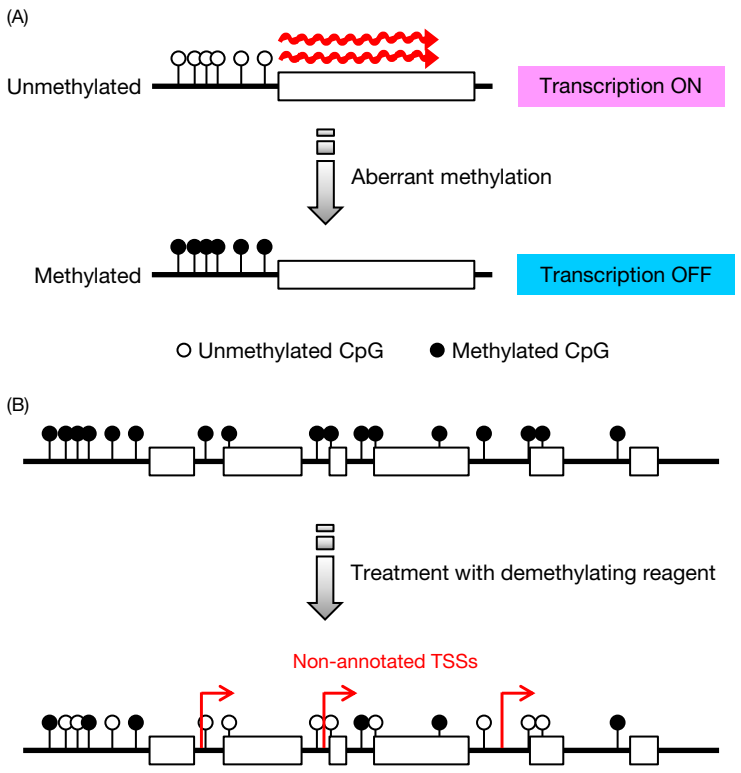

Fig. 4 Regulation of gene transcription by DNA methylation. (A) Repression of gene transcription by aberrant DNA methylation. Aberrant DNA methylation of a promoter CpG island completely represses transcription of its downstream gene (methylation-silencing), and can be deeply involved in the repression of various tumor-suppressor genes. (B) Generation of aberrant transcripts by DNA hypomethylation. Treatment of cells with a DNA demethylating reagent induced aberrant transcripts from thousands of non-annotated transcription start sites via demethylation of a gene body.

Global Hypomethylation

Global hypomethylation represents decreased 5-mC content in genomic DNA (Fig. 3), mostly due to demethylation of repetitive sequences (retrotransposons, Alu and LINE, and centromeric satellite DNA, α-satellite) highly methylated in normal cells. Global hypomethylation can lead to elevation of mutation rates, thus is involved in chromosomal instability. In addition, hypomethylation of promoter CpG islands of normally methylated genes can lead to their activation, including cancer-testis antigens, such as *MAGE-A1* and *NY-ESO-1*. Activation of oncogenes by hypomethylation has been proposed, but may need more robust analysis.

Recently, it has been reported that treatment of cells with a DNA demethylating agent induces aberrant transcripts from thousands of treatment-induced non-annotated transcription start sites (TSSs) via demethylation of the gene body (Fig. 4B). These aberrant transcripts can generate aberrantly truncated proteins, which are potentially involved in the dysregulation of cellular functions and immunogenicity. Global hypomethylation in cancer cells is proposed to lead to induction of such aberrant transcripts.

CpG Island Methylator Phenotype

A large number of CpG islands can be simultaneously hypermethylated in a cancer, and such status is referred to as the "CpG island methylator phenotype (CIMP)". The presence of cancers with the CIMP was first reported in colorectal cancers. Subsequently, cancers with the CIMP have been reported in various types of cancers, such as gastric cancers, glioblastomas, hepatocellular carcinomas, lung cancers, neuroblastomas, and renal cancers.

The CIMP is associated with specific cancer pathophysiology, such as patient prognosis and drug sensitivity, depending upon cancer types. In colorectal cancers, the CIMP is associated with old age, right-side location, and female patients. The association between the CIMP and patient prognosis in colorectal cancers is controversial, and associations with both good prognosis and poor prognosis have been reported. In neuroblastomas, the prognosis of patients with the CIMP is much poorer than those without. The predictive power of the CIMP is stronger than that of *MYCN* amplification, a clinically utilized prognostic marker. In lung cancers and renal cell carcinomas, the prognosis of patients with the CIMP is also poorer. In contrast, in glioblastomas and breast cancers, the prognosis of patients with the CIMP is better than that without.

As for the mechanisms of how the CIMP is induced, there had not been known. However, *IDH* mutations in glioblastomas and *SDH* (succinate dehydrogenase) mutations in gastrointestinal stromal tumors were recently shown to be causal for the CIMP. In contrast, an association between the CIMP and a *BRAF* mutation has been known for a long time in colorectal cancers, but its causal role has not been demonstrated.

Expression Changes of DNA Methylation Machineries in Cancers

Genes Involved in DNA Methylation

All the three DNMTs have been shown to be up-regulated in various types of cancers, such as breast cancers, colorectal cancers, and liver cancers. Therefore, the upregulation of DNMTs is a possible mechanism of aberrant DNA methylation induction. In addition, UHRF1 (Np95), which is required for maintenance of DNA methylation, is also upregulated in lung cancers and bladder cancers, and may be involved in aberrant methylation induction.

Genes Involved in Histone Modification

Expression changes of epigenetic machineries other than those directly involved in DNA methylation status have also been reported in various types of cancers. EZH2, a methyltransferase of histone H3 lysine 27 (H3K27), is upregulated in various types of cancers, such as prostate cancers, breast cancers, bladder cancers, gastric cancers, lung cancers, and liver cancers. The upregulation is often associated with aggressive phenotypes, such as the presence of metastasis and poor prognosis.

It is known that genes aberrantly methylated in cancer cells are frequently marked with trimethylation of H3K27 (H3K27me3) in their corresponding normal cells (as described in section "Aberrant DNA Methylation Induction by Environmental Stimuli"). Therefore, changes of H3K27me3 status by EZH2 upregulation may be possibly involved in aberrant DNA methylation induction.

Genetic Alterations of DNA Methylation Machineries in Cancers

Genes that Methylate DNA

Cancer genome analysis has revealed that various genes encoding epigenetic machineries are mutated, depending upon cancer types (Fig. 5). As for the genes that methylate DNA, DNMT3A mutations, mainly located within its C-terminal catalytic domain, especially at Arg882 (such as R882C, R882S, and R882H), have been reported in acute myeloid leukemia (AML). However, it had been unclear whether these *DNMT3A* mutations are a gain-of-function mutations or not (a loss-of-function mutations). Initially, it was reported that the *DNMT3A* mutations caused the reduction of its enzymatic (DNA methylation) activity, and were considered as loss-of-function mutations. This reduction of enzymatic activity was shown to lead to hypomethylation of *HOXB* cluster genes, critical genes for leukemogenesis.

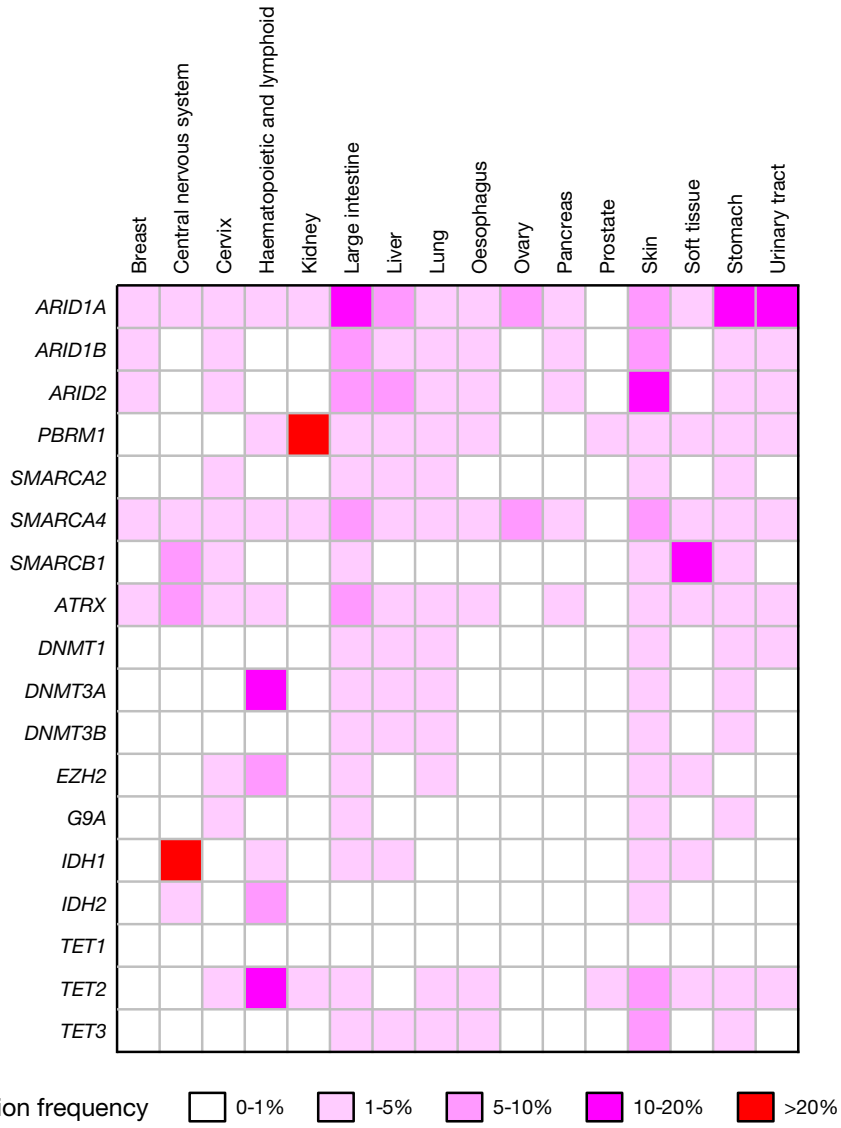

Fig. 5 Genetic alterations of genes encoding epigenetic machineries. Cancer genome analysis has revealed that various genes encoding epigenetic machineries are mutated in cancers. As for the genes involved in addition of DNA methylation, *DNMT3A* mutations mainly located within its C-terminal catalytic domain have been reported in AML. As for the genes involved in removal of DNA methylation, *TET2* mutations have been reported in several hematological cancers. Also, mutations of isocitrate dehydrogenase, *IDH1* and *IDH2*, whose gain-of-function product can inhibit TET activity has been reported in glioblastoma and AML. Therefore, mutations of these genes are considered to be involved in aberrant DNA methylation induction. Mutation frequencies were obtained from COSMIC (Catalogue of Somatic Mutations in Cancer; http://cancer.sanger.ac.uk/cancergenome/projects/census/).

Afterwards, gain of function by the *DNMT3A* mutation was also reported. R882H mutant DNMT3A protein, but not its wild-type protein, was shown to interact with polycomb repressive complex 1 (PRC1), which is involved in gene repression by inducing H3K27me3. In cells with the DNMT3A R882H mutation, myeloid differentiation-related genes, such as *Cebpa*, *Cebpe* and *PU.1*, are down-regulated, independently of DNA methylation. As for the other DNMTs, genetic alterations have not been documented.

Genes that Demethylate DNA

Mutations of genes encoding isocitrate dehydrogenase (IDH1 and IDH2) have been reported in glioblastoma and AML. IDH mutation is a typical gain-of-function mutation, and confers a novel enzymatic activity to convert α-ketoglutarate (α-KG) to (*R*)-2-hydroxyglutarate (2-HG) in addition to the impairment of its original enzymatic activity (conversion of isocitrate to α-KG). This oncometabolite, 2-HG, produced by mutant IDH protein inhibits the enzymatic activities of TET proteins. The accumulation of 5-mC by reduced TET activities is considered to be one of the molecular mechanism of aberrant DNA methylation induction.

In addition, *TET2* is known to be mutated in several hematological cancers, and the 5-hmC levels are reduced in the bone marrow of patients with *TET2* mutations. In solid tumors, 5-hmC levels are also reduced in several cancer types, such as melanoma, liver cancers, and lung cancers. *TET2* and *TET3* are actually mutated in these solid tumors, especially for *TET2* (Fig. 5). Therefore, *TET2* and *TET3* mutations are considered to be involved in aberrant DNA methylation induction.

Genes Related to Other Epigenetic Mechanisms

Epigenetic machineries involved in histone modifications are also mutated in cancers. Among various histone modifications, H3K27me3 in normal cells is known as a premark of aberrant DNA methylation induction (as described in section "Aberrant DNA Methylation Induction by Environmental Stimuli"). In lymphoma, *EZH2* is mutated predominantly at tyrosine641 (such as Y641H, Y641N, and Y641S). This mutation enhances histone methyltransferase activity of EZH2, and disturbs cellular H3K27me3 status. In addition, inactivating mutations of UTX (KDM6A), an H3K27 demethylase, have been reported in AML, colorectal cancers, and esophageal squamous cell carcinomas (ESCCs). Activation of an H3K27 methylase and inactivation of an H3K27 demethylase can affect H3K27me3 status, and thus these alterations are potentially involved in aberrant DNA methylation induction.

Genes Related to Chromatin Remodeling

A chromatin remodeling factor, such as SWI/SNF, is composed of multiple components, and regulates gene transcription via changing nucleosome occupancy. Genes encoding SWI/SNF components are frequently mutated in various types of cancers, and these mutations are considered to disrupt SWI/SNF functions, which is likely to lead to the alterations of nucleosome occupancy. The presence of nucleosome can protect genomic DNA from DNA methylation catalyzed by de novo type DNMTs, DNMT3A, and DNMT3B. Therefore, there is a possibility that SWI/SNF mutations might be one of the possible mechanisms of aberrant DNA methylation induction.

Aberrant DNA Methylation Induction by Environmental Stimuli

Inducers of Aberrant DNA Methylation

Aberrant DNA methylation can be detected not only in cancer cells but also in normal cells, even far before cancer development. As for the inducers of aberrant DNA methylation, aging has been known for a long time (Table 1). DNA methylation levels of genes encoding estrogen receptor in human colonic tissues were initially shown to increase, depending upon age. It has also been shown that some infectious agents can induce aberrant DNA methylation in various tissues. In the stomach, DNA methylation levels are up to 300-fold higher in people infected with *Helicobacter pylori* (*H. pylori*), an almost exclusive cause of gastric cancers, than those without. Also in the liver, aberrant DNA methylation is induced in people infected with hepatitis C virus (HCV). In addition, some chemicals (smoking) and a hormone (estrogen) are also reported to induce aberrant DNA methylation.

Aberrant DNA methylation can be accumulated in normal tissues for a long time. The degree of accumulation of aberrant methylation, namely methylation burden, is closely correlated with risk of cancer development in the stomach, esophagus, and mammary glands. This strongly indicates that the accumulation of aberrant DNA methylation in normal tissues leads to the formation of an epigenetic field for cancerization (epigenetic field defect), an apparently normal tissue but predisposed to carcinogenesis

Table 1 Inducers of aberrant DNA methylation

Inducing factors	*Tissues*
Aging	Colon
Bacterial infection	
Helicobacter pylori	Stomach
Viral infection	
Hepatitis B virus	Liver
Hepatitis C virus	Liver
Epstein-Barr (EB) virus	Stomach
Papillomavirus	Uterine cervix, Oropharynx
Parasitic infection	
Liver fluke	Bile duct
Schistosoma	Bladder
Smoking	Esophagus
Hormone	
Estrogen	Mammary epithelium (under the culture condition)

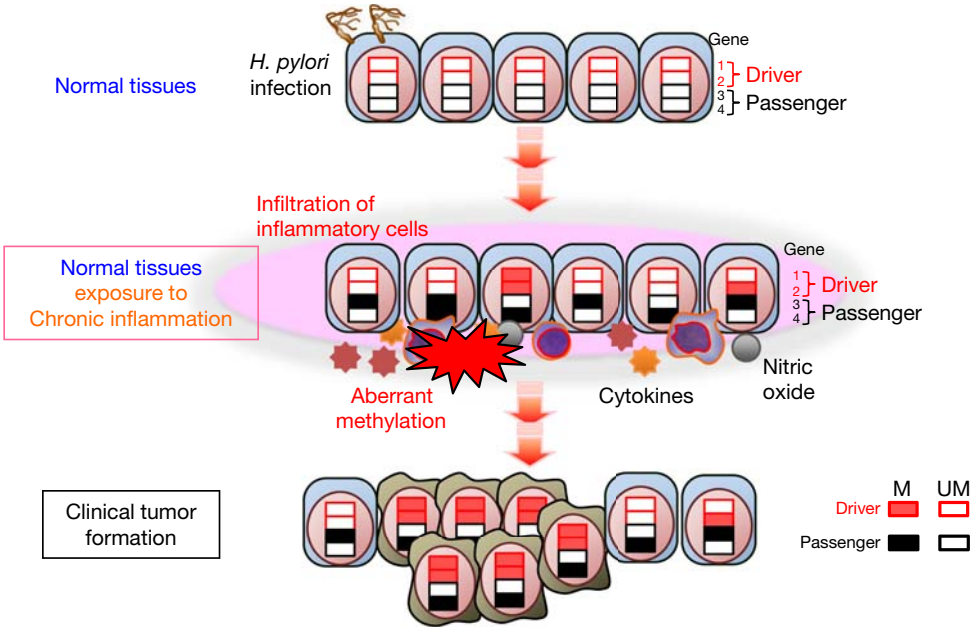

Fig. 6 Formation of an epigenetic field for cancerization. Aberrant DNA methylation can be detected not only in cancer cells but also in normal cells, even far before cancer development. It affects both driver and passenger genes. The accumulation of aberrant DNA methylation in normal tissues is associated with increased cancer risk, and produces a field for cancerization, a normal tissue predisposed to carcinogenesis.

(Fig. 6). The measurement of the degree of methylation burden is useful for cancer risk prediction, and the usefulness has been demonstrated even clinically for gastric cancer.

Importance of Chronic Inflammation in Aberrant DNA Methylation Induction

Chronic inflammation is especially important in aberrant DNA methylation induction. Suppression of chronic inflammation in the stomach of Mongolian gerbils by the administration of cyclosporin A (an immunosuppressant) markedly inhibited methylation induction while it did not affect colonization of *H. pylori*. This clearly showed that chronic inflammation, but not the *H. pylori* itself, is important for aberrant DNA methylation induction.

Mechanism of Target Gene Specificity

Aberrant DNA methylation is induced at specific genes, depending upon tissues and possibly methylation inducers. In gastric tissues, some genes are methylated at a high incidence (susceptible to methylation induction) in individuals with *H. pylori* infection while others are not (Fig. 7). As for the mechanism of the target gene specificity, methylation-susceptible genes were shown to have low transcription levels in normal cells. Then, it was also shown that genes aberrantly methylated in cancer cells are pre-marked by H3K27me3 in their corresponding normal cells (Fig. 8A). Further, methylation-resistant genes had high levels of RNA polymerase II (Pol II) binding at their nucleosome free regions (NFRs), even if they are not transcribed (Fig. 8B). Exposure to chronic inflammation can also induce an increase of H3K27me3 levels, which eventually leads to induction of aberrant DNA methylation (Fig. 8C). Tumor-suppressor genes can be silenced by aberrant DNA methylation, despite their high levels of Pol II binding at their NFRs in normal cells.

Perspective: Clinical Applications of Aberrant DNA Methylation

Aberrant DNA methylation can be utilized for cancer diagnosis and therapy. Measurement of aberrant DNA methylation specifically present in cancer cells can be utilized for cancer detection. The presence of such methylation can be measured using specimens that can be obtained with minimal invasion (tumor-derived cell-free DNA, cfDNA) or without invasion (urine, sputum, and stool). Measurement of DNA methylation in cancer biopsy specimens can be utilized for the prediction of cancer pathophysiology, such as drug sensitivity and patient prognosis. Measurement of DNA methylation in normal cells can be utilized for risk prediction of cancer development.

Aberrant DNA methylation can be removed using DNA demethylating drugs. Now two drugs, azacytidine (5-azacytidine) and decitabine (5-aza-2′-deoxycytidine), are clinically utilized for treatment of myelodysplastic syndrome (MDS) and a part of acute

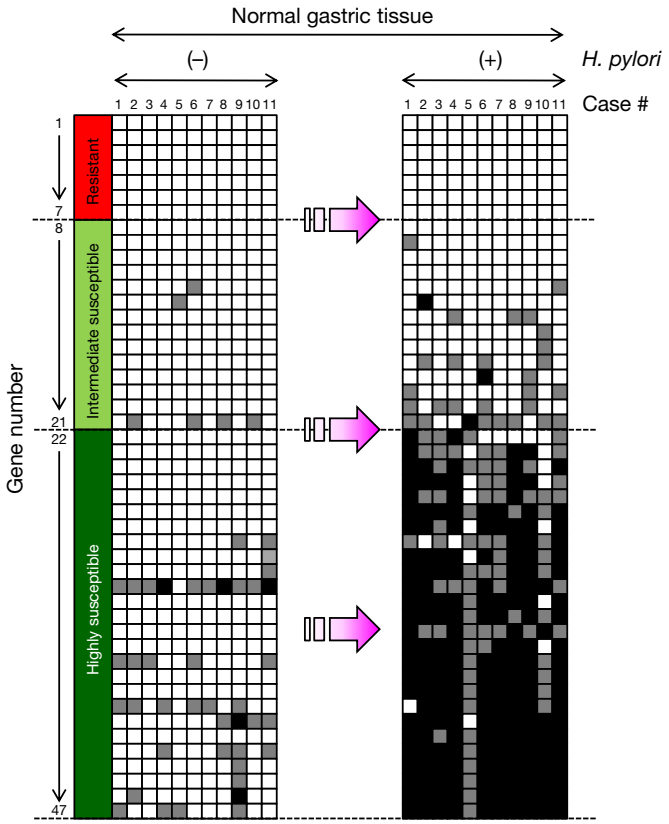

Fig. 7 Target gene specificity of aberrant DNA methylation induction. Aberrant DNA methylation is induced at specific genes, depending upon tissue types and possibly methylation inducers. For example, in gastric tissues, some genes were consistently methylated in different individuals exposed to *H. pylori*-triggered chronic inflammation (*Green*, highly susceptible to methylation induction) while the others were resistant (*Red*, resistance to methylation induction). Modified from Nakajima et al. *International Journal of Cancer* **124**, 905–910, 2009.

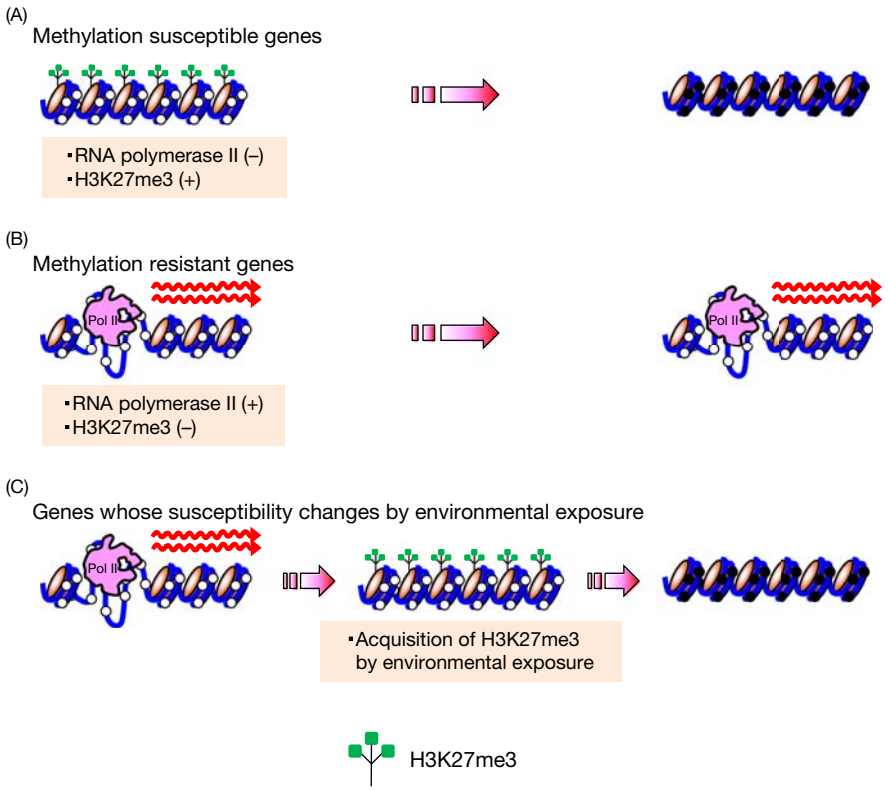

Fig. 8 Mechanisms of target gene specificity of aberrant DNA methylation induction. Genes aberrantly methylated in cancer cells are pre-marked by H3K27me3 in their corresponding normal cells (A). In contrast, methylation-resistant genes show high levels of RNA polymerase II (Pol II) binding at their nucleosome free regions (NFRs), even if they are not transcribed (B). Exposure to specific environmental factors can also induce an increase of H3K27me3 levels at a relatively early stage of the exposure, and this leads to aberrant DNA methylation after prolonged exposure (C).

myeloid leukemia (AML). Clinical trials for solid tumors are also being conducted, especially by focusing on a combination of a DNA demethylating drug and conventional chemotherapy (or another epigenetic drug). For example, a combination of azacytidine and entinostat (a histone deacetylase inhibitor) was shown to be effective for at least some patients with recurrent metastatic non-small cell lung cancers. Also, the efficacy of a combination of decitabine and carboplatin has been reported for carboplatin-resistant ovarian cancers, and a combination of decitabine and panitumumab (an anti-EGFR antibody) for metastatic colorectal cancers.

Epilogue

Recent development of technologies for genome-wide DNA methylation analysis revealed DNA methylation profiles in cancer cells that can be potentially used for diagnostic purposes. As inducers of aberrant DNA methylation, genes encoding epigenetic machineries are mutated and have altered expressions in cancers. The role of chronic inflammation is well known, and may be useful as a target of cancer prevention. Further efforts are required to deepen our understanding and to accelerate clinical applications of the findings in aberrant DNA methylation.

See also: Mutations in DNA Methyltransferases and Demethylases. DNA Methylation Changes in Cancer: Cataloguing. Defective 5-Methylcytosine Oxidation in Tumorigenesis. Chromatin Dynamics in Cancer: Epigenetic Parameters and Cellular Fate.

Further Reading

Abe, M., Ohira, M., Kaneda, A., Yagi, Y., Yamamoto, S., Kitano, Y., Takato, T., Nakagawara, A., Ushijima, T., 2005. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. Cancer Research 65, 828–834.

Delhommeau, F., Dupont, S., Della Valle, V., James, C., Trannoy, S., Masse, A., Kosmider, O., Le Couedic, J.P., Robert, F., Alberdi, A., Lecluse, Y., Plo, I., Dreyfus, F.J., Marzac, C., Casadevall, N., Lacombe, C., Romana, S.P., Dessen, P., Soulier, J., Viguie, F., Fontenay, M., Vainchenker, W., Bernard, O.A., 2009. Mutation in *TET2* in myeloid cancers. The New England Journal of Medicine 360, 2289–2301.

Hahn, M.A., Hahn, T., Lee, D.H., Esworthy, R.S., Kim, B.W., Riggs, A.D., Chu, F.F., Pfeifer, G.P., 2008. Methylation of polycomb target genes in intestinal cancer is mediated by inflammation. Cancer Research 68, 10280–10289.

Issa, J.P., Ottaviano, Y.L., Celano, P., Hamilton, S.R., Davidson, N.E., Baylin, S.B., 1994. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nature Genetics 7, 536–540.

Jones, P.A., Issa, J.P., Baylin, S., 2016. Targeting the cancer epigenome for therapy. Nature Reviews. Genetics 17, 630–641.

Juergens, R.A., Wrangle, J., Vendetti, F.P., Murphy, S.C., Zhao, M., Coleman, B., Sebree, R., Rodgers, K., Hooker, C.M., Franco, N., Lee, B., Tsai, S., Delgado, I.E., Rudek, M.A., Belinsky, S.A., Herman, J.G., Baylin, S.B., Brock, M.V., Rudin, C.M., 2011. Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. Cancer Discovery 1, 598–607.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., DiPersio, J.F., Mardis, E.R., Wilson, R.K., 2010. *DNMT3A* mutations in acute myeloid leukemia. The New England Journal of Medicine 363, 2424–2433.

Maeda, M., Nakajima, T., Oda, I., Shimazu, T., Yamamichi, N., Maekita, T., Asada, K., Yokoi, C., Ando, T., Yoshida, T., Nanjo, S., Fujishiro, M., Gotoda, T., Ichinose, M., Ushijima, T., 2017. High impact of methylation accumulation on metachronous gastric cancer: 5-year follow-up of a multicentre prospective cohort study. Gut 66, 1721–1723.

Maekita, T., Nakazawa, K., Mihara, M., Nakajima, T., Yanaoka, K., Iguchi, M., Arii, K., Kaneda, A., Tsukamoto, T., Tatematsu, M., Tamura, G., Saito, D., Sugimura, T., Ichinose, M., Ushijima, T., 2006. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. Clinical Cancer Research 12, 989–995.

Morin, R.D., Johnson, N.A., Severson, T.M., Mungall, A.J., An, J., Goya, R., Paul, J.E., Boyle, M., Woolcock, B.W., Kuchenbauer, F., Yap, D., Humphries, R.K., Griffith, O.L., Shah, S., Zhu, H., Kimbara, M., Shashkin, P., Charlot, J.F., Tcherpakov, M., Corbett, R., Tam, A., Varhol, R., Smailus, D., Moksa, M., Zhao, Y., Delaney, A., Qian, H., Birol, I., Schein, J., Moore, R., Holt, R., Horsman, D.E., Connors, J.M., Jones, S., Aparicio, S., Hirst, M., Gascoyne, R.D., Marra, M.A., 2010. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nature Genetics 42, 181–185.

Niwa, T., Tsukamoto, T., Toyoda, T., Mori, A., Tanaka, H., Maekita, T., Ichinose, M., Tatematsu, M., Ushijima, T., 2010. Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Research 70, 1430–1440.

Plass, C., Pfister, S.M., Lindroth, A.M., Bogatyrova, O., Claus, R., Lichter, P., 2013. Mutations in regulators of the epigenome and their connections to global chromatin patterns in cancer. Nature Reviews. Genetics 14, 765–780.

Suzuki, H., Yamamoto, E., Maruyama, R., Niinuma, T., Kai, M., 2014. Biological significance of the CpG island methylator phenotype. Biochemical and Biophysical Research Communications 455, 35–42.

Takeshima, H., Ushijima, T., 2010. Methylation destiny: Moira takes account of histones and RNA polymerase II. Epigenetics 5, 89–95.

Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J.G., Baylin, S.B., Issa, J.P., 1999. CpG island methylator phenotype in colorectal cancer. Proceedings of the National Academy of Sciences of the United States of America 96, 8681–8686.

Turcan, S., Rohle, D., Goenka, A., Walsh, L.A., Fang, F., Yilmaz, E., Campos, C., Fabius, A.W., Lu, C., Ward, P.S., Thompson, C.B., Kaufman, A., Guryanova, O., Levine, R., Heguy, A., Viale, A., Morris, L.G., Huse, J.T., Mellinghoff, I.K., Chan, T.A., 2012. *IDH1* mutation is sufficient to establish the glioma hypermethylator phenotype. Nature 483, 479–483.

Ushijima, T., Hattori, N., 2012. Molecular pathways: Involvement of helicobacter pylori-triggered inflammation in the formation of an epigenetic field defect, and its usefulness as cancer risk and exposure markers. Clinical Cancer Research 18, 923–929.

Weisenberger, D.J., Siegmund, K.D., Campan, M., Young, J., Long, T.I., Faasse, M.A., Kang, G.H., Widschwendter, M., Weener, D., Buchanan, D., Koh, H., Simms, L., Barker, M., Leggett, B., Levine, J., Kim, M., French, A.J., Thibodeau, S.N., Jass, J., Haile, R., Laird, P.W., 2006. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with *BRAF* mutation in colorectal cancer. Nature Genetics 38, 787–793.

Wilson, B.G., Roberts, C.W., 2011. SWI/SNF nucleosome remodellers and cancer. Nature Reviews. Cancer 11, 481–492.

Xiao, M., Yang, H., Xu, W., Ma, S., Lin, H., Zhu, H., Liu, L., Liu, Y., Yang, C., Xu, Y., Zhao, S., Ye, D., Xiong, Y., Guan, K.L., 2012. Inhibition of alpha-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes & Development 26, 1326–1338.

DNA Mismatch Repair: Mechanisms and Cancer Genetics☆

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Glossary

Deletion Loss of a segment of DNA from a chromosome, sometimes part of a gene or an entire gene(s).

Frameshift mutation Type of mutation in a gene resulting from insertion or deletion of additional base pairs, usually 1 or 2, that changes the reading frame of the gene downstream from the mutation.

Heterodimer Protein complex consisting of two different proteins.

Heterozygous Having two different alleles of a gene.

Homozygous Having two identical alleles of a gene.

Immunohistochemistry A method to detect the presence of a specific protein in a tissue sample using protein-specific antibodies.

Mispair DNA nucleotides paired in non-Watson–Crick base pairs. For example, G-T rather than the correct G-C or A-T.

Mutation A change in the original DNA sequence.

Neoantigen A newly formed antigen due to a mutation in a gene that results in the expression of a new protein sequence that is not present in normal tissues.

Template strand The strand of DNA used by a DNA polymerase as instructions to build a new DNA strand with the same information as the template.

Introduction

Mispaired DNA bases can occur as a result of misincorporation of incorrect nucleotides by DNA polymerases during DNA replication. DNA mismatch repair (MMR) is a conserved mechanism that corrects both base–base mispairs (G-T rather than G-C, for example) and small insertion/deletion loops that are caused by DNA polymerase slippage. The MMR machinery first recognizes the mispair and then excises the DNA strand containing the mispaired base followed by filling in the resulting single stranded gap in the DNA. Critically, MMR identifies the newly synthesized DNA strand and only targets this strand for excision and resynthesis. If repair was targeted to the template strand or if repair failed to occur before the next round of DNA replication, the mispaired base would result in a mutation (Fig. 1).

MMR is a major pathway for suppressing DNA replication errors. Defects in MMR result in increased cellular mutation rates, which means that MMR-defective cells accumulate mutations at higher rates than cells with functional MMR. Increased numbers of mutations and increased mutation rates have been observed in many cancers. The "mutator phenotype hypothesis" posits that increased mutation rates drive tumor evolution. These mutations can directly activate proto-oncogenes or inactivate tumor suppressor genes to initiate tumorigenesis as well as increase growth advantages of tumor subclones during cancer progression. Consistent with the predictions of this hypothesis, several cancer predisposition syndromes including Lynch syndrome (also called hereditary non-polyposis colorectal cancer or HPNCC) and biallelic MMR deficiency are caused by inherited mutations in MMR genes. It is also known that somatic MMR defects can underlie the development of sporadic cancers.

This article reviews our current understanding of MMR pathways and the proteins involved, while highlighting some major remaining questions in the field. Additionally, the cancer predisposition syndromes caused by inherited MMR defects and the occurrence of MMR defects in sporadic cancers are discussed. It should be noted that MMR also acts on (1) mispaired bases that are the result of chemical damage to DNA and (2) mispared bases that occur in the heteroduplex DNA intermediates formed during recombination, and MMR acts to prevent recombination between divergent but highly homologous DNAs as well as playing roles in somatic hypermutation and meiotic recombination; however, these aspects of MMR are not discussed in this article.

Mechanism of MMR

Scope and Comments on Nomenclature

Much of the early work on MMR mechanisms was performed using *Escherichia coli* as a model system. However, the MMR pathways of *E. coli* and other closely related Gammaproteobacteria have a number of mechanistic differences compared to the MMR pathways

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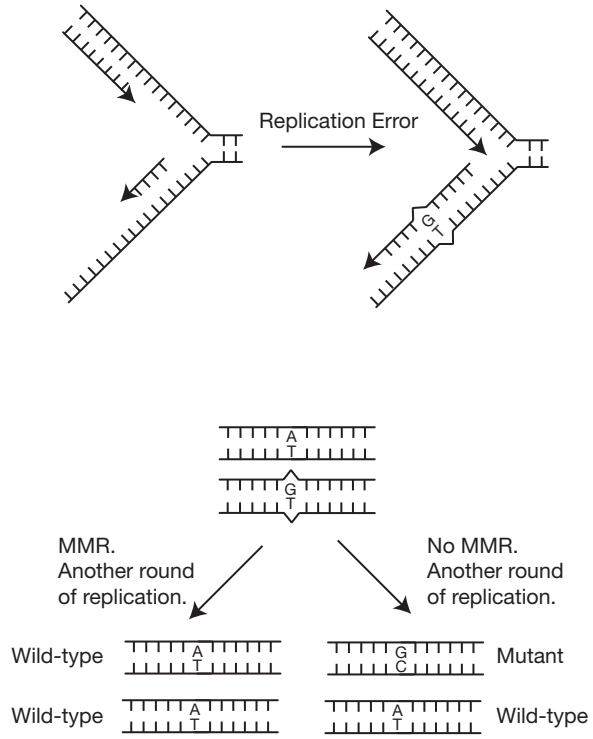

Fig. 1 Model for the formation and repair of mispaired based during DNA replication.

Table 1 Eukaryotic MMR proteins

H. sapiens/M. musculus	*S. cerevisiae*	*E. coli*	*Function in MMR*
Mispair recognition			
MSH2-MSH3	Msh2-Msh3	MutS	Mispair recognition protein. Msh2-Msh3 is also called MutSβ, and Msh2-Msh6 is also called MutSα. See text for more details
MSH2-MSH6	Msh2-Msh6		
Accessory factors			
MLH1-PMS2	Mlh1-Pms1	MutL	Mispair activated endonuclease that also recruits downstream proteins. In some bacteria, MutL does not have an endonuclease activity. Also called MutLα
MLH1-PMS1	Mlh1-Mlh2		Accessory factor for MMR acting through an unknown mechanism. Also called MutLβ
MLH1-MLH3	Mlh1-Mlh3		Can substitute for *S. cerevisiae* Mlh1-Pms1 in some cases. Acts primarily in resolution of meiotic crossovers. Also called MutLγ
Excision reaction			
EXO1	Exo1	[a]	5′ → 3′ dsDNA[b] exonuclease that excises DNA around the mispair
Resynthesis reaction/accessory proteins			
PCNA	PCNA	β Clamp	DNA polymerase processivity factor and required for activating the Mlh1-Pms1 (*S. cerevisiae*)/MLH1-PMS2 (*H. sapiens*) endonuclease and for DNA Pol δ
RFC	RFC	γ complex	Loads the β clamp/PCNA onto DNA
RPA	RPA	SSB	ssDNA[b] binding protein that acts in the excision and resynthesis reactions
DNA Pol δ	DNA Pol δ	[c]	DNA polymerase that resynthesizes excised DNA
DNA Pol ε	DNA Pol ε	[c]	Another DNA polymerase that can act during MMR
Ligase I	Ligase I	DNA ligase	Seals any nicks in the DNA

[a] *E. coli* uses a different excision mechanism involving a DNA helicase and one of four different ssDNA-specific endonucleases.

[b] dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

[c] *E. coli* uses DNA polymerase III, which is most analogous to DNA polymerase δ because of the requirement of the β clamp/PCNA processivity factor.

of eukaryotes and other bacteria, even though a number of MMR proteins are highly conserved between different organisms. Therefore, we will only discuss eukaryotic MMR proteins and mechanisms in this article. See **Table 1** for a list of the known *Saccharomyces cerevisiae* and human MMR proteins and their relationship to the known *E. coli* MMR proteins. The discussion of these proteins will focus on the human proteins and use the names of the human proteins as much as possible. However, MMR proteins and their function are the same in *S. cerevisiae* and human, and since studies of *S. cerevisiae* MMR have contributed enormously to our understanding of MMR mechanisms the discussion below will also draw upon insights developed through the study of *S. cerevisiae* MMR.

MutS-Related Proteins

Eukaryotes possess three homologs of bacterial *mutS* that encode proteins known to function in MMR: *MSH2*, *MSH3*, and *MSH6*. The protein products of these genes form two heterodimeric complexes, MSH2-MSH3 (also called MutSβ) and MSH2-MSH6 (also called MutSα), which have slightly different, but partially redundant roles in recognizing mispairs (Fig. 2). Base–base mispairs are primarily recognized by MSH2-MSH6, although MSH2-MSH3 can recognize a small number of these mispairs, and MSH2-MSH3 is solely responsible for recognizing large insertion/deletions. In contrast, both complexes act in the repair of small insertion/deletions, which are the most common type of DNA replication errors. Because of this partial redundancy and the importance of MSH2-MSH6 in base–base mispairs, MSH2-MSH6 appears to play a larger role in the suppression of mutations than MSH2-MSH3. Consequently deletion of *MSH3* causes only a small increase in mutation rates compared to deletion of *MSH6*. However, disruption of *MSH2* results in completely defective MMR and much higher mutation rates than disruption of either *MSH6* or *MSH3* because *MSH2* encodes a common subunit in both complexes.

The MSH2-MSH6 and MSH2-MSH3 mispair binding proteins have two composite ATP binding/hydrolysis sites. Binding of ATP, hydrolysis of ATP, and release of ADP at these sites modulates the interaction between these proteins and DNA as well with downstream MMR factors. The nucleotide-free and ADP-bound forms of MSH2-MSH6 and MSH2-MSH3 can bind to mispairs and induce a dramatic bend in the DNA at the site of the mispair. Remarkably, the structures of these complexes with mispair-containing DNAs has revealed that mispairs are recognized in a specific manner, such as the recognition of the thymidine base in a T:G mispair, which indicates that the orientation of mispair binding by MSH2-MSH6 or MSH2-MSH3 cannot be the source of strand-specificity for the downstream steps of MMR. In contrast, the ATP-bound form of these proteins cannot bind mispaired bases. Moreover, ATP binding by mispair-bound MSH2-MSH6 and MSH2-MSH3 triggers a conformational change whereby the proteins convert to a sliding clamp that releases from the mispair, eliminates the bend in the DNA, and slides along the DNA. ATP binding by MSH2-MSH6 and MSH2-MSH3 is also required for the recruitment of the MutL homolog complexes; however, some mutant versions of Msh2-Msh6 in *S. cerevisiae* are competent for recruitment but not sliding, suggesting that these two ATP-dependent states are not identical.

MutL-Related Proteins

Eukaryotes encode four genes related to bacterial *mutL* that encode proteins that act in MMR: *PMS1* (*MLH2* in *S. cerevisiae*), *PMS2* (*PMS1* in *S. cerevisiae*), *MLH1* and *MLH3*. The protein products of these genes form three heterodimeric complexes, MLH1-PMS2 (Mlh1-Pms1 in *S. cerevisiae*; also called MutLα), MLH1-PMS1 (Mlh1-Mlh2 in *S. cerevisiae*; also called MutLβ) and MLH1-MLH3 (also called MutLγ).

MLH1-PMS2 is the major MutL-related protein complex that functions in MMR. Mispair bound MSH2-MSH6 and MSH2-MSH3 recruit and load MLH1-PMS2 onto DNA (Figs. 2 and 3). There is evidence that either multiple molecules of MLH1-PMS2 are recruited per molecule of MSH2-MSH6 (or MSH2-MSH3) or that MLH1-PMS2 has a longer residence time on DNA than MSH2-MSH6 or MSH2-MSH3. Moreover, structural and single molecule studies suggest that MLH1-PMS2 may also form a clamp on the DNA. It should be noted that the MSH2-MSH6 sliding clamp is not required for MLH1-PMS2 recruitment, suggesting that sliding clamp formation and MLH1-PMS2 recruitment may represent different aspects of ATP binding-induced conformational changes. MLH1-PMS2 is an endonuclease that makes single strand breaks in DNA, and this activity is essential for MMR. Its endonuclease activity, which can be observed under a number of different reaction conditions, is highly activated in the presence of

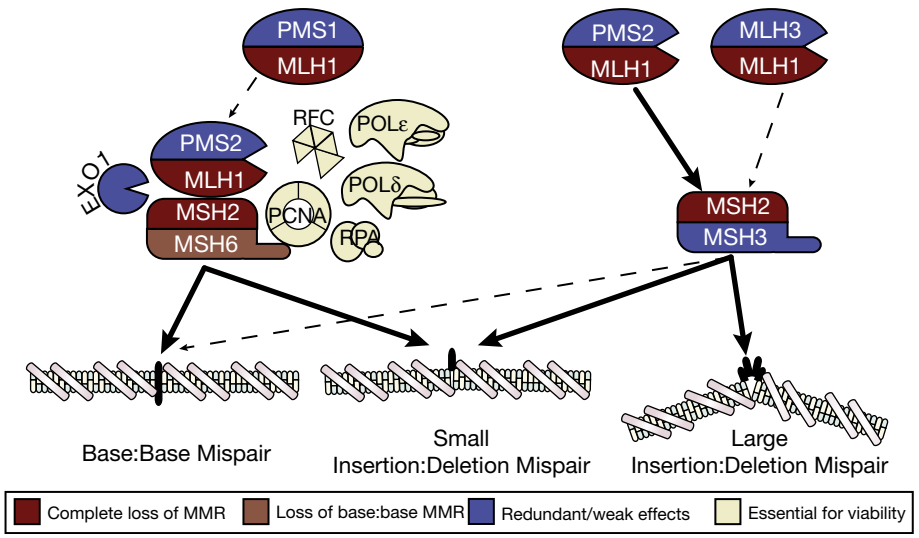

Fig. 2 Illustration of the known MMR proteins and their specificity for different mispaired bases.

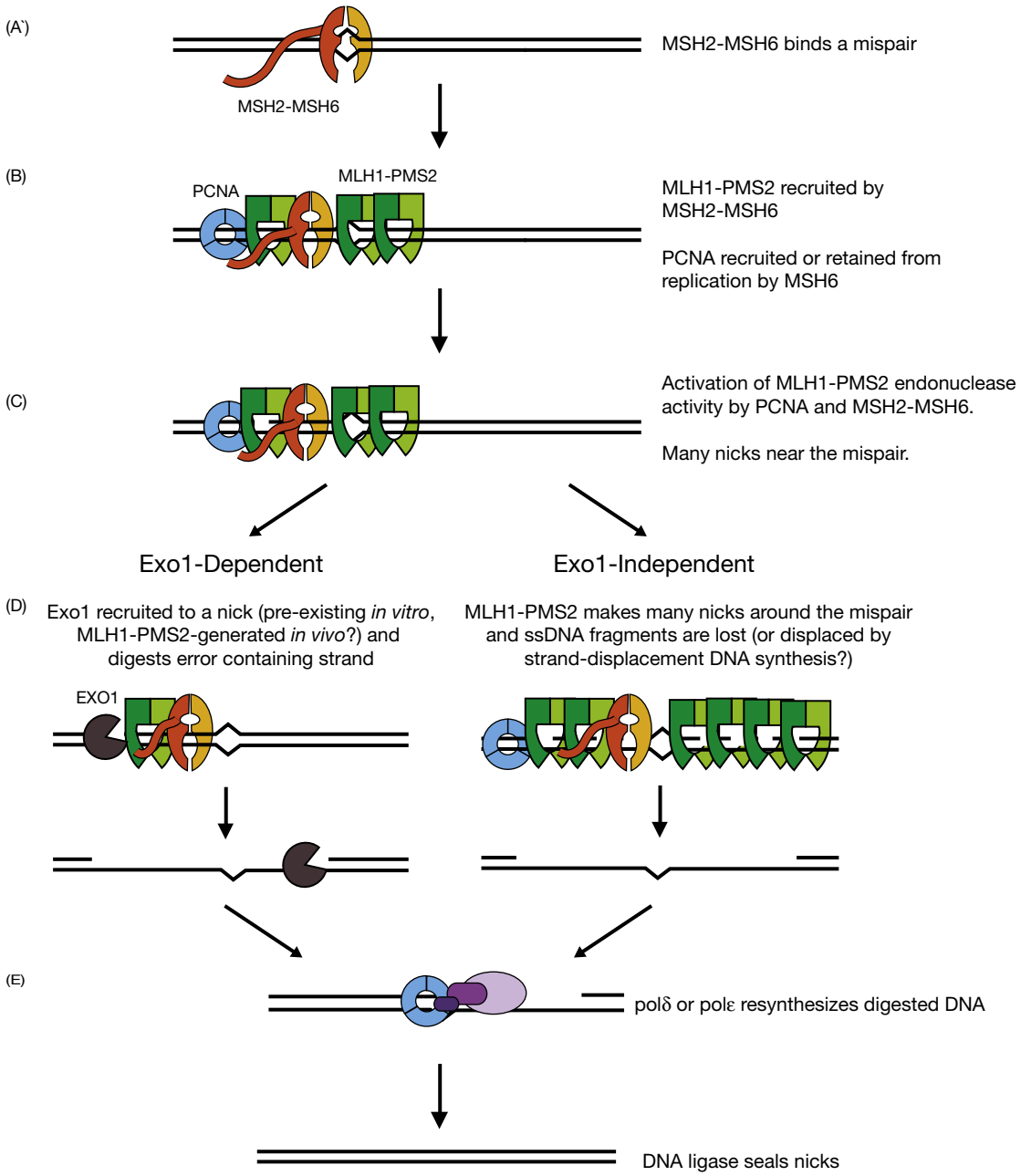

Fig. 3 Models for the mechanisms of Exo1-dependent and Exo1-independent MMR reactions.

MSH2-MSH6 or MSH2-MSH3, the proliferating clamp nuclear antigen (PCNA), replication factor C (RFC) and ATP on mispaired base containing DNA substrates that also contain a single strand break. Under these reaction conditions, MLH1-PMS2 makes single strand breaks (called nicks) only in the DNA strand that contains the pre-existing nick, although the mechanisms by which nicking is directed to the pre-nicked strand under these conditions are not yet understood. It is still unknown how eukaryotic systems distinguish between the newly synthesized and template strands to target MMR to the newly synthesized strand; however, it has been suggested that because the newly synthesized DNA strands can contain differing levels of nicks and PCNA may remain on the DNA after replication it may mark the newly replicated strand by an unknown mechanism and that PCNA-dependent strand-specific nicking by MLH1-PMS2 might then play a role in initiating strand-specific MMR.

The MLH1-MLH3 heterodimer is less important for MMR than MLH1-PMS2. Genetic studies in *S. cerevisiae* suggest that the MLH1-MLH3 heterodimer plays a small role in MMR that is partially redundant with that of MLH1-PMS2, and studies in mice support this idea (Fig. 2). Like PMS2, MLH3 contains an endonuclease active site; however, biochemical studies have not yet been able to demonstrate that MLH1-MLH3 has a mispair-dependent nicking activity like that of MLH1-PMS2. Other studies

have demonstrated that the major function of MLH1-MLH3 is in the resolution of meiotic crossing over intermediates and that the MLH1-MLH3 endonuclease may cleave Holliday junction recombination intermediates.

In contrast, less is known about the role of MLH1-PMS1. PMS1 does not appear to have an endonuclease active site like that of PMS2 or MLH3. In *S. cerevisiae*, Mlh1-Mlh2 (human MLH1-PMS1) can be recruited to DNA containing mispaired bases by both Msh2-Msh6 and Msh2-Msh3. Deletion of *S. cerevisiae MLH2* does not cause a mutator phenotype except under conditions of reduced Pms1 expression (human PMS2). These results suggest that the human MLH1-PMS1 complex functions in MMR as an MLH1-PMS2 accessory protein, though its exact function in this regard is unknown.

Identification of Other MMR Proteins

Exonuclease 1 (EXO1) was first implicated in MSH2-dependent MMR because it was identified as an MSH2-interacting protein. EXO1 was later found to interact with MLH1 through a peptide in EXO1 called a MIP (for MLH1 interacting peptide) box. EXO1 is a 5′ to 3′ dsDNA exonuclease that can also cleave branched DNA molecules. Considerable data support the idea that EXO1 functions in the mispair excision step of MMR under some conditions and that the interaction between EXO1 and MSH2 increases the extent of excision by EXO1 in response to a mispair. In contrast, the role of the interaction between EXO1 and MLH1 is less clear. Genetic studies show that loss of EXO1 only causes weak MMR defects, suggesting that other mechanisms for DNA strand excision during MMR must exist. Other exonucleases proposed to function in MMR are the endo/exonuclease FEN1/RAD27 and the 3′ to 5′ editing exonuclease functions of DNA polymerases δ (DNA Pol δ) and epsilon (DNA Pol ε); however, a role for these exonucleases in the excision step of MMR has not yet been definitively established. A combination of in vitro MMR reconstitution studies, genetic studies and studies of the MLH1-PMS2 endonuclease established a role for DNA Pol δ, DNA Pol ε, replication protein A (RPA), PCNA and RFC in MMR. Finally, recent studies identified WDHD1 and MCM9 as MSH2-interacting proteins; however, a role for these proteins in MMR has not been definitively established.

Reconstitution of MMR In Vitro

A number of insights into MMR mechanisms have been obtained through in vitro reconstitution studies using different combinations of proteins to catalyze MMR reactions. Two types of mispair-dependent excision/repair reactions have been reconstituted with human and *S. cerevisiae* proteins. In the first type of reaction, a combination of one of the mispair recognition factors MSH2-MSH6 or MSH2-MSH3, EXO1, DNA Pol δ, the single-stranded DNA binding protein RPA, PCNA and the PCNA loading factor RFC catalyze the repair of a circular mispaired substrate containing a single strand break (called a nick) on the 5′ side of the mispair. In this reaction, the mispair recognition factors stimulate excision by Exo1 from the nick past the mispair to produce a gap that is filled in by DNA Pol δ, PCNA, and RFC to repair the mispair. DNA Pol ε can substitute for DNA Pol δ in this first type of MMR reaction, although PCNA and RFC are no longer required under these conditions. In a second type of reaction, a combination of MSH2-MSH6 or MSH2-MSH3, MLH1-PMS2, EXO1, DNA Pol δ, RPA, PCNA and RFC promotes the repair of a circular mispaired substrate containing a nick on the 3′ side of the mispair. In this reaction, the MLH1-PMS2 endonuclease is activated in a mispair-dependent fashion by a combination of MSH2-MSH6 or MSH2-MSH3, PCNA and RFC to generate DNA nicks 5′ to the mispair. Once 5′-nicks are formed, repair appears to occur as observed in the 5′-nick directed MMR reactions. DNA Pol ε can substitute for DNA Pol δ in this second type of MMR reaction, although PCNA and RFC are still required due to their role in activating the MLH1-PMS2 endonuclease.

MMR can also occur in the absence of EXO1; however, our knowledge of EXO1-independent MMR mechanisms is not as well developed as it is for EXO1-dependent MMR. Several potential mechanisms have been suggested based on the results of biochemical and genetic studies. Biochemical reconstitution studies have shown that a combination of MSH2-MSH6, MLH1-PMS2, DNA Pol δ, RPA, PCNA and RFC can promote the repair of a circular mispaired substrate containing a nick on the 3′ side of the mispair. In this reaction, the MLH1-PMS2 endonuclease makes DNA nicks 5′ to the mispair and strand displacement synthesis by DNA Pol δ from the 5′ nick removes the nicked DNA strand from the 5′ nick to the 3′ nick, thus repairing the mispair. It should be noted that as yet there is no definitive genetic evidence supporting this mechanism. It is also unclear how this mechanism would work on the leading strand of the replication fork as nicks on the leading strand are much less frequent than nicks on the lagging strand, and human DNA Pol ε reportedly cannot support this reaction. A combination of biochemical, genetic and cell biology experiments showed that Exo1-independent MMR requires hyper-activation of the MLH1-PMS2 endonuclease leading to the hypothesis that iterative nicking of the DNA by MLH1-PMS2 may play a role in Exo1-independent MMR. Further study will be required to elucidate the mechanisms of Exo1-independent MMR.

Unanswered Questions About MMR Mechanisms

A model for eukaryotic MMR can currently be sketched out (**Fig. 3**); however, multiple steps remain speculative and are derived primarily from genetic inferences or are implicated primarily by biochemical reconstitution experiments that have not been verified in vivo. But even this model does not answer some important mechanistic questions. In order for MMR to prevent mutations that occur as a result of replication errors it must: (1) detect individual mispairs among a vast excess of normal basepairs even though the mispair binding proteins only have a modest selectivity for binding mispairs relative to base pairs; (2) target MMR to the newly synthesized DNA strand; and (3) complete MMR before the next round of DNA replication. A complete picture of how these steps are accomplished is not yet available.

Mispaired bases occur at very low frequencies and must be recognized and repaired in the presence of a vast excess of correct base pairs. However, MSH2-MSH6 and MSH2-MSH3 have only a 10–20 fold higher affinity for mispairs relative to their affinity for base pairs, suggesting there may be some mechanism to amplify the signal of the mispair. The conversion of MSH2-MSH6 and MSH2-MSH3 to sliding clamps allows additional molecules of these proteins to bind the mispair and then track along the DNA, which could be part of the mechanism by which the mispair signal is amplified to trigger repair in vivo. Another possible aspect of signal amplification may have been revealed by the formation of cytological *S. cerevisiae* Mlh1-Pms1 (human MLH1-PMS2) foci containing multiple molecules of *S. cerevisiae* Mlh1-Pms1 (human MLH1-PMS2) as a MMR intermediate. If the recruitment of MLH1-PMS2 protein involves a cycle of ATP hydrolysis by MSH2-MSH6 (or MSH2-MSH3), mispair re-recognition, and ATP binding, then the small energetic difference between mispairs and fully base paired DNA will be amplified.

The mispair binding proteins, MSH2-MSH6 and MSH2-MSH3, are known to colocalize with the DNA replication machinery where they function as mispair detectors for MMR. This colocalization is mediated by the interaction between MSH2-MSH6 (or MSH2-MSH3) and PCNA and is required for MMR under some conditions. Consistent with this, MMR is also temporally coupled to DNA replication during S-phase. Other mechanisms for coupling MMR to DNA replication likely exist but have not yet been definitively established; one possible mechanism is the reported interaction between MSH2-MSH6 and an S-phase specific chromatin modification through the deterostome-specific N-terminal PWWP domain of MSH6, although further studies of this possible mechanism are needed. S-phase coupling of MMR to DNA replication proteins during the S-phase in which the mispair occurred provides a potential mechanism for enhanced mispair recognition by MMR proteins and would be expected to ensure that mispairs are recognized and repaired before the next round of DNA replication.

It is less clear how MMR is targeted to newly replicated DNA strands. The unusual property of the MSH2-MSH6 (or MSH2-MSH3) and PCNA-dependent MLH1-PMS2 endonuclease that targets its endonuclease activity to nicked DNA strands is intriguing in this regard, as the newly replicated DNA strands, particularly the lagging DNA strand, would be expected to be marked by both nicks and PCNA. Key unresolved questions are: (1) what are the biochemical and structural mechanisms that phase MLH1-PMS2 to target it to nicked DNA strands; and (2) why would additional nicking actually be needed for MMR given that pre-existing nicks, particularly on the lagging strand, should be substrates for EXO1-mediated excision of mispairs. This potential strand recognition mechanism seems less applicable to MMR on the leading strand given the much lower density of nicks and PCNA that are expected on the leading strand. It has also been suggested that nicking at ribonucleotides that are misincorporated during DNA replication could provide a strand specificity signal, although this seems unlikely as defects that eliminate RNAseH2, the enzyme that would make these nicks, do not cause an MMR defect. Further investigation of the mechanisms of strand specificity during MMR are clearly needed.

MMR Defects and Cancer

Lynch Syndrome

A large number of hereditary cancer syndromes have been identified. Among the earliest of these to be described is Lynch syndrome (also called hereditary non-polyposis colorectal cancer, or HPNCC), which was first documented in the literature by Aldred Scott Warthin in 1913. Lynch syndrome is an autosomal dominant disorder characterized by inheritance of susceptibility to develop early onset cancers relative to sporadic cancers of the same type. Colorectal and endometrial cancers are the most common type of cancer associated with Lynch syndrome, other cancers are also associated with Lynch syndrome including ovarian, stomach, urological tract, small bowel, and hepatobiliary system and pancreatic, breast, prostate, brain (usually glioblastoma) and adrenocortical cancers have also been reported. At least two variants of Lynch syndrome have been reported. Muir–Torre syndrome is a variant of Lynch syndrome that is characterized by the presence of sebaceous neoplasms and/or keratoacanthomas in conjunction with the other cancers associated with Lynch syndrome. Turcot syndrome is characterized by an association between colorectal cancer and brain tumors and is due to either Lynch syndrome or familial adenomatous polyposis.

Linkage studies originally mapped Lynch syndrome to two loci, one on chromosome 2p and a second on chromosome 3p. The tumors from the patients analyzed in these studies showed an unusual phenotype—striking instability of mononucleotide and dinucleotide repeat sequences due to the accumulation of frameshift mutations in these sequences that are ubiquitous in the human genome, often referred to as microsatellite instability high or MSI-H. This phenotype was known to be caused by MMR deficiency in *E. coli* and *S. cerevisiae* and led to the initial demonstration that Lynch syndrome is caused by inherited heterozygous mutations in the MMR genes *MSH2* and *MLH1*. While *MSH2* and *MLH1* mutations are the most common cause of Lynch syndrome, candidate gene studies demonstrated that mutations in *PMS2* and *MSH6* are also present in Lynch syndrome, but at lower frequencies. Carriers of heterozygous *PMS2* mutations tend to have a milder phenotype relative to *MSH2* or *MLH1* carriers, including a lower frequency and later onset of cancer whereas carriers of heterozygous *MSH6* mutations often have a predominance of endometrial cancer. Convincing evidence for mutations in other MMR genes in Lynch syndrome is lacking. *MSH2* or *MLH1* are the only MMR genes in which mutations completely eliminate MMR, providing an explanation for the high prevalence of *MSH2* or *MLH1* defects in Lynch syndrome. In contrast, *MSH6* is partially redundant with *MSH3* and *PMS2* is partially redundant with *MLH3*, and hence defects in *MSH6* and *PMS2* cause partial MMR defects providing a possible explanation for their lower prevalence in Lynch syndrome.

Lynch syndrome was originally diagnosed using clinical criteria but is now diagnosed using both clinical criteria and molecular diagnostics, with the results from molecular diagnostics studies leading to a broadening of the clinical definition of Lynch

syndrome. There are presently three clinical criteria for Lynch syndrome, the Amsterdam criteria, the Amsterdam II criteria, and the Bethesda criteria (Table 2). The Amsterdam criteria primarily focused on a family history of colorectal cancer along with age of diagnosis. The Amsterdam II criteria, sometimes called the "Modified Amsterdam Criteria" included Lynch syndrome-associated cancers and tumor pathology in addition to colorectal cancer in the criteria for diagnosis of Lynch syndrome. Finally, the Bethesda criteria allowed for inclusion of smaller pedigrees, different Lynch syndrome-associated cancers, early age of diagnosis, multiple tumors and the presence of microsatellite instability in colorectal cancer resulting in a broader clinical definition of Lynch syndrome. The broader the definition of Lynch syndrome used, the more likely patients with MMR defects will be identified, but larger numbers of patients without MMR defects will be included in the cases identified.

Modern molecular and genetic testing methods have augmented and to some extent replaced clinical criteria for diagnosing Lynch syndrome. Testing of tumors from individuals suspected to have Lynch syndrome for MSI-H using accepted microsatellite markers and testing for loss of expression of MSH2, MSH6, MLH1 or PMS2 using immunohistochemistry (IHC) can effectively identify patents with candidate MMR defects; these approaches are often used to initially screen patients for potential MMR defects. It should be noted that *MSH6* defects can result in a less striking MSI-H phenotype with greater mononucleotide repeat instability than dinucleotide repeat instability compared to that caused by *MSH2*, *MLH1* or *PMS2* defects due to the redundancy between *MSH6* and *MSH3*. If abnormalities, such as loss of expression of an MMR gene or MSI-H, are observed or no data from tumor analysis is available for a suspected Lynch syndrome patient, then genetic testing for germline alterations focusing on the relevant genes is recommended. Genetic testing for *MSH2*, *MSH6*, *MLH1* or *PMS2* defects involves complete gene sequencing and analysis for the presence of deletion mutations and genome rearrangements affecting these genes and can also involve testing for *EPCAM* gene deletions that result in silencing of MSH2 expression and *MLH1* epimutations that result in silencing of MLH1 expression. Identification of a defect in an MMR gene constitutes a positive diagnosis of Lynch syndrome.

Biallelic Mismatch Repair Deficiency Syndrome (BMMR-D)

BMMR-D, also called constitutional MMR deficiency (CMMR-D), is a rare cancer susceptibility syndrome characterized by tumors of the gastrointestinal tract, skin lesions, brain tumors, leukemias and lymphomas, and rarely endometrial cancers usually occurring within the first two decades of life. BMMR-D is often connected to consanguinity, with over 50% of known cases coming from consanguineous families. Additionally, patients do not always have a family history of Lynch syndrome-like cancers. BMMR-D

Table 2 Clinical criteria for diagnosing Lynch syndrome

*Amsterdam I criteria**

A positive diagnosis of Lynch syndrome requires at least three relatives with colorectal cancer (CRC) and the presence of the following five criteria:

1. One relative must be a first degree relative of the other two
2. CRC must be present in two successive generations
3. One case of CRC must have been diagnosed when the patient was <50 years old
4. Familial adenomatous polyposis (FAP) has been eliminated as a possibility
5. Pathological examination has verified the tumors

*Amsterdam II criteria**

A positive diagnosis of Lynch syndrome requires at least three relatives diagnosed with a Lynch syndrome associated cancer (CRC, cancer of the endometrium, small bowel, ureter, brain, hepatobiliary tract, skin, stomach, or renal pelvis) and the presence of the following criteria:

1. One relative must be a first degree relative of the other two
2. Cancer must be present in two successive generations
3. One case of Lynch syndrome associated cancer must have been diagnosed when the patient was <50 years old
4. FAP has been eliminated as a possibility
5. Pathological examination has verified the tumors

*Revised Bethesda Guidelines for testing colorectal tumors for microsatellite instability (MSI)***

Because MSI-H is a hallmark of MMR deficient tumors, the Bethesda Guidelines suggest tumors from patients with suspected Lynch syndrome should be tested for MSI in the following situations:

1. If the patient is diagnosed with CRC at <50 years old
2. If the patient has synchronous, metachronous CRC, or other Lynch syndrome associated tumors at any age
3. If a patient of <60 years of age is diagnosed with CRC with MSI-H histology
4. If at least one first-degree relative is diagnosed with CRC or a Lynch syndrome related tumor at <50 years old
5. If at least two first- or second-degree relatives are diagnosed with CRC or a Lynch syndrome related tumor at any age

Information in this table was adapted from the following publications:

*Vasen, H. F. A., Watson, P., Mecklin, J-P., and Lynch, H. T. and the ICG-HNPCC. New Clinical Criteria for Hereditary Nonpolyposis Colorectal Cancer (HNPCC, Lynch Syndrome) Proposed by the International Collaborative Group on HNPCC. *Gastroenterology.* 116:1453–1456 (1999).

**Umar, A. et al. Revised Bethesda Guidelines for Hereditary Nonpolyposis Colorectal Cancer (Lynch Syndrome) and Microsatellite Instability. *J. Natl Cancer Inst.* 96: 261–268 (2004).

is caused by inheritance of two defective alleles of *MSH2* or *MLH1*, or more rarely, *PMS2*, and a small number of cases have been found to have homozygous *MSH6* defects. Genetic testing for BMMR-D generally follows the strategy used for Lynch syndrome; however, unlike Lynch syndrome, tumors of BMMR-D patients often do not present with MSI-H and consequently MSI-H is not a reliable method of diagnosing BMMR-D.

Sporadic Cancer

A variable fraction of many different types of sporadic cancers including some inflammation-associated colon cancers have MSI-H suggestive of an MMR defect; however, sequencing of MMR genes from these tumors rarely identified MMR gene defects. Biallelic *MLH1* promoter methylation and subsequent silencing of *MLH1* expression is recognized as the mechanism for inactivation of MMR in the majority of these sporadic MSI-H tumors. Approximately 20% of sporadic colorectal cancer is MSI-H and has *MLH1* promoter methylation, and *MLH1* promoter methylation is the primary cause of sporadic MSI-H endometrial cancers as well as other MSI-H sporadic cancers. Sporadic cancers with epigenetic silencing of *MLH1* are also frequently associated with activating mutations in the *BRAF* gene, which encodes a serine/threonine protein kinase in the mitogen-activated protein kinase (MAPK) pathway; *BRAF* is far less frequently mutated in Lynch syndrome. Why there is a relationship between the genetic and epigenetic alterations in these tumors is currently not understood. Analysis of sporadic cancers for MMR defects involves testing tumors for MSI-H and loss of expression of MMR genes by IHC followed by testing for *MLH1* promoter hypermethylation in those cases where there is loss of MLH1 or PMS2 expression; note that loss of MLH1 expression usually results in coordinate loss of expression of its partner protein, PMS2.

Causes and Treatment of MMR-Defective Cancers

Mutation of *MSH2* or *MLH1* or epigenetic silencing by hypermethylation of the *MLH1* promoter are the most common causes of total MMR defects, while mutation of *MSH6* causes an incomplete defect in MMR due to the partial redundancy of the MSH2-MSH6 and MSH2-MSH3 complexes. Regardless of the exact inherited defect present, it is clear that Lynch syndrome and other MMR related cancer predisposition syndromes result from loss of function mutations that cause partial or total defects in MMR and that early in the development of the cancer, the wild-type allele of the MMR gene in question is also lost, resulting in an MMR defect. Sporadic cancers that are MMR defective are also driven by early loss of MMR, primarily due to biallelic methylation, and hence silencing, of the *MLH1* promoter. Loss of MMR or reduction of MMR efficiency increases the rate of accumulating mutations in MMR-defective cells. This mutator phenotype increases the probability of the cells accumulating mutations that inactivate tumor suppressor genes or activate proto-oncogenes, which would then increase the rate of tumorigenesis. Tumorigenesis is well-known to be driven by the accumulation of mutations that inactivate tumor suppressor genes or activate proto-oncogenes, although due to the unique signature of mutations that arise due to MMR defects (e.g., high rates of frameshift mutations), the spectrum of defects in proto-oncogenes and tumor suppressor genes differs between MMR defective cancers and MMR proficient cancers of the same tissue types.

MMR defects can also cause resistance to chemotherapeutic drugs. For example, the standard treatment of colon cancer with fluorouracil-based compounds is less effective in MMR-defective tumors. Another example is that MMR-deficient malignant gliomas become resistant to the DNA methylating agent temozolomide. In the case of DNA damaging agents like temozolomide and possibly agents like fluorouracil that are incorporated into DNA, MMR can act on the damaged DNA, which contains lesions such as O^6-methylguanine-thymine mispairs, and induce apoptosis, or programmed cell death, in response to the drug. How MMR induces apoptosis is not clear; current hypotheses suggest that the MMR machinery either provides a direct signal for apoptosis or mediates a futile cycle of repair, which indirectly triggers apoptosis. Regardless, in the absence of MMR, this apoptotic response is defective, resulting in a reduced response to chemotherapeutic drugs. It is also possible that this reduced apoptotic response under conditions of normal cell growth may also contribute to the development of cancer, although studies in mutant mice have shown that this is not the major driver of the development of MMR-defective cancers.

Of critical importance is the development of effective treatments for MMR defective cancers. Some studies suggest that MMR defective colorectal cancers may be more sensitive to irinotecan and that MMR defective cells may be more sensitive to oxidative damage to DNA when the major pathways for oxidative DNA damage repair have been inhibited. Strikingly, MMR defective cancers appear to be highly responsive to immunotherapy, and MMR defects may provide a biomarker for responsiveness to immunotherapy. The FDA recently approved pembrolizumab, which is an antibody that inhibits the PD-1 immune checkpoint, for use against MSI-H or MMR deficient tumors. Pembrolizumab appears to work by allowing T cells in the adaptive immune system to recognize and eliminate cancer cells expressing neoantigens that arise in MMR-defective tumors due to the increased mutational burden of these tumors. Remarkably, the approval of pembrolizumab for MSI-H tumors is the first time a drug has been approved against a biomarker rather than against tumors derived from a specific tissue.

Summary

MMR is a conserved pathway that repairs base–base mismatches and small insertion/deletions caused by misincorporation errors during DNA replication. The MMR pathway works through mispair detection by the partially redundant complexes MSH2-MSH6 and MSH2-MSH3, followed by recruitment of downstream proteins that excise the error-containing DNA strand and resynthesize it

correctly. Two sub-pathways of MMR exist: the EXO1-dependent pathway that relies on DNA excision by EXO1, and a less well characterized EXO1-independent pathway. Defects in MMR lead to greatly elevated mutation rates that in turn can drive carcinogenesis. Lynch syndrome is a common hereditary cancer predisposition syndrome caused by heterozygous MMR defects that is most often characterized by early onset colorectal cancers and many other types of cancer. Another, rarer, cancer predisposition syndrome, BMMR-D is caused by homozygous MMR defects and results in a number of types of early onset cancers. The most common causes of these syndromes defects in *MSH2* or *MLH1*, both of which cause total MMR defects; less common causes are defects in *MSH6* and *PMS2*. MMR defective sporadic cancers of many types have also been characterized and are primarily due to silencing of the *MLH1* gene. The increased mutation rate caused by defective MMR increases the probability of inactivating mutations in tumor suppressor or proto-oncogenes, leading to increased development of cancer. Identifying MMR defects in patients has important implications for cancer surveillance in affected families and for cancer treatment, both in terms of avoiding drugs that MMR defective tumors are resistant to and using new treatments that may effectively target MMR defective tumors.

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See also: Lynch Syndrome.

Further Reading

Fox, E.J., Prindle, M.J., Loeb, L.A., 2013. Do mutator mutations fuel tumorigenesis? Cancer Metastasis Reviews 32, 353–361.
Goellner, E.M., Putnam, C.D., Kolodner, R.D., 2015. Exonuclease 1-dependent and independent mismatch repair. DNA Repair 32, 24–32.
Hampel, H., et al., 2005. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). New England Journal of Medicine 352, 1851–1860.
Jiricny, J., 2013. Postreplicative mismatch repair. Cold Spring Harbor Perspectives in Biology 5, a012633.
Kolodner, R.D., 2016. A personal historical view of DNA mismatch repair with an emphasis on eukaryotic DNA mismatch repair. DNA Repair 38, 3–13.
Le, D.T., et al., 2017. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 357, 409–413.
Le, D.T., et al., 2017. A blueprint to advance colorectal cancer immunotherapies. Cancer Immunology Research 5, 1–8.
Li, Z., Pearlman, A.H., Hsieh, P., 2016. DNA mismatch repair and the DNA damage response. DNA Repair 38, 94–101.
Lynch, H.T., Synder, C.L., Shaw, T.G., Heinen, C.D., Hitchins, M.P., 2015. Milestones of Lynch syndrome: 1895–2015. Nature Reviews Cancer 15, 181–194.
Palomaki, G.E., McClain, M.R., Melillo, S., Hampel, H.L., Thibodeau, S.N., 2009. EGAPP supplementary evidence review: DNA testing strategies aimed at reducing morbidity and mortality from Lynch syndrome. Genetic Medicine 11, 42–65.
Putnam, C.D., 2016. Evolution of the methyl-directed mismatch repair system in *Escherichia coli*. DNA Repair 38, 32–41.
Spies, M., Fishel, R., 2015. Mismatch repair during homologous and homeologous recombination. Cold Spring Harbor Perspectives in Biology 7, a022657.
Umar, A., et al., 2004. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. Journal of the National Cancer Institute 96, 261–268.
Vasen, H.F., Watson, P., Mecklin, J.P., Lynch, H.T., 1999. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative Group on HNPCC. Gastroenterology 116, 1453–1456.
Weissman, S.M., et al., 2012. Identification of individuals at risk for Lynch syndrome using targeted evaluations and genetic testing: National Society of Genetic Counselors and the Collaborative Group of the Americas on Inherited Colorectal Cancer joint practice guideline. Journal of Genetic Counseling 21, 484–493.

End of Life Support

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Glossary

Advance care planning Ongoing process of information exchange between health care providers and patients that enables individuals to make plans about their future health care consistent with their values, beliefs, and goals.
Advance directive An advance directive, ideally in the form of a legal document, provides specific direction to health care professionals about what actions to take when a person is no longer able to make and/or communicate their own health care choices.
Hospice care A philosophy of care that focuses on the relief suffering in all of its domains and supports for terminally ill patients and their loved ones.
Medical order for life sustaining therapy Used to issue a nonhospital DNR (do not resuscitate) and DNI (do not intubate) order, and must be honored by emergency medical services personnel, home care services personnel, hospice personnel, and hospital emergency services personnel. Physicians may also use the form for any patient in any setting to issue any orders for life-sustaining treatment.
Palliative care Approach that improves the quality of life of patients and their families facing the problems associated with life-threatening illness, through the prevention and relief of suffering by means of early identification and impeccable assessment and treatment of pain and other problems.

Introduction

Despite treatment advances, for many cancer is a lethal disease. Ensuring that patients and families have high-quality end of life care is integral to good cancer care. Communication about end of life preferences as well as high-quality symptom management, both physical and psychosocial, allows for the best quality of life through end of life. A palliative care team and/or a hospice team can provide expertise in assessing goals and ensuring appropriate symptom management at the end of life. Additional special topic areas that impact patients and families at the end of life include decisions regarding fluid and nutrition, and in some cases discussions around physician aid in dying (PAD), and palliative sedation.

Communication About Prognosis and End of Life Preferences

A life-limiting cancer diagnosis has a tremendous impact on both a patient and their family. As patients approach end of life they are often thinking through what is most important to them and planning forward with the time that they have remaining. A review of prognosis, a discussion of end of life preferences and documentation, and review of key medical and legal documents can help ensure that patients are receiving care that is in alignment with their wishes.

A Conversation About End of Life and Advance Care Planning

Advance care planning is the ongoing communication process by which patients make decisions about future health care needs. A diagnosis of cancer can expedite the need for advance care planning, as well as end of life discussions with medical providers. However, only in a minority of cases, do oncologists have these discussions early in a patient's clinical course. As a result, families have limited information to plan for end of life care and often receive intensive care (such as chemotherapy, hospitalizations, and intensive care admissions) at the end of life. Research demonstrates that early conversations regarding prognosis, goals, and end of life preferences improve the likelihood that patients and their families will receive high-quality end of life care.

The ideal setting for conversations around goals of care and end of life is in the ambulatory setting. This allows for a multidisciplinary approach and provides access to psychosocial and palliative care resources well in advance of an acute change or unexpected deterioration when patients may no longer have capacity to be involved in their own medical decisions. Susan Block, MD, a palliative care physician, has presented a simple framework for eliciting goals of care and end of life preferences. She asks four questions.

"Do you understand your prognosis? What are your fears about what is to come? What are your goals as time runs out? What trade-offs are you willing to make?"

These prompts can be a springboard for physicians to start difficult end of life conversations with their patients.

"Do you understand your prognosis?": **it** allows physicians to ascertain whether patients understand how much time they may have. While there is always uncertainty that underlies prognosis, physicians can provide data within a range that can increase accuracy and provide a framework for patients to consider their future. For example, physicians can frame time remaining as "years," "months to years," "weeks to months," "days to weeks" or "hours to days." The use of a range, rather than a specific amount of time, allows for uncertainty to coexist with information that can be helpful for patients and families to plan forward. It also allows for physicians to admit that they too do not know for sure, but can provide the best information within the scope of their medical knowledge. Prognosis is becoming even less clear with the advent of new therapies including immunotherapy and targeted agents that may improve prognosis dramatically, even when patients are clinically doing poorly and appear to be close to the end of life. With this in mind, a frequent dialogue, especially with each treatment change, can be helpful for both patient and provider. As patients move closer to the end of life, physicians can use physical cues and symptoms to help increase their prognostic accuracy. Common physical signs that end of life is approaching include increased fatigue leading to more time in bed, decreased appetite, progressive muscle wasting, and eventually decreased consciousness (**Table 1**).

"What are your fears about what is to come?" While many believe that a diagnosis of cancer immediately conjures up images of death and dying, what often troubles patients and families more than the prospect of death is suffering associated with end of life. Studies have shown that patients worry about symptoms at the end of life, such as pain and nausea. Additionally, they often have social concerns including how their loved ones will cope, whether they will be a burden to their families over time, and how finances will be managed. Finally, most confront spiritual questions around meaning and hope. An open-ended question around fears and worries can allow for meaningful discussions that will both guide medical treatment, lead to appropriate psychosocial and/or spiritual support, and provide the patient and family essential reassurance. A recent patient's most troubling fear was not his own illness, but concern about what would become of his wife, suffering from end-stage dementia, when he passed away. Early discussions with family that included planning for his wife's future provided the patient great relief and allowed him to face the dying process peacefully.

"What are your goals as time runs out?": Dr. Paul Kalanithi, a neurosurgeon who recently passed away due to metastatic cancer, wrote "The path forward would seem obvious if only I knew how many months or years I had left. Tell me three months, I'd just spend time with my family. Tell me one year, I'd have a plan (write that book). Give me 10 years, I'd get back to treating diseases." Goals change with a change in prognosis. While there is uncertainty in these discussions, asking this question supports a shared dialogue on what are the most pressing and important goals in the setting of limited time. Keeping these goals front and center helps ensure that treatment recommendations take into consideration and allow patients to do what is most important to them.

"What tradeoffs are you willing to make?": Ideally, treatment provides patients with more time and good quality of life. However, as illness progresses, this balance frequently shifts and treatment may prolong time at the cost of adverse effects on quality of life. Finally, there is a time point when treatment has little or no benefit with regard to time or quality of life. Early and continued discussions on potential trade-offs between treatment, survival and quality of life help physicians make treatment recommendations that are consistent with a patient and family's values and goals. One example is a gentleman in clinic whose main hobby was building model trains. This was a source of great joy for him, which he shared with his friends and grandchildren. When discussing treatment options, it became quite clear that any treatment with a significant risk of neuropathy posed an unacceptable risk to his quality of life. For him, it was important to continue building trains, even if that meant a decrease in longevity.

An essential conversation that incorporates these four questions provides guidance for patients, families, and medical teams and allows patients to focus on what is most important to them with the time they have remaining.

Advance Directives and Medical Order for Life-Sustaining Treatment

The process of advance care planning, through ongoing conversations between family and medical providers, is a critical building block to inform documents detailing medical interventions desired by the patient approaching end of life. These forms often

Table 1 Common symptoms at end of life

Prognosis	*Symptoms*
Weeks to months	Anorexia, fatigue, withdrawal from loved ones, muscle wasting, increasing time in bed
Days to weeks	Increased fatigue, confusion, restlessness, congestion, increased anorexia, hallucinations, secretions, variation in blood pressure, heart rate and respiratory rate, decreased urinary output
Hours to days	Irregular breathing, increased restlessness, cool blotchy extremities, weak pulse, decreased urine output, increased secretions
Minutes to hours	Gasping breathing, no awakening

include a legal document called an advance directive and a medical form that details choices around life-sustaining treatments (sometimes called a POLST (physician order for life-sustaining treatment) or a MOST (medical order for scope of treatment)).

Advance directives are a legal document that details how patients would want to be cared for if they are in a life-limiting situation and unable to convey their wishes. For example, patients may designate whether they would want life-prolonging interventions such as intubation or cardiopulmonary resuscitation. Patients can also designate a health care surrogate, a person who would make decisions on their behalf if the patient is no longer able to communicate. Advance care planning has been shown to decrease intensive care at the end of life and improve hospice utilization. It is important to note that the advance directive is a legal document and not a medical order. Medical personnel, for example, cannot follow the advance directive in the field.

A medical order for life-sustaining treatment known as either a POLST, MOLST (Medical/Physician Orders for Life Sustaining Treatment) or a MOST (Medical Orders for Scope of Treatment) specifies treatments that a patient would or would not want if he/she were to become seriously ill. Medical personnel must follow these orders. A medical order for life-sustaining treatment should be completed by patients who have made clear decisions about life-sustaining interventions and who have a serious illness that may lead to death within the subsequent year.

Each country has different legal and medical requirements regarding documentation of patient and family wishes at the end of life. The advance directive and the medical order for life-sustaining treatment are two common examples. The most crucial component is the conversation that occurs between family members and the medical team. Subsequent documentation can help to make this information available for others through the medical and legal record.

Symptoms at the End of Life

Physical symptoms commonly associated with advanced illness at the end of life include pain, dyspnea, delirium, secretions, and constipation. Patients may also struggle with psychological symptoms, most notably, anxiety, and depression. When possible clinicians can rely on patient history for symptoms at the end of life. However, as consciousness wanes, nonverbal cues are crucial to assessing symptoms. **Table 1** describes common physical symptoms and signs at the end of life. Optimal palliative or hospice care at the end of life focuses on relief of both physical and psychological symptoms.

Pain

Pain is a common and feared symptom at the end of life with a prevalence of 40%–50%. With this in mind, it is important to do regular assessments of pain. A numerical scale from 0 to 10 can be used to rate pain with a score of zero connoting no pain, and of 10 connoting severe pain. If patients are no longer verbal, using nonverbal cues (such as facial grimace, moaning, and restlessness) can provide cues that patients are in pain. Tylenol or antiinflammatories can be used to treat low levels of pain (0–3); can be given orally if the patient is able to swallow or rectally, if not. For moderate to severe pain, opioids and nonopioids in combination or opioids alone are recommended at the dose that adequately controls the pain. Specific considerations for the selection of analgesics, the route of administration, and drug dose and interval at the end of life include the patient's ability to swallow, body mass distribution, and organ dysfunction.

At the end of life, patients are often no longer able to swallow. Oral opioids at equianalgesic doses can be delivered via the transdermal, sublingual, buccal, rectal, subcutaneous, or intravenous route. Long-acting medications may be converted to equianalgesic doses that are delivered via a transdermal route or rectally. While these medications can also be converted to equianalgesic doses intravenously or subcutaneously, the least invasive and simplest means of delivery is preferred. Short-acting opioids may be converted to a concentrated liquid solution. For patients who have an enteral feeding tube, pain medications may be delivered via this route. However, it is important to note that long-acting opioids should never be crushed for administration via a feeding tube, as the total dose will be delivered rapidly (as if short acting). Finally, compounding pharmacies can make opioid suppositories or enemas. In the hospice setting, the subcutaneous route is frequently utilized to deliver concentrated doses of medications in low volumes (2–3 mL/h) when noninvasive options are not possible or inadequate for the needed total dose. See **Table 2** for common formulations and routes of opioids. As patients develop greater muscle wasting and weight loss, they may be less able to absorb medications that require fat for absorption (e.g., fentanyl). Additionally, as patients near end of life, kidney and liver function decline. Based on the metabolism of the prescribed analgesics, discontinuation of long-acting medications is recommended to avoid an unanticipated increase in blood drug concentration. Short-acting opioids, prescribed as needed, are safer as it is difficult to determine the correct dose and drug interval when metabolic function is actively declining. This approach requires continued evaluation of pain but is less likely to cause side effects such as sedation and delirium due to accumulation of opioid metabolites.

Adjuvant analgesics may also require rotation as patients decline. Patients who have a significant neuropathic component to their pain may benefit from rotation to methadone due to its effect on the *N*-methyl-D-aspartate (NMDA) receptor, a receptor that modulates neuropathic pain. Patients receiving antiinflammatory agents as a component of their pain regimen may need to be switched to a steroid such as dexamethasone. Steroids, while not recommended for long-term use due to side effects (proximal muscle wasting, mood disorders, abdominal pain, etc.), are potent antiinflammatory agents and helpful for short-term use. Dexamethasone can be given as a single daily dose once steady-state plasma levels are achieved, easier than the twice daily or three times

Table 2 Opioid equianalgesic doses and formulations

Medication	*Equianalgesic dosing Oral administration(PO)/Intravenous (IV)*	*Available formulations and direction*
		Short acting/long acting
Morphine	30 mg PO/10 mg IV	Oral, buccal, sublingual, rectal, intranasal, intravenous, intrathecal, epidural
		Short acting/long acting
Oxycodone	20 mg PO/not available	Oral
		Short acting
Hydromorphone	7.5 mg PO/1.5 mg IV	Oral, intravenous, rectal, subcutaneous, epidural, intrathecal
		Short acting and long acting
		Short acting—oral trans mucosal, nasal, and buccal
Fentanyl	Varies/50–100 μg IV	Long acting—transdermal
		Short acting
Tramadol	50–100 mg PO/not available	Oral
		Short acting/long acting
Oxymorphone	10 mg PO/1 mg IV	Oral, suppository, intravenous

Table 3 Common adjuvant medications for pain control and starting doses

Medication	*Indication*	*Starting doses/routes*
Acetaminophen and anti-inflammatories	Acetaminophen	4000 mg in 24 h (PO or IV)
Indications—bone pain, inflammation	Ibuprofen	2400 mg in 24 h (PO)
	Naproxen	1000 mg in 24 h (PO)
	Indomethacin	75–150 mg in 24 h (PO)
	Celecoxib	300 mg in 24 h (PO)
	Ketorolac	30–60 mg IV then 15–30 mg IV every 6 h
Bisphosphonates	Pamidronate	60–90 mg IV every 1–2 months
Indication—bone pain	Zoledronic acid	4 mg IV every 1–2 months
Antidepressants	Nortriptyline	10–25 mg daily (oral)
Indication—neuropathic pain	Desipramine	10–25 mg daily (oral)
	Venlafaxine	75 mg (oral)
	Duloxetine	30 mg (oral)
Antiepileptics	Gabapentin	100 mg three times daily (oral)
Indication—neuropathic pain	Pregabalin	50 mg three times daily (oral)

a day dosing of most oral nonsteroidal antiinflammatory agents. Table 3 provides a list of common adjuvant analgesics used for pain as well as the common indications for their use.

Continued pain assessment is essential to ensure patients have adequate pain relief. A palliative care team and/or hospice team is a helpful resource for making decisions about needed interventions for symptom control and for recommendations regarding which medications require adjustment or discontinuation as patients come closer to the end of life.

Nonpharmacologic approaches to pain include physical techniques such as physical therapy, assist devices, and massage. Physical therapists are helpful in deciding which techniques may be most beneficial based on the patient's clinical status and cognitive function. Additionally, for patients who are still alert and responsive, cognitive behavioral interventions such as breathing exercises, mindfulness, and spiritual practices may contribute to pain control. Finally, complementary approaches such as acupuncture, reiki, homeopathic, or ayurvedic treatments can be quite useful for the treatment of cancer pain. A thoughtful patient and family history may identify nonpharmacologic approaches that have previously been useful to the patient for symptom control. Reminding families of these techniques can empower families to help care for their loved ones.

Dyspnea

Dyspnea is described as the feeling of difficulty or distressed breathing, of "air hunger." Up to 70% of patients with cancer experience dyspnea at the end of life. Dyspnea is a subjective experience and may occur in the absence of pulmonary findings or objective changes in oxygenation or respiratory rate. The evaluation of dyspnea for patients with advanced cancer begins with a discussion about prognosis and the patient's desire to undergo evaluation for potential reversible causes. For the patient who has a prognosis of months and is out of bed close to 50% of the time (WHO Performance Status of 2–3) assessment for, and treatment of, potentially reversible causes of dyspnea is reasonable if consistent with the patient's goals. Common causes of dyspnea include bronchospasm, hypoxia, and anemia. Treating the underlying cause in this situation is appropriate via inhalers, oxygen, or transfusions. However, as

patients decline further, the burden of evaluation and treatment may be unacceptable. In addition, the underlying etiology of the dyspnea may no longer be reversible. In this case, the backbone of treatment is opioids. Opioids may decrease the feeling of air hunger by decreasing the ventilatory response to hypercapnia, through a decrease in preload and pulmonary congestion secondary to opioid-related vasodilation, a change in central perceptions or by decreasing opioid-related anxiety. The exact mechanism is unknown. The initial dose of opioids for the treatment of dyspnea is lower than that used for treating pain. An initial dose of just 1–2 mg of intravenous morphine or 5 mg of oral morphine may improve symptoms. Inhaled opioids (e.g., using a nebulized solution) have been evaluated for treatment of dyspnea, with mixed results. Much like for pain, a continuous symptom requires around the clock medication. Opioids may be delivered as an intravenous infusion or orally, depending on the patient's overall status and site of care. When dyspnea is accompanied by anxiety, the addition of benzodiazepines may further decrease the sensation of shortness of breath. Although oxygen is frequently prescribed for dyspnea, oxygen is only of use if hypoxia is a major contributor to the dyspnea. Nonpharmacologic treatments include relaxation, breathing training, facial cooling (via a fan), changes in position, and a decrease in ambient temperature.

Delirium

Delirium is a disturbance in consciousness that may occur suddenly or over hours to days and is characterized by cognitive dysfunction, emotional disturbances, and disorganized behavior. Symptoms tend to wax and wane and are often most severe at night and in settings unfamiliar to the patient. There are three types of delirium: hyperactive, hypoactive, and mixed. The hyperactive type is generally the easiest to recognize as patients present with agitation and restlessness and may experience rapid mood changes and hallucinations. Hypoactive delirium is underrecognized, especially in patients with advanced illness. The primary symptoms of this type of delirium, drowsiness, lethargy, and decreased engagement with the environment, may be misconstrued as progressive decline from the underlying cancer. Mixed delirium has symptoms of both of the prior types and the symptoms may fluctuate rapidly. Delirium occurs in up to 80% of patients at the end of life and may be reversible. Potential causes of delirium include hypoxia, infection, fever, uncontrolled pain, organ dysfunction, brain metastases, medication changes, constipation, urinary retention, or dehydration. Much like many symptoms in patients with advanced illness, the first consideration is determination of the balance between the burden of assessment to identify the underlying cause and its treatment and the patient's prognosis. Reversible causes should be evaluated and treated as appropriate for the patient's goals. A reversible cause is not always identified even when a work-up is undertaken.

Terminal delirium, or terminal restlessness, is a hyperactive delirium that occurs in some dying patients. It is sometimes called the "difficult road to death" and is generally associated with a prognosis of hours to days. Terminal delirium is an exceedingly distressing experience for families. Like all delirium, symptoms are managed aggressively to promote patient and family comfort. Family members caring for a patient with delirium have an increased risk of anxiety. However, education of family members on how to best care for their loved one, as well as continued emotional support via hospice, palliative care, and other professional caregivers can mitigate the negative effects of delirium on both patients and their loved ones.

Pharmacologic interventions for delirium include antipsychotic agents such as haloperidol, olanzapine, and quetiapine. Benzodiazepines, such as lorazepam, are not front-line therapy, because they may lead to paradoxical increase in agitation, especially in the elderly. However, they can be used with caution in conjunction with antipsychotic agents, when sedation is warranted and in the setting of irreversible delirium which may be more resistant to standard therapy (Table 4). Nonpharmacologic interventions include reorientation to the environment, reduction in stimulating factors, and improving sleep.

Constipation and Bowel Management

Constipation, a common symptom at the end of life, has a prevalence of 40%–80% and is a source of patient and family suffering. Causes of constipation are pharmacologic, metabolic, neurologic, and structural (Table 5). Additionally, decreased physical activity and changes in diet at the end of life frequently exacerbate constipation.

Table 4 Medications to manage delirium-titrate dose to effect

Typical antipsychotics	*Starting dose*	*Routes*
Haloperidol	0.5–2 mg every 2–12 h	PO, IV, SC
Chlorpromazine	12.5–50 mg every 4–6 h	PO, IV, SC, PR
Atypical antipsychotics		
Respiradone	0.5 mg daily	PO
Olanzapine	2.5–5 mg daily	PO
Seroquel	25 mg daily	PO
Anxiolytics/sedatives		
Lorazepam	0.5–2 mg q 4 h	IV, PO, SL, PR
Midazolam	0.5–1 mg/h	IV
Propofol	10 mg bolus ➔ 10 mg/h	IV

Table 5 Common causes for constipation at the end of life

Pharmacologic	Antacids, antiepileptics, cancer chemotherapeutic drugs, anticholinergics, antiparkinsonian, antidepressants, diuretics, opioids, neuroleptics, antitussives
Metabolic	Dehydration, hypercalcemia, diabetes, hypokalemia, uremia, hypothyroidism
Neurologic	Cerebral tumors, spinal cord or nerve involvement
Structural	Tumor mass, strictures, painful ano-rectal conditions
Functional and diet	Decreased oral intake, inactivity, sedation

Table 6 Approach to treatment of constipation

First line treatment ➔ combination of a softener (polyethylene glycol, or lactulose) and a stimulant (senna)
↓
No improvement in symptoms ➔ rectal suppository and enema, consider a peripheral acting opioid antagonist (in patients who are taking opioids) such as methylnaltrexone
↓
No improvement in symptoms ➔ manual evacuation, consider further diagnostic evaluation, discussion of goals of treatment

For patients who are taking in fluids and/or solids, an evaluation of constipation begins with a detailed history of the patient's oral intake and diet, bowel habits, recent medication changes, and other new symptoms. The physical examination can readily rule out fecal impaction and can provide clues to other potential causes including spinal cord compression or malignant bowel obstruction, for example. For patients who are still passing bowel movements, but less frequently or with greater difficulty because of hard stool, combining a stool softener with a motility agent is most effective. Use of a combination of agents is important in the end of life setting, since many of these patients do not have normal bowel contractility either due to physical inactivity or from side effects of medications. As a result, a stool softener alone may not enable patients to expel the stool. Ducosate is one widely available stool softener. Laxatives are categorized by their mechanism of action as lubricants (e.g., mineral oil), hyperosmotic agents (e.g., polyethylene glycol and magnesium citrate), or stimulants (senna and dulcolax). Specifically for opioid-induced constipation, peripheral opioid antagonists such as methylnaltrexone may be effective in relieving constipation but are expensive and require subcutaneous administration (Table 6).

As for most symptoms, prevention is generally easier than treatment. A daily oral regimen of stool softeners, with or without a stimulant, is sufficient to prevent constipation. However, if constipation is refractory to an escalating oral regimen, the addition of agents by rectum (suppository or enema) is indicated. Enemas introduce fluid into the rectum and intestine and stimulate a bowel movement via this mechanism. Suppositories may produce their effect as hyperosmotic agents, increasing intraluminal water and softening stool or as a stimulant.

Secretions

In the last hours of life, patients may develop noisy, and gurgling respirations; commonly known as the "death rattle." This "rattle" occurs due to the patient's inability to expectorate or swallow secretions. The "rattle" results from the air passing through the secretions pool with respirations. This is a symptom that is associated with a very short survival, generally hours to days. While these audible respirations are not thought to cause distress or discomfort for patients, the sounds are frequently disturbing for family members and caregivers. Initial treatment is nonpharmacologic and includes repositioning and decreasing fluid intake (if being delivered intravenously). Occasionally, oropharyngeal suctioning is attempted, but is not likely to be effective, since the secretions are usually much deeper than can be reached by the suctioning device. The backbone of pharmacologic interventions is anticholinergic agents, such as hyoscine, atropine, scopolamine, and glycopyrrolate. There is limited data to support one agent over the other, though it appears that earlier intervention results in better outcome regardless of which of these agents is initiated. Glycopyrrolate is often preferred in patients who are conscious since it does not cross into the central nervous system. Medications can be given via multiple routes: transdermal (e.g., a scopolamine patch), sublingual/oral (e.g., atropine), or as an IV infusion. Scopolamine often takes 4–8 h to work, supporting the need to initiate treatment as soon as symptoms begin.

It is often difficult to control secretions at the end of life. Most often, patients who develop audible secretions are no longer conscious and are unlikely to be distressed. Families benefit from support, education, and reassurance that their loved one is not likely suffering.

Psychological Symptoms

Depression and Anxiety

The prevalence of depression at the end of life is between 25% and 75%. Risk factors for depression include a prior history of depression, prior suicide attempts, social stresses, family history of depression, as well as poorly controlled symptoms. Depression causes

suffering, reduces quality of life, impairs the ability to find meaning, and may shorten survival. Patients at the end of life can be screened for depression with a simple screening tool. The PHQ-2 instrument for depression screening asks the following questions: over the past 2 weeks, how often have you been bothered by any of the following?

1. Little interest or pleasure in doing things.
2. Feeling down depressed or hopeless.

This tool has a sensitivity of 91% and a specificity of 86% based on the studies of patients with cancer receiving palliative care. For patients who are depressed it is important to screen for suicide. Asking the question, "Are you having suicidal thoughts?" is a good starting point and is an entry for asking more detailed questions regarding plan and intent. Treatment of depression includes pharmacologic and nonpharmacologic approaches. Pharmacologic approaches are diverse and can include selective serotonin reuptake inhibitors (SSRIs), serotonin, norepinephrine reuptake inhibitors, tricyclic antidepressants, or stimulants. The choice of medication should be based on prognosis and comorbid conditions. Many of the first-line antidepressants, such as the SSRIs, take 4–6 weeks to be effective and are only be useful for patients with a relatively long prognosis. For those patients closer to the end of life, a low-dose stimulant such as methylphenidate or modafinil may be appropriate for hypoactive symptoms. For patients with agitated depression, a neuroleptic such as risperidone may be a good choice. An option for patients with sleep difficulties or weight loss is mirtazapine, which has a relatively short time to efficacy and may also improve sleep and stimulate weight gain. Tricyclic antidepressants may be a good option for patients with pain and depression (**Table 7**).

Anxiety is quite common at the end of life and can affect patients both physically and psychologically. It can present as agitation, restlessness, insomnia, hyperventilation, or panic disorder. Management of anxiety at the end of life is primarily through the use of benzodiazepines. Short-acting drugs, such as alprazolam, are useful for acute symptoms, but for patients with a longer duration of symptoms, a longer acting benzodiazepine, such as clonazepam, is recommended.

Nonpharmacologic therapies are effective for anxiety and depression for some patients. For patients who are able to be out of bed for a proportion of each day, a gentle exercise program or yoga may improve mood. As patients become less mobile, they may benefit from structured counseling and interventions such as cognitive behavioral therapy and mindfulness-based stress reduction. Finally, existential and spiritual distress at the end of life may require spiritual care. Palliative and hospice care teams routinely provide chaplaincy and for others, religious counselors may be available.

Palliative Care and Hospice

Palliative care and hospice teams provide symptom management and end of life care. Palliative care is defined as "an approach that improves the quality of life of patients and their families facing the problems associated with life-threatening illness, through the prevention and relief of suffering by means of early identification and impeccable assessment and treatment of pain and other

Table 7 Medications for management of depression and anxiety at end of life

Medication	*Starting dose*	*Time to onset*	*Side effects*	*Other benefits*
Selective serotonin reuptake inhibitors		4–6 weeks	GI	
Citalopram	10 mg		Insomnia, agitation	
Escitalopram	5 mg			
Fluoxetine	5 mg			
Fluvoxamine	25 mg			
Paroxetine	10 mg			
Sertraline	12.5 mg			
Serotonin–norepinephrine reuptake inhibitors		4–6 weeks	GI distress,	Neuropathic pain
Venlafaxine	75 mg		Insomnia, agitation	
Duloxetine	10 mg			
Tricyclic antidepressants		2–3 weeks	Sedation	Pain
Nortryptline	10 mg		Anti-cholinergic	
Amitryptiline	25 mg		Insomnia, agitation	
Despiramine	10 mg		Cardiac arrhythmia	
Imipramine	25 mg		Weight gain	
Doxepin	10 mg		Orthostatic hypotension	
Stimulants		1–2 days	Cardiac arrhythmia	Fatigue
Modafinil	100 mg		Agitation	
Ritalin	10 mg			
Noradrenergic and specific serotonin inhibitor				
Mirtazapine	7.5 mg	1–2 weeks	Drowsiness, weight gain	Weight gain, sleep
Dopamine–norepinephrine reuptake inhibitors		1–2 weeks	Weight gain, insomnia agitation,	Activating agent
Wellbutrin	75 mg		GI distress	

problems, physical, psychosocial and spiritual" (WHO). The integration of palliative care early in a patient's care has been shown to improve mood and quality of life. Early integration has also been shown to improve the quality of end of life care with a reduction in chemotherapy use, hospitalizations, and intensive care. The American Society of Clinical Oncology (ASCO) guidelines recommend the integration of palliative care at diagnosis for all patients with a metastatic or recurrent cancer diagnosis and/or a high symptom burden.

Palliative care can be provided by a patient's primary providers (i.e., oncologist and primary care doctor) or by a specialist palliative team or a hospice team. Scope of practice includes symptom management, goals of care discussions and support, and end of life care. A specialist palliative care team is interdisciplinary and generally includes a physician, a nurse, a social worker, and a chaplain. The team works together to create a unified plan with the patient and family that takes into account their medical, social, psychological, and spiritual needs.

Patients usually transition from palliative care to hospice care when their prognosis is less than six limited and they are no longer receiving disease-modifying therapies. Hospice care, like palliative care, is provided by an interdisciplinary team. Hospice recognizes the patient and family as the unit of care. Hospice can provide end of life care both in a patient's home and in a facility. The focus is on symptom management and quality of life.

Special Topics

Hydration and Nutrition at the End of Life

Several studies have examined the role of artificial nutrition at the end of life. A Cochrane meta-analysis concluded that there is insufficient evidence to recommend artificial nutrition at the end of life. Additionally, enteral feeding requires invasive procedures with risk of infection, bleeding, and pain, as well as complications of feeding such as diarrhea, aspiration, and electrolyte abnormalities. Parenteral feeding also has unique risks including infection, glucose control abnormalities, hepatic dysfunction, volume overload, and cholecystitis. With these risks in mind, artificial nutrition is not recommended at the end of life.

Artificial hydration is more controversial than feeding; some proponents believe that it may help with delirium at the end of life. However, there are recognized risks from hydration at the end of life, including volume overload, ascites, and pulmonary edema. If hydration is to be prescribed, low volumes are recommended with the goal of relieving symptoms, rather than delivering a set amount of fluid. Continuous hydration is generally not recommended.

At the end of life, patients often do not feel hungry, so it is appropriate to provide food and fluid based on the patient's desires. This is often called "pleasure feeding" and may involve small quantities offered at frequent intervals. If patients are having difficulty with swallowing, patients still benefit from frequent mouth care (keeping the oral cavity moist and clean) since xerostomia is a troubling symptom for patients with advanced illness. Discontinuation of oral intake by patients is a major source of emotional distress for families. Families may feel as if they are starving their loved one. It is important to reassure families that loss of appetite is an expected symptom of advanced disease. Providing suggestions to families of activities, other than feeding, with which they can demonstrate their love may be helpful. Alternate family activities might include massage, reading together, games, or legacy projects. Support and education by the hospice or palliative care team can be very reassuring for patients and families.

Palliative Sedation

The emancipation principle of palliative care states that one should "spare no scientific or clinical effort to free dying persons from twisting and racking pain that invades, dominates and shrivels their consciousness and leaves them no psychic or mental space for the things they want to think and say and do before they die." While most symptoms are able to be controlled, there are situations at the end of life when patients' symptoms become refractory, where further invasive or noninvasive approaches are incapable of providing adequate relief, are associated with excessive morbidity, or cannot provide relief in a tolerable time frame. In these situations, palliative sedation may be considered to relieve suffering at the end of life.

Palliative sedation is a procedure that utilizes sedative medications, in a supervised setting, to reduce the awareness of intolerable suffering by a terminally ill patient who has a very limited prognosis (hours to days). If palliative sedation is being considered, involvement of a multidisciplinary team is recommended. A hospice or palliative care team will evaluate the patient and consider any potential remaining opportunities for symptom control to ensure that reversible causes of discomfort have been addressed. If no alternative approaches or treatments are identified, informed consent is obtained for palliative sedation from the patient or health care surrogate. The consent discussion includes an overview of the patient's general condition, acknowledgment that prior treatments have not successfully controlled symptoms, current prognosis, rational for palliative sedation, recommended medications to achieve sedation, potential alternatives, expected effects of sedation, and risks of sedation. If a patient is unable to give consent, and there is no health care proxy available, it is the physician's responsibility to use all measures needed (including sedation), to relieve suffering.

The medications commonly used in this setting are anxiolytics and barbiturates. Sedating medications are delivered intravenously and titrated to symptom relief. Neuroleptics may be used for patients with delirium. Opioids are continued, in addition to the sedation, for patients with pain.

Care for the family and the medical team taking care of the patient are imperative during the palliative sedation procedure. Family often finds comfort in being with their loved ones, but may need reassurance that sedation is an acceptable course of action.

Family also benefits from knowing what to expect as the process proceeds. The medical team also often requires reassurance. Health care providers may feel like they are inadvertently "killing" the patient. They may express personal distress and struggle with a decision to move forward with palliative sedation. Education for staff and debriefing at team meetings may provide much needed support for staff.

PAD and Euthanasia

PAD refers to the provision of a prescription of a lethal dose of medication by a physician for a terminally ill, competent person who intends to use the medication to end his or her own life. The practice has been legalized in several countries and in several states in the United States. In most cases, there is a protocol that patients must undergo to confirm the terminal nature of their illness, competence of the patient, and lack of coexisting psychiatric illness.

Euthanasia refers to the administration of a lethal dose of medication by a physician to a patient to relieve suffering. This is legal in only a few countries such as the Netherlands, Belgium, Luxembourg, and Colombia. Generally, euthanasia is considered for competent and conscious patients who have severe symptoms and terminal illness.

In both of these settings, the intent of PAD or euthanasia is to end life. This is quite different from palliative sedation, where the primary intent is to relieve suffering.

Conclusion

End of life support begins with thorough conversations regarding patient and family goals of care and documentation of wishes. The involvement of a multidisciplinary team such as a palliative care team or a hospice team can ensure that patients receive appropriate symptom management throughout their end of life experience. In special situations, patients may access PAD and/or euthanasia. All patients deserve the best quality of life possible through the end of life, with intensive symptom control and care for the family.

Prospective Vision

Care for patients at the end of life is an area of great interest as people continue to live longer and often live with comorbid illness. Novel therapeutics, such as immunotherapy and targeted therapy, are changing the landscape of cancer and bring forward the potential of caring for cancer as a chronic rather than life-limiting illness. The financial toxicity for patients at the end of life will only become more challenging, as patients live longer and often have to devote more financial resources toward their health care. A new system of health care delivery with a focus on prevention and interventions that improve quality of care, in addition to longevity, will be critical moving forward.

Further Reading

Back, A.L., Arnold, R.M., Quill, T.E., 2003. Hope for the best, and prepare for the worst. Annals of Internal Medicine 138 (5), 439–443.
Back, A.L., Arnold, R.M., Tulsky, J.A., Baile, W.F., Fryer-Edwards, K.A., 2005. On saying goodbye: Acknowledging the end of the patient–physician relationship with patients who are near death. Annals of Internal Medicine 142 (8), 682–685.
Byock, I., 2013. The best care possible. A physicians' quest to transform care through the end of life. In: Avery Publishing Group, New York.
Cherny, N., Portenoy, R., 1994a. Sedation in the treatment of refractory symptoms: Guidelines for evaluation and treatment. Journal of Palliative Care 10 (2), 31–38. Summer.
Earle, C.C., Neville, B.A., Landrum, M.B., et al., 2004. Trends in the aggressiveness of cancer care near the end of life. Journal of Clinical Oncology 22, 315–321.
Ferris, F., von Gunten, C., Emanuel, L., 2003. Competency in end-of-life care: Last hours of life. Journal of Palliative Medicine 6, 605–613.
Fitzgerald, P., Miller, K., Li, M., Rodin, G., 2015. Depressive disorders. Chapter 37. In: Holland, J., Breitbart, W., et al. (Eds.), Psycho-oncology, 3rd edn. Oxford University Press, Oxford.
Gawande, A., 2014. Being mortal: Medicine and what matters in the end, 1st edn. Metropolitan books/Henry Holt and Company, New York.
Grady, C., 2012. Advance directives, decision making and withholding and withdrawing treatment. Chapter 57. In: Berger, A., Shuster, J., Von Roenn, J. (Eds.), Principles and practice of supportive. Oncology, 4th edn. Williams and Wilkins, Lippincott.
Greenberg, D.B., 2012. Treating depression at the end of life. Chapter 41. In: Berger, A., Shuster, J., Von Roenn, J. (Eds.), Principles and Practice of Supportive. Oncology, 4th edn. Williams and Wilkins, Lippincott.
Greer, J.A., MacDonald, J., Traeger, L., 2015. Anxiety disorders. Chapter 39. In: Holland, J., Breitbart, W., et al. (Eds.), Psycho-Oncology, 3rd edn. Oxford University Press, Oxford.
Hagerty, R.G., Butow, P.N., Ellis, P.A., et al., 2004. Cancer patient preferences for communication of prognosis in the metastatic setting. Journal of Clinical Oncology 22, 1721–1730.
Huskamp, H.A., Keating, N.L., Malin, J.L., et al., 2009. Discussions with physicians about hospice among patients with metastatic lung cancer. Archives of Internal Medicine 169, 954–962.
Irwin, S.A., Buckholtz, G.T., Pirello, R.D., Hirst, J.M., Ferris, F.D., 2012. Recognizing and managing delirium. Chapter 40. In: Berger, A., Shuster, J., Von Roenn, J. (Eds.), Principles and practice of supportive. Oncology, 4th edn. Williams and Wilkins, Lippincott.
Johnston, S.C., Pfeifer, M.P., McNutt, R., 1995. The discussion about advance directives: Patient and physician opinions regarding when and how it should be conducted—End of life study group. Archives of Internal Medicine 155, 1025–1030.
Kalanithi, K., 2016. When breath becomes air. Bodley Head, New York.
Levy, M.H., 1996. Pharmacologic treatment of cancer pain. The New England Journal of Medicine 335, 1124–1132.
Lynn, J., 2001. Serving patients who may die soon and their families the role of hospice and other services. JAMA 285 (7), 925–932.

Quill, T.E., Arnold, R.M., Platt, F., 2001. "I wish things were different": Expressing wishes in response to loss, futility, and unrealistic hopes. Annals of Internal Medicine 135 (7), 551–555.
Ross, D.D., 2001a. Management of common symptoms in terminally ill patients: Part II. Constipation, delirium and dyspnea. American Family Physician 64 (6), 1019–1026.
Ross, D.D., 2001b. Management of common symptoms in terminally ill Patients: Part I. Fatigue, anorexia, cachexia, nausea and vomiting. American Family Physician 64 (5), 807–815.
Tulsky, J.A., Arnold, R.M., 2012. Communication during transitions in care. Chapter 48. In: Berger, A., Shuster, J., Von Roenn, J. (Eds.), Principles and practice of supportive. Oncology, 4th edn. Williams and Wilkins, Lippincott.
Twaddle, M.L., Kelley, S.A., 2012. Hospice. Chapter 46. In: Berger, A., Shuster, J., Von Roenn, J. (Eds.), Principles and practice of supportive. Oncology, 4th edn. Williams and Wilkins, Lippincott.
von Gunten, C.F., Ferris, F.D., Emanuel, L.L., 2000. Ensuring competency in end-of-life care: Communication and relational skills. JAMA 284 (23), 3051–3057. https://doi.org/10.1001/jama. 284.23.3051.
Yun, Y.H., Lee, C.G., Kim, S.Y., et al., 2004. The attitudes of cancer patients and their families toward the disclosure of terminal illness. Journal of Clinical Oncology 22, 307–314.
Cherny, N.I., Portenoy, R.K., 1994b. Sedation in the management of refractory symptoms: guidelines for evaluation and treatment. Journal of Palliative Care 10, 31–38.
Roy DJ, Journal of Palliative Care, 1990, vol. 6, pg. 3-4.

Relevant Websites

http://theconversationproject.org/ - The Conversation Project.
https://www.capc.org/ - The Center to Advance Palliative Care (CAPC).
http://aahpm.org/ - American Academy of Hospice and Palliative Medicine.
https://www.mypcnow.org/fast-facts - Palliative Care Network of Wisconsin. Fast Facts and Concepts.
https://www.asco.org/practice-guidelines/cancer-care-initiatives/palliative-care-oncology - American Society of Clinical Oncology. Palliative Care in Oncology.

Endometrial Cancer: Pathology and Genetics

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Epidemiology

Endometrial cancer, consisting predominantly of endometrial carcinoma (EC), is the 6th cancer in incidence in women worldwide. However, this malignancy is not among the 10 most common causes of cancer mortality globally, and ranks 10th in developed countries. This reflects the fact that endometrial cancer predominantly affects women in developing countries, where access to medical treatment is better. Additionally, most uterine corpus carcinomas are diagnosed at earlier stage and consist of grade 1–2 endometrioid carcinomas, tumors that are less aggressive compared to carcinomas in other organs. Disease incidence is increasing in developed countries, including both Europe and the United States.

Risk factors which predispose to development of endometrial cancer include obesity, diabetes, nulliparity, history of colon/breast carcinoma, ovulatory failure, exposure to endogenous or exogenous estrogen and tamoxifen treatment. Oral contraceptive use confers protection. Exposure to endogenous or exogenous estrogen may result from hormone-producing tumors, such as sex cord stromal tumors of the ovary, and from medical administration of estrogens, for example, in patients with polycystic ovary syndrome, respectively (Rahaman et al., 2017). Increased exposure to estrogen primarily affects type I carcinomas (see below).

Genetics

Several genetic syndromes are associated with increased risk for developing EC, accounting in total for 6% of EC cases, the most common of which is Lynch syndrome. Lynch syndrome, which also predisposes women to colon cancer, ovarian cancer and other malignancies, is inherited in an autosomal dominant manner which is caused by mutation in one of the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, and *PMS2*, or the epithelial cell adhesion molecule gene *EPCAM*, which regulates the expression of *MSH2*. Over 50% of women affected by the syndrome will present with a gynecological cancer. The risk for developing EC is in the range of 15%–60% depending on the gene involved.

Cowden syndrome is caused by mutation in the tumor suppressor gene *PTEN* (phosphatase and tensin homolog), and is associated with an up to 28% risk for developing EC, as well as high risk for developing breast, renal and thyroid cancer. Other syndromes that are more rarely implicated in EC include Li-Fraumeni syndrome, in which the *TP53* gene is mutated, Peutz-Jeghers syndrome, in which patients carry mutation in the tumor suppressor gene *STK11*, and PPAP, polymerase proofreading-associated polyposis, in which the *POLD1* and *POLE* genes, encoding subunits of DNA polymerases δ and ε, respectively, are mutated (Spurdle et al., 2017; Ring et al., 2017). Recently published recommendations advocate screening of all patients with newly diagnosed EC for Lynch syndrome using MMR immunohistochemistry (IHC) (Randall et al., 2017).

Morphology

The 2014 WHO classification of EC includes the following entities (Kurman et al., 2014):

Endometrioid carcinoma (EEC).
Mucinous carcinoma (MC).
Serous carcinoma (SC).
Clear cell carcinoma (CCC).
Neuroendocrine tumors (low-grade and high-grade).
Mixed cell adenocarcinoma.
Undifferentiated carcinoma.
Dedifferentiated carcinoma.

The detailed morphology of each tumor is discussed in the WHO classification, as well as in several other recent publications (Lax, 2017; Hanley et al., 2017). Briefly, EC consist of cells that resemble endometrial glands, occasionally with a papillary growth pattern, and a variable degree of atypia. They are graded 1–3 based on the percentage of tumor which has a solid growth pattern (**Fig. 1** A and B). Secretory changes (**Fig. 1**C) and squamous metaplasia may be seen. MC has >50% component with mucinous differentiation. SC have high-grade atypia and grow in glandular, papillary or solid pattern, with scalloping of the inner aspects of the glandular structures (**Fig. 1**D). CCC consists of overtly atypical cells with high-grade nuclei and clear or eosinophilic cytoplasm, often with hobnailing, growing in tubolocystic, solid or papillary architecture (**Fig. 1**E). Mixed tumors are not uncommon and should contain at least 5% of each component in order to be diagnosed as such. Carcinomas in which all cells combine two histological phenotypes, most often endometrioid and serous, additionally occur. Undifferentiated carcinomas are high-grade tumors which cannot be assigned to any of the above-mentioned histotypes, whereas in dedifferentiated carcinomas a component of grade 1–2 EEC can be identified (**Fig. 1**F and H). Carcinosarcomas (CS), previously perceived to be mesenchymal tumors, are now classified as mixed epithelial-mesenchymal tumors, but widely accepted to originate from high-grade carcinomas (**Fig. 1**I and J).

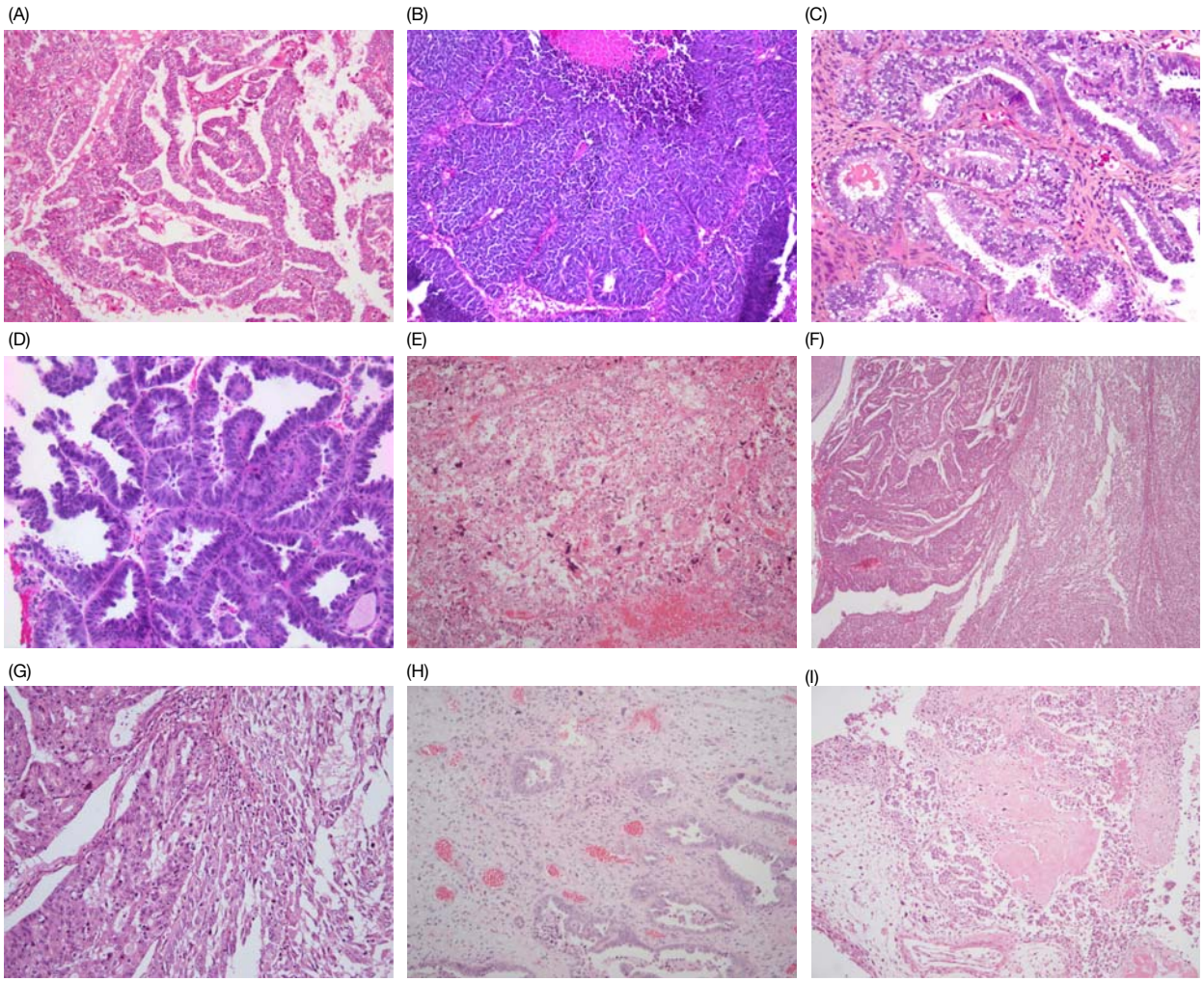

Fig. 1 Morphology. (A) Grade 1 endometrioid carcinoma. (B) Grade 3 endometrioid carcinoma. (C) Grade 1 endometrioid carcinoma with secretory features. (D) Serous carcinoma. (E) Clear cell carcinoma. (F and G) Dedifferentiated carcinoma. (H and I) Carcinosarcoma.

EC has been classically divided into type I and type II tumors. Type I EC consists of EEC, is estrogen-dependent, accounts for the majority of EC, is often associated with endometrial hyperplasia, and has relatively good prognosis. Type II tumors include non-endometrioid carcinomas (NEEC), that is, SC and CCC, which are clinically aggressive tumors, and are estrogen-independent, often developing in an atrophic endometrium. Activation of distinct molecular pathways has been identified in each of these groups (see below) (Yeramian et al., 2013; Matias-Guiu and Davidson, 2014; Murali et al., 2014). While some authors perceive CS as a distinct entity which should be excluded from the carcinoma category (Vaidya et al., 2006; Amant et al., 2005), there is a clear rationale in including these tumors in the type II group, given their cellular origin (Piulats et al., 2017).

While this dichotomous classification of EC holds true in most cases, it has its limitations. As discussed above, mixed tumors do occur. Additionally, some grade 1–2 EEC (G1EEC, G2EEC) are clinically aggressive. Finally, the relatively indolent clinical course of so-called type I tumors is limited to G1EEC and G2EEC. Grade 3 EEC (G3EEC) are recognized as clinically aggressive tumors, although the issue of whether they are as aggressive as SC and CCC has not been conclusively settled (Hamilton et al., 2006; Alkushi et al., 2010; Boruta et al., 2004; Park et al., 2013; Ayeni et al., 2013; Soslow et al., 2007; Voss et al., 2012).

In addition to the difficulty in assigning a clinically inclusive category for all EEC, there are also challenges in the differential diagnosis of the various EC histotypes. G3EEC and SC may have solid growth pattern and high-grade atypia, and SC may have a predominantly glandular growth pattern, leading to sub-optimal inter-observer reproducibility even among experts (Gilks et al., 2013), although several characteristics in distinguishing these tumors from each other have been published (Soslow, 2013; Clement and Young, 2004; Bartosch et al., 2011).

Difficulties may occasionally arise also in the differential diagnosis of SC from G1EEC and G2EEC, as well as in deciding whether the presence of tumor cells with clear cytoplasm truly represents CCC. In view of the overlapping morphology, ancillary methods, including both immunohistochemical staining for marker expression and molecular techniques have been frequently applied in order to reach a more conclusive diagnosis.

Immunohistochemical Markers

No single marker is by itself is able to differentiate between EEC, SC, and CCC. Consequently, panels of variable size and content have been assessed by different authors, and the choice of panels in everyday practice is similarly heterogeneous. Markers which have been tested in EC typing include p53, p16, estrogen receptor (ER), progesterone receptor (PR), monoclonal CEA (mCEA), PTEN, the MMR proteins hMLH1, hMSH2, hMSH6, and hPMS2, the insulin-like growth factor II (IGF-II) mRNA-binding protein family (IMP; IGFBP) members IMP2 and IMP3, Ki-67, β-catenin, the intestinal mucosal protein TFF3, The *ARID1A* protein product BAF250A, hepatocyte nuclear factor-1β (HNF1β), napsin A and high-mobility group AT-hook 2 (HMGA2) (Bartosch et al., 2011; Reid-Nicholson et al., 2006; Yemelyanova et al., 2009; Zheng et al., 2008; Li et al., 2007; Zhang et al., 2011; Mhawech-Fauceglia et al., 2010, 2013; Schlosshauer et al., 2002; Allo et al., 2014; McCluggage et al., 2012; Bárcena and Oliva, 2011; Fadare and Liang, 2012). Results have often been variable for markers tested by several groups.

In the most comprehensive of these studies, Han et al. analyzed the diagnostic role of 12 proteins, including ER, PR, p16, p53, Ki-67, PTEN, β-catenin, vimentin, IMP3, TFF3, ARID1A, and HNF1β in this differential diagnosis. Expression of TFF3, ARID1A loss and β-catenin expression had a specificity of 100%, but relatively low sensitivity (37%, 33%, and 7%, respectively) in diagnosing G3EEC. p53, p16 and IMP3 stained 94%, 80%, and 63% of SC compared to 26%, 11%, and 11% of G3EEC, respectively. The combination of ER, PR, p16, p53, vimentin, PTEN and IMP3 was 100% concordant with morphology, whereas the combination of ER, p16, and p53 was the most informative when applying a 3-marker panel (Han et al., 2013).

The author's preferred markers in the differential diagnosis between SC and EEC are p16, p53, PTEN, and WT1, despite the fact that the latter is often only focally expressed in uterine SC. ER and PR may be used as adjuncts, but have lower discriminating power. ARID1A, HNF1β, and napsin A are useful when CCC is a differential diagnosis. However, loss of ARID1A and nuclear expression of HNF1β may be seen in other histotypes, particularly in EEC. Examples of immunostains are shown in **Fig. 2**.

Molecular Analyses

In addition to differences in their morphology and marker protein expression profile, the different histotypes of EC have a unique molecular signature. Comparative analyses of type I and II EC, the latter with a focus on SC, have shown that EEC often has mutations in *PTEN*, *KRAS*, *PIK3CA*, *CTNNB1*, *ARID1A*, and *FGFR*, as well as microsatellite instability (MI), whereas type II tumors more often carry mutations in *TP53*, *PIK3R2*, and *PPP2R1A* and amplifications of *PIK3CA*, *CCND1*, and *CCNE1* (Yeramian et al., 2013). The possibility to use these differences in molecular profile for diagnostic purposes has been assessed by different groups. McConechy et al. found the use of a 9-gene test, including *ARID1A*, *PPP2R1A*, *PTEN*, *PIK3CA*, *KRAS*, *CTNNB1*, *TP53*, *BRAF*, and *PPP2R5C* to be useful in classifying EC (McConechy et al., 2012), and the validity of this signature was further assessed in a follow-up study with correlation to morphology and IHC profiles (Hoang et al., 2013).

The use of DNA ploidy as an ancillary technique with a diagnostic and prognostic role in EC has been advocated by some authors, and has recently been applied in several studies (Werner and Salvesen, 2014; Nastic et al., 2017; Proctor et al., 2017). However, this method has failed to gain widespread acceptance and is not in use in most institutions.

The fact that dividing all EC into type I or type II tumors is an oversimplification was further highlighted by the Cancer Genome Atlas (TCGA) study, an integrated genomic, transcriptomic and proteomic characterization of 373 EC, including 307 EEC, 53 SC and 13 mixed tumors. Somatic copy number alterations (SCNA) divided the studied tumors into four clusters, of which clusters 1–3 consisted almost entirely of EEC, divided by SNCA rates and 1q amplification, the latter associated with worse progression-free survival (PFS). Most SC and mixed tumors clustered in cluster four with a small number (36) of EEC, the majority of which were G3 EEC, and this group was characterized by extensive copy number alterations, few DNA methylation changes, low ER/PR levels, and frequent TP53 mutations, as well as MYC, ERBB2, and CCNE1 amplifications. This group had the worst survival. The majority of EEC had frequent mutations in PTEN, CTNNB1, PIK3CA, ARID1A, ARID5B, and KRAS, but few copy number alterations or TP53 mutations, whereas a smaller group of EC had increased mutation frequency and hotspot mutations in POLE. The latter group, consisting mainly of G3EEC, had excellent prognosis. Based on the combination of somatic nucleotide substitutions, microsatellite status (stability vs. instability; MSS vs. MSI) and SCNAs, EC were classified into four categories: POLE ultramutated, microsatellite instability hypermutated, copy-number low, and copy-number high (Cancer Genome Atlas Research Network et al., 2013).

Several follow-up studies based on the TCGA study have been published in recent years. Meng and co-workers analyzed 53 G3EEC, 25 SC, 16 CCC and 5 dedifferentiated carcinomas for the presence of *POLE* mutations by Sanger sequencing. *POLE* mutations were found in 8/53 (15%) G3EEC, of which only one had deficient MMR, but none of the other entities, and none of the patients with *POLE*-mutated tumors had disease progression (Meng et al., 2014).

Analysis of the morphology of these 8 *POLE*-mutated tumors and 8/17 *POLE*-mutated tumors from the TCGA cohort showed that the majority of these tumors had high-grade architecture and nuclear grade, as well as high mitotic counts. While all tumors except one had areas of endometrioid morphology, many tumors showed morphological heterogeneity and ambiguity. Many were rich in tumor-infiltrating lymphocytes and/or peri-tumoral lymphocytes. The majority of tumors had *PTEN*, *ARID1A*, and *KRAS* mutations (Hussein et al., 2015).

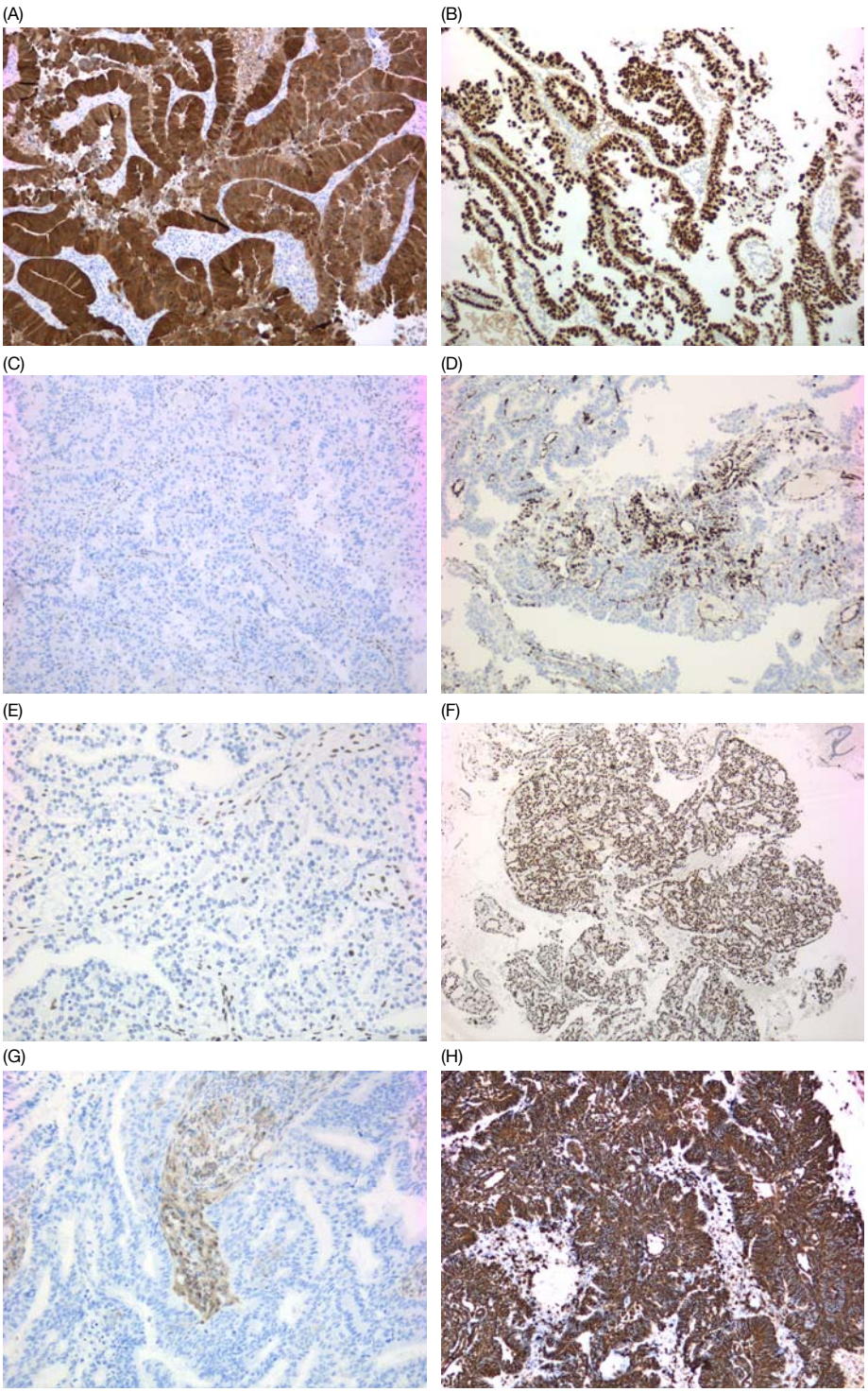

Fig. 2 Immunohistochemistry. (A) p16 staining in serous carcinoma. (B) Aberrant p53 (overexpression) in serous carcinoma. (C) Aberrant p53 ("null" pattern) in serous carcinoma. (D) WT1 stain in serous carcinoma. (E) Loss of ARID1A in clear cell carcinoma. (F) HNF1β stain in clear cell carcinoma. (G) Loss of PTEN in endometrioid carcinoma. (H) Vimentin expression in endometrioid carcinoma. (I) Focal CEA expression in endometrioid carcinoma. (J and M) Mismatch repair (MMR) proteins. Loss of MLH1 (J), intact MSH2 (K) and MSH6 (L), loss of PMS2.

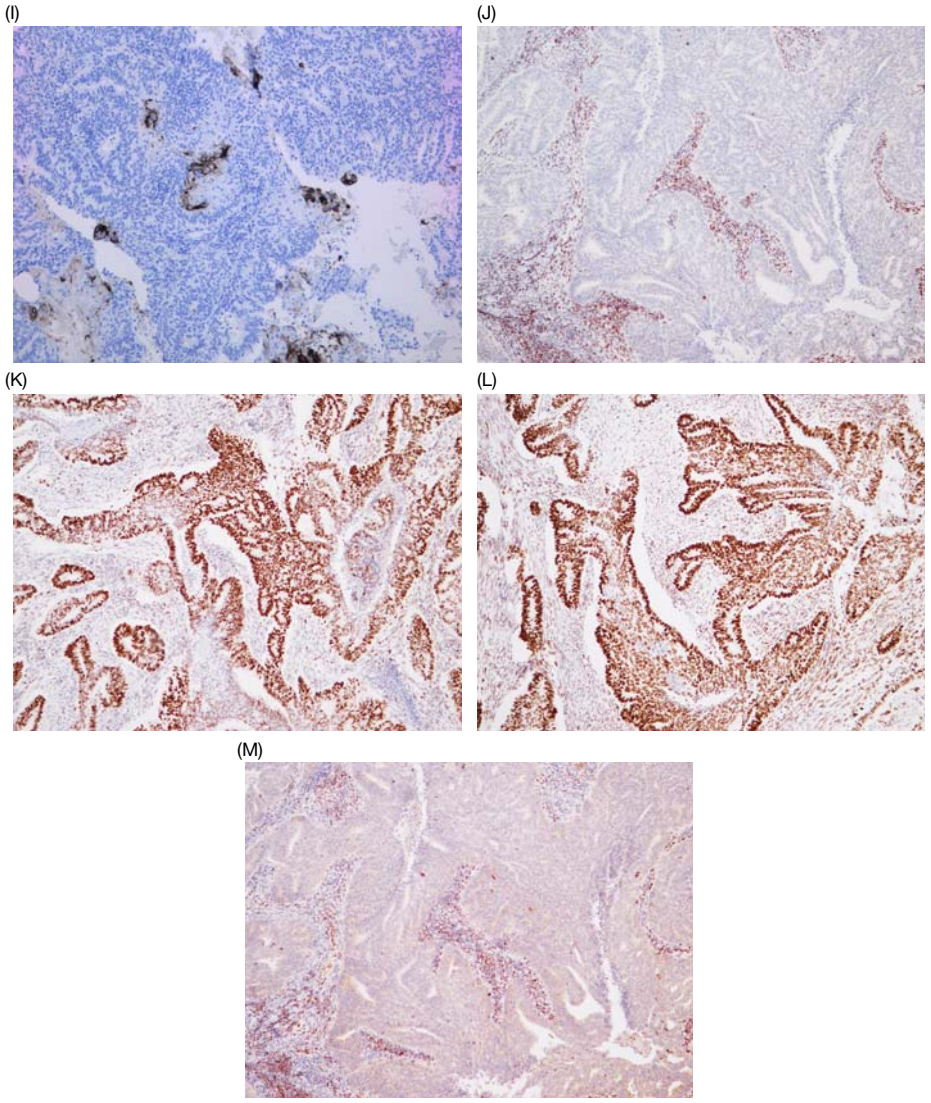

Fig. 2 (*continued*).

Billingsley et al. analyzed 544 EEC and found *POLE* mutations in 30 of them (5.6%). *POLE* mutations were more frequent in younger patients (< 60 years). However, no association with MS status or with PFS was observed. Association with longer overall survival (OS) was observed, but was lost in multivariate analysis (Billingsley et al., 2015).

Talhouk and co-workers analyzed 143 EC, including 119 EEC and 24 serous/mixed EC and found that MMR and p53 status by immunohistochemistry, combined with POLE mutation status, strongly correlate to clinicopathologic parameters and provide independent additional prognostic information (Talhouk et al., 2015). The usefulness of this classification was recently reproduced in analysis of 319 new EC cases (215 EEC, 104 non-EEC), in which this 3-marker combination was independently associated with OS, PFS and disease-specific survival (DSS) in multivariate analysis (Talhouk et al., 2017).

In an additional study related to this 3-paramter classification system, 151 EC were divided into *POLE*-mutated, MMR-deficient, p53 wildtype (wt) and p53 abnormal and morphologically classified by seven gynecologic pathologists into G1-2EEC, G3EEC, MC, SC, CCC, dedifferentiated carcinoma, CS, mixed carcinoma and other. Consensus among all seven pathologists was highest for p53 wt tumors (90%), intermediate for *POLE*-mutated and MMR-deficient tumors (65% and 58%, respectively) and lowest for abnormal p53 cases (39%). A large number of EEC and SC failed to fall in into the p53 wildtype (wt) and p53 abnormal categories, respectively, highlighting the need for ancillary techniques in this differential diagnosis (Hoang et al., 2017). The difficulties in reconciling the morphological and the genomic differential diagnosis of EEC and SC was highlighted in another study, in which inter-observer agreement between two gynecologic pathologists was better for copy-number low tumors than for the other TCGA categories (Hussein et al., 2016).

Schultheis et al. performed an in-depth analysis of *TP53* mutations in 228 EC from the TCGA dataset. Mutations were found in 64 (28%) tumors, were more frequent in SC compared to EEC (88% vs. 15%, respectively) and were associated with poor survival.

No association between the type of *TP53* mutation (i.e., missense, frameshift, or nonsense) and tumor type was found. However, TP53 hotspot mutations were more common in SC than in EEC. Somatic frameshift or nonsense "null mutations" that result in negative p53 protein expression and are diagnostically more challenging than overexpression were found in 22% of the mutated tumors (Schultheis et al., 2016).

Two other genes that are central in the biology of EC are *ARID1A* and *CTNNB1*.

ARID1A is member of a family of chromatin remodelers belonging to the polymorphic BRG-/BRM-associated factor (BAF), the Switch/Sucrose Non-Fermentable (SWI/SNF) complex, and Polybromo-associated BAF (PBAF). Mutations in genes belonging to these complexes have been found in >20% of cancers. *ARID1A*, a tumor suppressor located on chromosome 1p36.11, is the most commonly mutated among these genes, with mutations reported in various carcinomas, most frequently in ovarian CCC (50% of tumors) and in uterine and ovarian EEC (30%–40% of tumors), as well as Burkitt lymphoma, medulloblastoma and neuroblastoma. Mutations are typically nonsense or frameshift and result in loss of expression of the ARID1A (a.k.a. BAF250A) protein (Hodges et al., 2016; Takeda et al., 2016; Bitler et al., 2015; Gounaris and Brenton, 2015). Analysis of 190 high-grade EC, including 82 G3EEC, 88 SC, 10 CCC and 10 mixed tumors, showed loss of BAF250a, the protein product of ARID1A, in 55 (29%) tumors, the majority of which were G3EEC. BAF250a loss was significantly associated with MMR protein loss and intact p53. BAF250a loss was significantly associated with better PFS in the entire cohort, but only MMR and p53 status was informative of survival in separate analysis of G3EEC and SC. Analysis of the TCGA data showed that *ARID1A* mutations were negatively associated with *TP53* mutations and unrelated to MMR gene mutations (Allo et al., 2014).

The Wnt/β-catenin signaling pathway is deregulated in 10%–45% EC, mainly EEC, via inactivating mutations in the *CTNNB1* gene encoding β-catenin, or epigenetic regulation of the Wnt pathway (Dellinger et al., 2012). Liu and co-workers performed unsupervised clustering of gene expression profiling of 271 EEC from the TCGA series and identified four clusters that were statistically significantly associated with tumor stage, grade, MSI status and the three mRNA clusters characterized in the TCGA study. Clusters III and IV contained most of the G3ECC and stage III–IV tumors, while clusters I and II consisted mainly of G1-2EEC and stage I–II tumors. Cluster II cases were enriched for *CTNNB1* exon 3 mutations (87%; 47/54) of these mutations were significantly associated with Wnt/β-catenin signaling activation. In G1-2EEC tumors, high expression levels of *CTNNB1*, *MYC*, and *CCND1* was associated with poor OS. This clustering into four groups was confirmed in an independent dataset (Liu et al., 2014).

The role of epigenetic regulation of gene expression is now recognized as central in cancer biology, and this topic has recently been reviewed in two comprehensive papers by Bartosch et al. (2017a,b). Promoter hypermethylation has been reported for hormone receptors, *MLH1*, *MGMT*, *CDKN2A* (encoding p16) and molecules belonging to the phosphatidylinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), fibroblast growth factor (FGF) and WNT pathways. Hypomethylation has been less frequently studied, but has been reported for *PAX2*, *S100A4*, *PARP1*, *BMP*, *BORIS*, and *14-3-3 sigma* (Bartosch et al., 2017a).

Wentzensen et al. analyzed 148 EC and 23 normal endometria for methylation profile using 1500 probes for 807 genes. A total of 114 CpG sites on 37 genes that had significant differences in methylation at a cut-off of $P \leq 10^{-7}$ were identified. Eight genes (*ADCYAP1*, *ASCL2*, *CDH13*, *HS3ST2*, *HTR1B*, *MME*, *NPY* and *SOX1*) chosen for validation were confirmed to be discriminators between the two sample types in analysis of a second series of 69 EC and 40 normal endometria, as well as based on the TCGA data. HTR1B and SOX1 were shown to effectively differentiate SC from normal endometrium (Wentzensen et al., 2014).

Other molecules involved in epigenetic regulation of EC are those modifying histones, including histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs). Several of these molecules have been shown to be differentially expressed in EC compared to normal endometrium, and members of all four classes, including the HDACs members HDAC1, HDAC2 and Sirtuin 1 (SIRT1), the HAT Elongator complex protein 3 (ELP3), the HMT Enhancer of zeste homolog 2 (EZH2), and the HDM Lysine-specific histone demethylase 1 (LSD1), have been associated with aggressive behavior in EC. A large number of microRNA have also been shown to be deregulated, both over- and under-expressed in EC (Bartosch et al., 2017b).

The TCGA project has focused on molecular characteristics of EEC and SC, leaving the genomic characterization of CCC less explored. In a recent paper, DeLair and co-workers studied 32 pure CCC, including 16 FIGO stage I and 16 FIGO stage III or IV tumors, using IHC and massively parallel sequencing targeting of 300 cancer-related genes. Uterine CCC were shown to be a heterogeneous group which includes tumors that may be molecularly classified in all four TCGA groups. By IHC, abnormal protein expression of p53, ARID1A and at least one MMR member, most commonly MSH6, was found in 11 (34%), 7 (22%), and 6 (19%) tumors. At the molecular level, two ultramutated tumors with *POLE* mutation were found, while the remaining cases harbored *TP53* (46%), *PIK3CA* (36%), *PPP2R1A* (36%), *FBXW7* (25%), *ARID1A* (21%), *PIK3R1* (18%), and *SPOP* (18%) mutations, many of them in hotspot locations. *CCNE1* and *ERBB2* amplification and *DAXX* homozygous deletion was found in 5/28, 3/28, and 3/28 analyzed tumors, respectively. Based on the observed heterogeneity, the authors questioned the validity of classifying all CCC as high-grade/type II tumors (DeLair et al., 2017).

CS constitute 4.3% of uterine cancers and are highly aggressive tumors with a high recurrence rate of 37% and 5-year survival at 65% or less even at FIGO stage I. Current treatment has not led to major improvements in the outcome of patients with this malignancy (Cherniack et al., 2017).

A comprehensive molecular analysis of 57 uterine CS was recently published (Gibson et al., 2016). The most frequent mutations found were in *TP53*, detected in 91% of tumors. Additional mutations were detected in the PI3K pathway genes *PIK3CA* (35%), *PTEN* (19%), and *PIK3R1* (11%). *PTEN* and *TP53* mutations co-existed in 8/11 tumors harboring the former. Other mutated genes included *FBXW7* (28%), *PPP2R1A* (28%), *CDH4* (18%), *KRAS* (12%), *ARID1A* (12%), *RB1* (11%), *ARHGAP35* (11%), *ZBTB7B*

(11%), *SPOP* (7%), and *U2AF1* (4%). The majority of these genes, though not all, have been previously shown to be mutated in EC. Analysis of RNA sequencing data showed kinase-domain gene fusions in *NUP210-MAST1*, *CENPP-WNK2*, and *DDX6-ALK* in three tumors. Gene amplifications were observed in *TERC* (3q26.2), *FGFR3* (4p16.3), *MYC* (8q24.21), *KAT6A* (10q22.2), *MDM2* (12q15), *ERBB2* (17q12), *CCNE1* (19q12), *BCL2L1* (20q11.21), and RIT1 (1q22). A 76-gene signature was used to assign an epithelial-to-mesenchymal transition (EMT) score. Proteomics analysis was not informative with respect to clustering of tumors.

Prediction, Prognosis and Targeted Therapy

The difficulty in classifying EC based on morphology, IHC and molecular analysis owes to remarkable heterogeneity both across histological types and within morphologically similar tumors, as well as the existence of tumors combining the features of more than one histotype. Further complicating this issue is the observation that approximately half of the genetic changes observed in metastatic EC are not present in the primary tumor (Gibson et al., 2016). This heterogeneity has in turn limited the ability to identify predictive and prognostic markers that are reproducible and to identify therapeutic targets for this cancer. Consequently, at present no molecule is approved as therapy target for patients with EC, with the exception of hormone receptors.

In addition to clinicopathologic parameters, numerous biomarkers have been reported to have a prognostic role in EC at the protein and/or molecular level, including ER and PR, PTEN, Stathmin, L1CAM, β-catenin, molecules in the PI3K and MAPK pathway, vascular endothelial growth factor (VEGF), POLE, p53, epidermal growth factor receptor (EGFR), HER2, FGFR2, MSI/DNA repair, DNA ploidy (Matias-Guiu and Davidson, 2014; Werner and Salvesen, 2014; Binder and Mutch, 2014; Buhtoiarova et al., 2016; McAlpine et al., 2016). Candidate predictive markers, of which many are also targetable molecules, include ER and PR, Stathmin, L1CAM, HER2, the PI3K and MAPK pathways, KRAS, EGFR, FGFR and VEGF, as well as metabolic treatment using metformin and modulation of the microenvironment, including the immune response (Hoang et al., 2013; Binder and Mutch, 2014; Buhtoiarova et al., 2016; McAlpine et al., 2016; Lheureux and Oza, 2016; MacKay et al., 2017; Morice et al., 2016). Clinical trials focusing on some of these targets are discussed in several of these publications (Lheureux and Oza, 2016; MacKay et al., 2017; Eritja et al., 2017).

Two biomarkers of interest which have not been discussed in this article in the context of molecular changes, but are of interest at the protein level, are Stathmin and L1CAM. These are discussed below as examples of potentially targetable molecules in EC.

Stathmin (a.k.a. oncoprotein 18/Op18, LPA18 or metablastin) is a 149 amino acid microtubule-destabilizing phosphoprotein which mediates its effect via sequestration of free tubulin or induction of mitotic catastrophe. Stathmin has been reported to be expressed in many cancers, in which it has generally been associated with aggressive clinical behavior (Belletti and Baldassarre, 2011; Biaoxue et al., 2016a,b).

Stathmin expression is associated with aggressive clinical behavior and activation of the PI3K pathway in EC (Salvesen et al., 2009), and Stathmin expression was reported to be a better marker of PI3K pathway activation than AKT or p-AKT in this cancer (Trovik et al., 2010). In a follow-up study with a total of 1076 patients, Stathmin expression was significantly related to non-endometrioid histology, high grade, and aneuploidy, independently predicted lymph node metastasis, and was significantly associated with poor disease-specific survival (Trovik et al., 2011). Stathmin expression was further shown to be related to poor response to paclitaxel both in vitro and in clinical specimens (Werner et al., 2014). Stathmin expression was recently reported to be associated with shorter PFS and OS in the GOG-177 cohort of 69 patients, particularly in patients who received adriamycin/cisplatin only (Reyes et al., 2017). Conversely, in a recent multi-parameter analysis of 460 EC (10.8% cases not informative for Stathmin), Stathmin expression was not significantly related to survival (Karnezis et al., 2017).

L1CAM is a 200–220 kDa transmembrane glycoprotein belonging to the immunoglobulin (Ig) supergene family that is involved in development of nervous system via regulation of neuronal migration. L1CAM has in recent years been reported to be expressed in different cancers, including various carcinomas, melanoma and gastrointestinal stromal tumor (GIST) and is informative of clinical outcome in several of them (Altevogt et al., 2016). The possibility of targeting L1CAM has been assessed in several experimental models (Grünberg et al., 2005; Gast et al., 2008).

L1CAM was shown to be overexpressed in SC and CCC compared to EEC and its expression in the latter group, observed in 78/272 tumors, was related to poor survival. L1CAM expression was further showed to be related to absence of ER, PR and E-cadherin and development of EMT (Huszar et al., 2010). This was reproduced in a multicenter study of 1021 stage I EC, the majority of which were EEC, in which L1CAM was a strong predictor of relapse and death of disease (Zeimet et al., 2013). Similar results were observed in pooled analysis of EC ($n = 865$) from two randomized controlled trials (PORTEC-1 and -2) (Bosse et al., 2014). In a multicenter study by the ENITEC consortium, in which 1199 EC were analyzed, L1CAM was expressed in of 93/935 (10%) stage I EEC, 28/160 (18%) advanced-stage EEC, and 78/104 (75%) non-endometrioid tumors. L1CAM expression was significantly associated with advanced stage, nodal involvement, high grade, non-endometrioid histology, lymphovascular space invasion, and distant recurrence in the entire cohort, and with poor survival in patients with EEC (van der Putten et al., 2016). Similarly, analysis of 805 EC, in which 121 tumors (15%) were L1CAM-positive, demonstrated significant association with advanced stage, high grade, non-endometrioid histology, lymphovascular space invasion, cervical stromal invasion, positive peritoneal cytology and age >65 years, as well as poor disease-specific survival in patients with EEC, which was confirmed in a Cox multivariate analysis (Pasanen et al., 2016). L1CAM was additionally reported to be associated with the p53 abnormal molecular subtype based on the ProMisE classification, as well as with poor survival, in the study by Karnezis et al. (2017). In contrast, L1CAM was not significantly

associated with the risk of relapse or death of any cause in a recent analysis of 388 stage I EEC, although L1CAM was significantly associated with risk of relapse in patients who were not treated with chemotherapy (Smogeli et al., 2016).

A cut-off at 50% rather than 10%, as has been used in the above-mentioned studies, has recently been proposed in order to identify high-risk EC patients (Van Gool et al., 2016).

Prospective Vision

The majority of EC can be cured by surgery. While this group consists predominantly of G1-2EEC, it is evident that not all so-called type II EC are truly clinically aggressive cancers. At the same time, some so-called type I EC do recur, of which some prove lethal. Major improvements in our understanding of this cancer have been made in recent years, mainly based on highly informative molecular analyses, particularly the TCGA study. Difficulty in reconciling the morphologic heterogeneity of EC with expression patterns by IHC and molecular heterogeneity nevertheless remains, a fact which makes the identification of a widely-accepted diagnostic panel which correlates with clinical behavior highly desirable. Whether this will be a limited assay, such as the ProMisE approach, or a more comprehensive assay, such as the 53-gene signature suggested by Dai and co-workers (2016), remains to seen, although it is likely that the former, or a comparable assay, will be adopted for practical reasons. Analyses of biomarker expression, with the aim of applying targeted therapy, are likely to benefit from the identification of patient sub-groups with tumors that express the target proteins and in which these proteins have a clinical role. As in other cancers, large multi-institutional studies are a cornerstone of this research and its clinical implementation.

References

Alkushi, A., Köbel, M., Kalloger, S.E., Gilks, C.B., 2010. High-grade endometrial carcinoma: Serous and grade 3 endometrioid carcinomas have different immunophenotypes and outcomes. International Journal of Gynecological Pathology 29 (4), 343–350.

Allo, G., Bernardini, M.Q., Wu, R.C., et al., 2014. ARID1A loss correlates with mismatch repair deficiency and intact p53 expression in high-grade endometrial carcinomas. Modern Pathology 27 (2), 255–261.

Altevogt, P., Doberstein, K., Fogel, M., 2016. L1CAM in human cancer. International Journal of Cancer 138 (7), 1565–1576.

Amant, F., Cadron, I., Fuso, L., Berteloot, P., de Jonge, E., Jacomen, G., Van Robaeys, J., Neven, P., Moerman, P., Vergote, I., 2005. Endometrial carcinosarcomas have a different prognosis and pattern of spread compared to high-risk epithelial endometrial cancer. Gynecologic Oncology 98 (2), 274–280.

Ayeni, T.A., Bakkum-Gamez, J.N., Mariani, A., McGree, M.E., Weaver, A.L., Haddock, M.G., Keeney, G.L., Long 3rd, H.J., Dowdy, S.C., Podratz, K.C., 2013. Comparative outcomes assessment of uterine grade 3 endometrioid, serous, and clear cell carcinomas. Gynecologic Oncology 129 (3), 478–485.

Bárcena, C., Oliva, E., 2011. WT1 expression in the female genital tract. Advances in Anatomic Pathology 18 (6), 454–465.

Bartosch, C., Manuel Lopes, J., Oliva, E., 2011. Endometrial carcinomas: A review emphasizing overlapping and distinctive morphological and immunohistochemical features. Advances in Anatomic Pathology 18 (6), 415–437.

Bartosch, C., Lopes, J.M., Jerónimo, C., 2017a. Epigenetics in endometrial carcinogenesis—Part 1: DNA methylation. Epigenomics 9 (5), 737–755.

Bartosch, C., Lopes, J.M., Jerónimo, C., 2017b. Epigenetics in endometrial carcinogenesis—Part 2: Histone modifications, chromatin remodeling and noncoding RNAs. Epigenomics 9 (6), 873–892.

Belletti, B., Baldassarre, G., 2011. Stathmin: A protein with many tasks. New biomarker and potential target in cancer. Expert Opinion on Therapeutic Targets 15, 1249–1266.

Biaoxue, R., Xiguang, C., Hua, L., Shuanying, Y., 2016a. Stathmin-dependent molecular targeting therapy for malignant tumor: The latest 5 years' discoveries and developments. Journal of Translational Medicine 14 (1), 279.

Biaoxue, R., Hua, L., Wenlong, G., Shuanying, Y., 2016b. Overexpression of stathmin promotes metastasis and growth of malignant solid tumors: A systemic review and meta-analysis. Oncotarget 7 (48), 78994–79007.

Billingsley, C.C., Cohn, D.E., Mutch, D.G., Stephens, J.A., Suarez, A.A., Goodfellow, P.J., 2015. Polymerase ε (POLE) mutations in endometrial cancer: Clinical outcomes and implications for lynch syndrome testing. Cancer 121 (3), 386–394.

Binder, P.S., Mutch, D.G., 2014. Update on prognostic markers for endometrial cancer. Women & Health 10 (3), 277–288.

Bitler, B.G., Fatkhutdinov, N., Zhang, R., 2015. Potential therapeutic targets in ARID1A-mutated cancers. Expert Opinion on Therapeutic Targets 19, 1419–1422.

Boruta 2nd, D.M., Gehrig, P.A., Groben, P.A., Bae-Jump, V., Boggess, J.F., Fowler Jr., W.C., Van Le, L., 2004. Uterine serous and grade 3 endometrioid carcinomas: Is there a survival difference? Cancer 101 (10), 2214–2221.

Bosse, T., Nout, R.A., Stelloo, E., Dreef, E., Nijman, H.W., Jürgenliemk-Schulz, I.M., Jobsen, J.J., Creutzberg, C.L., Smit, V.T., 2014. L1 cell adhesion molecule is a strong predictor for distant recurrence and overall survival in early stage endometrial cancer: Pooled PORTEC trial results. European Journal of Cancer 50 (15), 2602–2610.

Buhtoiarova, T.N., Brenner, C.A., Singh, M., 2016. Endometrial carcinoma: Role of current and emerging biomarkers in resolving persistent clinical dilemmas. American Journal of Clinical Pathology 145 (1), 8–21.

Cancer Genome Atlas Research Network, Kandoth, C., Schultz, N., Cherniack, A.D., Akbani, R., Liu, Y., Shen, H., Robertson, A.G., Pashtan, I., Shen, R., Benz, C.C., Yau, C., Laird, P.W., Ding, L., Zhang, W., Mills, G.B., Kucherlapati, R., Mardis, E.R., Levine, D.A., 2013. Integrated genomic characterization of endometrial carcinoma. Nature 497, 67–73.

Cherniack, A.D., Shen, H., Walter, V., Stewart, C., Murray, B.A., Bowlby, R., Hu, X., Ling, S., Soslow, R.A., Broaddus, R.R., Zuna, R.E., Robertson, G., Laird, P.W., Kucherlapati, R., Mills, G.B., Cancer Genome Atlas Research Network, Weinstein, J.N., Zhang, J., Akbani, R., Levine, D.A., 2017. Integrated molecular characterization of Uterine Carcinosarcoma. Cancer Cell 31 (3), 411–423.

Clement, P.B., Young, R.H., 2004. Non-endometrioid carcinomas of the uterine corpus: A review of their pathology with emphasis on recent advances and problematic aspects. Advances in Anatomic Pathology 11 (3), 117–142.

Dai, D., Thiel, K.W., Salinas, E.A., Goodheart, M.J., Leslie, K.K., Gonzalez Bosquet, J., 2016. Stratification of endometrioid endometrial cancer patients into risk levels using somatic mutations. Gynecologic Oncology 142 (1), 150–157.

DeLair, D.F., Burke, K.A., Selenica, P., Lim, R.S., Scott, S.N., Middha, S., Mohanty, A.S., Cheng, D.T., Berger, M.F., Soslow, R.A., Weigelt, B., 2017. The genetic landscape of endometrial clear cell carcinomas. The Journal of Pathology 243 (2), 230–241.

Dellinger, T.H., Planutis, K., Tewari, K.S., Holcombe, R.F., 2012. Role of canonical Wnt signaling in endometrial carcinogenesis. Expert Review of Anticancer Therapy 12 (1), 51–62.

Eritja, N., Yeramian, A., Chen, B.J., Llobet-Navas, D., Ortega, E., Colas, E., Abal, M., Dolcet, X., Reventos, J., Matias-Guiu, X., 2017. Endometrial carcinoma: Specific targeted pathways. Advances in Experimental Medicine and Biology 943, 149–207.

Fadare, O., Liang, S.X., 2012. Diagnostic utility of hepatocyte nuclear factor 1-Beta immunoreactivity in endometrial carcinomas: Lack of specificity for endometrial clear cell carcinoma. Applied Immunohistochemistry & Molecular Morphology 20 (6), 580–587.
Gast, D., Riedle, S., Issa, Y., Pfeifer, M., Beckhove, P., Sanderson, M.P., Arlt, M., Moldenhauer, G., Fogel, M., Krüger, A., Altevogt, P., 2008. The cytoplasmic part of L1-CAM controls growth and gene expression in human tumors that is reversed by therapeutic antibodies. Oncogene 27 (9), 1281–1289.
Gibson, W.J., Hoivik, E.A., Halle, M.K., Taylor-Weiner, A., Cherniack, A.D., Berg, A., Holst, F., Zack, T.I., Werner, H.M., Staby, K.M., Rosenberg, M., Stefansson, I.M., Kusonmano, K., Chevalier, A., Mauland, K.K., Trovik, J., Krakstad, C., Giannakis, M., Hodis, E., Woie, K., Bjorge, L., Vintermyr, O.K., Wala, J.A., Lawrence, M.S., Getz, G., Carter, S.L., Beroukhim, R., Salvesen, H.B., 2016. The genomic landscape and evolution of endometrial carcinoma progression and abdominopelvic metastasis. Nature Genetics 48 (8), 848–855.
Gilks, C.B., Oliva, E., Soslow, R.A., 2013. Poor interobserver reproducibility in the diagnosis of high-grade endometrial carcinoma. The American Journal of Surgical Pathology 37 (6), 874–881.
Gounaris, I., Brenton, J.D., 2015. Molecular pathogenesis of ovarian clear cell carcinoma. Future Oncology 11, 1389–1405.
Grünberg, J., Novak-Hofer, I., Honer, M., Zimmermann, K., Knogler, K., Bläuenstein, P., Ametamey, S., Maecke, H.R., Schubiger, P.A., 2005. In vivo evaluation of 177Lu- and 67/64Cu-labeled recombinant fragments of antibody chCE7 for radioimmunotherapy and PET imaging of L1-CAM-positive tumors. Clinical Cancer Research 11 (14), 5112–5120.
Hamilton, C.A., Cheung, M.K., Osann, K., Chen, L., Teng, N.N., Longacre, T.A., Powell, M.A., Hendrickson, M.R., Kapp, D.S., Chan, J.K., 2006. Uterine papillary serous and clear cell carcinomas predict for poorer survival compared to grade 3 endometrioid corpus cancers. British Journal of Cancer 94 (5), 642–646.
Han, G., Sidhu, D., Duggan, M.A., Arseneau, J., Cesari, M., Clement, P.B., Ewanowich, C.A., Kalloger, S.E., Köbel, M., 2013. Reproducibility of histological cell type in high-grade endometrial carcinoma. Modern Pathology 26 (12), 1594–1604.
Hanley, K.Z., Birdsong, G.G., Mosunjac, M.B., 2017. Recent developments in surgical pathology of the uterine corpus. Archives of Pathology & Laboratory Medicine 141 (4), 528–541.
Hoang, L.N., McConechy, M.K., Köbel, M., Han, G., Rouzbahman, M., Davidson, B., Irving, J., Ali, R.H., Leung, S., McAlpine, J.N., Oliva, E., Nucci, M.R., Soslow, R.A., Huntsman, D.G., Gilks, C.B., Lee, C.H., 2013. Histotype-genotype Correlation in 36 high-grade endometrial carcinomas. The American Journal of Surgical Pathology 37 (9), 1421–1432.
Hoang, L.N., Kinloch, M.A., Leo, J.M., Grondin, K., Lee, C.H., Ewanowich, C., Köbel, M., Cheng, A., Talhouk, A., McConechy, M., Huntsman, D.G., McAlpine, J.N., Soslow, R.A., Gilks, C.B., 2017. Interobserver agreement in endometrial carcinoma histotype diagnosis varies depending on the cancer genome atlas (TCGA)-based molecular subgroup. The American Journal of Surgical Pathology 41 (2), 245–252.
Hodges, C., Kirkland, J.G., Crabtree, G.R., 2016. The many roles of BAF (mSWI/SNF) and PBAF complexes in cancer. Cold Spring Harbor Perspectives in Medicine 6 (8), a026930.
Hussein, Y.R., Weigelt, B., Levine, D.A., Schoolmeester, J.K., Dao, L.N., Balzer, B.L., Liles, G., Karlan, B., Köbel, M., Lee, C.H., Soslow, R.A., 2015. Clinicopathological analysis of endometrial carcinomas harboring somatic POLE exonuclease domain mutations. Modern Pathology 28 (4), 505–514.
Hussein, Y.R., Broaddus, R., Weigelt, B., Levine, D.A., Soslow, R.A., 2016. The genomic heterogeneity of FIGO grade 3 endometrioid carcinoma impacts diagnostic accuracy and reproducibility. International Journal of Gynecological Pathology 35 (1), 16–24.
Huszar, M., Pfeifer, M., Schirmer, U., Kiefel, H., Konecny, G.E., Ben-Arie, A., Edler, L., Münch, M., Müller-Holzner, E., Jerabek-Klestil, S., Abdel-Azim, S., Marth, C., Zeimet, A.G., Altevogt, P., Fogel, M., 2010. Up-regulation of L1CAM is linked to loss of hormone receptors and E-cadherin in aggressive subtypes of endometrial carcinomas. The Journal of Pathology 220 (5), 551–561.
Karnezis, A.N., Leung, S., Magrill, J., McConechy, M.K., Yang, W., Chow, C., Kobel, M., Lee, C.H., Huntsman, D.G., Talhouk, A., Kommoss, F., Gilks, C.B., McAlpine, J.N., 2017. Evaluation of endometrial carcinoma prognostic immunohistochemistry markers in the context of molecular classification. Journal of Pathology: Clinical Research 3 (4), 279–293.
Kurman, R.J., Carcangiu, M.L., Herrington, C.S., Young, R.H. (Eds.), 2014. WHO classification of tumours of female reproductive organs. IARC, Lyon, France.
Lax, S.F., 2017. Pathology of endometrial carcinoma. Advances in Experimental Medicine and Biology 943, 75–96.
Lheureux, S., Oza, A.M., 2016. Endometrial cancer-targeted therapies myth or reality? Review of current targeted treatments. European Journal of Cancer 59, 99–108.
Li, C., Zota, V., Woda, B.A., Rock, K.L., Fraire, A.E., Jiang, Z., Lu, D., Xu, B., Dresser, K., Lutman, C.V., Fischer, A.H., 2007. Expression of a novel oncofetal mRNA-binding protein IMP3 in endometrial carcinomas: Diagnostic significance and clinicopathologic correlations. Modern Pathology 20 (12), 1263–1268.
Liu, Y., Patel, L., Mills, G.B., Lu, K.H., Sood, A.K., Ding, L., Kucherlapati, R., Mardis, E.R., Levine, D.A., Shmulevich, I., Broaddus, R.R., Zhang, W., 2014. Clinical significance of CTNNB1 mutation and Wnt pathway activation in endometrioid endometrial carcinoma. Journal of the National Cancer Institute 106 (9), dju245.
MacKay, H.J., Levine, D.A., Bae-Jump, V.L., Bell, D.W., McAlpine, J.N., Santin, A., Fleming, G.F., Mutch, D.G., Nephew, K.P., Wentzensen, N., Goodfellow, P.J., Dorigo, O., Nijman, H.W., Broaddus, R., Kohn, E.C., 2017. Moving forward with actionable therapeutic targets and opportunities in endometrial cancer: NCI clinical trials planning meeting report on identifying key genes and molecular pathways for targeted endometrial cancer trials. Oncotarget 8 (48), 84579–84594.
Matias-Guiu, X., Davidson, B., 2014. Prognostic biomarkers in endometrial and ovarian carcinoma. Virchows Archiv 464 (3), 315–331.
McAlpine, J.N., Temkin, S.M., Endometrial, M.H.J., 2016. Cancer: Not your grandmother's cancer. Cancer 122 (18), 2787–2798.
McCluggage, W.G., Connolly, L.E., McBride, H.A., Kalloger, S., Gilks, C.B., 2012. HMGA2 is commonly expressed in uterine serous carcinomas and is a useful adjunct to diagnosis. Histopathology 60 (4), 547–553.
McConechy, M.K., Ding, J., Cheang, M.C., Wiegand, K.C., Senz, J., Tone, A.A., Yang, W., Prentice, L.M., Tse, K., Zeng, T., McDonald, H., Schmidt, A.P., Mutch, D.G., McAlpine, J.N., Hirst, M., Shah, S.P., Lee, C.H., Goodfellow, P.J., Gilks, C.B., Huntsman, D.G., 2012. Use of mutation profiles to refine the classification of endometrial carcinomas. The Journal of Pathology 228 (1), 20–30.
Meng, B., Hoang, L.N., McIntyre, J.B., Duggan, M.A., Nelson, G.S., Lee, C.H., Köbel, M., 2014. POLE exonuclease domain mutation predicts long progression-free survival in grade 3 endometrioid carcinoma of the endometrium. Gynecologic Oncology 134 (1), 15–19.
Mhawech-Fauceglia, P., Herrmann, F.R., Rai, H., Tchabo, N., Lele, S., Izevbaye, I., Odunsi, K., Cheney, R.T., 2010. IMP3 distinguishes uterine serous carcinoma from endometrial endometrioid adenocarcinoma. American Journal of Clinical Pathology 133 (6), 899–908.
Mhawech-Fauceglia, P., Yan, L., Liu, S., Pejovic, T., 2013. ER+/PR+/TFF3+/IMP3− immunoprofile distinguishes endometrioid from serous and clear cell carcinomas of the endometrium: A study of 401 cases. Histopathology 62 (7), 976–985.
Morice, P., Leary, A., Creutzberg, C., Abu-Rustum, N., Darai, E., 2016. Endometrial cancer. Lancet 387 (10023), 1094–1108.
Murali, R., Soslow, R.A., Weigelt, B., 2014. Classification of endometrial carcinoma: More than two types. The Lancet Oncology 15 (7), e268–78.
Nastic, D., Shanwell, E., Wallin, K.L., Valla, M., Måsbäck, A., Mateoiu, C., Lidang, M., Liakka, A., Lappi-Blanco, E., Grove, A., Davidson, B., Carpen, O., Bertelsen, B.I., Bak, J., Abusland, A.B., Selling, J., Carlson, J.W., 2017. A selective biomarker panel increases the reproducibility and the accuracy in endometrial biopsy diagnosis. International Journal of Gynecological Pathology 36 (4), 339–347.
Park, J.Y., Nam, J.H., Kim, Y.T., Kim, Y.M., Kim, J.H., Kim, D.Y., Sohn, I., Lee, S.W., Sung, C.O., Kim, K.R., 2013. Poor prognosis of uterine serous carcinoma compared with grade 3 endometrioid carcinoma in early stage patients. Virchows Archiv 462 (3), 289–296.
Pasanen, A., Tuomi, T., Isola, J., Staff S, Bützow, R., Loukovaara, M., 2016. L1 cell adhesion molecule as a predictor of disease-specific survival and patterns of relapse in endometrial cancer. International Journal of Gynecological Cancer 26 (8), 1465–1471.
Piulats, J.M., Guerra, E., Gil-Martín, M., Roman-Canal, B., Gatius, S., Sanz-Pamplona, R., Velasco, A., Vidal, A., Matias-Guiu, X., 2017. Molecular approaches for classifying endometrial carcinoma. Gynecologic Oncology 145 (1), 200–207.
Proctor, L., Pradhan, M., Leung, S., Cheng, A., Lee, C.H., Soslow, R.A., Gilks, C.B., Talhouk, A., McAlpine, J.M., Danielsen, H.E., Hoang, L.N., 2017. Assessment of DNA Ploidy in the ProMisE molecular subgroups of endometrial cancer. Gynecologic Oncology 146 (3), 596–602.

van der Putten, L.J., Visser, N.C., van de Vijver, K., Santacana, M., Bronsert, P., Bulten, J., Hirschfeld, M., Colas, E., Gil-Moreno, A., Garcia, A., Mancebo, G., Alameda, F., Trovik, J., Kopperud, R.K., Huvila, J., Schrauwen, S., Koskas, M., Walker, F., Weinberger, V., Minar, L., Jandakova, E., Snijders, M.P., van den Berg-van Erp, S., Matias-Guiu, X., Salvesen, H.B., Amant, F., Massuger, L.F., Pijnenborg, J.M., 2016. L1CAM expression in endometrial carcinomas: An ENITEC collaboration study. British Journal of Cancer 115 (6), 716–724.

Rahaman, J., Ku, K., Cohen, C.J., 2017. Endometrial cancer. In: Bast Jr., R.C., Croce, C.M., Hait, W.N., Hong, W.K., Kufe, D.W., Piccart-Gebhart, M., Pollock, R.E., Weichselbaum, R.R., Wang, H., Holland, J.F. (Eds.), Holland-Frei cancer medicine, 9th edn. Wiley-Blackwell, Hoboken, NJ, pp. 1305–1316.

Randall, L.M., Pothuri, B., Swisher, E.M., Diaz, J.P., Buchanan, A., Witkop, C.T., Bethan Powell, C., Smith, E.B., Robson, M.E., Boyd, J., Coleman, R.L., Lu, K., 2017. Multi-disciplinary summit on genetics services for women with gynecologic cancers: A Society of Gynecologic Oncology White Paper. Gynecologic Oncology 146 (2), 217–224.

Reid-Nicholson, M., Iyengar, P., Hummer, A.J., Linkov, I., Asher, M., Soslow, R.A., 2006. Immunophenotypic diversity of endometrial adenocarcinomas: Implications for differential diagnosis. Modern Pathology 19 (8), 1091–1100.

Reyes, H.D., Miecznikowski, J., Gonzalez-Bosquet, J., Devor, E.J., Zhang, Y., Thiel, K.W., Samuelson, M.I., McDonald, M., Stephan, J.M., Hanjani, P., Guntupalli, S., Tewari, K.S., Backes, F., Ramirez, N., Fleming, G.F., Filiaci, V., Birrer, M.J., Leslie, K.K., 2017. High stathmin expression is a marker for poor clinical outcome in endometrial cancer: An NRG oncology group/gynecologic oncology group study. Gynecologic Oncology 146 (2), 247–253.

Ring, K.L., Garcia, C., Thomas, M.H., Modesitt, S.C., 2017. Current and future role of genetic screening in gynecologic malignancies. American Journal of Obstetrics and Gynecology 217 (5), 512–521.

Salvesen, H.B., Carter, S.L., Mannelqvist, M., Dutt, A., Getz, G., Stefansson, I.M., Raeder, M.B., Sos, M.L., Engelsen, I.B., Trovik, J., Wik, E., Greulich, H., Bø, T.H., Jonassen, I., Thomas, R.K., Zander, T., Garraway, L.A., Oyan, A.M., Sellers, W.R., Kalland, K.H., Meyerson, M., Akslen, L.A., Beroukhim, R., 2009. Integrated genomic profiling of endometrial carcinoma associates aggressive tumors with indicators of PI3 kinase activation. Proceedings of the National Academy of Sciences of the United States of America 106 (12), 4834–4839.

Schlosshauer, P.W., Ellenson, L.H., Soslow, R.A., 2002. Beta-catenin and E-cadherin expression patterns in high-grade endometrial carcinoma are associated with histological subtype. Modern Pathology 15 (10), 1032–1037.

Schultheis, A.M., Martelotto, L.G., De Filippo, M.R., Piscuglio, S., Ng, C.K., Hussein, Y.R., Reis-Filho, J.S., Soslow, R.A., Weigelt, B., 2016. TP53 mutational spectrum in endometrioid and serous endometrial cancers. International Journal of Gynecological Pathology 35 (4), 289–300.

Smogeli, E., Davidson, B., Cvancarova, M., Holth, A., Katz, B., Risberg, B., Kristensen, G., Lindemann, K., 2016. L1CAM as a prognostic marker in stage I endometrial cancer: A validation study. BMC Cancer 16, 596.

Soslow, R.A., 2013. High-grade endometrial carcinomas—Strategies for typing. Histopathology 62 (1), 89–110.

Soslow, R.A., Bissonnette, J.P., Wilton, A., Ferguson, S.E., Alektiar, K.M., Duska, L.R., Oliva, E., 2007. Clinicopathologic analysis of 187 high-grade endometrial carcinomas of different histologic subtypes: Similar outcomes belie distinctive biologic differences. The American Journal of Surgical Pathology 31 (7), 979–987.

Spurdle, A.B., Bowman, M.A., Shamsani, J., Kirk, J., 2017. Endometrial cancer gene panels: Clinical diagnostic vs. research germline DNA testing. Modern Pathology 30 (8), 1048–1068.

Takeda, T., Banno, K., Okawa, R., et al., 2016. ARID1A gene mutation in ovarian and endometrial cancers (review). Oncology Reports 35, 607–613.

Talhouk, A., McConechy, M.K., Leung, S., Li-Chang, H.H., Kwon, J.S., Melnyk, N., Yang, W., Senz, J., Boyd, N., Karnezis, A.N., Huntsman, D.G., Gilks, C.B., McAlpine, J.N., 2015. A clinically applicable molecular-based classification for endometrial cancers. British Journal of Cancer 113 (2), 299–310.

Talhouk, A., McConechy, M.K., Leung, S., Yang, W., Lum, A., Senz, J., Boyd, N., Pike, J., Anglesio, M., Kwon, J.S., Karnezis, A.N., Huntsman, D.G., Gilks, C.B., JN, M.A., 2017. Confirmation of ProMisE: A simple, genomics-based clinical classifier for endometrial cancer. Cancer 123 (5), 802–813.

Trovik, J., Wik, E., Stefansson, I., Carter, S.L., Beroukhim, R., Oyan, A.M., Kalland, K.H., Akslen, L.A., Salvesen, H.B., 2010. Stathmin is superior to AKT and phospho-AKT staining for the detection of phosphoinositide 3-kinase activation and aggressive endometrial cancer. Histopathology 57 (4), 641–646.

Trovik, J., Wik, E., Stefansson, I.M., Marcickiewicz, J., Tingulstad, S., Staff AC, Njolstad, T.S., MoMaTec Study Group, Vandenput, I., Amant, F., Akslen, L.A., Salvesen, H.B., 2011. Stathmin overexpression identifies high-risk patients and lymph node metastasis in endometrial cancer. Clinical Cancer Research 17 (10), 3368–3377.

Vaidya, A.P., Horowitz, N.S., Oliva, E., Halpern, E.F., Duska, L.R., 2006. Uterine malignant mixed mullerian tumors should not be included in studies of endometrial carcinoma. Gynecologic Oncology 103 (2), 684–687.

Van Gool, I.C., Stelloo, E., Nout, R.A., Nijman, H.W., Edmondson, R.J., Church, D.N., MacKay, H.J., Leary, A., Powell, M.E., Mileshkin, L., Creutzberg, C.L., Smit, V.T., Bosse, T., 2016. Prognostic significance of L1CAM expression and its association with mutant p53 expression in high-risk endometrial cancer. Modern Pathology 29 (2), 174–181.

Voss, M.A., Ganesan, R., Ludeman, L., McCarthy, K., Gornall, R., Schaller, G., Wei, W., Sundar, S., 2012. Should grade 3 endometrioid endometrial carcinoma be considered a type 2 cancer-a clinical and pathological evaluation. Gynecologic Oncology 124 (1), 15–20.

Wentzensen, N., Bakkum-Gamez, J.N., Killian, J.K., Sampson, J., Guido, R., Glass, A., Adams, L., Luhn, P., Brinton, L.A., Rush, B., d'Ambrosio, L., Gunja, M., Yang, H.P., Garcia-Closas, M., Lacey Jr., J.V., Lissowska, J., Podratz, K., Meltzer, P., Shridhar, V., Sherman, M.E., 2014. Discovery and validation of methylation markers for endometrial cancer. International Journal of Cancer 135 (8), 1860–1868.

Werner, H.M., Salvesen, H.B., 2014. Current status of molecular biomarkers in endometrial cancer. Current Oncology Reports 16 (9), 403.

Werner, H.M., Trovik, J., Halle, M.K., Wik, E., Akslen, L.A., Birkeland, E., Bredholt, T., Tangen, I.L., Krakstad, C., Salvesen, H.B., 2014. Stathmin protein level, a potential predictive marker for taxane treatment response in endometrial cancer. PLoS One 9 (2), e90141.

Yemelyanova, A., Ji, H., Shih, I.M., Wang, T.L., Wu, L.S., Ronnett, B.M., 2009. Utility of p16 expression for distinction of uterine serous carcinomas from endometrial endometrioid and endocervical adenocarcinomas: Immunohistochemical analysis of 201 cases. The American Journal of Surgical Pathology 33 (10), 1504–1514.

Yeramian, A., Moreno-Bueno, G., Dolcet, X., Catasus, L., Abal, M., Colas, E., Reventos, J., Palacios, J., Prat, J., Matias-Guiu, X., 2013. Endometrial carcinoma: Molecular alterations involved in tumor development and progression. Oncogene 32, 403–413.

Zeimet, A.G., Reimer, D., Huszar, M., Winterhoff, B., Puistola, U., Azim, S.A., Müller-Holzner, E., Ben-Arie, A., van Kempen, L.C., Petru, E., Jahn, S., Geels, Y.P., Massuger, L.F., Amant, F., Polterauer, S., Lappi-Blanco, E., Bulten, J., Meuter, A., Tanouye, S., Oppelt, P., Stroh-Weigert, M., Reinthaller, A., Mariani, A., Hackl, W., Netzer, M., Schirmer, U., Vergote, I., Altevogt, P., Marth, C., Fogel, M., 2013. L1CAM in early-stage type I endometrial cancer: Results of a large multicenter evaluation. Journal of the National Cancer Institute 105 (15), 1142–1150.

Zhang, L., Liu, Y., Hao, S., Woda, B.A., Lu, D., 2011. IMP2 expression distinguishes endometrioid from serous endometrial adenocarcinoma. The American Journal of Surgical Pathology 35 (6), 868–872.

Zheng, W., Yi, X., Fadare, O., Liang, S.X., Martel, M., Schwartz, P.E., Jiang, Z., 2008. The oncofetal protein IMP3: A novel biomarker for endometrial serous carcinoma. The American Journal of Surgical Pathology 32 (2), 304–315.

Enhancers in Cancer: Genetic and Epigenetic Deregulation

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Glossary

Bromodomain A protein domain of about 110 amino acid residues that binds acetylated lysine. Bromodomain-containing proteins can bind acetylated histones and recruit transcription regulators to modulate gene expression.

Chromatin immunoprecipitation (ChIP) A technique used to determine the localization of a protein of interest to specific DNA sequences in vivo. In combination with high-throughput sequencing (ChIP-seq), it can be used to study the genome-wide distribution of proteins of interest.

Chromosome conformation capture (3C, 4C, and Hi-C) Molecular biology methods for studying chromatin looping and the three-dimensional structure of chromatin in vivo.

CRISPR/Cas9 A genome editing method derived from a prokaryotic immune system called CRISPR (Clustered Regularly Interspersed Palindromic Repeats). By delivering the RNA-guided Cas9 nuclease and a guide RNA into cells, specific DNA sequences could be targeted by Cas9 to generate double strand break.

Enhancer hijacking A process in cancer where the repositioning of inter- or intra-chromosomal enhancers to a close proximity of proto-oncogenes leads to the activation of these genes.

Global run-on sequencing (GRO-seq) An assay aiming to study the position and amount of nascent RNA associated with transcriptionally engaged RNA polymerase II (RNA Pol II) in a genome-wide fashion. This method is often used to investigate the promoter-proximal pausing of RNA Pol II in cells but it is also a sensitive method for detecting eRNA transcription.

Genome-wide association study (GWAS) An analysis method that compares single nucleotide polymorphisms (SNPs) in people with and without certain traits to help understand the relationship between genetic variants and diseases.

Single nucleotide polymorphism (SNP) A type of genetic variation which represents a difference in a single nucleotide located at a specific position in the genome. Although most SNPs have little effects on human health, some are highly associated with pathogenesis.

YEATS domain A protein domain of about 80–120 amino acids that binds acetylated lysine. Unlike the bromodomain, the YEATS domain is found in only a handful of proteins in humans.

Introduction

Enhancers are regulatory noncoding DNA sequences that can activate genes across a long distance. The coining of the term "enhancer" came from the finding that a segment of SV40 DNA could activate the β-globin gene 200-fold on a recombinant plasmid in HeLa cells. Subsequently, functional studies in model organisms including fruit-flies and mice demonstrated that enhancers were critical regulators of organismal development through spatiotemporal gene expression and specification of cell identity. Genome-wide studies, using recently developed next generation sequencing techniques, have revealed that enhancers are omnipresent in the human genome and harbor distinct epigenetic features. In addition, the capacity to carry out functional studies of enhancer function is growing enormously due to advancements in genome engineering using tools such as CRISPR/Cas9.

Transcriptional deregulation of proto-oncogenes and tumor suppressor genes is integral to the transition from healthy cells to malignancy as they acquire cancer hallmarks. An emerging theme is that mutation, aberrant methylation, or genomic rearrangement of enhancers drives transcription deregulation in human malignancies, suggesting that therapies addressing enhancer malfunction will benefit treatment regimens. In this article, we discuss the mechanisms of enhancer mediated transcription regulation and the different ways through which enhancer malfunction leads to cancer.

Enhancer Biology

Features of Enhancers

Enhancers such as those in the locus control region (LCR) of the human β globin gene are able to drive the expression of genes across a long distance. Located 60 kilobases (kb) upstream of the human β globin gene, the LCR of β globin contains five DNase I hypersensitive sites (HS), and controls the expression of the five genes in the β globin locus during embryonic development. Mutations in the LCR can lead to thalassemia, a disease characterized by insufficient hemoglobin function. In general, the temporal and spatial regulation of tissue specific genes is driven by enhancers. For example, the ordered expression of the homeotic (Hox) genes are controlled through a cohort of enhancers located upstream and downstream of their gene clusters during development. The

orientation of the enhancers does not seem to be critical for transcription activation but their localization in particular local chromatin environments can regulate their activity.

Traditionally, the identification and characterization of enhancers have been very challenging, as the noncoding DNA in which they typically reside takes up more than 97% of the human genome. The recent development of genome-wide sequencing techniques such as global run-on sequencing (GRO-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) reveals that active enhancers produce transcripts called enhancer RNA (eRNA). Furthermore, deep sequencing indicates that enhancers are characterized by DNase I hypersensitivity and DNA hypomethylation.

Core histone modifications mark the activity of enhancers in specific cell types and tissues. Active enhancers are marked by mono-methylation at lysine 4 and acetylation at lysine 27 of histone H3 (H3K4me1 and H3K27ac), while poised enhancers are decorated with H3K4me1 and tri-methylation at lysine 27 of histone H3 (H3K27me3). Bioinformatics analyses of ChIP-seq studies have led to the identification of groups of closely spaced enhancers variably named transcriptional initiation platforms (TIPs), stretch enhancers, or more commonly super-enhancers. Super-enhancers are characterized by the enrichment of master transcription factors, a high level of occupancy of the Mediator complex, and they are comprised of $\sim$10 kb genomic regions on average. Super-enhancers are found to be associated with key cell identity genes but not housekeeping genes. Super-enhancers have been identified near oncogenes such as *c-Myc* and *TAL1* in cancer cells but not normal cells, suggesting that tumors acquire super-enhancers at oncogenes during pathogenesis. Super-enhancers overlap with many previously characterized LCRs, indicating that it is possible that these extended regulatory regions are clusters of regular enhancers. Do individual enhancers within a super-enhancer region have synergistic effects on nearby genes? To answer this question, the α-globin regulatory region, a typical super-enhancer, has been carefully analyzed. Individual deletion of the five enhancers in the α-globin super-enhancer demonstrated that the enhancer elements act in an additive manner. None of the five enhancers were solely responsible for the activation of the α-globin gene, and the effect of compound deletion was similar to that of the additive results of single-enhancer deletions. This suggests that individual enhancers in one super-enhancer region may work independently from each other. However, in other cases, enhancers in a cluster may work as "shadow enhancers," a term used to describe the discovery of seemingly functionally redundant enhancers in *Drosophila* for genes that already have known enhancers. In mammals, two "shadow enhancer" like regulatory elements are present in the HoxA gene cluster, with the removal of either enhancer having little effect whereas deletion of the two enhancers significantly impairs HoxA gene activation. It is likely that the "shadow enhancer" mechanism is frequently utilized to guarantee the well-regulated expression of important genes during development.

Enhancer-Promoter Communication

How do cis-regulatory elements located several 100 kb away from a promoter regulate their target genes? A series of experiments using DNA fluorescence in situ hybridization (FISH) and chromosome conformation capture (3C) derived techniques revealed that promoters and their regulatory elements form long-range loops that are correlated with transcription levels of the loop-associated genes. Enhancer-promoter loops can be formed prior to gene activation and remain stable in different development stages. Such preformed loops may be necessary for the genes to be turned on, however, it is difficult to experimentally prove the importance of loop formation as genetic perturbation of contact points or depleting cells of factors promoting looping are likely to have secondary effects. To solve this issue, Deng and colleagues artificially recruited GATA1 cofactor LDB1 to silent loci in erythroid cells and found forced looping could activate genes independently of GATA1, suggesting that enhancer-promoter communication per se drives transcription.

CTCF and cohesin are essential for the formation and stabilization of enhancer-promoter loops (**Fig. 1**). CTCF was initially characterized as an insulator binding factor that blocks the enhancer-promoter interaction if placed between the two elements. However, recent studies indicate that CTCF is also enriched at the boundaries of topologically associated domains (TADs), the building units of the three-dimensional (3D) chromatin structure, and over-represented at the border of sub-TAD regions, suggesting a possible role of CTCF in maintaining long-range interactions and genome architecture. Indeed, rapid depletion of CTCF resulted in a dramatic loss of chromatin loops. Interestingly, the orientation of CTCF binding sites (BSs) seem to be important for the formation of long-range loops, which are preferably formed between convergently oriented CTCF BSs.

Cohesin is a complex of proteins that forms a ring-like structure that is important for sister chromatid cohesion during mitosis, physically interacts with CTCF, and colocalizes with CTCF on the genome, suggesting that CTCF and cohesin cooperate to regulate long-range loops. As with CTCF, rapid depletion of cohesin subunits leads to dissolution of chromatin loops. Cohesin interacts with the Mediator complex and recruits Mediator to enhancers not bound by CTCF. Such recruitment is thought to maintain enhancer-promoter interactions and therefore is important for cell fate determination.

Transcription Factors Modulate Enhancer Activity

The activity of an enhancer is directly controlled by the transcription factors (TFs) and epigenetic modifiers bound to it. Therefore, the TFs' expression levels and binding patterns determine the tissue specific expression of developmental genes. However, enhancers harboring binding motifs of cell-type specific TFs may not be bound by these TFs, as the local chromatin environment, such as a TF binding site being embedded within a nucleosome, or residing within heterochromatin, can limit the accessibility of these proteins to enhancer elements. Unlike general TFs, pioneering TFs such as FoxA, GATA, PU.1, and Oct4 can directly bind their preferred sequences even when embedded within a nucleosome, and are therefore thought to play important roles in recruiting cofactors

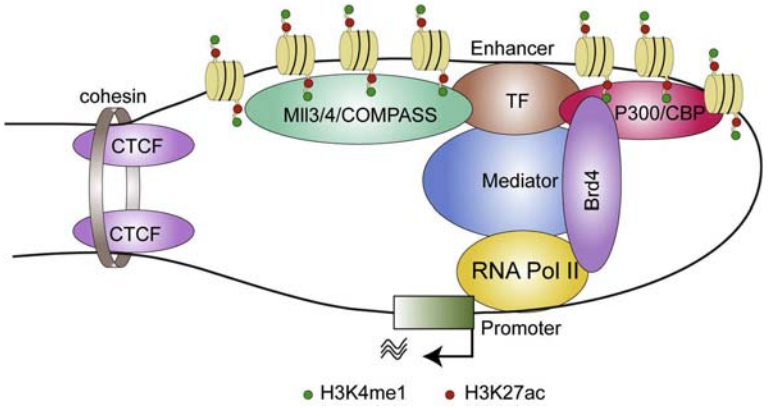

Fig. 1 A model of transcription activation through chromatin looping and enhancers. The interaction between CTCF and cohesin facilitates the formation of chromatin loops . Transcription factors (TF) such as pioneering TFs bind to enhancers and recruit epigenetic modifiers Mll3/4/COMPASS and P300/CBP, which deposit H3K4me1 and H3K27ac respectively at enhancer nucleosomes. Chromatin decompaction at enhancers and histone modifications allow the recruitment of the Mediator complex and chromatin binding proteins such as Brd4, which further assembles basic transcription machineries at the gene promoter and activates transcription.

to decompact chromatin, establish long-range interactions between promoters and enhancers, and activate specific gene expression patterns.

Enhancer-binding TFs regulate gene expression partly through their interactions with the Mediator complex, which is central to the transcription initiation and elongation of RNA polymerase II (RNA Pol II) (Fig. 1). The large protein complex has an impact on basic transcription machineries such as the preinitiation complex (PIC), RNA Pol II pausing factors, and transcription elongation factors. The 26 subunits of Mediator interact with various enhancer-binding TFs such as nuclear receptors, P53, and Myc. We refer readers to the review by Allen and Taatjes on the role of the Mediator complex in transcription regulation for further reading. Mediator appears to have an important role in enhancer-promoter interaction. The Mediator complex interacts with cohesin and colocalizes with cohesin at enhancers. Mediator and cohesin are more enriched at short-range chromatin loops smaller than 100 kb, suggesting that the formation of the short-range looping events depends on the Mediator complex. Depletion of Mediator component Med12 leads to a reduction of looping efficiency as indicated by FISH. It is worth noting that depletion of the Mediator complex would impair both enhancer activity and RNA Pol II initiation at promoters, thus whether the loss of promoter-enhancer interaction is driven by reduction in transcription activity or directly by Mediator depletion is unclear.

Epigenetic Enzymes and Enhancers

Enhancer-associated epigenetic marks are deposited by a specific group of enzymes. In *Drosophila* and mammals, Trr, Mll3, and Mll4 are the enzymes responsible for depositing H3K4me1 at enhancers (Fig. 1). In yeast, all three states of H3K4 methylation (H3K4me1, H3K4me2, and H3K4me3) are implemented by Set1, which is the catalytic core of the large histone methyltransferase complex named "COMplex of Proteins ASsociated with Set1" (COMPASS). COMPASS is also present in higher eukaryotes with three *Drosophila* homologs, named Set1, Trx, and Trr. In mammals, Set1A (KMT2F) and Set1B (KMT2G) are homologs of Drosophila Set1, Mll1 (KMT2A), and Mll2 (KMT2B) are homologs of Trx, and Mll3 (KMT2C) and Mll4 (KMT2D) are homologs of Trr. Genetically removing each COMPASS leads to embryonic lethality in mice, each with distinct phenotypes, suggesting that this family of H3K4 methyltransferases is critical, and that each member of the COMPASS family plays distinct roles during mammalian development. Such variability may be attributed, in part, to the functional differences of mammalian COMPASS in catalyzing H3K4 methylation in vivo.

Set1A/COMPASS and Set1B/COMPASS are mainly responsible for the bulk level of H3K4me2 and H3K4me3. Mll1/COMPASS contributes to promoter and intergenic H3K4me2 and H3K4me3 in a context dependent manner, while Mll2/COMPASS regulates H3K4me3 at a subset of enhancers and at promoters of a specific group of genes called bivalent genes in mouse embryonic stem cells (mESCs). Mll3/COMPASS and Mll4/COMPASS deposit H3K4me1 at enhancers in most cell types. A number of proteins such as TFIID, BPTF, and CHD1 are able to bind H3K4 methylation, however whether H3K4 methylation functions through these binding proteins is poorly studied. Intriguingly, several lines of evidence indicate that the COMPASS family of methyltransferases has both catalytic and noncatalytic functions. It is conceivable that large protein complexes like COMPASS may recruit factors required for enhancer activation. Indeed, the histone demethylase UTX, which targets H3K27 methylation, is a subunit of both Mll3 and Mll4 COMPASS. The simultaneous methylation at H3K4 and demethylation at H3K27 may have additive effects on opening chromatin and activating transcription. In addition, the H3K4 demethylases LSD1, KDM5C, and KDM5D are found to be localized at enhancers in specialized contexts, where they function to repress enhancers, thus providing an additional layer for regulating gene expression.

The active enhancer mark H3K27ac is catalyzed by the histone acetyltransferases P300 and CBP in vivo. Histone acetylation is thought to decompact chromatin as it neutralizes the negative charge of DNA and loosens histone DNA interactions to expose DNA to TFs and RNA Pol II. Apart from relaxing chromatin, histone acetylation can be recognized by bromodomain proteins and YEATS domain proteins, leading to a profound impact on downstream transcription regulation (Fig. 1). BRD4, one of the BET (bromodomain and extra terminal domain) family members, binds the transcription elongation complex P-TEFb to enhance transcription. The YEATS domain proteins AF9 and ENL are components of another transcription elongation complex named super elongation complex (SEC) that also contains P-TEFb. SEC is required for the rapid induction of transcription in response to environmental and developmental cues. Therefore, perturbing these binders of histone acetylation may help rectify the deregulated transcription program in cancer.

The poised enhancer mark of H3K27me3 is implemented by polycomb repressive complex 2 (PRC2). Initially characterized at bivalent promoters in mESCs, H3K27me3 is correlated with transcriptional repression. It has been proposed that the canonical PRC1 complex recognizes H3K27me3, modifies histone H2A with ubiquitination on lysine 119 (H2A119ub), and causes chromatin compaction as well as transcriptional silencing. Other evidence indicates that PRC2 recruitment and H3K27me3 placement may depend on a non-canonical PRC1 that lacks the H3K27me3 recognition module. In summary, although the functions of epigenetic marks at enhancers are not fully understood at this time, it is evident that the further elucidation of how epigenetic enzymes are recruited to enhancers will greatly facilitate the understanding of enhancer biology, and how its misregulation leads to transcription deregulation in cancer.

Long Noncoding RNAs and eRNAs in Modulating Enhancer Activity

Although only 3% of the human genome contains protein-coding sequences, many noncoding DNA regions are transcribed to generate so called noncoding RNA (ncRNA). Two types or noncoding RNAs, eRNA and long noncoding RNA (lncRNA), have been heavily studied in enhancer biology. eRNAs are unspliced, non-polyadenylated, and bidirectionally transcribed from enhancer DNA, while lncRNAs are spliced, polyadenylated, and transcribed from canonical promoters. Transcription from cis-regulatory regions, such as the LCRs of β globin, MHC class II, and human growth hormone (hGH), were discovered long before the era of next generation sequencing. Transcription from the hGH LCR is important as demonstrated by the insertion of a RNA Pol II termination element in the LCR leading to attenuation of transcription of its downstream gene. Genome-wide analysis of RNA Pol II ChIP-seq and RNA-seq data revealed that indeed Pol II resides on many enhancers throughout the genome and that eRNA production precedes the activation of the adjacent protein-coding gene, suggesting a potential instructive role of eRNA on the transcription of its target genes. Furthermore, depletion of eRNAs has been shown to lead to downregulation of expression of nearby genes in several different cases. Multiple models have been proposed to explain the mechanisms underlying the functions of eRNA, including the in *trans* model where eRNA recruits co-activators such as the Mediator complex to activate nearby genes, and the in *cis* model where eRNA traps transcription factors such as YY1 to the enhancer locus to keep chromatin accessible and active.

It is reported that there are over 58,000 lncRNA genes in the human genome. Most of the known functions of lncRNA are involved in nuclear structure, chromatin interactions, transcription regulation, and RNA processing. For the roles of lncRNA in normal biological processes and cancer, we refer readers to the review by Long et al. for further reading. In addition to the function of the lncRNA transcript, the lncRNA promoter-proximal and genic regions are often found to contain enhancer elements. The *cis* versus *trans* effects of lncRNAs are hard to discern as the transcription activity at the locus of a lncRNA gene may be required for the expression of nearby genes, while the lncRNA transcript could be dispensable, as has been shown for the *Lockd-Cdkn1b* locus, as well as the *Uph-Hand2* locus. Future studies that combine genome-wide screening and locus-specific studies, including deletion of genomic segments, forced induction of premature transcription termination, and targeted degradation of the transcript, will shed new light on the functions of lncRNAs and their genomic loci in transcription regulation.

Genetic Changes in Enhancers Lead to Cancer

Mutations such as point mutations, amplifications, deletions, insertions, and rearrangements of enhancer sequences have been associated with many types of cancer. Many of these mutations lead to misregulation of proto-oncogenes and tumor suppressor genes, which drive oncogenesis and tumor progression. Below, we discuss examples of these types of enhancer mutations in cancer.

Enhancer Hijacking

The first example of enhancer hijacking was described in Burkitt lymphoma where the proto- oncogene *c-Myc* on chromosome 8 is translocated near to an enhancer at the immunoglobulin heavy chain (IgH) locus on chromosome 14, resulting in the overexpression of *c-Myc*. Subsequently, similar translocation of the *Bcl2* gene proximal to the *IgH* enhancer have been reported by several groups, suggesting that the overexpression of proto-oncogenes by enhancer hijacking could be a general mechanism of lymphomagenesis. In recent years, enhancer hijacking has been observed in various types of cancers including medulloblastoma, neuroblastoma, leukemia, lung cancer, and colorectal cancer.

In general, genome rearrangements that lead to enhancer hijacking have two types of structure (Fig. 2): (1) interchromosomal translocations that juxtapose an enhancer to a proto-oncogene, such as the *Myc-IgH* translocation in Burkitt lymphoma, or the

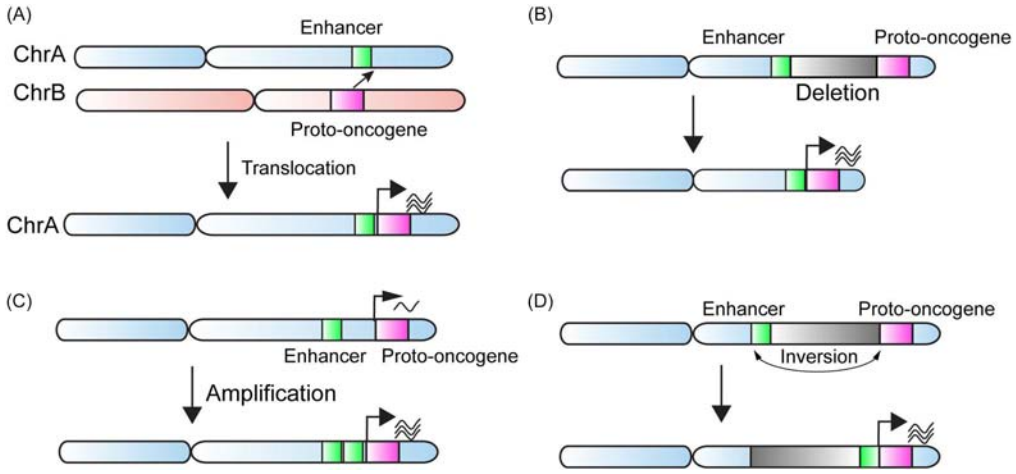

Fig. 2 Inter-chromosomal and intra-chromosomal rearrangements in cancer cells lead to enhancer hijacking. (A) Inter-chromosomal translocation juxtaposes an enhancer to a proto-oncogene and drives its expression. (B–D) Enhancers activate proto-oncogenes through intra-chromosomal deletion (B), amplification (C), and inversion (D).

Bcl2-IgH translocation in follicular lymphoma; (2) intrachromosomal rearrangements, including deletion, duplication, and inversion of genomic regions, which can bring an enhancer to the proximity of a proto-oncogene, as in the case of the *Gata2* enhancer translocation that activates *Evi1* to drive acute myeloid leukemia (AML), and the *Ddx31* enhancer's translocation to drive *Gfi1b* expression in medulloblastoma. Recent findings that CTCF binding sites are frequently mutated in cancer, and that somatic mutations in boundaries of topological domains are enriched in cancer, shed new light on how enhancers can be hijacked through the alteration of chromatin architecture. Hypermethylation at a CTCF BS in *IDH* mutant gliomas leads to loss of insulation between topological domains and aberrant activation of the proto-oncogene *PDGFRA* by a constitutive enhancer. In 293T cells, mutating CTCF BSs at domain boundaries found to be deleted in T-ALL, resulted in ectopic interactions between *TAL1* and *LMO2* genes and distal enhancers to increase the expression of these two proto-oncogenes. Interestingly, tandem duplication of the *IGF2* locus in colorectal cancer samples creates a de novo topological domain containing a super-enhancer with the duplicated *IGF2* gene, causing *IGF* upregulation. Both improvements in the detection of genome rearrangements and future studies to understand the mechanisms of the rearrangement, will advance our understanding of the degree to which enhancer hijacking contributes to oncogenesis.

Mutation of Enhancers in Cancer

Mutations in enhancer sequences include point mutations, insertions, small deletions, and focal amplifications. These mutations are found in many cancers and can result in upregulation of proto-oncogenes or inactivation of tumor suppressor genes. Point mutations in an enhancer may affect TF binding to the enhancer. cis-Regulatory regions of proto-oncogene *c-Myc* have been heavily studied, which has led to the identification of cancer-related single nucleotide polymorphisms (SNPs). For example, the SNP rs6983267, located within a TCF binding motif at an enhancer of *c-Myc*, has tight association with colorectal cancer and prostate cancer. The risk region loops to the *c-Myc* gene and it is highly occupied by TCF7L2, whose binding to the mutated enhancer is linked to the activation of *c-Myc* and carcinogenesis. At least 17 cancer-associated SNPs, including rs6983267, have been identified in the noncoding regions of the 8q24 locus. These SNPs in enhancers could play a role in regulating enhancer activity, chromatin looping, and c-Myc expression in colorectal cancer, breast cancer, ovarian cancer, prostate cancer, chronic lymphoblastic leukemia, and B-cell lymphoma. Apart from the 8q24 locus, the SNP rs339331, located at the *Rfx6* enhancer, strengthens the binding of HoxB13 to the enhancer in prostate cancer, leading to overexpression of Rfx6. SNP rs9383590, found at the *Esr1* enhancer, affects the recruitment of GATA3 to the enhancer in breast cancer, altering the activity of the enhancer as well as promoter-enhancer interactions. On the other hand, the SNP rs2168101, located within a super-enhancer of *Lmo1*, leads to abrogation of GATA3 binding and reduced H3K27ac levels at the super-enhancer, and is associated with decreased expression of *Lmo1* in neuroblastoma. These data suggest that a single mutation can modulate the activity of a large cluster of enhancers to drive oncogenesis. Although the improvement in genome-wide association studies (GWAS) has identified increasing numbers of risk related SNPs in noncoding regions, it is worth noting that whether these SNPs have causative effects on the expression of proto-oncogenes is largely unknown. Future studies that endogenously mutate these SNPs in cancer cells, for example, using CRISPR/Cas9, will elucidate the functional relevance between enhancer SNPs and cancer.

Similar to point mutations, small insertions in cis-regulatory elements have been found to be very frequent in various cancer types including T-ALL, breast cancer, neuroblastoma, lung cancer, colorectal cancer, melanoma, glioblastoma, B-cell lymphoma, and pancreatic cancer. Insertions could alter the activity of enhancers to activate proto-oncogenes, or reduce the expression of tumor suppressors. In T-cell acute lymphoblastic leukemia (T-ALL), genome-wide sequencing reveals a 2–18 bp insertion upstream of the

proto-oncogene *TAL1* in patient-derived cell lines. The insertion creates a binding site for the transcription factor MYB, thereby activating a super-enhancer that drives the overexpression of *TAL1*. Importantly, when the insertion is deleted by CRISPR/Cas9-guided genome engineering, H3K27ac at the super-enhancer was diminished and *TAL1* expression was drastically reduced. Similar insertions and regulatory mechanisms have been found at the *LMO2* super-enhancer, where MYB and TAL1 ectopically bind to the altered enhancers to drive expression of *LMO2* in T-ALL.

In addition to insertions that create novel TF binding sites, enhancers can be focally amplified such that their activity is significantly increased to upregulate a proto-oncogene. Focal amplifications have been found at different enhancers of *c-Myc* in AML, T-ALL, lung adenocarcinoma, and endometrial carcinoma. A statistical study of copy number variation in 29 tumor types found 55 focally amplified noncoding regions, six of which contain super-enhancers, including those regulating *Klf5*, *USP12*, *PARD6B*, and *c-Myc* expression. The amplified *c-Myc* super-enhancer has increased physical contact with *c-Myc*, indicating that focal amplification not only increases the activity of enhancers but also strengthens the enhancer-promoter communication. Further inhibition of enhancer activity by CRISPR inhibition and deletion of enhancer elements demonstrate that the transcription activity at the amplified enhancers is required to turn on the *c-Myc* gene.

It is noteworthy that although enhancers are frequently mutated or hijacked in tumor samples and cancer cell lines, there is not enough evidence to prove a causal relationship between enhancer mutation and oncogenesis. Enhancer mutations clearly contribute to the misregulation of oncogenes and tumor suppressor genes but their functions need to be further tested to determine which ones are true cancer drivers.

Epigenetic Alteration at Enhancers Leads to Cancer

Transcription factors and chromatin modifiers play important roles in enhancer malfunction as many of these molecules control a large cohort of enhancers and genes in specific cell types. In this section, we will focus on the major enhancer regulators and their roles in enhancer misregulation and oncogenesis.

Mll3 and Mll4/COMPASS in Cancer

Mll3 and Mll4, the major enzymes placing H3K4me1 at enhancers, are central for enhancer activity (refer to Sze and Shilatifard for further reading). Importantly, a cancer genome sequencing study by Lawrence et al. found that Mll3 and Mll4 are among the most frequently mutated genes across more than 20 cancer types. Human Mll3 is 4911 amino acids in length and Mll4 contains 5537 amino acids. Despite their large size, Mll3 and Mll4 only harbor a few known functional domains including PHD domains, which can bind histones or other proteins, and a SET domain which catalyzes H3K4 methylation. As the cancer-related mutations of Mll3 and Mll4 are distributed throughout the two proteins, it is likely that these methyltransferases lose their physiological functions during tumorigenesis either through loss of chromatin recruitment or loss of methyltransferase activity. Such loss-of-function may lead to enhancer deactivation, oncogenesis, and cancer progression. Observations in mice null for the SET domain of Mll3 support a tumor suppressor role of Mll3/COMPASS with half of the Mll3 SET-deleted mice developing urothelial tumors within 4 months after birth. Furthermore, the tumorigenic process is shortened in a p53 heterozygous background, suggesting that Mll3/COMPASS plays a role in DNA damage repair. Such a notion is consistent with the role of Mll4/COMPASS in maintaining genome stability as Mll4 mutant cells show increased DNA damage and mutation rates. The tumor suppressor role of Mll4/COMPASS has also been shown during mouse B cell development and in a mouse model of lymphomagenesis. Deletion of Mll4 early in B cell development leads to increased germinal center formation, upregulation of cell cycle regulators, and increased proliferation of B cells. In addition, depletion of Mll4 cooperates with Bcl2 to accelerate lymphomagenesis. Mechanistically, many of the follicular lymphoma and diffuse large B cell lymphoma-associated Mll4 mutations impair the methyltransferase activity of Mll4/COMPASS, and Mll4 deletion results in reduced H3K4 methylation at enhancers of tumor suppressor genes in B cell lymphoma.

Enhancer activation of tumor suppressor genes may be a general mechanism for the role of Mll3/Mll4/COMPASS in repressing cancer development; nevertheless, multiple lines of evidence suggest that these enhancer regulators play pro-cancer roles in different contexts. For example, Mll4 deletion leads to impairment of hematopoietic stem cells (HSCs) repopulation in vivo. Moreover, in contrast to its tumor suppressor role in B cell lymphoma, Santos et al. have found that Mll4 can facilitate Mll1-AF9 leukemia, with deletion of Mll4 leading to increased oxidative stress and DNA damage in the Mll1-AF9 leukemia cells, which led to increased myeloid differentiation. In breast cancer cells, gain-of-function (GOF) P53 mutant proteins bind to the promoters of Mll1 and Mll4 to activate their transcription, which leads to increased levels of H3K4 methylation and transcription of proto-oncogenes such as Hox genes. Interestingly, depletion of Mll1 or inhibition of Mll1 activity impairs the growth of P53 GOF breast cancer cells but has no effect in P53 WT breast cancer cells. Although the function of Mll4 in P53 GOF breast cancer cells was not investigated, the upregulation of Mll4 by GOF P53 mutants suggests that enhancer activation through Mll4 may participate in promoting the tumorigenicity of these cells. The oncogenic role of Mll4/COMPASS in ER-positive breast cancer cells was further demonstrated in the PI3K-AKT pathway, where AKT inhibits Mll4 activity, which leads to decreased ER recruitment. PI3Kα inhibition resulted in a more active Mll4, suggesting that the therapeutic efficacy of PI3Kα inhibitors in ER-positive breast cancer can be enhanced by Mll4 inhibition. Mll3 may also have an oncogenic role in breast cancer cells, as the pioneering TF FoxA1 can recruit Mll3 to activate enhancers in ER-positive breast cancer, while depletion of Mll3 results in loss of H3K4me1 at enhancers, reduced ER induced-gene expression, and decreased ER-triggered cell growth.

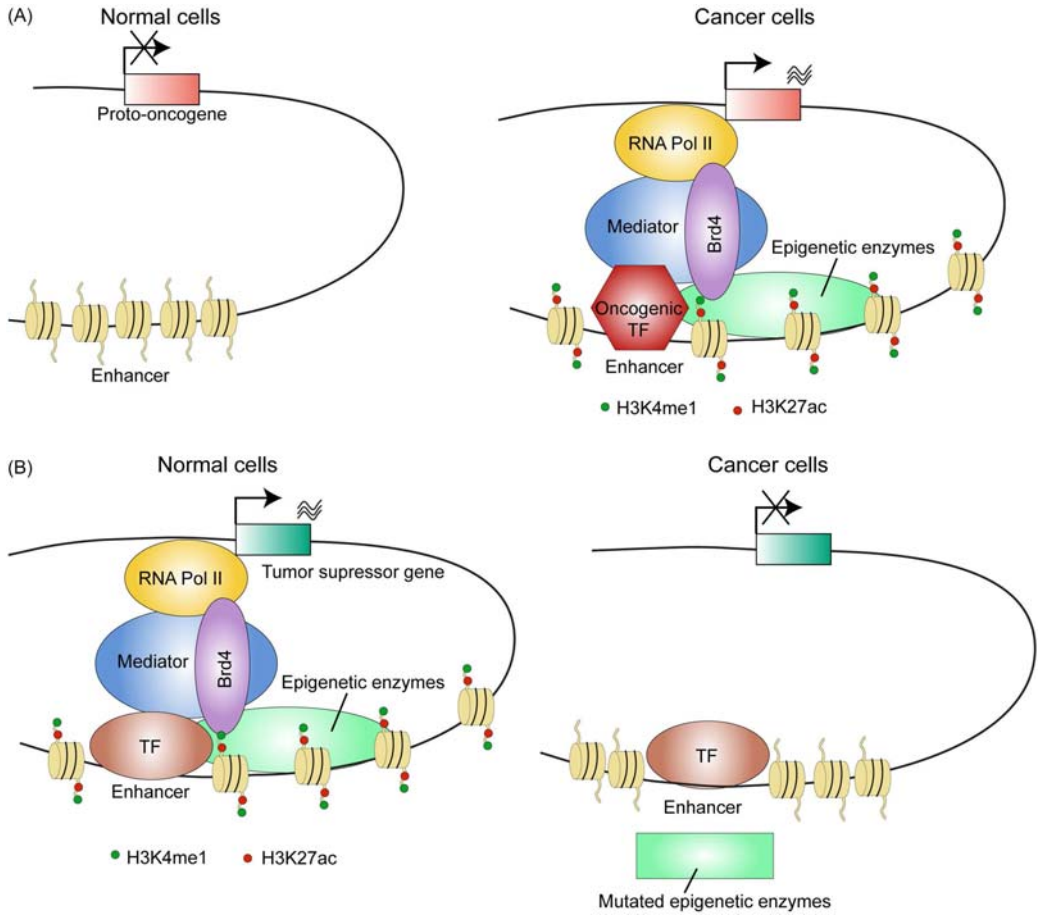

Fig. 3 Oncogenic and tumor suppressing roles of epigenetic enzymes. (A) A model of oncogenic functions of epigenetic modifiers. In cancer cells, oncogenic transcription factors could recruit epigenetic enzymes such as Mll3/4/COMPASS and P300/CBP to the enhancers of proto-oncogenes. Chromatin is decompacted, nucleosomes are modified with H3K4me1 and H3K27ac, and proto-oncogenes are activated. (B) A model of tumor suppressing functions of epigenetic modifiers. In cancer cells with loss-of-function mutations of Mll3/Mll4 or P300/CBP, the chromatin at enhancers of tumor suppressor genes cannot be modified and enhancers are not accessible to basic transcription machineries, leading to transcription repression of these genes.

What factors determine if Mll3/Mll4/COMPASS function as oncogenes or tumor suppressors? It is possible that Mll3 and Mll4 can acquire gain-of-function somatic mutations to drive enhancer activation of downstream proto-oncogenes or tumor suppressor genes. On the other hand, loss-of-function mutations in Mll3 and Mll4 may lead to deactivation of proto-oncogenes or tumor suppressor genes (Fig. 3). We suspect that the transcription factors that recruit Mll3/Mll4/COMPASS in specific cell types are critical for the functions of these important enhancer regulators. Future studies characterizing the consequence of COMPASS mutations in specific types of cancer in combination with studying the recruitment mechanisms of COMPASS will be helpful to solve this challenging question.

The Role of UTX in Cancer

UTX/KDM6A is one of the components of Mll3/Mll4/COMPASS, and it is also highly mutated in multiple cancers, including AML, T-ALL, colorectal cancer, glioblastoma, lung cancer, pancreatic cancer, bladder cancer, and renal carcinoma. The mutation rate of UTX in cancer is higher in its JmjC domain, which is the domain responsible for its demethylase activity, suggesting that the enzymatic function of UTX is important during tumorigenesis. Indeed, tumor-suppressor roles of UTX are supported by studies of fibroblast proliferation and hematopoiesis. Depletion of UTX causes increased proliferation in human fibroblasts, while UTX overexpression leads to a reduction in proliferation. Deletion of UTX, however, led to enlarged spleens, myeloid dysplasia, and chromosomal aberrations of bone marrow cells in female mice. Moreover, two recent studies from independent groups found that UTX acts as a tumor suppressor in T-ALL. Bone marrow transplantation of UTX-deleted and NOTCH1-overexpressing HSCs resulted in an increased leukemic blast number in peripheral blood, aggravated leukemia cell filtration, and downregulation of tumor suppressor genes. In a different NOTCH1-overexpressing transplantation model, depletion of UTX by shRNA accelerated T-ALL development and led to repression of tumor suppressors with increased H3K27me3 levels. Thus, like Mll3 and Mll4, the

function of UTX in cancer seems to be context dependent. Interestingly, UTX was reported to play an oncogenic role in TAL1-positive but not in TAL1-negative T-ALLs. It was further shown that inhibition of UTX activity by small molecule GSK-J4 kills TAL1-positive T-ALLs in vitro and in xenograft models. The pro-tumor roles of UTX was also observed in glioblastoma stem cells (GSCs) tolerant to receptor tyrosine kinase (RTK) inhibitors. In comparison with naive GSCs, these so called persister-like GSCs had upregulated levels of lysine demethylases and exhibited a genome-wide redistributed H3K27me3 profile. The growth rate of persister-like GSCs but not naive GSCs was drastically reduced by UTX deletion and H3K27 demethylase inhibition, highlighting an oncogenic role of UTX in persister-GSCs.

UTX protein gets degraded when Trr is depleted in *Drosophila* cells, indicating that the integrity of Trr/COMPASS is required for UTX stability and that it is possible that there is little free UTX outside of Trr/COMPASS. However, how much Trr/Mll3/Mll4/COMPASS function depends on UTX during animal development and tumorigenesis is largely unknown. The simultaneous methylation of H3K4 and demethylation of H3K27 by Mll3/Mll4/COMPASS may be required for enhancer activation, but since the function of UTX is dependent on Mll3 and Mll4, the loss-of-function (LOF) of UTX may not generate as severe phenotypes as LOF of Mll3 and Mll4. Indeed, Mll4 null mice die before E9.5, whereas many UTX null male mice are viable and fertile. It is worth noting that UTX has a male-specific paralog UTY, which lacks demethylase activity. Similar to UTX, UTY also co-immunoprecipitated with Rbbp5, indicating that UTY could be part of Mll3/Mll4/COMPASS. Interestingly, male mice null for both UTX and UTY die in utero, suggesting a non-catalytic function of UTX that remains elusive.

The Functions of P300/CBP and Acetylation Readers in Cancer

As pervasive histone acetyltransferases, P300 and CBP are implicated in almost every aspect of transcription. P300/CBP are highly mutated in bladder urothelial carcinoma, endometrial carcinoma, head and neck squamous cell carcinoma, and lung squamous cell carcinoma based on cancer genome sequencing studies. They are considered tumor suppressors based on a series of early studies. The first evidence of tumor suppressor functions for P300/CBP came from studies of Rubinstein-Taybi syndrome (RTS), a rare genetic disorder characterized by broad thumbs, toes, facial abnormalities, and mental retardation, which is likely driven by heterozygous LOF mutations in CBP or P300. Patients with RTS have an increased likelihood to develop cancer, including AML, non-Hodgkin lymphoma, and neuroblastoma, suggesting that P300/CBP LOF contributes to tumorigenesis. Furthermore, mice with CBP haploinsufficiency develop hematologic malignancies. Interestingly, loss of heterozygosity of CBP was observed in tumors dissected from CBP heterozygous mice, and chimeric CBP or P300 null mice develop hematological tumors that are exclusively derived from the CBP or P300 mutant cell population, further supporting a tumor suppressor role of CBP. It was later shown that reintroduction of WT P300 in cancer cell lines lacking normal P300 resulted in suppression of cell growth, while expressing P300 in P300 WT cells had a minimal effect on growth rate. The interactions of P300/CBP with tumor suppressing transcription factors such as BRCA1 may explain their anti-tumor role, as these tumor suppressing TFs could recruit P300/CBP to activate downstream tumor suppressor genes. Recent identification of inactivating mutations in P300/CBP in B-cell lymphoma and relapsed ALL further support their tumor suppressor roles. Indeed, CBP haploinsufficiency cooperates with BCL2 deregulation to drive B-cell lymphomagenesis in vivo, consistent with its LOF mutation in B-cell lymphoma.

Like Mll3/4 COMPASS, P300/CBP plays an oncogenic role in some contexts. Both the MOZ-CBP fusion protein produced by a recurrent translocation in the M4/M5 subtype of AML, and the Mll-CBP fusion protein found in therapy-related myeloproliferative disease, are potential oncogenic proteins which induce malignancy. In colorectal cancer cells after DNA damaging, P300 deletion leads to a more stable P53 level, increased apoptosis, and decreased tumor size in a xenograft model. In some cases, the activity of P300/CBP can be augmented by oncogenic proteins to activate other oncogenes. For instance, the kinase IKKα was reported to phosphorylate CBP and enhance its activity such that NFκB-mediated gene expression is increased while P53-mediated genes are repressed due to the preference of phosphorylated CBP's binding to NFκB over P53. In another scenario, P300 can acetylate the AML1-ETO fusion protein in acute myelogenous leukemia, which enhances the oncogenic capability of the fusion protein. Importantly, inhibition of P300/CBP activity can block AML1-ETO induced leukemogenesis, indicating an oncogenic function of P300 in this context.

In recent years, the role of lysine acetylation binding proteins such as the bromodomain and extra terminal (BET) domain family of proteins and YEATS domain containing proteins are intensively studied in cancer and other diseases. There are four members of BET domain family: BRD2, BRD3, BRD4, and BRDT. Except for BRDT which is expressed in testis, BRD2–4 are ubiquitously expressed and play a role in a series of biological processes including TF recruitment, chromatin remodeling, transcription elongation, and cell proliferation. In NUT midline carcinoma, a very aggressive squamous cell epithelia cancer, *BRD4* and sometimes *BRD3* are translocated to the gene *NUTM1* on chromosome 15, producing the NUT-BRD fusion protein, which drives cell growth, potentially through a feed-forward cycle involving recruitment of P300 to enhancers by the NUT part and binding to acetylated histones by the BRD4 portion.

RNAi screens revealed that BRD4 depletion inhibits AML growth and suppresses Myc regulated genes, indicating that Myc could be a downstream target of BRD4 in AML. Using a proteomic strategy, an independent study identified that BET proteins interact with the PAF complex (PAFc) and super elongation complex (SEC), both of which are involved in regulating transcription elongation, and found that inhibition of BET proteins can specifically inhibit the growth of Mll-fusion leukemia, potentially through suppressing the expression of *Bcl2*, *CDK6*, and *Myc*. In addition, Myc suppression by BRD4 inhibition has been observed in different types of cancer including multiple myeloma and lymphoma.

Recent genome-wide CRISPR screens identified that the YEATS domain protein ENL is required for the proliferation of Mll-fusion leukemia and the degradation of ENL attenuates the recruitment of SEC to oncogenes such as *Myb*, *Meis1*, *Hoxa10*, and *Myc*. Similarly, an independent study showed that ENL depletion reduces AML proliferation in vitro and in a xenograft model, which led to reduced RNA Pol II recruitment to oncogenes. Importantly, the interaction between the YEATS domain of ENL and histone acetylation is crucial for ENL's function. Furthermore, BET protein inhibition and mutation of the ENL YEATS domain had an additive effect on AML proliferation and progression, suggesting that the recruitment through binding acetylated histones is critical for tumor growth, and that inhibition of this binding could be further investigated for drug development in cancer therapy.

The Role of CTCF and Cohesin in Cancer

CTCF and cohesin are key to the establishment and/or maintenance of chromatin loops and TAD structure. The LOF of CTCF or cohesin in cancer may cause activation of oncogenes through altering chromatin loops (Fig. 4). CTCF is frequently mutated in endometrial carcinoma, head and neck squamous cell carcinoma, and breast cancer. More than half of the known missense CTCF mutations in cancer are located in its zinc fingers (ZFs), suggesting that the DNA binding capability of CTCF is impaired in cancers harboring these mutations. Tumor specific mutations in the ZF regions of CTCF disrupt CTCF's recognition toward its binding sites and result in the CTCF dependent transcription in reporter assays, suggesting a tumor suppressor role of CTCF. Moreover, overexpression of CTCF in B-cell lymphoma leads to apoptosis, downregulation of *c-Myc* oncogene, and upregulation of P53 target genes. CTCF's role in tumor suppression is further demonstrated in vivo using CTCF hemizygous knockout mice, which have a significantly higher chance to succumb to different types of cancer including uterine tumors, T cell lymphoma, and B cell lymphoma by 100 weeks of age in comparison to their WT counterparts. Furthermore, CTCF hemizygous knockout mice have accelerated development of Kras-driven lung cancer. The tumor suppressor function of CTCF may be due to its role in DNA double-strand break (DSB) repair as CTCF is recruited to the sites of DSB, and depletion of CTCF causes delayed kinetics of DSB repair. In summary, although CTCF's tumor suppressor role is well established, whether CTCF LOF leads to oncogenesis primarily through disrupting chromatin looping and chromatin structure is largely unknown.

Cohesin is frequently mutated in both solid tumors and hematological malignancies. Among all the cohesin complex subunits, the cohesin subunit STAG2 is the most mutated in tumors including bladder cancer, Ewing sarcoma, myelodysplastic syndrome (MDS), and AML. STAG2 mutations in glioblastoma, Ewing Sarcoma, and melanoma mostly lead to truncation of the protein. Depletion of STAG2 in the colorectal cancer cell line HCT116 leads to defects in sister chromatid cohesion while targeted correction of the endogenous mutant STAG2 in glioblastoma cells restores sister chromatid cohesion and reduces genome instability. The role of cohesin in leukemia has been reported by several independent studies. Viny and colleagues conditionally knocked out cohesin subunit Smc3 in mouse HSCs and found that Smc3 hemizygous knockout mice have increased HSC self-renewal and succumb to AML much earlier than WT mice when carrying the *Flt3-ITD* allele. Mullenders and colleagues generated shRNA mouse models in which cohesin subunits RAD21, STAG2, and SMC3 were respectively depleted and demonstrated that cohesin depletion skews HSC differentiation and leads to myeloproliferative neoplasms in aged animals. Mazumdar and colleagues took a different approach, making mutants of cohesin subunits found in AML and overexpressing them in leukemia cell lines and primary human hematopoietic stem and progenitor cells (HSPCs). They found that cohesin mutants have dominant negative functions as they impair myeloid differentiation, increase serial replating of human HSPCs, and enforce the expression of stem cell genes. All three studies found that chromatin accessibility was altered upon depletion of cohesin or expression of mutant cohesin subunits, suggesting that genome architecture may be altered by cohesin LOF in AML with cohesin mutations. On the other hand, Galeev et al. and Fisher et al. identified cohesin in an RNAi screen as a modifier of hematopoietic self-renewal and differentiation. Interestingly, depletion of Hoxa7 or Hoxa9 is able to restore the HSPC differentiation capability impaired by cohesin depletion, indicating that these

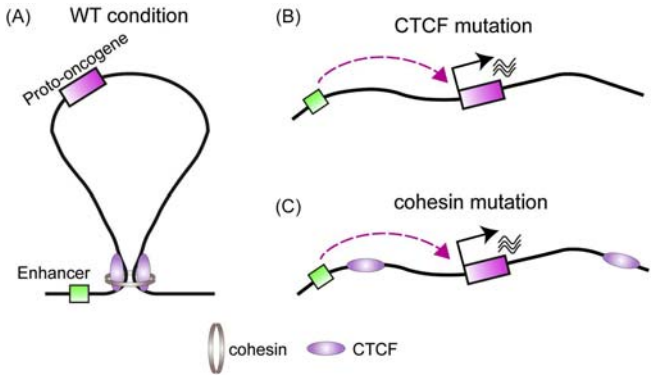

Fig. 4 Proto-oncogene activation through CTCF and cohesin loss-of-function. (A) A model showing that chromatin looping mediated by CTCF and cohesin blocks the communication between an enhancer and the promoter of a proto-oncogene in wildtype (WT) cells. (B and C) In cancer cells carrying loss-of-function mutations in CTCF (B) or cohesin (C), ectopic enhancer-promoter communication is established, which activates the proto-oncogene.

important AML oncogenes may be downstream of cohesin's regulation. Collectively, these findings demonstrate a tumor suppressor role for cohesin in solid and hematological malignancy.

Conclusions and Perspectives

Transcription deregulation is a common theme of cancer pathogenesis. Enhancer malfunction plays an essential role in gene misregulation of cancer cells. In this review, we discussed the malfunction of enhancers both in *cis* and in *trans*. Both mutations in enhancers and enhancer regulating proteins are frequently found in human cancer, suggesting that enhancer malfunction is one of the major drivers of oncogenesis and tumor progression. However, the functional relationship between the cancer-related mutations and oncogenesis is not clear and studies that focus on the roles of mutations are still lacking. Future studies using the CRISPR/Cas9 technique in cell lines and animals to create specific cancer-associated mutations will be very helpful to understand the functions of the mutated DNA sequences or mutant proteins in cancer.

Whether the oncogenic or tumor-suppressing role of enhancer-regulating epigenetic enzymes depends on the enzymatic activities remain largely unknown. For example, catalytic-independent regulation of enhancer activity by the H3K4 methyltransferase Mll3/Mll4/COMPASS has been shown in noncancer biological systems. The further dissection of catalytic-dependent and independent functions of chromatin modifiers on enhancer activation will be essential to understand the mechanisms of oncogenesis and to develop novel cancer therapies. Furthermore, the direct link between promoter-enhancer looping and regulators of chromatin architecture in cancer is not well established. CTCF and cohesin's tumor suppressor roles in cancer have been linked with DNA damage repair. Although deletions in CTCF/cohesin binding sites have an impact on chromatin architecture, the effects of CTCF and cohesin mutations on looping during carcinogenesis are not known. Hi-C and 4C-seq experiments in the mutant cell lines as well as the rapid depletion of these proteins using the auxin inducible degradation (AID) system will elucidate the causal relationship between chromatin looping and regulation of cancer associated genes and allow disambiguating loss of enhancer function due to cohesin mutation from DNA instability due to loss of sister chromatid cohesion.

It is noteworthy that enhancers may impact their target genes through basic transcription machineries during pathogenesis, as recent findings indicate that the pausing of RNA pol II at gene promoters may be modulated by their enhancers. Such a mechanism could be utilized by transcription factor binding at enhancers to regulate oncogenes or tumor-suppressor genes during cancer pathogenesis. Our understanding of enhancer malfunction in cancer prompts the development of novel therapies for cancer treatment. The BET inhibitor JQ1 has already been shown to suppress the growth of different malignancies including lung cancer, leukemia, neuroblastoma, melanoma, prostate cancer, breast cancer, and diffuse intrinsic pontine gliomas in mouse models. Similarly, a recently developed specific inhibitor of P300/CBP has been found to be effective in mouse models of hematological malignancies and prostate cancer. We believe that the generation of compounds that target other epigenetic modifiers such as Mll3/Mll4/COMPASS, UTX, and the histone acetylation binding protein ENL, will significantly advance the treatment of cancer.

See also: Defective 5-Methylcytosine Oxidation in Tumorigenesis. DNA Methylation Changes in Cancer: Cataloguing. Mod Squad: Altered Histone Modifications in Cancer. Mutations in DNA Methyltransferases and Demethylases.

Further Reading

Allen, B.L., Taatjes, D.J., 2015. The mediator complex: A central integrator of transcription. Nature Reviews Molecular Cell Biology 16, 155–166.
Deng, W., Lee, J., Wang, H., et al., 2012. Controlling long-range genomic interactions at a native locus by targeted tethering of a looping factor. Cell 149, 1233–1244.
Fujisawa, T., Filippakopoulos, P., 2017. Functions of bromodomain-containing proteins and their roles in homeostasis and cancer. Nature Reviews Molecular Cell Biology 18, 246–262.
Ghirlando, R., Felsenfeld, G., 2016. CTCF: Making the right connections. Genes & Development 30, 881–891.
Herz, H.M., Mohan, M., Garruss, A.S., et al., 2012. Enhancer-associated H3K4 monomethylation by Trithorax-related, the Drosophila homolog of mammalian Mll3/Mll4. Genes & Development 26, 2604–2620.
Kandoth, C., McLellan, M.D., Vandin, F., et al., 2013. Mutational landscape and significance across 12 major cancer types. Nature 502, 333–339.
Lawrence, M.S., Stojanov, P., Mermel, C.H., et al., 2014. Discovery and saturation analysis of cancer genes across 21 tumor types. Nature 505, 495–501.
Long, Y., Wang, X., Youmans, D.T., Cech, T.R., 2017. How do lncRNAs regulate transcription? Science Advances 3, eaao2110. https://doi.org/10.1126/sciadv.aao2110.
Piunti, A., Shilatifard, A., 2016. Epigenetic balance of gene expression by Polycomb and COMPASS families. Science 352, aad9780. https://doi.org/10.1126/science.aad9780.
Shilatifard, A., 2012. The COMPASS family of histone H3K4 methylases: Mechanisms of regulation in development and disease pathogenesis. Annual Review of Biochemistry 81, 65–95.
Smith, E., Shilatifard, A., 2014. Enhancer biology and enhanceropathies. Nature Structural and Molecular Biology 21, 210–219.
Spitz, F., Furlong, E.E., 2012. Transcription factors: From enhancer binding to developmental control. Nature Reviews Genetics 13, 613–626.
Sze CC and Shilatifard A (2016) MLL3/MLL4/COMPASS family on epigenetic regulation of enhancer function and cancer. Cold Spring Harbor Perspectives in Medicine 6: pii: a026427
Zhou, S., Treloar, A.E., Lupien, M., 2017. Emergence of the noncoding cancer genome: A target of genetic and epigenetic alterations. Cancer Discovery 6, 1215–1229.

Environmental and Occupational Exposures

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Introduction

Many environmental exposures have been established as causes of human cancer. Some established examples are listed in Table 1. Early indications for potential carcinogenic effects are often case reports of unexpected, typically rare cancers among people with well-defined exposures, often among occupational groups. It should be noted that the term "environmental" in the chapter encompasses both the workplace and ambient general environment. The initial indications of cancer risks potentially related to environmental exposures have often been prompted by case reports, or clusters, of relatively rare cancers occurring among workers with characteristic exposures. Cancer clusters have also been reported from the general population whose environmental carcinogen exposures result from contaminated air, water, or food supplies. Classic examples of exposure/disease associations prompted by case reports are asbestos and mesothelioma, benzene and acute myeloid leukemia, and vinyl chloride and liver angiosarcoma among exposed workers. Case reports can then lead to epidemiologic studies that are designed to estimate the strength and specificity of associations in the population, dose-response gradients, and possible synergistic effects with other causes of the cancer under investigation. Epidemiologic studies of well-defined occupational groups are especially informative regarding cancer risks related to environmental exposures because the workplace typically represents the "high dose" setting. When there is consistent epidemiologic evidence derived from multiple studies examining an etiologic relation, public health agencies can undertake surveillance programs that identify new cases and measure exposure levels in workplaces and the general environment. Ultimately, regulatory agencies rely on epidemiologic evidence to set permissible exposure limits in the workplace and ambient environment.

The components involved in identify environmental carcinogens, starting with initial case reports, and culminating in setting exposure standards to prevent cancer are summarized in Fig. 1. It should be noted that the process includes contributions by clinicians, epidemiologists, environmental exposure scientists, toxicologists, biostatisticians, risk assessors, and policy makers.

Illustrative Examples

The following examples of environmental chemicals were chosen to illustrate the approaches for and concepts underlying recognition of carcinogenic potential from the epidemiologic perspective. Each of the chemicals has been classified by the International Agency of Research on Cancer [IARC] as a confirmed human carcinogen (category 1), although the strength of etiologic evidence varies by cancer site. Considerations of differences in sources of exposure, bio-persistence, and presumed carcinogenesis mechanisms also contribute to challenges in carcinogen characterization.

Formaldehyde

Formaldehyde is a simple one'carbon molecule that is produced commercially for use as a biocide, preservative, and basic chemical in the manufacture of common materials such as plastics, building materials, glues and fabrics, and many household and consumer products, including medicines, health and beauty aids. Formaldehyde is also a product of organic matter combustion. Formaldehyde is a widespread environmental exposure worldwide. Major exposure sources include some laboratories, indoor air (e.g., carpets), vehicle emissions, cigarette smoke, and workplaces manufacturing or using resins, various wood products (e.g. particle board), adhesives, textiles, and numerous other products. Inhalation is the predominant route of exposure to exogenous formaldehyde.

Table 1 Some established[a] environmental and occupational carcinogens

Agent	*Sources*	*Major cancer site(s)*
Arsenic	Smelters, mines, drinking water	Bladder, lung, skin
Asbestos	Mining, insulation	Lung, pleura, peritoneum
Benzene	Solvents, petroleum products	Leukemias, lymphomas
Dioxin	Herbicides	Multiple (promoter)
Formaldehyde	Foam insulation, embalming, tobacco smoke	Nasophayrnx, sinonasal, leukemias
Polychlorinated biphenyls	Insulation materials, flame retardants	Melanoma, non-Hodgkin lymphoma
Radon	Underground mines, tunnels	Lung
Silica	Mining, ceramics, construction	Lung
Vinyl chloride	Polyvinyl chloride manufacturing	Liver
Wood dust	Furniture making, lumber, construction	Sinonasal

[a]Classified by IARC as confirmed carcinogens (Category 1).

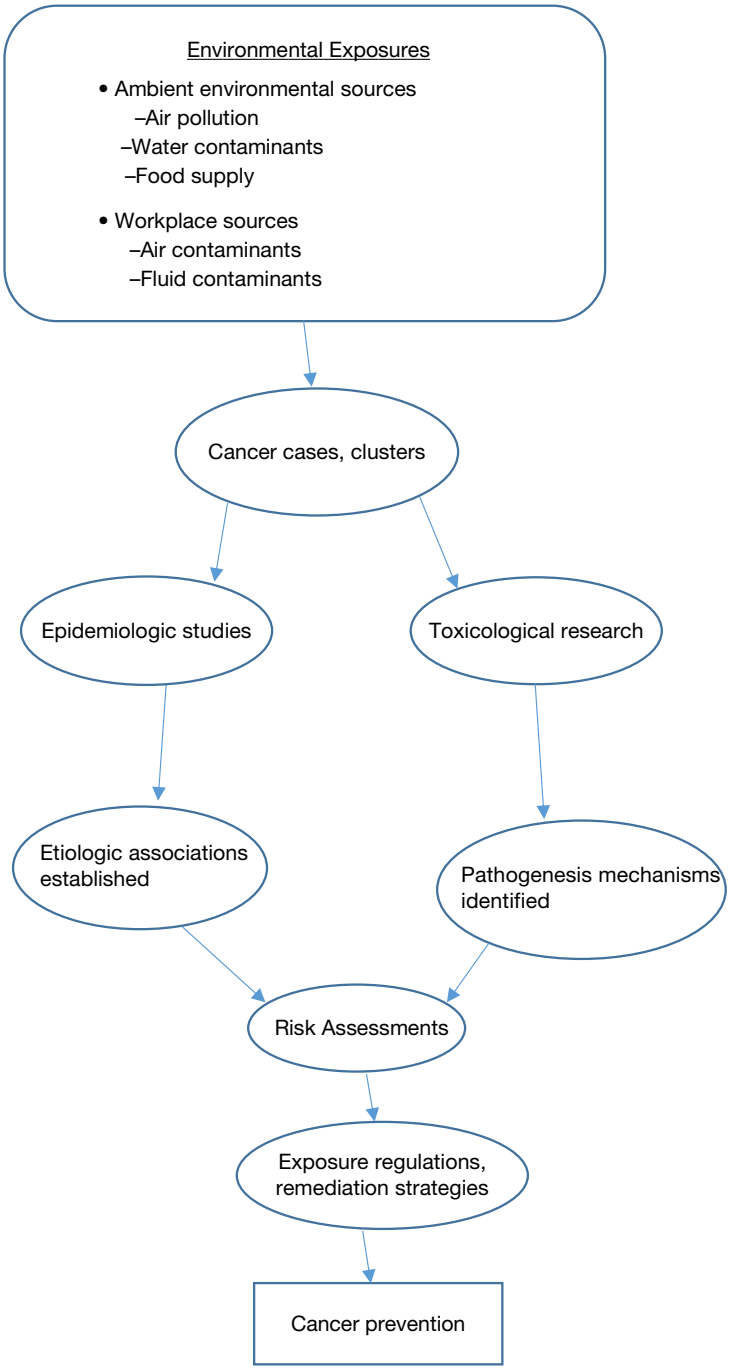

Fig. 1 Identification of environmental carcinogens and methods for prevention.

Concerns about the carcinogenicity of formaldehyde were prompted in the early 1980s by the induction of nasal tumors in rats exposed at high concentrations. As a consequence, the focus of early epidemiologic studies was on nasal and upper airway tract cancers, based on the understanding that formaldehyde is rapidly metabolized at the site of contact. There have also been numerous epidemiologic studies of associations with other malignancies, including the leukemias and lymphomas. IARC has classified formaldehyde as a confirmed (category 1) human carcinogen, based largely on evidence for etiologic associations with nasopharyngeal cancer and myeloid leukemia, malignancies that have very different sets of putative causes and pathogenesis mechanisms. The nasal upper airway passage is a primary initial target for inhaled formaldehyde, which strengthens arguments for biological plausibility for an etiologic relation with nasopharyngeal cancer. In contrast, findings from experimental toxicokinetic studies indicate that exogenous formaldehyde from environmental sources does not reach the bone marrow, which is the prime target site for the lymphohematopoietic malignancies.

Findings from an occupational cohort study of cancer mortality among over 25,000 workers in U.S. formaldehyde-exposed industries (e.g., urea formaldehyde foam insulation) conducted by the U.S. National Cancer Institute have been especially influential in establishing an association with myeloid leukemia and other lymphohematopoietic malignancies. The investigators conducted an occupational cohort mortality in which workers' historical exposures were reconstructed by linking occupational history data with a job-specific formaldehyde exposure matrix based on historical measurements taken in the workplaces. As shown in **Table 2**, a much stronger association with myeloid leukemia was detected for peak exposure than for cumulative exposure, which is the standard dose metric in epidemiologic research. Interpretation of the findings is therefore complicated because of the absence of an established carcinogenesis mechanism for peak formaldehyde exposure.

Wood Dust

Since the 1960s, there have been numerous case reports of nasal cancers among furniture workers and other occupational groups exposed to wood dust. Sinonasal adenocarcinoma has been identified as mostly strongly related nasal cancer to wood dust.

Reliable data on dose-response trends are limited in the epidemiologic literature, as few studies have generated quantitative wood dust exposure data based on industrial hygiene measurements for dose estimation. Moreover, the rarity of sinonasal cancer in most occupational cohorts has hindered dose-response estimation. A case-control study of adenocarcinoma nested in the German wood industry involved quantitative wood dust exposure estimation and a distinction between hard and soft wood exposure. The cases were identified from worker illness and injury claims among employees in wood-working industries, such as lumber preparation and furniture making. Controls were also wood working employees who had experienced work-related accidents. The dose-response findings are summarized in **Table 3**.

There are some suggestions associations with SNC risk varies by exposure to different types of wood dust, usually classified as "hard" or "soft" wood (IARC 2012). Comparing high vs. low level hard wood dust exposures in the aforementioned German case-control study yielded a strong association (Relative Risk = 3.98 [95% Confidence Interval 1.92–8.25]), whereas a seemingly "protective" effect was observed for high vs. low level soft wood dust exposure (Relative Risk = 0.34 [95% Confidence Interval: 0.17–0.65]).

In summary, exposure to wood dust has been convincingly linked epidemiologically to excess risk for SNC, especially of adenocarcinoma histology. It is reasonable to presume that there is an underlying dose-response etiologic relation that perhaps may differ

Table 2 Associations between cumulative and peak exposures to formaldehyde and myeloid leukemia mortality among U.S. formaldehyde products industry workers[a]

	No. deaths	*Relative risk (95% CI)*
Cumulative exposure (ppm[b]-years)		
>0–<1.5 [ref]	26	1.00 (–)
1.5–<5.5	8	0.82 (0.36–1.83)
>5.5	10	1.02 (0.48–2.16)
Peak exposure (ppm)		
>0–<2.0	14	1.00 (–)
2.0–<4.0	11	1.30 (0.58–2.92)
	19	1.78 (0.87–3.64)

[a]Beane Freeman et al. (2009).
[b]parts per million in air.

Table 3 Association of wood dust exposure and adenocarcinoma among German wood workers[a]

Cumulative exposure (mg/m^3 × years)[c]	*Cases*	*Controls*	*Rel. risk (95% CI)*[b]
<140 [ref]	36	120	1.00 (–)
140–<200	29	48	1.72 (0.77–3.87)
≥200	21	36	4.20 (1.69–10.43)

[a]Pesch et al. (2008).
[b]Odds ratio (95% Confidence interval) adjusted for age, smoking, exposure to varnishes or stains.
[c]Milligrams per cubic meter air.

by type of wood (e.g., soft vs. hard), and by source (e.g., furniture making, construction). However, sparse data on dose-response trends by type of wood and source can complicate causal attribution and standard setting.

Polycyclic Aromatic Hydrocarbons (PCBs)

Polycyclic aromatic hydrocarbons are bio-persistent compounds whose main commercial applications have been as coolants and insulating fluids in electrical transformers, waterproofing flame retardants, and plasticizers in paints and cements. Two hundred nine (209) PCB congeners have been identified, based on the extent and location of chlorination. From a carcinogenesis standpoint, PCB congener groupings have been developed to represent chemical structural and toxicological features: estrogenic, dioxin-like, immunotoxic, and induction of phenobarbital and cytochrome P450s.

PCB exposures occur in occupational settings, (e.g., electrical capacitor manufacturing), from contaminated air, soil or water, and from dietary sources (e.g., seafood). Exposures typically occur as mixtures of congeners. For logistical reasons, many epidemiologic studies have based exposure assessments on plasma concentrations, rather than concentrations measured in environmental media. Tissue concentrations in target organs (e.g., breast) have also been evaluated in relation to cancer risk in some studies. Physiological PCB concentrations change over time due to exposure variability and metabolism; consequently, repeated measures of PCBs would be most desirable epidemiologically. This has seldom been achievable, however. Instead, physiological concentrations are generally divided into pre- and post-diagnostic time intervals. It is logical to assume that pre-diagnostic concentrations are most relevant etiologically, as they should best reflect exposures prior to cancer occurrence and would not be influenced by the disease process.

It should be evident from the foregoing description of PCBs that assessing epidemiologic evidence for carcinogenicity is complicated by variability in chemical features, routes of exposure, and presumed toxicological mechanisms.

IARC (2016) concluded that PCBs can cause malignant melanoma and non-Hodgkin lymphoma, and that there is suggestive evidence for causation of breast and prostate cancers. The following two examples of epidemiologic studies of malignant melanoma are illustrative of epidemiologic studies whose findings provided mixed evidence for an etiologic association.

Gallagher et al. (2011) conducted a population-based case-control study in British Columbia, Canada that included 80 cases and 310 controls matched on age and gender. PCB exposures were based on lipid-adjusted plasma measurements of 14 congeners. As shown in Table 4, there was a strong dose-response trend for total PCB levels, adjusted for potential confounders, including total recreational sun exposure. This study had the advantage of assessing exposures from multiple sources, as indicated by plasma concentrations. However, the plasma samples were post-diagnostic and specific sources of PCB exposure and their relative contributions could not be determined.

A cohort mortality study conducted by the U.S. National Institute for Occupational Safety and Health addressed risks for multiple cancer outcomes in relation to PCB exposures (Ruder et al. 2014). The cohort included nearly 25,00 workers from three electrical capacitor plants. PCB exposures were assessed by a job-exposure matrix that classified jobs according to semi-quantitative estimates of inhalation exposure. The results, summarized in Table 4, do not indicate a dose-response relation, and in fact, might be construed as evidence for a "protective" effect of PCB exposures. Data interpretation is limited, however, by the absence of direct quantitative exposure measurements, and reliance on mortality, rather than incidence of malignant melanoma (Table 5).

Prospective Vision

Identifying etiologic relations between environmental exposures and malignant diseases is a critical first step in cancer prevention in the population. The strongest evidence is obtained when the cancer of concern has few or no other established causal factors, such as the case of asbestos and mesothelioma, or when cancer rates increase dramatically after introduction of an environmental exposure. A well-known example of the latter is arsenic contamination of drinking water in Bangladesh resulting from greatly increased installation of residential tube wells for drinking water that led to sharply elevated rates of cancers of the skin, bladder, kidney, and lung. More typical are situations where cancer hazards related to environmental exposures become evident over time, as epidemiologic and toxicological research develops.

Table 4 Polcyhlorinated biphenyl (PCB) pre-diagnostic plasma levels and risk of malignant melanoma[a]

Exposure quartiles (ng/g)[b]	*Cases*	*Controls*	*Rel Risk (95% CI)*[c]
<98.01 [ref]	18	76	1.00 (–)
98.01–148.71	11	77	1.36 (0.45–4.09)
148.72–213.44	12	77	1.27 (0.39–4.12)
>213.44	29	75	6.02 (2.00–18.17)

[a]Gallagher et al. (2011).

[b]nanograms per gram plasma lipid.

[c]Odds ratio (95% Confidence Interval), adjusted for age, gender, education, skin reaction to repeated sun exposure, total recreational sun exposure hours.

Table 5 Polychlorinated biphenyl exposure and malignant melanoma mortality among US electrical capacitor workers[a]

Cumulative exposure[b]	*No. deaths*	*RR (95% CI)*[c]
0–<40,000 [ref]	21	1.00 (–)
40,000–<150,000	5	0.34 (0.13–0.92)
150,000–<600,000	10	0.78 (0.37–1.68)
≥600,000	3	0.20 (0.06–0.70)

[a]Ruder et al. (2014).
[b]PCB exposure units × days.
[c]Standardized rate ratio (95% Confidence Interval).

Ideally, decisions regarding control or elimination of exposures to an environmental agent should be based on consistently strong evidence derived from high-quality research. Challenges that epidemiologists often confront are limitations of exposure assessment for the agent of concern and co-occurring carcinogens that may be confounders. By way of illustration, characterizing PCB exposures is complicated for several reasons. There are 209 PCB congeners with various toxicity features, and there are multiple sources of exposure that include the workplace, ambient environment, and the food chain. Blood or target tissue PCB concentrations can vary greatly over time, and seldom indicate contributing exposure sources. Reliance on exposure surrogates, such as occupational, residential, or dietary history, due to the absence of personal exposure measurement data is a major limitation in many epidemiologic studies of environmental carcinogens.

Case reports and cancer clusters will likely remain the primary triggers for in-depth epidemiologic investigations and disease surveillance. Updating existing epidemiologic databases will also be extremely valuable to determine whether cancer risks have been affected by temporal changes in exposure, or by replacement of an established carcinogen with a presumed safer agent. Future research will clearly benefit by incorporation of improved methods for measuring environmental exposures at the individual level, advances in techniques to link exposure data with population-based cancer incidence data, such as Geographical Information System modeling, and increased collaboration with toxicologists and other experimental scientists engaged in research to predict carcinogenesis pathways. Moreover, further development of biomarkers of genetic variability, including DNA polymorphisms and blood proteins indicative of pre-clinical carcinogenesis, will be valuable for identifying subgroups of the population who are especially susceptible to cancers resulting from environmental exposures.

See also: Cancer Risk Reduction Through Lifestyle Changes.

Further Reading

Acheson, E.D., Cowdell, R.H., Hadfield, E., Macbeth, R.G., 1968. Nasal cancer among workers in the furniture industry. British Medical Journal 2, 587–596.
Beane Freeman, L.E., Blair, A., Lubin, J.H., et al., 2009. Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries: the National Cancer Institute Cohort. Journal of the National Cancer Institute 101 (10), 751–761.
Binazzi, A., Ferrante, P., Marinaccio, A., 2015. Occupational exposure and sinonasal cancer: a systematic review and meta-analysis. BioMed Central Cancer 15, 49–65.
Boffeta, P., Hashim, D., 2017. Exposure to silicon carbide and cancer risk: a systematic review. International Archives of Occupational and Environmental Health 90, 1–12.
Checkoway, H., Boffetta, P., Mundt, D.J., et al., 2012. Critical review and synthesis of the epidemiologic evidence on formaldehyde exposure and risk of leukemia and other lymphohematopoietic malignancies. Cancer Causes & Control 23, 1747–1766.
Emeville, E., Giusti, A., Coumoul, X., et al., 2015. Associations of plasma concentrations of dichlorodiphenyldichloroethylene and polychlorinated biphenyls with prostate cancer: a case-control study in Guadeloupe (French West Indies). Environmental Health Perspectives 123, 317–323.
Gallagher, R.P., MacArthur, A.C., Lee, T.K., et al., 2011. Plasma levels of polychlorinated biphenyls and risk of cutaneous malignant melanoma: a preliminary study. International Journal of Cancer 128, 1872–1880.
Pesch, B., Pierl, C.B., Gebel, M., et al., 2008. Occupational risks for adenocarcinoma of the nasal cavity and paranasla sinuses in the German wood industry. Occupational and Environmental Medicine 65, 191–196.
Ruder, A.V., Hein, M.J., Hopf, N.B., Waters, M.A., 2014. Mortality among 24,865 workers exposed to polychlorinated biphenyls (PCBs) in three electrical capacitor manufacturing plants: a ten-year update. International Journal of Hygiene and Environmental Health 217, 176–187.
Smith, A.H., Lingas, E.O., Rahman, M., 2000. Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. Bulletin of the World Health Organization 78, 1093–1103.
World Health Organziation, International Agency for Research on Cancer [IARC], 2012. IARC monographs on the evaluation of carcinogenic risks to humans. In: A review of human carcinogens part F: chemical agents and related occupations, vol. 100. International Agency for Research on Cancer, Lyon.
World Health Organziation, International Agency for Research on Cancer [IARC], 2012. IARC monographs on the evaluation of carcinogenic risks to humans. In: Arsenic, metals, fibers, and dusts: a review of human carcinogens, vol. 100C. International Agency for Research on Cancer, Lyon, France.
World Health Organziation, International Agency for Research on Cancer [IARC], 2016. IARC monographs on the evaluation of carcinogenic risks to humans. In: Polychlorinated biphenyls and polybrominated biphenyls, vol. 107. International Agency for Research on Cancer, Lyon, France.

Environmental Exposures and Epigenetic Perturbations

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Glossary

Carcinogenesis A complex multistep process of the turning a normal cell into the cancer cell.

Epigenetics The study of phenomena and mechanisms that cause chromosome-bound changes in gene expression that do not involve changes in DNA sequence.

Nucleosome The fundamental unit of chromatin that consists of 146 base pairs of DNA coiled in two superhelical turns around an octamer of core histones.

DNA methylation The addition of methyl groups from the universal methyl group donor *S*-adenosyl-L-methionine to carbon five on cytosine residues and results in the formation of 5-methylcytosine in DNA.

Histone modification A posttranslational covalent modification, for example, methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation, of the amino-terminal tails of histones.

Genotoxic carcinogens Agents that interact with DNA, either directly or after metabolic activation, causing the formation of covalent DNA adducts as part of their mechanism of carcinogenicity

Nongenotoxic carcinogens A diverse group of carcinogens that cause cancer by mechanisms other than directly damaging DNA.

Abbreviations

5,10-MTHF 5,10-Methylenetetrahydrofolate
5-hmeC 5-Hydroxymethylcytosine
5-meC 5-Methylcytosine
5-MTHF 5-Methyltetrahydrofolate
8-oxo-dG 8-Oxo-7-hydroxydeoxyguanosine
BPDE Anti-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
CDH1 Cadherin 1
CDKN2A Cyclin-dependent kinase inhibitor 2A
DAPK (DAP)-kinase
DNMT1 DNA methyltransferase 1
DNMT3A DNA methyltransferase 3A
DNMT3B DNA methyltransferase 3B
ERCC3 Excision repair cross-complementation group 3
FOXE1 Forkhead box E1
GATA2 GATA binding protein
GSH Glutathione
H3K27 Histone H3 lysine 27
H3K9 Histone H3 lysine 9
H3K9me2 Histone H3 lysine 9 dimethylation
H3S10ph Histone H3 serine 10 phosphorylation
H4K16 Histone H4 lysine 16
H4K20me3 Histone H4 lysine 20 trimethylation
HCY Homocysteine
HIC1 Hypermethylated in cancer 1
HNF1a HNF1 homeobox A
IARC International Agency for Research on Cancer
KRAS KRAS proto-oncogene, GTPase
LINE-1 Long interspersed nuclear element 1
LOX Lysyl oxidase
MAGEA1 Melanoma antigen family A, 1
MGMT O^6-Methylguanine-DNA methyltransferase

MTHFR Methylenetetrahydrofolate reductase
NNK 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
PARP-1 Poly (ADP-ribose) polymerase 1
PAX2 Paired box 2
PPARA Peroxisome proliferator activated receptor alpha
PROX1 Prospero homeobox 1
RARβ$_2$ Retinoic acid receptor β_2
RASSF1A Ras association domain family member 1
RUNX3 Runt-related transcription factor-3
SAH *S*-adenosyl-L-homocysteine
SAM *S*-adenosyl-L-methionine
TCA cycle Tricarbocyclic acid cycle
TET Ten-eleven-translocation
THF Tetrahydrofolate
TOPOIIα DNA topoisomerase II

Cancer is a disease characterized by the acquisition of multiple heritable abnormal cellular phenotypes, including persistent uncontrolled cell proliferation, resistance to cell death, deregulated metabolism, compromised immune response, and genomic instability, driven by mutations from exogenous and endogenous damage to genomic DNA. Because of that, traditionally, the research on cancer has been focused on investigating the role of DNA damage and genetic aberrations in cancer causation and progression. However, after many years of extensive research it is clear that DNA damage is "necessary but not sufficient" for tumor induction and additional factors contribute to carcinogenesis. One of these factors associated with the carcinogenic process is epigenetic alterations, research on which has greatly evolved over the recent years, despite the fact that the importance of epigenetic component in the carcinogenesis was suggested almost a half of a century ago. Currently, it is well-established that cancer is a genetic and epigenetic disease, and both genetic and epigenetic alterations cooperate and complement each other at every stage of cancer development.

Brief Overview of Epigenetics

The term "epigenetics" defines the phenomena and mechanisms that cause chromosome-bound changes in gene expression without changes in the primary DNA sequence. The epigenetic phenomena include cytosine DNA methylation, posttranslational modifications of histone proteins, and nucleosome positioning along DNA.

In the mammalian genome, negatively charged linear DNA is compacted into chromatin, a complex of DNA and proteins, and assembled into chromosomes (Fig. 1). The fundamental repeating units of chromatin are nucleosomes. Approximately 80% of the genomic DNA is packaged into nucleosomes and 20% is associated with the short-linker regions connecting neighboring nucleosomes. Nucleosome consists of 146 base pairs of DNA coiled in two superhelical turns around an octamer of core histones formed by two copies of each histone monomers H2A, H2B, H3, and H4·Histones are evolutionary conserved proteins that consist of a globular carboxy-terminal domain critical to nucleosome formation and a positively charged amino-terminal tail that can bind tightly to DNA.

The chromatin structure is dynamic and subject to chemical modifications that can occur anywhere in the genome. Cytosine DNA methylation, which is the addition of methyl groups from the universal methyl group donor *S*-adenosyl-L-methionine (SAM) to carbon five on cytosine residues and results in the formation of 5-methylcytosine (5-meC) in DNA, is a major and much-studied epigenetic modification. This reaction is mediated by a family of DNA methyltransferases (DNMTs), which includes the maintenance DNA methyltransferase DNMT1 and two de novo DNA methyltransferases, DNMT3A and DNMT3B.

In the genome of somatic mammalian cells, methylation of DNA occurs almost exclusively at CpG dinucleotides, and approximately 70%–90% of them are methylated; however, the distribution of the methylated CpG sequences across the genome is uneven (Fig. 1). In normal cells, most of the methylated CpG dinucleotides are located in repetitive sequences and in exonic, intronic, and intergenic unique sequences, while the unmethylated high-density CpG sites are primarily found in gene promoters. Methylation of DNA is a dynamic and well-balanced process between DNA methylation and DNA demethylation reactions. DNA methylation is initiated and established by de novo DNA methyltransferases DNMT3A and DNMT3B and maintained during DNA replication by the maintenance DNA methyltransferase DNMT1. DNA demethylation is achieved through two different mechanisms: (i) a "passive" replication-dependent mechanism during cell division and (ii) an "active" replication-independent mechanism. During active DNA demethylation, a family of ten-eleven-translocation (TET) proteins sequentially oxidizes 5-meC to

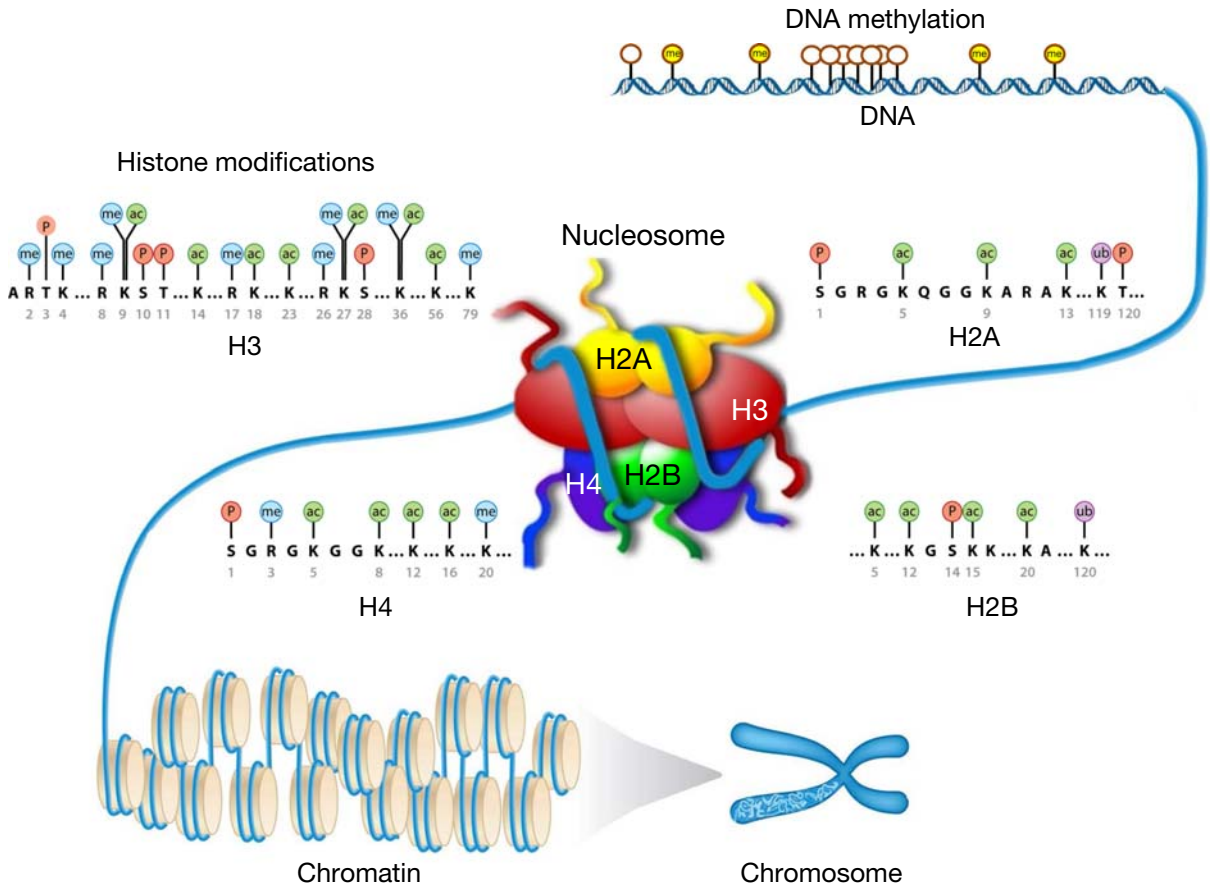


Fig. 1 The structure of the mammalian epigenome. Epigenome, a complex regulatory network that modulates the chromatin structure and the function of the genome, consists of chemical modifications to the DNA and histone proteins. The changes in the epigenome result in alterations in the structure of chromatin and altered expression of genetic information.

5-hydroxymethylcytosine (5-hmeC) and 5-carboxycytosine, which are later removed and replaced by cytosine via base excision DNA repair mechanism.

A second major epigenetic mechanism, which is tightly and interdependently connected to cytosine DNA methylation, is post-translational covalent modifications of the amino-terminal tails of histones. This includes methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation. Multiple interdependent histone modifications may occur on a given histone tail (Fig. 1). These histone modifications are established by "writers" (e.g., histone acetylases, methylases, and phosphorylases) that introduce a chemical modification, interpreted by "readers", proteins that bind specifically to a modified histone (e.g., bromodomains and chromodomains), and removed by "erasers" (e.g., histone deacetylases, demethylases, and phosphatases), which remove a chemical modification from histone proteins.

The primary function of cytosine DNA methylation and histone modifications is to establish the defense of the genome by controlling the accurate chromatin structure and transcription. DNA methylation occurs, mainly, but not exclusively, at regulatory regions functions as a transcriptional "OFF-ON" switch. The occurrence of DNA methylation at unmethylated CpG sites inhibits transcription, whereas demethylation of methylated CpG sequences activates transcription. In contrast, various types of modifications either on the same (e.g., trimethylation and acetylation at lysine residue 27 of histone 3) or different histone amino-terminal tails (e.g., trimethylation at lysine residue 4 of histone 3 and trimethylation at lysine residue 20 of histone 4), or the same type of modification at different amino acid residues on the same histone amino-terminal tail (e.g., trimethylation at lysine residues 4 and 27 of histone 3), have different effects on gene expression. For example, while trimethylation of lysine residues 4 on histone 3 is associated with active gene transcription, trimethylation of lysine 27 at the same histone amino-terminal tail is associated transcriptional silencing.

Well-balanced and tightly regulated epigenetic mechanisms are essential for the maintenance of normal cell homeostasis, normal cell functioning, and, especially, for proper control of the expression of genetic information. The disruption of this fine balance is one of the key events associated with carcinogenesis.

Epigenetic Alterations Induced by Carcinogen Exposure

All carcinogenic agents can be classified based on the mechanism of cancer causation as genotoxic or nongenotoxic carcinogens. Genotoxic carcinogens are agents that interact with DNA to cause damage to DNA or form covalent DNA adducts that result in mutations and genetic aberrations. In contrast, nongenotoxic carcinogens are agents that cause tumor formation by mechanisms other than direct damage to DNA. Despite the different mechanisms of action, evidence suggests that exposure to genotoxic and nongenotoxic carcinogens can alter the cellular epigenome. A link between epigenome alterations induced by environmental carcinogens and tumor development may be related to (i) major exposure-related cancer-specific epigenetic abnormalities, the consequences of which can be observed in the resulting tumors, and (ii) the fact that carcinogen-induced epigenetic perturbations may create an environment susceptible to cancer-prone mutations in the cells.

Table 1 lists the examples of major epigenetic alterations induced by well-known chemical and physical environmental agents classified as Group 1, "a known human carcinogen" or Group 2B, "a possible human carcinogen", by the International Agency for Research on Cancer (IARC). Direct evidence for the carcinogenicity of these agents was obtained in epidemiological and experimental studies.

Epigenetic Effects of Environmental and Occupational Carcinogens

Benzene

Benzene is a natural constituent of crude oil and a "high production volume" industrial chemical. Wide usage of benzene in a variety of industries, including the production of petrochemicals, and its presence as a common component of vehicle exhaust are public health concerns worldwide. Exposure to benzene has been associated with the development of acute myeloid and chronic lymphocytic leukemia driven by genotoxic and epigenotoxic abnormalities, including noticeable changes in DNA methylation. Specifically, exposure to benzene at low concentrations was linked to reduced 5-meC levels at repetitive DNA elements and demethylation of the melanoma antigen family A, 1 (*MAGEA1*) gene in humans. On the other hand, benzene-induced genome instability may be the result of compromised DNA repair caused by epigenetically mediated inhibition of DNA repair genes, including the poly (ADP-ribose) polymerase 1 (*PARP-1*), the excision repair cross-complementation group 3 (*ERCC3*) genes, and DNA topoisomerase II (*TOPOIIα*).

Benzo[α]pyrene

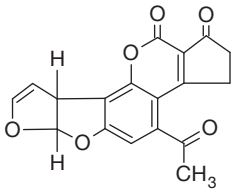
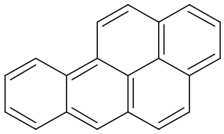
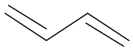
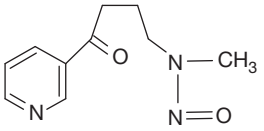
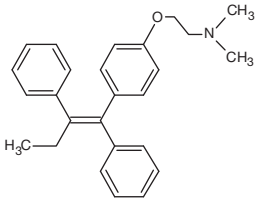
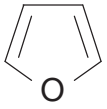
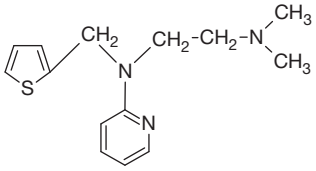
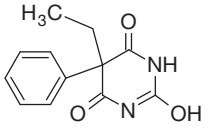
Benzo[*a*]pyrene, a representative polycyclic aromatic hydrocarbon, is one of the human genotoxic carcinogens (IARC Group 1) showing tumorigenic potential in virtually all in vivo experimental animal model systems. The carcinogenic activity of benzo[*a*]pyrene is associated with an induction of tightly interconnected genotoxic and nongenotoxic epigenetic alterations. In particular, exposure to benzo[*a*]pyrene results in extensive and selective formation of anti-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) adducts at the major hot-spot mutation codons 157, 248, and 273 in the human tumor suppressor *P53* gene, and at codon 14 in human *KRAS* oncogene. CpG methylation at these codons greatly increases the formation of the aforementioned genotoxic benzo[*a*]pyrene-DNA adducts.

Furthermore, the presence of BPDE-DNA adducts affects global and gene-specific DNA methylation by impairing the functioning of DNA methyltransferases. As a consequence, hypermethylation of critical cancer-related cyclin-dependent kinase inhibitor 2A (*CDKN2A*; $p16^{INK4A}$), retinoic acid receptor β_2 ($RAR\beta_2$), hypermethylated in cancer 1 (*HIC1*), and glutathione-*S*-transferase genes are frequently detected upon benzo[*a*]pyrene exposure.

1,3-Butadiene

1,3-Butadiene, one of the most common representatives of conjugated genotoxic dienes, is a "high-production-volume" chemical and ubiquitous environmental pollutant. 1,3-Butadiene is human genotoxic carcinogen (IARC Group 1). The major mechanism of 1,3-butadiene carcinogenicity is associated with the formation of DNA adducts, mainly N7-(2,3,4-trihydroxybut-1-yl)guanine and 1,4-bis-(guan-7-yl)-2,3-butanediol crosslinks, and protein adducts. Similar to other classic genotoxic carcinogenesis models, the formation of DNA adducts is necessary, but not sufficient to cause the development of cancer. Several experimental studies have established the importance of an epigenotoxic component, in addition to a genotoxic component, in 1,3-butadiene carcinogenicity. Specifically, exposure to 1,3-butadiene caused loss of global cytosine DNA methylation in the lung and liver, the two main target organs for tumor development induced by exposure to 1,3-butadiene. Additionally, exposure to 1,3-butadiene resulted in a reduction in trimethylation of histone H3 at lysine 9 and lysine 27 and histone H4 at lysine 20 in the liver and deacetylation of histone H3 at lysine 56 and histone H4 at lysine 16 in the lung. The presence of these epigenetic abnormalities has been reported in several types of human cancer.

Table 1 Environmental carcinogens and epigenetic alterations.

		Epigenetic alterations	
Carcinogen	*IARC carcinogen classification*	*DNA methylation*	*Histone modifications*
Genotoxic carcinogens			
Aflatoxin B1 	Group 1	*Hypomethylation*: genomic *Hypermethylation*: *MGMT, CDKN2A, RASSF1A*	*Increased:* H3S10ph *Reduced*: H3K9ac
Benzo[*a*]pyrene 	Group 1	*Hypermethylation*: *CDKN2A, RAR* β_2*, HIC1*	
1,3-Butadiene 	Group 1	*Hypomethylation*: genomic	*Reduced*: H3K9me3, H3K27me3, H4K20me3, H3K56ac, H4K16ac
Nicotine-derived nitrosamine ketone 	Group 1	*Hypermethylation*: *DAPK, CDKN2A, RAR* β*, GATA2, RUNX3, LOX*	
Tamoxifen 	Group 1	*Hypomethylation*: genomic, LINE-1, *PAX2* *Hypermethylation*: *MGMT*	*Reduced*: H4K20me3
Ultraviolet radiation	Group 1	*Hypomethylation*: genomic *Hypermethylation*: *CDKN2A, RASSF1A, CDH1*	
Nongenotoxic carcinogens			
Inorganic arsenic	Group 1	*Hypomethylation*: genomic *Hypermethylation*: *P53, CDKN2A, RASSF1A, DAPK*	*Increased:* H3K9me2, H3S10ph *Reduced*: H4K16ac
Furan 	Group 2B	*Hypomethylation*: genomic *Hypermethylation*: *CDKN2A, RASSF1A, FOXE1*	*Increased:* H3K9me3, H3K27me3 *Reduced*: H3K9ac, H3K27ac, H3K56ac
Methapyrilene 		*Hypomethylation*: genomic	*Reduced*: H3K9ac
Phenobarbital 	Group 2B	*Hypermethylation*: region-specific *Hypomethylation*: genomic	

Nicotine-Derived Nitrosamine Ketone

Nicotine-derived nitrosamine ketone (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNK) is a potent tobacco human genotoxic lung carcinogen (IARC Group 1). The molecular mechanisms of NNK-induced lung tumorigenesis are associated with genotoxic and epigenotoxic events. The major epigenetic alteration induced by NNK exposure is hypermethylation of the promoter region of a number of tumor suppressor and cancer-related genes, including the death-associated protein (DAP)-kinase (*DAPK*), *CDKNA2*, *RARβ*, GATA binding protein 2 (*GATA2*), runt-related transcription factor-3 (*RUNX3*), and lysyl oxidase (*LOX*) genes. Importantly, NNK induces unique promoter hypermethylation signatures in the *RAR-beta* and *RUNX3* genes that can differentiate lung tumors associated with NNK exposure from spontaneous tumors or tumors induced by other carcinogens. The underlying molecular mechanisms behind NNK-induced tumor suppressor gene promoter hypermethylation are associated with (i) NNK-induced accumulation of the DNMT1 in the nucleus, and (ii) enhanced binding of DNMT1 to promoter regions of the tumor suppressor genes.

Arsenic

Inorganic arsenic is one of the most common elements in the Earth's crust and an abundant environmental contaminant. The primary sources of human exposure to arsenic include contaminated food and drinking water. Inorganic arsenic was one of the earliest identified human carcinogens (IARC Group 1). Chronic exposure to arsenic may cause the development of skin, lung, bladder, and liver cancer. The molecular mechanisms of arsenic carcinogenicity include, but are not limited to, the induction of oxidative stress, chromosomal aberrations, disruption of signaling pathways, and epigenetic alterations. Arsenic-induced epigenetic dysregulation contributing to its carcinogenicity consists of pronounced alterations in cytosine DNA methylation and histone modifications, among which cytosine demethylation of DNA is the most prominent and consistent finding. This is evidenced by paralleling the extent of genome-wide DNA demethylation and malignant cell transformation documented in a number of independent in vitro and in vivo studies. Additionally, an extensive amount of data has demonstrated region-specific DNA demethylation (e.g., demethylation of repetitive elements, oncogenes, and cancer-related genes) during arsenic-induced malignant cell transformation in vitro and arsenic-induced tumorigenesis in vivo.

The main mechanisms of DNA demethylation in response to arsenic exposure may be attributed to (i) a reduced cellular availability of SAM because of its absolute requirement for the biomethylation of inorganic arsenic; (ii) induction of oxidative stress and oxidative damage to DNA that severely compromises the ability of DNA methyltransferases to methylate target cytosines; (iii) activation of DNA repair pathways that promote active demethylation of DNA; and (iv) downregulation of DNA methyltransferases.

In addition to global and gene-specific DNA hypomethylation, inorganic arsenic exposure causes methylation-induced transcriptional silencing in a number of tumor suppressor genes and cancer-related genes, including *P53*, *CDKN2A*, Ras association domain family member 1(*RASSF1A*), and *DAPK*.

Aberrations in posttranslational global and gene-specific histone modifications are additional epigenetic events associated with arsenic carcinogenicity. A number of independent studies have documented that arsenic induces higher levels of dimethylation of histone H3 at lysines 9, phosphorylation of histone H3 serine 10, and deacetylation of histone H4 lysine 16.

Ultraviolet Radiation

Exposure to ultraviolet (UV) radiation, a human Group 1 carcinogen and one of the main constituents of natural sunlight, is the main environmental etiological risk factor for the development of basal cell carcinoma, cutaneous squamous cell carcinoma, and melanoma in humans. Ultraviolet irradiation directly damages DNA, causing the formation of cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts. Cyclobutane pyrimidine dimers specifically are responsible for the vast majority of C → T mutations induced by UV exposure. The underlying mechanism of ultraviolet radiation carcinogenicity consists of tightly and interdependently connected genotoxic and epigenotoxic components. First, similar to benzo[*a*]pyrene, the cyclobutane pyrimidine dimers are preferentially formed in DNA at dipyrimidine with methylated cytosines. Second, damage to DNA induced by ultraviolet radiation exposure causes a broad range of epigenotoxic alterations, including abnormalities in DNA methylation and histone modifications. One of the major epigenetic alterations caused by ultraviolet radiation exposure is demethylation of DNA that may be attributed to (i) formation of cyclobutane pyrimidine dimers, presence of which compromises the methylating ability of DNA methyltransferases and (ii) loss of methylated cytosines during DNA repair. Additionally, ultraviolet radiation induces hypermethylation of *CDKN2A*, *RASSF1A*, and cadherin 1 (*CDH1*) tumor-suppressor genes indirectly, mainly due to the compensatory elevated expression and activity of DNA methyltransferases and induction of inflammatory-mediated 5-halogenated cytosine intermediates that can mimic 5-meC and direct maintenance DNA methyltransferase DNMT1 to methylate previously unmethylated CpG sites.

Ultraviolet radiation, similarly to ionizing radiation, causes major chromatin conformational and functional alterations. These chromatin changes are tightly associated with alterations in global histone modifications, mainly those involved in DNA damage response and repair.

Ionizing Radiation

Ionizing radiation is an abundant environmental human genotoxic carcinogen (IARC Group 1). Additionally, there are a progressively rising number of people exposed to ionizing radiation either due to their occupation or due to medical diagnostic imaging and radiation treatments. It is well established that DNA damage is the main mechanism associated with ionizing radiation tumorigenicity; however, ionizing radiation also causes major aberrations in the cellular epigenome, including alterations in DNA methylation, histone modifications, and chromatin accessibility. Among these epigenetic abnormalities, a loss of global DNA methylation, driven by either demethylating mechanisms similar to those caused by ultraviolet radiation orionizing radiation-induced damage of 5-meC, and aberrant DNA damage-related histone modifications are the major epigenetic alterations.

Epigenetic Effects of Food Contaminants

Aflatoxin B_1

Aflatoxin B_1 is an abundant food contaminant and human genotoxic carcinogen (IARC Group 1). Aflatoxin B_1 is one of the main risk factors for the development of hepatocellular carcinoma, one of the most aggressive human cancers. The carcinogenicity of aflatoxin B_1 is mainly attributed to its genotoxicity, especially to the ability of aflatoxin B_1-DNA adducts to induce G:C → T:A transversion mutations in the human tumor suppressor *P53* gene. In addition to a genotoxic component of aflatoxin B_1 carcinogenicity, epigenetic alterations, including transcriptional silencing of O^6-methylguanine-DNA methyltransferase (*MGMT*), *CDKN2A*, and *RASSF1A* tumor-suppressor genes mediated by promoter hypermethylation, are typical epigenetic events usually observed in liver carcinogenesis induced by aflatoxin B_1 exposure. Additional cancer-related epigenetic events of aflatoxin B_1-carcinogenicity include global and gene-specific DNA demethylation, increased phosphorylation of histone H3 at serine 10, and deacetylation of a transcription activating mark histone H3 at lysine 9.

Furan

Furan is a volatile heterocyclic organic industrial chemical and food contaminant found in a wide spectrum of common human foods. Furan is a liver toxicant and carcinogen in mice and rats. The carcinogenic effects of furan have been attributed to genotoxic and nongenotoxic mechanisms; however, the lack of evidence for furan genotoxicity in vivo suggests that nongenotoxic mechanisms may be involved in furan liver carcinogenicity. Indeed, accumulated data showed the occurrence of sustained cytosine DNA hypomethylation, promoter region hypermethylation in the tumor suppressor genes *CDKN2A*, *RASSF1A*, and forkhead box E1 (*FOXE1*), and deacetylation of histone H3 at lysines 9 and 56 in the liver upon exposure to furan. Importantly, these epigenetic alterations are tightly interconnected (e.g., gene-specific hypermethylation was accompanied by increased trimethylation at lysine 9 and lysine 27 of histone H3, transcription silencing marks, whereas gene-specific hypomethylation was paralleled with deacetylation of histone H3 lysine 9 and lysine 27). This indicates that epigenetic alterations are not a random event and play a fundamental role in furan-related hepatobiliary carcinogenesis.

Epigenetic Effects of Pharmaceuticals

Tamoxifen

Tamoxifen is a nonsteroidal selective estrogen receptor modulator widely used to treat and prevent occurrence and re-occurrence of breast cancer in women; however, despite this indisputable benefit, the use of tamoxifen has been shown to increase the risk of endometrial cancer in women and to induce liver cancer in rats.

The existing experimental data revealed that a critical mechanistic component of tamoxifen rat liver carcinogenesis is its binding to DNA and the formation of (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen and (*E*)-α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen DNA adducts. Similarly, emerging evidence suggests that the occurrence of tamoxifen-associated endometrial tumors in humans may be partially due to DNA adduct formation. Among other factors contributing to the tumorigenic activity of tamoxifen, epigenetic alterations may play a major role. This was evidenced by a presence of global loss of DNA methylation, decreased methylation of repetitive DNA elements such as LINE-1, and reduced level of histone H4 lysine 20 trimethylation in the livers of tamoxifen-exposed rats. Additionally, substantial demethylation of the paired box 2 (*PAX2*) gene and hypermethylation in the promoter region of the *MGMT* gene have been reported in tamoxifen-induced endometrial tumors in humans.

Phenobarbital

Phenobarbital, a commonly used antiseizure and sedative drug, was one of the first therapeutic drugs to be investigated for its liver cancer-inducing potential with a nongenotoxic mode-of-action. Exposure to phenobarbital results in rapid and progressive accumulation of both types of DNA demethylation and DNA hypermethylation abnormalities in the livers of experimental animals, especially in tumor-prone B6C3F1 mice.

Methapyrilene

Methapyrilene, a histamine antagonist that belongs to the class of pyridine chemicals, was widely used in many nonprescription drugs until 1979, when it was demonstrated to cause the development of liver tumors in rats. Because of the absence of DNA adducts in the livers of rats treated with methapyrilene, the hepatocarcinogenicity of methapyrilene has been attributed to nongenotoxic mechanisms, mainly to induction of mitochondrial dysfunction and disruption of the cellular epigenome. Exposure to methapyrilene caused target organ-specific epigenetic alterations that consisted of a reduction in the levels of cytosine DNA methylation, and global and gene-specific deacetylation of histone H3 lysine 9. These epigenetic alterations were induced by methapyrilene at the concentration that exhibited no cytotoxicity and were associated with reduced expression of critical cancer-related genes, including prospero homeobox 1 (*PROX1*), HNF1 homebox A (*HNF1A*), and peroxisome proliferator activated receptor alpha (*PPARA*), providing a mechanistic link between methapyrilene-induced epigenetic aberrations and liver carcinogenesis.

Mechanisms of Carcinogen-Induced DNA Methylation Alterations

The molecular events that lead to carcinogen-induced epigenetic abnormalities are elusive, and it is likely that multiple processes and pathways are involved (Fig. 2).

DNA Integrity and Epigenetic Alterations

Genotoxic DNA Adducts and Epigenetic Alterations

DNA damage and epigenetic alterations are two of the key tightly linked characteristic events of the carcinogenic process caused by carcinogen exposure. With respect to genotoxic carcinogens, the formation of DNA adducts, especially with guanine or cytosine

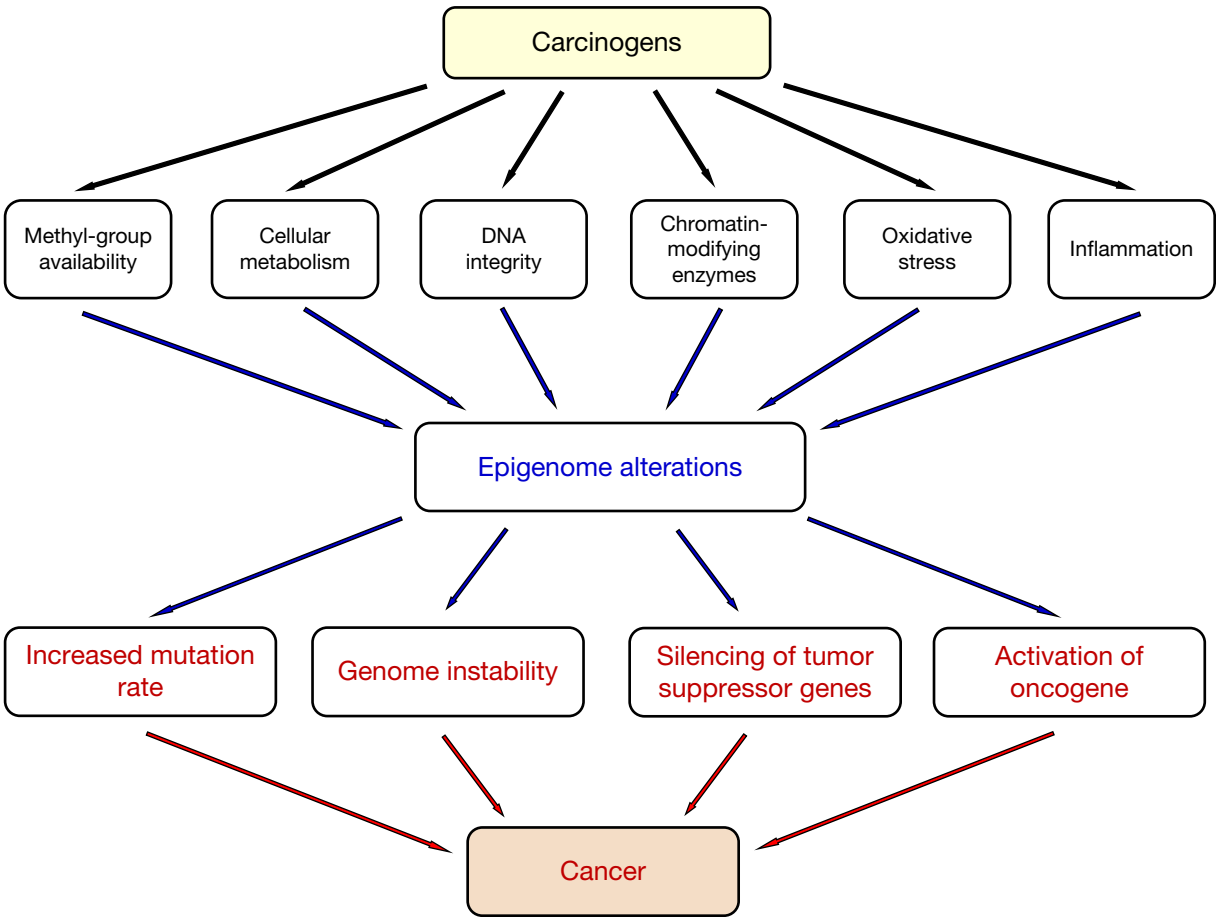


Fig. 2 Epigenetic alterations induced by environmental carcinogens and cancer development. Exposure to carcinogens induced a number of molecular alterations that affect the cellular epigenome. These alterations compromise the stability of the genome and expression of genetic information, any or all of these events may trigger neoplastic cell transformation and promote cancer development.

nucleobases at CpG dinucleotides, may diminish the ability of DNA methyltransferases to methylate a target cytosine resulting in the demethylation of DNA. For instance, the presence of benzo[*a*]pyrene-2′-deoxyguanosine or cyclobutane pyrimidine dimers in DNA inhibit methylation of DNA. Also, miscoding properties of many DNA adducts, for example, (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen, or deamination of cyclobutane pyrimidine dimers may cause sequence variations at CpG dinucleotides, which could lead to the loss of 5-meC from CpG sites in DNA.

In addition to carcinogen-DNA damage-mediated changes in DNA cytosine methylation, carcinogen exposure affects histone proteins, evidenced by the formation of anti-BPDE-histone adducts with lysine residues and crosslinks between the glutathione conjugate of *cis*-2-butene-1,4-dial and lysine 107 of histone H2B. Moreover, a number of histone modifications, including the phosphorylation of the histone H2AX and changes in methylation of histone H3 lysine 9, histone H3 lysine 27, and histone H4 lysine 20, and acetylation of histone H3 lysine 9, histone H3 lysine 56, and histone H4 lysine 16, are mediated by the DNA damage response and DNA repair.

Oxidative DNA Damage and Epigenetic Alterations

A common carcinogenesis-related event, which is specific for both genotoxic and nongenotoxic carcinogens, is the induction of oxidative stress. Oxidative stress is a multistressor condition that arises when the generation of reactive oxygen species exceeds the capacity of the cellular antioxidant defense system to control their detoxification. As a result dynamic cellular "redox equilibrium" is compromised. Reactive oxygen intermediates directly damage DNA, resulting in a variety of lesions, among which 8-oxo-7-hydroxydeoxyguanosine (8-oxo-dG) is the most prevalent and universal oxidative DNA lesion. The replacement of guanine with 8-oxo-dG in CpG dinucleotides substantially impairs DNA methylation of adjacent cytosines by (i) inhibiting the binding of the methyl-CpG binding protein 2 (MECP2) to DNA and (ii) diminishing the ability of DNA methyltransferases to methylate a target cytosine in a mechanism similar to genotoxic carcinogen-induced DNA adducts. A similar mechanism of DNA demethylation is specific also for 5-hydroxymethylcytosine, another major oxidative DNA lesion.

DNA Repair and Epigenetic Alterations

Finally, an activation of DNA repair processes and removal of carcinogen-induced DNA lesions could also lead to the demethylation of DNA via replacing 5meC with regular cytosine.

Chromatin Modifying Enzymes and Epigenetic Alterations

One intriguing carcinogenesis-related epigenetic phenomena is global demethylation of DNA accompanied by an increased expression and activity of DNA methyltransferases. This upregulation of DNA methyltransferases may be viewed as a compensatory cellular reaction aiming to restore the normal pattern of DNA methylation and to preserve the integrity of the epigenome and the genome. Despite this upregulation, DNA methyltransferases are unable to restore the original DNA methylation pattern. This may be attributed to the greater binding affinity of DNA methyltransferases to carcinogen-induced DNA lesions resulting in the sequestration of the enzyme. On the other hand, the inability of the DNA methyltrasferases to maintain a proper cytosine methylation status could trigger compensatory upregulation of DNA methyltransferases and lead to random hypermethylation of normally unmethylated promoter CpG islands.

Intracellular Metabolism and Epigenetic Alterations

One of the hallmark alterations during the carcinogenic process is a profound deregulation of the intracellular metabolism. Exposure to carcinogenic agents greatly contributes to this deregulation. The proper functioning of many chromatin-modifying enzymes and epigenetic processes depends greatly on cofactors or cosubstrates generated by cellular metabolism. This tightly links a number of intracellular metabolic pathways and epigenome and, therefore, altered intracellular metabolism, especially tricarboxylic acid cycle (TCA cycle; Krebs cycle), one carbon, lipid, and iron metabolic pathways, induced by carcinogen exposure can cause a variety of epigenetic abnormalities and contribute to cancer development.

One Carbon Metabolism and Epigenetic Alterations

SAM is the major methyl donor for all cellular methylation reactions. The biosynthesis of SAM from L-methionine and adenosine is catalyzed by methionine adenosyltransferase in the cytosol of all mammalian cells and depends on the status of methionine, choline, folic acid, and vitamin B_{12}. SAM is the link to three key metabolic pathways: *trans*-methylation, trans'sulfuration, and polyamine synthesis.

In the *trans*-methylation pathway, SAM donates its methyl group to a large variety of acceptor biomolecules, including proteins and nucleic acids, in reactions catalyzed by methyl-transferases. In this *trans*-methylation pathway, after donation of a methyl group, SAM is converted to *S*-adenosyl-L-homocysteine(SAH) which is subsequently hydrolyzed in a reversible reaction to homocysteine

and adenosine by *S*-adenosylhomocysteine hydrolase. SAH is a potent competitive inhibitor of methylation reactions, and prompt removal of adenosine and homocysteine is required to prevent accumulation of SAH. Homocysteine undergoes remethylation to methionine in the *trans*-methylation pathway or conversion to cysteine in the trans'sulfuration pathway. Remethylation of homocysteine to form methionine is mediated by two enzymes: methionine synthase, which requires normal levels of folate and vitamin B_{12}, and betaine-homocysteine methyltransferase, which is dependent on betaine, a metabolite of choline. Methionine synthase-catalyzed homocysteine remethylation requires 5-methyltetrahydrofolate (5-MTHF), which is derived from 5,10-methylenetetrahydrofolate (5,10-MTHF) in a reaction catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR). 5-MTHF is then converted to tetrahydrofolate (THF) as it donates its methyl group and then is converted to 5,10-MTHF to complete the folate cycle.

Another route of homocysteine removal is the irreversible trans'sulfuration pathway, which involves two pyridoxal phosphate-dependent enzymes, cystathionine β-synthase and cystathionine y-lyase. Serine can condense enzymatically with homocysteine to generate cystathione by cystathionine β-synthase. Cystathione is then cleaved by cystathionine y-lyase to generate α-ketobutyrate and cysteine, which can be shunted into glutathione (GSH) production.

Glutathione, the most abundant cellular physiological antioxidant, is greatly depleted during tumorigenesis, mainly by enhanced metabolism of exogenous and endogenous chemicals and their metabolites and by induction of oxidative stress. Consequently, this results in the increased need for glutathione synthesis, activation of the trans'sulfuration pathway causing a reduction in the synthesis of methionine and SAM, and, consequently, perturbing DNA and histone methylation reactions.

Inflammation and Epigenetic Alterations

Inflammation is another main hallmark of carcinogenesis and the main pathophysiological event of carcinogen exposure. In addition to a broad range of cancer-inducing and cancer-promoting molecular alterations, carcinogen-induced inflammation causes several epigenetic alterations, among which gene-specific hypermethylation is of special interest since it provides another important mechanism of cancer-related genes. In particular, the formation of inflammation-induced 5-halogenated cytosine intermediates in DNA, including 5-chlorocytosine and 5-bromocytosine, mimics 5-meC and direct DNA methyltransferases to target unmethylated cytosine causing abnormal cytosine hypermethylation at unmethylated CpG sites.

Prospective Vision

Increasing human exposure to the growing number of carcinogens in the form of environmental, agricultural, industrial contaminants, pharmaceutical products, and food additives is a high-priority concern to all public health agencies worldwide. The major challenge in carcinogenicity testing is associated with the fact that the production of chemicals greatly exceeds our testing capacity.

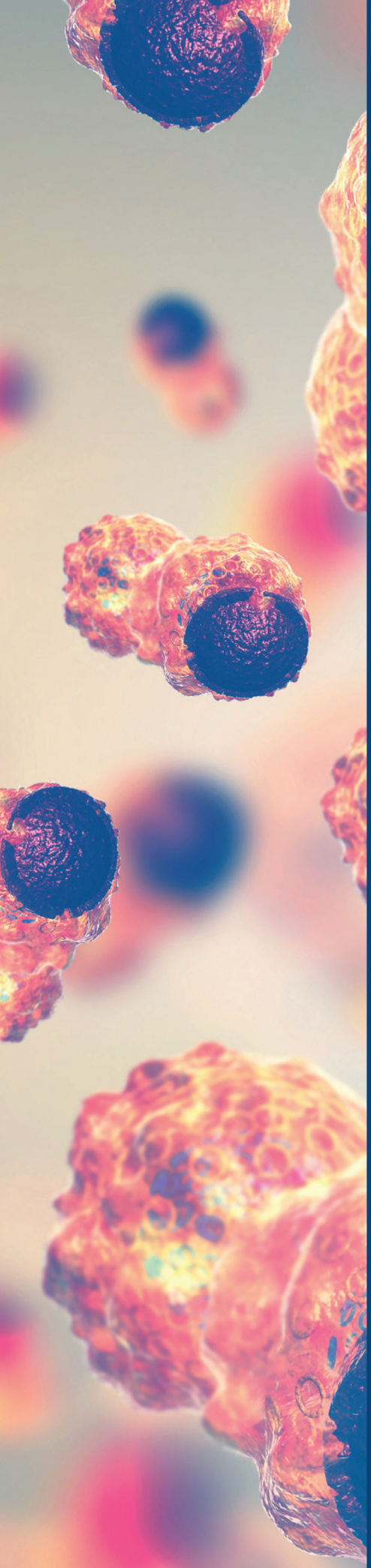
The current primary "gold standard" approach for determining the carcinogenic potential of chemicals in humans is the 2-year, 2-species rodent carcinogenicity bioassay. This is the most reliable assay in the assessment of carcinogenicity. The value of this assay may be illustrated by the fact that all human carcinogens have produced positive results in at least one animal model. However, because of substantial limitations of rodent bioassays, including the time and resources required, there is a crucial need for alternative testing strategies to increase testing efficiency. The process of carcinogenicity assessment and proper cancer risk management consist of several fundamental steps, including hazard identification and characterization, exposure assessment, and health risk evaluation. A key point in this process is an identification of hazard, which relies on the ability to detect adverse effects of exposure early, in other words on biomarkers of exposure.

The measurement of DNA damage, gene mutations, and chromosome damage is frequently assessed as the primary approach for identification of genotoxic human carcinogens; however, some shortcomings in the use of this approach still exist. Additionally, predicting the carcinogenic potential of nongenotoxic compounds is extremely challenging due to the diversity in modes of action that may lead to tumorigenesis. Recognition of the fundamental role of epigenetic abnormalities in cancer development and the results of human epidemiological and exploratory in vivo studies showing the induction of a spectrum of epigenetic alterations by both genotoxic and nongenotoxic carcinogens suggest that they may be used as biomarkers in the evaluation of carcinogenicity. Incorporation of epigenetic biomarkers in the carcinogenicity testing guidelines holds a number of advantages over traditionally used methods. Specifically, the following features of epigenetic alterations favor their use as biomarkers in the studies for the investigation of carcinogenic potential of environmental exposures: (a) early appearance; (b) stability; (c) target organ specificity; (d) applicability to physical and chemical genotoxic and nongenotoxic carcinogens; and (e) a greater number of detectable epigenetic abnormalities than the genetic aberrations. Furthermore, evaluation of epigenetic alterations in carcinogenicity assessment promises to substantially enhance testing efficiency, increase identification of carcinogenic hazard, and improve risk management strategies aimed to reduce or prevent human exposure to carcinogenic agents.

See also: DNA Methylation Changes in Cancer: Mechanisms. Environmental and Occupational Exposures.

Further Reading

Bernstein, B.E., Meissner, A., Lander, E.S., 2007. The mammalian epigenome. Cell **128**, 669–681.
Cerda, S., Weitzman, S.A., 1997. Influence of oxygen radical injury on DNA methylation. Mutation Research **386**, 141–152.
Chen, Z., Li, S., Subramaniam, S., Shyy, J.Y.-J., Chien, S., 2017. Epigenetic regulation: A new frontier for biomedical engineers. Annual Review of Biomedical Engineering **19**, 195–219.
Felenfeld, G., Groudine, M., 2003. Controlling the double helix. Nature **421**, 448–453.
Hou, L., Zhang, X., Wang, D., Baccarelli, A., 2012. Environmental exposures and human epigenetics. International Journal of Epidemiology **41**, 79–105.
Garcia-Giménez, J.L., Romá-Mateo, C., Pérez-Machdo, G., Peiró-Chova, L., Pallardó, F.V., 2017. Role of glutathione in the regulation of epigenetic mechanisms in disease. Free Radical Biology & Medicine **112**, 36–48.
Kaelin, W.G., McKnight, S.L., 2013. Influence of metabolism on epigenetics and disease. Cell **153**, 56–69.
Lee, D.-H., Jacobs, D.R., Porta, M., 2009. Hypothesis: A unifying mechanism for nutrition and chemicals as lifelong modulators of DNA hypomethylation. Environmental Health Perspectives **117**, 1799–1802.
Loeb, L.A., Harris, C.C., 2008. Advances in chemical carcinogenesis: A historical review and prospective. Cancer Research **68**, 6863–6872.
Luch, A., 2005. Nature or nurture—Lessons from chemical carcinogenesis. *Nature Reviews. Cancer* **5**, 113–125.
Madugundu, G.S., Cadet, J., Wagner, J.R., 2014. Hydroxyl-radical-induced oxidation of 5-methylcytosine in isolated and cellular DNA. Nucleic Acids Research **42**, 7450–7460.
Marczylo, E.L., Jacobs, M.N., Gant, T.W., 2016. Environmentally induced epigenetic toxicity: Potential public health concerns. Critical Reviews in Toxicology **46**, 676–700.
Pfeifer, G.P., 2006. Mutagenesis at methylated CpG sequences. Current Topics in Microbiology and Immunology **301**, 259–281.
Poirier, M.C., 2016. Linking DNA adduct formation and human cancer risk in chemical carcinogenesis. Environmental and Molecular Mutagenesis **57**, 499–507.
Valinluck, V., Sower, L.C., 2007. Inflammation-mediated cytosine damage: A mechanistic ling between inflammation and the epigenetic alterations in human cancers. Cancer Research **67**, 5583–5586.



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Dr. Boffetta is the editor or associate editor of 5 scientific journals and member of the editorial board of 10 additional journals; he is a member of review panels of NIH and several medical research agencies in Europe. He has edited 13 books and is Editor in Chief of the new edition of Elsevier's *Encyclopedia of Cancer*. He has published over 1250 peer-reviewed publications; his publications have been quoted more than 90000 times; his h-index is 148.

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Pierre Hainaut holds a PhD in Biology (Zoology) from University of Liège, Belgium (1987). After postdocs in Nice (France, 1988–90), Cambridge, and York (United Kingdom, 1990–94), he joined the International Agency for Research on Cancer (IARC, World Health Organization) in 1995, where he held the post of Head of Molecular Carcinogenesis from 1999 to 2011. In 2012, he joined the International Prevention Research Institute (Lyon, France) and became Professor at the Strathclyde Institute of Pharmacy and Biomedical Science (Glasgow, United Kingdom). In 2014, he was awarded a Chair of Excellence in Translational Research from University Grenoble-Alpes (Grenoble, France).

His research focuses on *TP53* mutations and p53 protein regulation in cancer and chronic diseases. From 1994 to 2011, he has led the development of the international IARC TP53 database, a source of information on the causes and consequences of mutations affecting the *TP53* suppressor gene in cancer. His work addresses the mechanisms of *TP53* mutagenesis as well as the prognostic and predictive significance of *TP53* mutations in lung, liver, and oesophageal cancers. His studies on p53 regulation have focused on the role of environmental mutagens in *TP53* mutagenesis, on the biochemical mechanisms of p53 control by oxidation-reduction and by metabolism, and on the identification of p53 isoforms as factors acting as dominant inhibitors of p53 functions in cancers without *TP53* mutations. His current activities focus on germline *TP53* mutation and on the diversity of genetic and nongenetic factors that modulate the penetrance of the Li–Fraumeni Syndrome, as well as on the mechanisms that maintain optimal p53 protein balance in cells and tissues over lifetime. He is the author of over 450 publications and 50 book chapters. He is Editor of the Cancer Biology section of *Current Opinion in Oncology*. He has co-edited books on p53 (*25 Years of p53 Research*, 2005, 2007, *p53 in the Clinics*, Springer), a textbook on molecular epidemiology (*Molecular Epidemiology: Principle and Practice*, IARC Press, 2011) and two-volume textbook on human biobanking (*Human Biobanking, Principle and Practice*, 2017, 2018).

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Fred T. Bosman MD PhD, studied Medicine at the University of Leiden (MD 1971), where he also earned his PhD degree (in cytogenetics, 1976), and trained as a pathologist. He was staff pathologist at the University of Leiden, Professor and Chair of Pathology at the Faculty of Medicine of the University of Maastricht, Faculty of Medicine of the Erasmus University in Rotterdam, Director of the University Institute of Pathology, and Professor of Pathology at the University Medical Center (CHUV) of Lausanne in Switzerland, now emeritus. He is Honorary Fellow of the Royal College of Pathologists (United Kingdom) and foreign correspondent of the Royal Netherlands Academy of Sciences.

Fred Bosman's research activities (combining diagnostic and experimental pathology) focused on the biology of digestive tract cancer, notably Barrett's esophagus and colorectal cancer, with a strong emphasis on the development of molecular diagnostics. He has written over 350 original publications and over 50 book chapters. Fred Bosman was Series co-editor of the 4th edition of the WHO Series *Classification of Human Tumours*, the international standard for tumor classification, and co-editor of the Volume on *Tumours of the Digestive Tract*.

Graham A. Colditz, MD, DrPH, FAFPHM, is an internationally recognized leader in cancer prevention. As an epidemiologist and public health expert, he has a longstanding interest in the preventable causes of chronic disease, particularly among women. He focuses his research on early life and adolescent lifestyle, growth, and breast cancer risk. He is also interested in approaches to speed translation of research findings into prevention strategies that work. Dr. Colditz developed the award-winning Your Disease Risk website (www.yourdiseaserisk.wustl.edu) which communicates tailored prevention messages to the public. He has published over 1100 peer-reviewed publications, six books and six reports for the Institute of Medicine, National Academy of Sciences. His h-index is over 220.

In October 2006, on the basis of professional achievement and commitment to public health, Dr. Colditz was elected to membership of the National Academy of Medicine, an independent body that advises the US government on issues affecting public health. In 2011, he was awarded the American Cancer Society Medal of Honor for cancer control research. In 2012 he received the AACR-American Cancer Society Award for Research Excellence in Cancer Epidemiology and Prevention. He also received awards in 2014 for cancer prevention research from ASCO and from AACR. During 2016 he served on the Implementation Science Work Group of the Blue-Ribbon Panel to advise the National Cancer Moonshot. He received the 2018 Daniel P. Schuster Award for Distinguished Work in Clinical and Translational Science, Washington University School of Medicine. He was also elected as a Fellow, American Association for the Advancement of Science

Carlo La Vecchia received his MD from the University of Milan and a Master of Science degree in Medicine (epidemiology) from Oxford University. Presently, he is Professor of Medical Statistics and Epidemiology at the Faculty of Medicine at the University of Milan. Dr. La Vecchia serves as an editor for numerous clinical and epidemiologic journals. He is among the most renowned and productive epidemiologists in the field with over 2040 peer-reviewed papers in the literature and is among the most highly cited medical researchers in the world, according to ISI HighlyCited.com, the developer and publisher of the Science Citation Index (2003, 2017, H index 153, H10 index 1543, second Italian in Clinical Medicine). Dr. La Vecchia is Adjunct Professor of Medicine at Vanderbilt Medical Center and the Vanderbilt-Ingram Cancer Center (2002-18).

Gerd. P. Pfeifer received a PhD degree in biochemistry from Goethe University in Frankfurt, Germany. After postdoctoral training, he became a faculty member at the Beckman Research Institute of the City of Hope in Duarte, California, where he spent much of his career working on cancer research. In 2014, Dr. Pfeifer joined the new Center for Epigenetics at the Van Andel Research Institute in Grand Rapids, MI, United States, as a Professor of Epigenetics. Dr. Pfeifer has authored more than 300 publications, has held an NIH MERIT award, and was elected Fellow of the American Association for the Advancement of Science in 2015. Research in Dr. Pfeifer's laboratory has been concerned with genetic and epigenetic mechanisms of human carcinogenesis, with emphasis on DNA methylation and genetic toxicology.

Marco Alessandro Pierotti graduated in 1973 in Biological Sciences at the University of Milan, Italy, and started working at the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) in Milan. From 1978 to 1980 he was Visiting Investigator at the Laboratory of Chemical Cancerogenesis of the NCI-NIH Bethesda (MD, United States) and Postdoctoral Research Fellow at the Laboratory of Viral Oncology of the Memorial Sloan-Kettering Institute in New York. In 2006, Dr. Pierotti was appointed Scientific Director of the Fondazione IRCCS Istituto Nazionale dei Tumori in Milan, where, since 1970, he had already held various positions, including Director of the Department of Experimental Oncology.

Since 1988, he has been Professor of Molecular Genetics of Cancer at the Postgraduate School of Oncology, University of Milan Medical School and co-director of the Laboratory of Molecular Diagnosis at the INT. In September 2014 he resigned from his position at INT to take the position of President and CEO of Nerviano Medical Sciences (NMS) srl, one of the biggest oncological pharma companies in Europe. In April 2015 he left the company and took the position of Scientific Coordinator of the Institute of Pediatric Researches (IRP) in Padua, Italy, devoted to study molecular aspects of the main pediatric diseases with particular focus on pediatric onco-hematology. The Institute was created by a private Charity, The City of Hope Foundation of Monte Malo (Vi).

Since 2000 he is Senior Group leader of the Molecular Genetics of Cancer group at the Institute FIRC of Molecular Oncology (IFOM, Milan). Past President (2006–08) of the Italian Cancer Society, Dr. Pierotti is a member of the American Association for Cancer Research and of its Advisory Board and the Laboratory Research Awards Selection Committee. He has also been President (2006–08) of the European Association for Cancer Research (EACR) and in this role was among the founders of the European CanCer Organisation (ECCO) where he was appointed as member of the Policy Committee.

In recent years, he was the Italian Representative at the Scientific Committee of the International Agency for Research on Cancer (IARC), Lyon. From 2006 to 2014 he was Scientific Secretary of Alleanza Contro il Cancro (ACC), promoted by the Italian Ministry of Health. In 2008 he was Member of the Evaluation of the Research Program Functional and Structural Genomics for DKFZ. He was an expert for the Oncology Research Projects of the European Community and was a consultant in oncology research for the Ministries of Research of different Countries. From 2006 to 2014 he was Chair of the regional Project, The Region Lombardy Oncological Network (ROL), selected in 2014 by the EC as one of the best examples of oncological network. His appointments in the Organisation of European Cancer Institutes (OECI) started in 2007 when he took the position of Vice-President. From 2008 he was the President-elect and then the President of the Organization. Finally, in 2014 he was appointed OECI Executive Secretary.

Over the years, Dr. Pierotti has been Principal Investigator or Head of several national and international research grants, funded by both private and public bodies. His authorship includes over 470 publications that deal with various aspect of experimental oncology including studies on immunology, biochemistry, and molecular biology using both experimental and human tumors.
In addition, since its fifth edition he is the first author of the chapter on "Oncogenes" in the most reputed textbook *Cancer Medicine* (Holland-Frei). The metrics of his scientific activity is summarized by an H index of 96 and total citations of 37.256 (Google scholar June 2018).

Professor **Thomas Tursz**, born in Kraków, Poland, in 1946, died in Paris, France, on April 27 2018. He was Professor of Oncology at the Faculty of Medicine Paris-Sud since 1986 and General Director of the Institut Gustave Roussy (1994–2010). He was the leader of the French Doctoral School of Oncology which he founded in 1999, and President of the French Federation of Comprehensive Anticancer Centres (FNCLCC) from 2004 to 2010. He was highly involved in the European Organization for the Research and Treatment of Cancer (EORTC) as both Chairman of the Scientific Advisory Committee (2003–06) and Vice President of the Board (2006–09). His experience as President of the FNCLCC was crucial for the Organization of European Cancer Institutes (OECI) when he acted as President from 2002 to 2005.

His scientific interests included the biology of virus-induced tumors, as well as immunological responses including the role of thioredoxin in lymphocytes infected by Epstein–Barr virus. In the clinical research area, he conducted a number of important clinical trials in breast cancer, lung cancer, and soft-tissue sarcoma. He had a particular interest in cytokines and gene therapy, and his clinical research activities were further disseminated to the European level when he was the Chairman of the Sarcoma Group of the EORTC (1993–96).

Prof. Tursz received several prestigious awards, such as the Prix de Cancérologie from the French National League Against Cancer (1979), the Bernard Halpern Immunology Award (1983), the Rosen Oncology Award (1989), the Grand Prix in Oncology from the Academy of Medicine (1992), the Hamilton Fairley Award for clinical research (1998), and the Prix de Rayonnement Français (2001). He was the author of 350 international scientific publications. He was also an esteemed member of the Editorial Board of *Molecular Oncology* ever since its creation in 2007.

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HOW TO USE THE ENCYCLOPEDIA

Structure of the Encyclopedia

All articles in the encyclopedia are arranged alphabetically as a series of entries.

There are four features to help you easily find the topic you are interested in: an alphabetical contents list, cross references, a full subject index, and contributors.

1. Alphabetical contents list: The alphabetical contents list, which appears at the front of each volume, lists the entries in the order that they appear in the encyclopedia. So that they can be easily located, entry titles generally begin with the key word or phrase indicating the topic, with any generic terms following. For example, "Multiple Myeloma: Pathology and Genetics" is the entry title rather than "Pathology and Genetics of Multiple Myeloma".
2. Cross references: Virtually all the entries in the encyclopedia have been extensively cross-referenced. The cross references which appear at the end of an entry, serve three different functions:
   i. To draw the reader's attention to related material on other entries
   ii. To indicate material that broadens and extends the scope of the article
   iii. To indicate material that covers a topic in more depth

Example
The following list of cross-references appears at the end of the entry "Carcinogen—DNA Adducts".

> ***See also:*** Cancer Risk Reduction Through Lifestyle Changes. Cell Responses to DNA Damage. Genetic Instability. Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2. Molecular Epidemiology and Cancer Risk. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics.

3. Index: The index appears at the end of volume 3 and includes page numbers for quick reference to the information you are looking for. The index entries differentiate between references to a whole entry, a part of an entry, and a table or figure.
4. Contributors: At the start of each volume there is a list of the authors who contributed to all volumes.

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PREFACE

Cancer holds a special status in biology, medicine, and society. It offers formidable challenges to basic, clinical, and population science; it ranks at the top of medical research priorities and healthcare costs in most countries. The general public is continuously exposed to news of discoveries promising to defeat the disease in the near future. Indeed, ground-breaking advances are being made, from preventive vaccines to genome-guided personalized medicine, sophisticated imaging and surgical technologies, and systemic therapies aimed at awakening natural immune responses against cancer. These novel therapeutic approaches make cure a real possibility for a growing number of patients. They also launch cancer care into a new era of maintaining the disease under control for an indefinite period of time, turning it into a form of chronic disease. At the same time, evidence-based prevention and early detection strategies have opened a new window on the natural history of the disease, enabling effective intervention well ahead of diagnosis. As a result, the first two decades of this millennium have witnessed a marked decrease in the mortality and, in some instances, the incidence of cancers that have dominated the death toll in more developed countries during the second half of the 20th century.

A turning point in our understanding of cancer is the deciphering of the human genome and its spin-off endeavors aimed at exploring the genomic landscape and architecture of human cancers. These discoveries are causing a major overhaul of our vision of cancer as a dynamic, rapidly evolving, and heterogeneous disease at the individual level. Harnessing this complexity requires mastering increasingly complex sources of data at molecular, cellular, systemic, personal, environmental, and societal level, heralding the emergence of big-data science in cancer diagnosis and treatment. However, this exceptional acceleration in knowledge and solutions cannot hide the fact that cancer remains a global scourge that exerts a massive burden on humankind and societies worldwide, in particular in societies in transition and in low-resource contexts.

Today, cancer crystallizes many of the major societal challenges pertaining to lifestyles, sustainable development and environmental policies, demography and population aging, access to education and healthcare, sharing of resources and knowledge, and protection of persons and personal information. The information on cancer available at a fingertip is overwhelming in volume, complexity, veracity, and velocity. We worked on the Third Edition of Elsevier's *Encyclopedia of Cancer* with this rapidly changing background. Rather than aiming at developing a comprehensive framework encompassing all aspects, we attempted to address the literal meaning of the greek terms ἐγκύκλιος παιδεία, which means "general education". While we retained some articles from the previous edition, which, at the time of the publication, represented an exceptional achievement of Dr. J. Bertino, we largely modified the structure and the list of chapters, and the possibility of continuous update of the articles has been a great incentive for us and for the authors of the chapters. This new edition of the Encyclopedia consists of six major parts: (i) mechanisms of cancer, (ii) hallmarks of cancer, (iii) causes of cancer, (iv) cancer prevention and control, (v) diagnosis and treatment of specific cancers, and (vi) pathology and genetics of specific cancers. This repartition is necessarily artificial and is complemented by the extensive cross-references between articles. The repartition, however, reflects our effort to identify discrete topics that would best address the needs of a wide community of readers.

The primary target readership of the Encyclopedia comprises medical and other health science students, as well as non-specialized physicians and other health practitioners. Cancer researchers, oncologists, and other cancer professionals may find the articles pertaining to their specific field to be too short, over-simplistic, and perhaps obsolete; they too, however, may benefit from articles on topics other than their own. The Encyclopedia also offers an easy way to navigate across concepts and topics that should be appealing to readers from other communities, including social sciences or stakeholders in public decision-making.

We were fortunate to work with a formidable team of section editors, including Fred Bosman, Graham Colditz, Carlo La Vecchia, Gerd Pfeifer, and Marco Pierotti. An additional section editor was Professor Thomas Tursz, who passed away prematurely during the preparation of the Encyclopedia. Thomas was a great colleague and mentor, and a major figure in oncology in France and internationally. We had the privilege to involve him in the last project of his long career, and we want to dedicate this work to him. We wish to thank the many article authors, who agreed to contribute to the success of this international endeavor, and in particular Dr. Katarzyna Szymańska, who drafted several cancer-specific articles. Finally, we want to thank the staff at Elsevier, whose patience and perseverance helped us bringing the project to the final stage. All these individuals are responsible for the many strengths of the new edition of the *Encyclopedia of Cancer*, while weaknesses are mainly ours.

Paolo Boffetta
Pierre Hainaut

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Epigenetic Therapy

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Glossary

Bromodomain A protein domain that recognizes acetylated lysines.
Chromatin Histone protein DNA complex.
Epigenetics Heritable changes in gene expression not attributed to genomic sequence.
Euchromatin Loosely structured or less tightly packed chromatin regions that are enriched for actively transcribed genes.
Heterochromatin Tightly packed chromatin that is less accessible to protein factors and repressed gene transcription.
Methyltransferase Enzymes that transfer a methyl group from *S*-adenosylmethionine to DNA or protein substrates.
Xenograft A tissue or cell graft from a different species than the recipient. For instance human cancer cells xenografted into a mouse.

Nomenclature

ac Acetylation
bp Base pair
K Lysine
me1, me2, me3 Mono-, di- and tri-methylation
nt Nucleotide

Epigenetic Dysregulation in Cancer

Heritable changes in gene expression not due to the underlying DNA sequence are maintained and regulated by epigenetic mechanisms. These mechanisms allow for cellular identity and context differences in gene expression when there is no DNA sequence variation. The epigenome is dynamically regulated by the three dimensional organization of the genome. In eukaryotic cells, DNA is complexed with histone proteins to form the nucleosome, consisting of ~145 bp of DNA wrapped around an octamer of the four core histone proteins: H2A, H2B, H3, and H4. Nucleosomes are the basic repeating units that allows folding of chromosomal DNA into the higher order structure of chromatin, maintenance of genome structure during condensation of chromatin into chromosomes and segregation of chromatids during cell division. Nucleosomes have a general inhibitory effect on RNA transcription and are important for maintaining the structural integrity of DNA, protecting it from damage during interphase. Nucleosome positioning allows for the occlusion of critical DNA binding sites from transcription factors that inhibits the formation of the pre-initiation complex. In regions of the genome that are transcriptionally inert, heterochromatin consists of nucleosomes tightly packed together to form a higher order solenoid structure. In contrast, euchromatin may be bound by nucleosomes with significant nucleosome free regions that can be accessed by sequence specific transcription factors, co-factors and the transcriptional machinery.

The existence of overexpressed and chimeric transcription factors in human cancer has been known for several decades but only recently, with the advent of next generation genome sequencing, have we discovered that mutations affecting the transcriptional apparatus are some of the most common in human tumors. For example, pan-cancer sequencing efforts found that along with frequently occurring mutations in well-known oncogenes such as Ras, p53, and PTEN, a number of chromatin regulators are frequently deregulated across the cancer spectrum. Mutations have been detected in DNA methyltransferases that participate in gene silencing and heterochromatin promotion, transcription factors that bind DNA, cofactors that recruit the polymerase machinery, chromatin remodeling co-factors such as subunits of the SWI/SNF complex and enzymes that govern posttranslational modification of histones such as acetyl- and methyltransferases. Furthermore, molecules that recognize covalent histone modifications (marks) may be affected in cancer, causing aberrant interpretation of such modifications and the histone residues modified during gene regulation may even become mutated. Chromatin integrity can also be altered in cancer by inactivation of either cohesin complex subunits that link distal enhancers with proximal promoters or replication independent histone chaperones that place specific forms of histone at sites of transcription.

Epigenetic marks are dynamic information carriers. Chromatin can store and transmit heritable information in the patterns of DNA methylation or histone posttranslational modifications. Protein complexes that carry out these epigenetic processes can be classified as writers, readers, and erasers. Epigenetic writers catalyze the addition of epigenetic marks onto either DNA or histones,

such as methylation of N-terminal histone "tails" extending from the nucleosome structure. The recognition of a mark by reader protein complexes facilitates tethering of enzymatic activities that may alter the chromatin architecture further or regulate enzymatic processes such as transcription factor binding and RNA polymerase processivity. Erasers such as histone deacetylases remove epigenetic marks. Unlike genetic events, epigenetic regulation is a dynamic and reversible process. Anti-cancer therapies that target these classes of epigenetic mechanisms are currently showing promise in clinical trials both as single agents and in combination with other therapies.

Anti-Cancer Strategies That Target DNA Methylation

DNA Methylation in Cancer

In mammals, DNA methylation almost exclusively occurs on cytosines that precede a guanine (CpG) and often plays a key role in regulation of gene expression. Endogenous palindromic methylation patterns are maintained in the genome and transmitted through the germline. CpG dinucleotides are often concentrated within "islands" of CpG-rich DNA regions located near transcription start sites (TSSs). Hypermethylation of CpG islands may repress expression of the corresponding gene by preventing binding of transcription factors and recruiting methyl-CpG binding proteins that interact with repressive histone modifying enzymes. This initial model for the function of cytosine methylation has been augmented by more recent genome-wide studies which demonstrated that methylation of enhancers can modulate the function of these elements, methylation of insulator sequences can affect the ability of different regions of chromatin to interact with each other and methylation of gene bodies enhances high-fidelity gene transcription. Changes in both global and individual gene methylation patterns are often found in cancer and aberrant methylation patterns have been used to differentiate tumor subtypes. In general, global DNA hypomethylation is observed in tumors due to loss of repeat region methylation and hypomethylation of specific loci. However, hypermethylation of specific CpG rich regions has been frequently reported in malignant transformation leading to silencing of tumor suppressors such as Rb and p16. Furthermore, aberrant methylation patterns and hypomethylation outside CpG islands has been reported to contribute to increased expression of proto-oncogenes such as Hox11, ERBB2, Bcl-2, and Ras in a variety of malignancies. The enzymes responsible for establishing and maintaining these patterns of DNA methylation are DNA methyltransferases (DNMTs). DNMT3A and DNMT3B establish de novo DNA methylation patterns that are maintained by DNMT1, which can recognize methylation in DNA and assures methylation of daughter strands after replication. Demethylation of DNA is facilitated by the TET enzymes which convert methyl cytosine into hydroxymethylcytosine which is not recognized by DNMT1. DNA methylation patterns may be upset in cancer due to loss of function mutations of DNMT3A or TET enzymes as well as chronic inflammation and exposure to heavy metals. One current thread of epigenetic therapeutics attacks methylation in cancer.

Therapeutic Strategies Targeting DNA Methyltransferase

The cytosine analogues 5′-azacytidine (Azacitidine, Aza) and 5-aza-2′-deoxycytidine (decitabine) were originally developed as high-dose anti-leukemia cytotoxic agents. These compounds are azanucleosides with nitrogen at the C-5 position of their pyrimidine ring that when incorporated into DNA cause irreversible binding with DNMT1, leading to DNMT1 degradation and genome-wide DNA hypomethylation. Decitabine is mostly incorporated into DNA, but about 80%–90% of Aza is incorporated into RNA and some of its effects may be due to altered composition of RNA which is now known to also be covalently modified. Classical studies from the 1980s and 1990s revealed that DNMT inhibitors (DNMTi) may reactivate silenced genes such as fetal globin in adult red cell precursors. The ability of DNMTi to reactivate silenced tumor suppressor genes in cancer cells was a prime motivation for testing these agents in hematological and other malignancies. As a result, Aza and decitabine were approved for the treatment of myelodysplastic syndrome (MDS) (**Table 1**). These agents typically cause a slow improvement in blood counts in these pre-leukemia patients due to suppression of the malignant dysplastic cell population and emergence of increased normal hematopoiesis. In addition, a second-generation analogue, SGI-110 whose active metabolite is decitabine, is currently being tested in clinical trials for acute myeloid leukemia (AML), MDS, ovarian cancer and hepatocellular carcinoma (**Table 1**). While hypomethylating agents can reverse promoter methylation and reactivate silenced tumor suppressor gene expression in some instances, many reports attribute their anticancer activity to other mechanisms. For instance, treatment with decitabine causes the formation of DNA-DNMT adducts, subsequent double-stranded DNA breaks and G2 arrest. In addition, reports suggest that DNMTs may associate with histone modifying enzymes and a global increase of histone H3 and H4 acetylation has been observed after treatment with Aza. Furthermore, decitabine has been reported to stimulate expression of normally silent endogenous retroviral sequences in colon cancer cell lines to cause a host antiviral response including activation of interferon response genes independent of promoter demethylation. Taken together these results indicate that DNA methyltransferase inhibitors can have cooperative functions that contribute to their cytotoxic effect.

Cooperation of DNA Hypomethylating Agents With Immunotherapy

Recently one of the most promising therapeutic anticancer strategies has been to combine single agent epigenetic therapies with other epigenetic or chemotherapies. The benefit of this approach is that often lower drugs doses can be used that may limit side

Table 1 Select epigenetic therapies targeting DNA methylation or histone deacetylation

Drug	*Target*	*Cancer type*	*Clinical status*
Single agent therapy			
5-Azacytidine (AZA)	DNMT1	MDS	Approved
5-Aza-2′-deoxycytidine (Decitabine)	DNMT1	MDS	Approved
SGI-110 (Guadecitabine)	DNMT1	Clinical trials: MDS, AML, ovarian, HCC	NCT01261312 NCT01752933
Belinostat	Pan HDAC	PTCL	Approved
Panobinostat	Pan HDAC	MM	Approved
Romidepsin	HDAC1, 2	CTLC, PTCL	Approved
Vorinostat (SAHA)	Pan HDAC	CTLC	Approved
Entinostat	HDAC1,2,3	NSCLC, HL, solid tumors	NCT001349959 NCT00866333
Ricolinostat (ACY-1215)	HDAC6	MM, lymphoma	NCT02091063
Combination therapy			
AZA/Romidepsin/PD-1 Ab		CRC	NCT02512172
SGI-110/GVAX/CY		CRC	NCT01966289
AZA/entinostat/nivolumab		NSCLC	NCT01928576
Entinostat/interleukin-2		Kidney	NCT01038778
Decitabine/dendritic cell vaccine		High grade glioma	NCT02332889
ACY-1215/paclitaxel		Breast	NCT02632071

Abbreviations: *ALL*, Acute lymphocytic leukemia; *AML*, acute myelogenous leukemia; *CLL*, chronic lymphocytic leukemia; *CRC*, colorectal cancer; *CTLC*, cutaneous T-cell lymphoma; *HCC*, hepatocellular carcinoma; *HL*, Hodgkin's lymphoma; *MDS*, myelodysplastic syndrome; *MM*, multiple myeloma; *NSCLC*, non-small cell lung cancer; *PTCL*, peripheral T-cell lymphoma.

effects and reduce the potential for development of drug resistance. A more recent emphasis of clinical cancer therapy research has been directed towards testing DNMT inhibition in combination with immunotherapy.

DNA demethylating agents may induce an antiviral, antiproliferative state that reactivates tumor antigen presentation and alters apoptotic cell signaling cascades. In some circumstances evidence suggests that 5-azacytidine induces expression of typically silent endogenous retroviral sequences by demethylation of normally heavily methylated and inactivated long terminal repeat regions to promote the production of dsRNA and activation of the host anti-viral defense mechanism including interferon production. Significantly, inhibition of DNA methylation has been reported to sensitize a mouse melanoma model to anti-CTLA4 therapy and Aza induced an immune response expression signature in non-small cell lung cancer cells (NSCLC) that included increased expression of PD-L1 (CD274). These findings have led to subsequent clinical trials testing Aza in combination with anti-PD1 immune checkpoint inhibitors such as nivolumab and pembrolizumab. Epigenetic agents have also been reported to benefit cell-based immunotherapies. For example, decitabine has been reported to induce expression of NY-ESO-1 (CTAG1B) in glioblastoma cells which could be targeted with antigen specific lymphocytes in vivo. Thus, combination therapies that include demethylating agents to increase antigen production in cancer cells have emerged as a promising anti-cancer strategy to boost the effectiveness of either antibodies directed against immune checkpoint regulators or cell based immunotherapy (**Table 1**).

Targeting Defective DNA Demethylation

Defects in CpG demethylation cause DNA hypermethylation that may alter gene expression and promote tumorigenesis. Mutations and translocations of *TET2* were observed in MDS, myeloproliferative syndrome and in AML are associated with poor prognosis. Loss of function of *TET2* might rationally be approached by use of demethylating agents and some clinical data suggests that *TET2* mutant MDS may be more amenable to Aza. Aberrant loss of DNA demethylation is also associated with recurrent site specific heterozygous mutations of the enzymes isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) which are found in gliomas, AML, chondrosarcomas and cholangiocarcinoma. The mutant IDH1/2 protein reduces α-ketoglutarate (αKG) to 2-hydroxygluterate (2-HG), a competitive inhibitor of TET proteins. The TET enzymes contain the Jumonji C domain representing an α-ketoglutarate dependent dioxygenase that converts 5′-methylcytosine to 5′-hydroxymethylcytosine, an important intermediate in cytosine demethylation. IDH1/2 mutations are associated with a pattern of aberrant hypermethylation and gene expression in AML, a block of white cell differentiation and enhanced self-renewal. Pharmacological agents (Ivosidenib and enasidenib) that target mutant IDH1 and IDH2 respectively were recently approved for the treatment of AML. These agents block the production of 2-HG by the mutant enzymes, thus restoring TET2 function and reverting DNA methylation and gene expression patterns towards the normal state. This represents a form of differentiation therapy, analogous to the use of retinoic acid in acute promyelocytic leukemia, in that the mature granulocytes that appear in the circulation of patients treated with these agents are derived from the malignant clone. Complete remissions occur without the bone marrow aplasia typical of cytotoxic chemotherapy, making this therapy more tolerable for elderly leukemia patients.

Anticancer Strategies That Target Histone Acetylation

Histone Acetylation in Cancer

Lysine acetylation of histones is important for regulation of chromatin structure, transcription and DNA repair. Two competing enzyme families regulate this highly dynamic modification, histone lysine acetyltransferases (HATs) and histone deacetylases (HDACs). Approximately 30 HATs have been identified and classified into two groups based on their subcellular location. The mainly nuclear type-A HATs acetylate chromatin bound histones and nuclear proteins while the cytoplasmic type-B HATs acetylate newly translated histones. Type-A HATs are further categorized into families based on structural and functional homology. HATs transfer the acetyl group from acetyl-CoA to the amino group of a histone lysine thereby neutralizing the positive charge of lysine and diminishing the interaction of histone with DNA. In general this causes a more relaxed and accessible chromatin structure that favors binding of proteins such as transcription factors, and thus acetylation of chromatin is associated with transcriptional activation while deacetylation is associated with gene repression. In many cancers chromosomal translocations (e.g., MLL-CBP and MOZ-CBP) or mutations (e.g., p300/CBP) of HATs have been reported. Deletions or mutations that inactivate CBP have been reported in about 40% of diffuse large B-cell and follicular lymphomas and this is associated with defective acetylation of Bcl6 and p53, potentially contributing to lymphomagenesis. Cells defective in CBP appear to require the related p300 acetyltransferase protein for survival and hence inhibitors of HATs may be useful in specific settings. HAT inhibitors are currently at the preclinical stage of development. One promising p300-HAT inhibitor, C646, was reported to specifically inhibit growth of lung and hematopoietic cancer cells and more recently A-485, an even more potent and drug like inhibitor that competes with acetyl-coA, was effective in models of castration-resistant prostate cancer.

Histone Deacetylase Inhibitors as Cancer Therapy

HDACs play a key role in gene expression by removal of the activating histone acetylation and may also have other roles in the cell by controlling acetylation of non-histone and non-nuclear proteins (Fig. 1). HDACs may be aberrantly recruited to target genes through overexpressed transcription factors or chimeric transcription factors such as those created by chromosomal fusions in AML. These latter findings motivated the targeting of these proteins for cancer therapy. HDACs are classified into four groups based

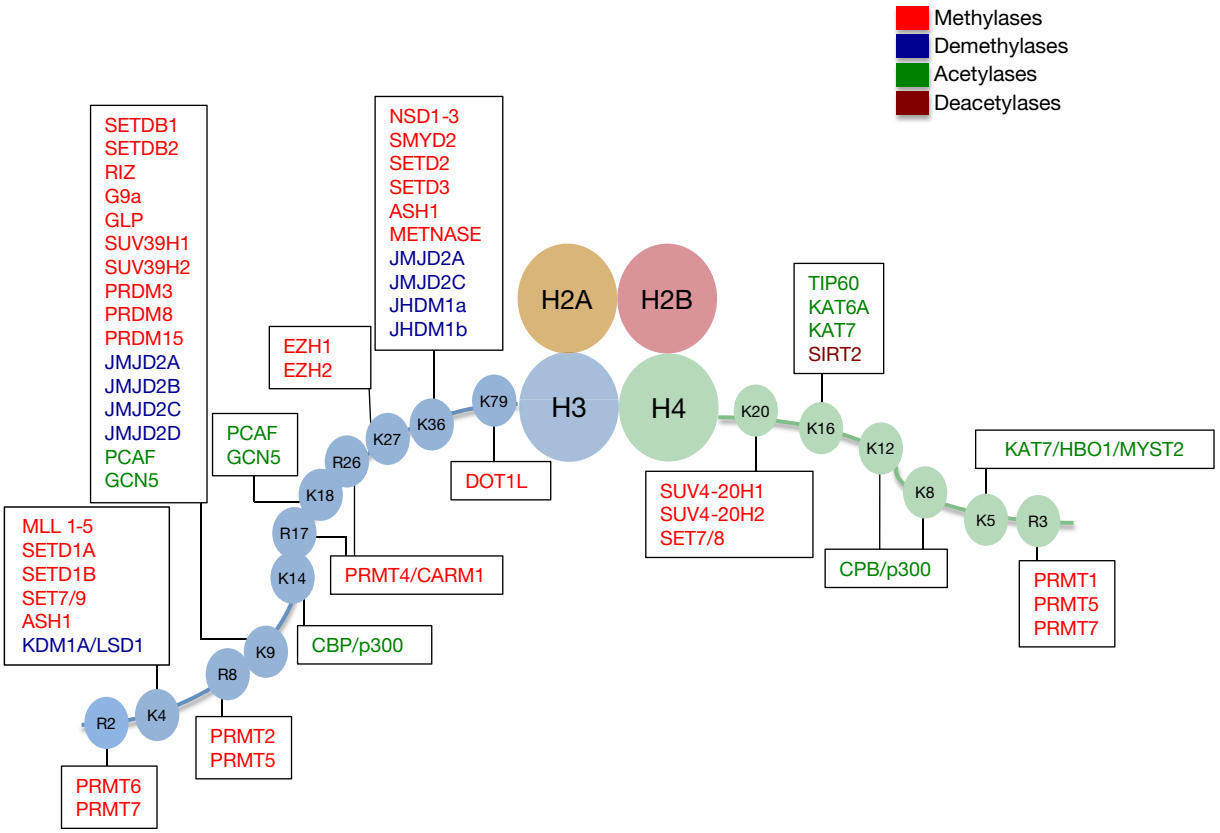

Fig. 1 Histone modifying enzymes and their targets. The lysine (K) and arginine (R) residues on histone H3 and H4 tails are targets for modification by epigenetic mark writers (methyltransferases and acetyltransferases) and erasers (demethylases and deacetylases). Some amino acid residues can serve as targets for several different types of epigenetic modifications as depicted.

on homology and structure. HDACs in classes I, II, and IV are Zn^{++}-dependent while class III HDACs are nicotine adenine dinucleotide (NAD)-dependent sirtuins. Class I (HDACs 1–3, and 8) and class II (HDACs 4–7, 9, and 10) HDACs have been reported to play roles in tumorigenesis. Many synthetic or natural product HDAC inhibitors (HDACi) have been developed as cancers therapies. These HDACi can be organized into four groups based on their chemical structure: hydroxamates, benzamides, cyclic peptides or short-chain fatty acids (**Table 1**). Several nonselective and broad spectrum HDACi target the Zn^{2+} ion in the active site of Zn^{2+} dependent HDACs to inhibit their enzymatic activity. These include the hydroxamate class agents vorinostat, belinostat, and panobinostat. Vorinostat has been approved to treat patients with cutaneous T-cell lymphoma (CTCL) and induces cell cycle arrest, apoptosis and sensitivity to chemotherapy. Belinostat and panobinostat have been approved for treatment of peripheral T-cell lymphomas (PTCL) and multiple myeloma (MM) respectively and are in clinical trials to treat solid tumors. These nonselective HDACi can reverse aberrant epigenetic chromatin changes to reactivate tumor suppressor genes such as p21. In addition, HDACi may block acetylation of non-histone proteins such as transcription factors that contribute to cell cycle regulation, differentiation and apoptosis in cancer cells. These initially approved HDACi have pleotropic actions and the exact mechanism for their antitumor effect remains uncertain. Additional non-transcriptional mechanisms may include generation of excess reactive oxygen species, hyperacetylation of chaperone proteins causing oncoprotein instability, DNA damage due to highly accessible, hyperactylated chromatin and mitotic anomalies due to hyperacetylation of critical regulatory proteins. Selective HDACi include romidepsin that targets HDAC1 and 2 and ricolinostat, an HDAC6 specific inhibitor. Romidepsin has been approved for use in patients with CTCL or PTCL while ricolinostate is in clinical trials for patients with MM or lymphoma. Interestingly, HDAC6 is a cytoplasmic deacetylase that regulates the acetylation of histone chaperones and also binds ubiquitinated proteins. HDAC6 inhibition leads to the accumulation of unfolded ubiquitinated proteins and cell death. Thus, ricolinostat has been especially promising for MM since these cells are dependent on clearance of misfolded/aggregated proteins due to high immunoglobulin production. Many clinical trials are ongoing to test the efficacy of HDACi against various cancers (**Table 1**), and understanding how HDACi affect mechanisms of cancer cell survival is an active area of basic and translational research.

Combination Therapies That Include Histone Deacetylase Inhibition

In addition to being used as a single agent therapy, many reports indicate that low dose application of HDACi improves the ability of Aza to reactivate expression of genes in vitro, specifically in cancer cells with hypermethylated promoter CpG islands. Because of these data, several ongoing studies are testing combinations of DNMTi and HDACi in the treatment of hematologic malignancies and solid tumors. One of the most promising applications of combination HDACi and hypomethylating agents has been with NSCLC patients where Phase I/II trials indicate that low doses of 5-azacytidine and entinostat were associated with improved progression free and overall survival in a small cohort of patients with a complete and a partial response observed in one patient. The evidence that the effect of HDACi/demethylation combination is due to reactivation of gene expression is mixed. Alternative explanations could be due to the ability of HDAC inhibitors to hyperacetylate chromatin and sensitize cells to DNA damaging effects of Aza or decitabine. Another possibility is that the combination can briskly reactivate endogenous retroviruses and induce an antiproliferative, antiviral response in the cancer cell.

Anticancer Strategies That Target Histone Methylation

Histone Methylation in Cancer

Post-translational modification of histone N-terminal and C-terminal tails that extend beyond the nucleosome core influence downstream biological processes such as transcription, replication, and chromosomal stability. Reversible methylation of histone tails is orchestrated by lysine methyltransferases (KMTs) and arginine methyltransferases (PRMTs) while lysine and arginine demethylases (KDMs and PRDMs) remove these marks (**Fig. 1**). Histone methyltransferases (HMTs) catalyze transfer of a methyl group to the basic amino acids lysine, arginine and histidine present in histone tails. Histone lysine residues can be modified to mono-, di- or tri-methylated forms (me1, me2 or me3) and histidine can be mono-methylated but this modification is rare. Arginine may be mono or di-methylated and dimethylation may be either symmetrical, meaning that methyl groups are added to both nitrogen atoms in the side chain or asymmetrical in which two methyl groups are added to only one of the side chain nitrogen atoms.

Except for DOT1L1, which has an unique enzymatic domain, the enzymatic activity of all KMTs resides in the Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) catalytic domain (SET) domain while type I and type II PRMTs share a well conserved *S*-adenosyl methionine (SAM)-binding catalytic core of about 350 amino acids. These enzymatic domains have pockets that bind SAM to be used as a donor co-factor for the transfer of methyl groups to substrates. Quantitative analysis of methylated histone residues by mass spectrometry demonstrates that all histone proteins (H1, H2A, H2B, H3, and H4) may be methylated, and the most frequently methylated histones are H3 and H4. Commonly observed lysine methylation sites include K4, K9, K27, K36, and K79 on histone H3, K20 on histone H4, and K26 on histone H1. Major arginine methylation sites are R2, R8, R17, R26 on histone H3, R3 on histone H4 and R11 and R29 on histone H2A. Lysine methylation causes an increase in hydrophobicity with no net change in charge, in contrast to acetylation that neutralizes the positive charge of lysine. These methylated histones can be recognized by chromatin "reader" molecules and further recruit other molecules to alter chromatin and/or transcription states. Chromatin regulators may contain one or many different types of chromatin reader modules and often chromatin "writers" recognize the very modification they make, attracting more molecules of the enzyme to chromatin and allowing chromatin modifications

to spread along the length of chromatin. Bromodomain and PHD domains recognizes acetylated histone. Methyl-lysine may be recognized several different classes of domains including chromo, PWWP, WD40, and MBT, while methyl-arginine is recognized by the Tudor domains. As discussed below some of these domains represent therapeutic targets.

Demethylation of histones is accomplished by two main classes of histone demethylases: the flavin adenine dinucleotide (FAD) dependent amine oxidases and the Fe(II)/2-oxoglutarate (2-OG) dependent Jumonji C domain family. In addition, arginine residues within proteins can be converted to citrulline by peptidyl arginine deiminases (PADs) to block arginine monomethylation. A major group of deiminated proteins are the core histones: H2A, H3, and H4. Analogous to histone lysine acetylation, histone citrullination neutralizes the positive charge of arginine and is associated with an open chromatin structure.

Dynamic changes in histone methylation state can occur based on cell type, tissue type or cell cycle phase and are important for the response to factors such as DNA damage, mitogen signaling and environmental stress. The balance between the methylated and demethylated states of histones at specific lysine residues can regulate transcriptional activity (Fig. 2). For instance, lysine methylation of histone H3 at amino acid residue 4 (H3K4), 36 (H3K36), and 79 (H3K79) are associated with activation of gene expression while methylation at lysine 9 (H3K9), 20 (H3K20), and 27 (H3K27) are associated with repression of gene expression. Dysfunctional histone methylation and the resultant aberrant gene expression has been linked to a range of malignancies. Loss or gain of KMT or KDM activity can result from missense mutation, deletion, amplification or chromosomal rearrangement affecting the genes encoding these enzymes. KMT or KDM dysfunction has often been reported to be linked with tumor grade, chromosomal instability, mutation burden and clinical outcome. Given the importance of these enzymes in regulating gene expression, and the fact that methylation is reversible, there has been considerable effort towards developing pharmacological inhibitors for methyltransferases and demethylases in hopes of restoring histone methylation balance in cancer cells leading either to either cell death or enhanced response to chemotherapy or other therapies.

Anti-Cancer Therapeutic Strategies Targeting Histone Methyltransferases

Inhibitors of DOT1L methyltransferase

DOT1L is a mammalian ortholog of yeast disruptor of telomeric silencing-1 (Dot1) gene, and plays an important role in regulating various biological functions including cell cycle, development and DNA repair. DOT1L is the only known methyltransferase that methylates H3K79 and has an important role in leukemia associated with rearrangement of the MLL (Mixed lineage leukemia) gene. Translocations of MLL/KMT2, which encodes a HMT specific for H3K4me3, yields an in frame fusion of the N terminus of MLL that is devoid of HMT activity with other nuclear proteins such as AF4, AF9, AF10 or ENL. These fusion proteins recruit DOT1L and other MLL related factors to increase DOT1L mediated methylation of H3K79 which promotes transcription elongation. This methylation mark serves as a signal for active transcription and is thought to be responsible for oncogenic expression of HOXA9 and MEIS1 in leukemias. Since the H3K79 methylation signature of MLL-rearranged leukemia is distinct from other forms of AML, targeting DOT1L may be a useful therapeutic strategy for MLL-rearranged leukemias.

Most methyltransferase inhibitors identified to date compete with the methyl donor *S*-adenosyl methionine (SAM) for binding to the enzyme and developing drugs specific for any one HMT has proven a challenge. Several small molecule inhibitors developed

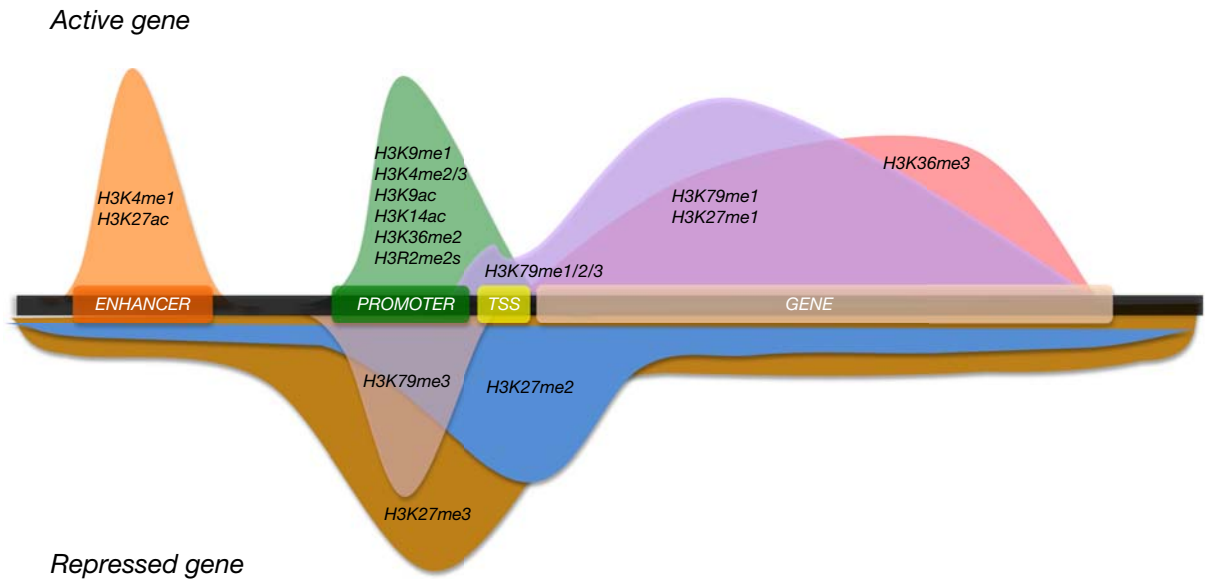

Fig. 2 Distribution of histone marks across a gene. Enrichment of widely studied histone modification marks associated with gene activation (upward peaks) and gene repression (downward peaks) at the enhancer, promoter and the gene body regions is represented. TSS = Transcription start site.

Table 2 Select epigenetic therapies targeting histone tail methylation

Drug	*Target*	*Cancer type*	*Clinical status*
HMT inhibitors			
GSK3326595	PRMT5	Solid tumors, non-HL	NCT02783300
Pinometostat (EPZ-5676)	DOT1L	Clinical trials: hematologic malignancies	NCT01684150
Tazemetostat (EPZ-6438)	EZH2	Clinical trials: solid tumors, DLBCL, HL, non-HL	NCT02889523
HDM inhibitors			
ORY-1001	LSD1	Clinical trials: AML	
GSK2879552	LSD1	Clinical trials: AML, relapsed/refractory small cell lung carcinoma	NCT02034123 NCT02034123
Tranylcypromine	LSD1	Clinical trials: AML, MDS	NCT02273102
4SC-202	HDAC-LSD1	Clinical Trials: hematologic malignancies	NCT01344707

Abbreviations: *AML*, Acute myelogenous leukemia; *DLBCL*, diffuse large B-cell lymphoma; *HL*, Hodgkin's lymphoma; *MDS*, myelodysplastic syndrome.

to inhibit DOT1L function by targeting the interaction between DOT1L and SAM. Two potent inhibitors developed by Epizyme are in clinical trials (**Table 2**). EPZ004777 selectively binds DOT1L and other methyltransferases that utilize SAM as a methyl-group donor and was shown to selectively eliminate rearranged-MLL tumor cells in vitro as well as increase survival of mice. A derivative of EPZ004777 known as Pinometostat (EZ-5676) effectively inhibits the proliferation of MLL-rearranged cells and decreases expression of HOX genes associated with H3K79 methylation and DOT1L function. Mouse xenograft models showed significant reduction and regression of tumors upon EZ-5676 treatment. Due to poor pharmacokinetic properties and toxicity of EPZ004777 it was eliminated in earlier phases of clinical development. However, the derivative EZ-5676 exhibits less toxicity, has completed Phase I trial (ClinicalTrials.gov identifier: NCT01684150 and NCT02141828) and is currently in further development (**Table 2**). Both the drugs specifically target the MLL-rearranged leukemia cells with little to no effect on other leukemia cells. EZ-5676 has also been tested in combination with other therapies including DNMT3 inhibitors like 5-azacytidine, daunorubicin or cytarabine and with inhibitors of menin, a critical cofactor for MLL-fusion associated leukemia. In general, these preclinical studies demonstrate a synergistic effect between EZ-5676 and other therapies in killing of tumor cell lines.

Inhibitors of EZH2 methyltransferase

Enhancer of zeste2 (EZH2) is the catalytic component of the polycomb repressive complex 2 (PRC2) that is responsible for trimethylation of H3K27 associated with chromatin compaction and transcriptional repression. EZH2 contains a SET catalytic domain, is an important regulator in several cellular pathways including cell cycle regulation, X-chromosome inactivation and metastasis. EZH2 is generally overexpressed in metastatic tumors relative to normal tissues or primary tumor specimens and promotes cancer cell growth and an epithelial-mesenchymal transition (EMT). Gain of function mutations of EZH2 are found in diffuse large B cell lymphoma of the germinal center type and more rarely in thyroid cancer and malignant melanoma. These heterozygous mutations represent a change of function that enhances the ability of the enzyme to cause trimethylation of H3K27 and in lymphoma this leads to aberrant repression of tumor suppressor genes and late B cell genes, thus locking the B cell in a state of continuous proliferation at the germinal center stage of differentiation. Loss of function and deletion of EZH2 is found in MDS and AML and is associated with global decreases in the repressive H3K27me3 mark that activates oncogene expression (**Fig. 2**). Thus, depending on the cellular context or function of dysregulated gene expression, EZH2 can have either an oncogenic or tumor suppressor function.

Several pharmacological inhibitors of EZH2 have been developed and studied both in vitro and in clinical trials. The first widely studied inhibitor of EZH2 was 3-deazaneplanocin A (DZNep), an adenosine analogue that interferes with the methionine cycle causing the level of *S*-adenosyl-L-homocysteine (SAH) to increase and inhibit SAM-dependent methylases. In addition, DZNep induced degradation of PRC2 complex members including EZH2 to reactivate epigenetically silenced gene expression in cancer cells and induce apoptosis. Recently, significant efforts have been made to develop inhibitors that are more selective and effective EZH2 inhibitors than DZNep. Perhaps the most promising agent currently undergoing clinical trials is Tazemetostat (EPZ-6438), an orally bioavailable EZH2 inhibitor developed by Epizyme (**Table 2**). Tazemetostat effectively inhibited tumor growth in xenograft models and is currently being tested in Phase 1 and 2 clinical trials (ClinicalTrials.gov: NCT01897571 and NCT 02601950) for B-cell lymphomas and synovial sarcomas. Tazemetostat is also under study for the treatment of sarcomas associated with rearrangements or mutations of genes encoding the SWI/SNF chromatin remodeling complex. Preclinical studies showed that the lack of SWI/SNF activity leads to a failure of expression of specific genes which can be overcome by inhibition of the repressive action of EZH2 and the PRC2 complex. Another EZH2 inhibitor under investigation in Phase I clinical trial is CPI-1205 developed by Constellation Pharmaceuticals (ClinicalTrials.gov: NCT02395601). A SAM-competitive inhibit with high specificity for EZH2, CP-1205 effectively reduced H3K27me3 in vitro and inhibited tumor growth in animal studies. GlaxoSmithKline also developed small molecule compounds that demonstrate inhibition of EZH2 in preclinical studies. GSK343 exhibited high specificity for enzymatic inhibition of both EZH1 and EZH2 while GSK126 specifically inhibited proliferation of diffuse large B-cell lymphoma (DLBCL) cells that have a gain of function EZH2 mutant. Unfortunately, while GSK126 worked well in cell line and animal models to decrease H3K27 methylation levels, it failed in early phase clinical trials due to unsatisfactory efficacy (Clinicaltrials.gov: NCT02082977). Other

SAM competitive inhibitors of EZH2 have been identified by high throughput screens and been the subject of preclinical studies. For instance the compound EI1 developed by Novartis is reported to specifically inhibit EZH2 compared to other HMTs including EZH1, and EPZ005687, developed by Epizyme, inhibited H3K27 methylation in lymphomas bearing activating EZH2 mutations. In addition, UNC1999 was the first orally bioavailable SAM competitive inhibitor of EZH1 and EZH2 identified, and preclinical studies have demonstrated its effectiveness at decreasing H3K27 methylation and promoting cell death of DLBCL cell lines.

Because combination therapies are preferable to prevent cancer cell escape from any single agent and secondary EZH2 mutations have been observed in cell lines that acquired resistance to EL1, several preclinical studies have investigated the efficacy of combination therapies that include EZH2 inhibition. In a non-Hodgkin lymphoma (NHL) model combining Tazemetostat with a standard therapy cocktail containing cyclophosphamide, hydroxyldaunorubicin, oncovin and predinosone (CHOP) promoted synergistic cell killing of EZH2 mutant germinal center NHL cell lines. In this study, significant synergy was observed between Tazemetostat and Predinosone, a glucocorticoid receptor agonist, suggesting a cooperation between glucocorticoid mediated gene regulation and EZH2-mediated chromatin remodeling. The combination of Tazemetostat and Predinosone is in Phase 1/2 clinical trials for patients with DLBCL (Clinicaltrials.gov: NCT01897571). In addition, GSK126 was observed to cooperate with etoposide to promote cell death in prostate cancer cell lines. Furthermore, EZH2 inhibitors increased cell sensitivity to topoisomerase inhibition specifically in BRG1 or EGFR mutant NSCLC cell lines. Together, these preclinical data demonstrate the potential of combining EZH2 inhibition therapy with traditional chemotherapies.

Inhibitors of arginine methyltransferases

Nine PRMTs have been identified in mammalian cells. PRMTs 1–4, 6, and 8 are type I PRMTs that catalyze the monomethylation and asymmetric dimethylation of arginine residues. PRMT5 and 9 are type II PRMTs that catalyze mono and symmetric dimethylation of arginine. PRMT7 is a type III PRMT that only catalyzes monomethylation of arginine. Arginine methylation regulates protein-protein interactions with Tudor domain-containing proteins and asymmetric or symmetric dimethylated arginine motifs bind to distinct subsets of Tudor domain containing proteins. In general, asymmetric dimethylation of histones has been associated with transcriptional activation while symmetric dimethylation is linked to transcriptional repression. Arginine methylation can be further regulated by the composition of PRMT containing protein complexes. For instance PRMT1 substrate specificity can be regulated by binding to BTG family proteins, PRMT3 methyltransferase activity is inhibited by association with tumor suppressor DAL-1, PRMT4/CARM1 has the ability to methylate nucleosomal histone H3 when part of the nucleosomal methylation activator complex and PRMT5 can be activated by MEP50 binding to associate with the SWI/SNF chromatin remodeling complex.

Several studies have associated PRMT activity with tumorigenesis. For instance, increased PRMT1 expression promotes survival and invasion, while loss of PRMT1 leads to cell cycle arrest in cancer cell lines. PRMT1 was reported to methylate and promote the activity of the AML1-ETO fusion found in about 15% of AML. Furthermore, PRMT1 has been reported to be required for the transformation activity of MLL fusion proteins such as MLL-EEN or MLL-GAS7 in MLL rearranged leukemias. PRMT1 is the primary cellular arginine methylase since it is broadly expressed in both the cytoplasm and nucleus and is responsible for almost all global asymmetric arginine dimethylation. PRMT1 substrates include histone H4 (H4R4), transcription factors, transcription initiation factors and RNA splicing factors. PRMT1 was also reported to have roles in DNA damage repair, RNA splicing, signal transduction as well as gene expression. In addition, PRMT4/CARM1 and PRMT6 expression as well as asymmetric dimethylation of arginine was found to be elevated in many cancers. Significantly, PRMT4 was required for initiation and maintenance of AML-ETO or MLL-AF9 driven leukemia in mouse models. Overexpression or increased activity of type II PRMT5 has also been observed particularly in hematopoietic malignancies, and deletion or inhibition of PRMT5 relieves the differentiation block characteristic of MLL-rearranged leukemia in vitro and in vivo.

Extensive efforts have been made to develop PRMT inhibitors as anti-cancer agents. Early studies utilized SAM analogues such as sinefungin as pan-methylase competitive inhibitors to study PRMT function. More recently several pharmacological compounds have been identified that more potently and selectively inhibit PRMTs. These compounds include AMI-1 that selectively inhibits type I PRMTs as well as inhibitor 6e and C7280948 that specifically inhibit PRMT1. In addition, AMI-408 has can inhibit PRMT1 and decrease transformation of AML cells expressing an MLL-GAS7 or MOZ-TIF2 fusion. Furthermore, EPZ015666 (GSK3235025) is a selective inhibitor of PRMT5 that decreases symmetric dimethylation of arginine and exhibits anti-tumor activity in mantle cell lymphoma xenograft models. In addition, EPZ015666 delayed disease progression and improved survival of MLL-fusion AML xenografts by inducing myeloid cell differentiation without affecting expression of MLL-fusion oncogene targets. This compound primarily inhibited arginine dimethylation of non-histone targets. Many preclinical studies have identified and characterized PRMT inhibitors as anticancer agents in cell lines and animals and GSK3326595, a PRMT5 inhibitor, is currently in Phase I trials for patients with solid tumors and non-Hodgkin's lymphoma (**Table 2**). Studies with PRMT inhibitors have been important to dissect the mechanism and function of arginine methylation in cancer but also demonstrate the challenges to develop HMT inhibitors that are specific and efficacious enough to have a clinical impact.

Anticancer Therapeutic Strategies Targeting Histone Demethylases

Since the discovery of the first lysine specific demethylase in 2004, the number of histone demethylases identified has expanded to include more than 30 enzymes that can be classified into two families based on their mechanisms: the flavin-dependent KDM1 family (also called LSDs) and the Fe(II) and 2-oxogluterate (2OG)-dependent Jumonji (JmjC) family consisting of the KDM2 to KDM8 subfamilies. In addition, a subset of JmjC lysine demethylases have been reported to demethylate arginine in vitro but

the biological activity of these enzymes in cells is presently unclear. The substrate specificity of KDMs is often dictated by their binding partners which can change in different cellular contexts. Mutations or aberrant expression of demethylases may be observed in cancer and function to deregulate chromatin structure and gene expression. Thus, inhibition of demethylases that are overexpressed or activated by mutations in cancer may rebalance the methylation load and trigger cell death mechanisms in tumor cells.

Inhibitors of KDM1 demethylases

The KDM1 family is comprised of KDM1A (LSD1) and closely related KDM1B (LSD2) that for mechanistic reason are only able to demethylate mono- and di-methylated residues. KDM1A (LSD1) has been reported to demethylate H3K4me1/me2, H3K9me1/me2, DNMT1, E2F1 and p53 while KDM1B (LSD2) demethylates H3K4me1/me2. KDM1A may be overexpressed in both solid tumors and hematologic malignancies. Because the catalytic domain of the KDM1 family is related to monoamine oxidases (MOAs) that catalyze the oxidation of neurotransmitters, the first generation of inhibitors tested for inhibition of KDM1 were relatively nonspecific MOA inhibitors first approved for depression and other psychiatric disorders. Many of these MOA inhibitors function by irreversibly binding the KDM1 cofactor flavin adenine dinucleotide (FAD) within the active site of the demethylase. MOA inhibitors that have been characterized to inhibit KDM1 and are being evaluated in clinical trials include phenelzine, tranylcypromine (TCP or PCPA), and pargyline (**Table 2**). In addition several TCP derivatives have been developed with improved potency and selectivity for KDM1A that are being tested in preclinical studies, Phase 1 and 2 trials. These TCP derivatives include the compounds ORY-1001 and GSK2879552 which have been reported to be effective in cell lines and mouse models of human tumors and are in Phase 1 clinical trials for AML and small cell lung cancer (**Table 2**). Significantly, reports suggest that KDM1A inhibition sensitizes cells to all-trans-retinoic acid (ATRA), and KDM1A inhibitors TCP, GSK2879552, INCB059872 or IMG-7289 are currently being evaluated in combination with ATRA in clinical trials for patients with AML, MDS or myelofibrosis (NCT02273102, NCT02842827, NCT03136185, and NCT02177812). Several other agents have been developed to specifically inhibit KDM1A in cell lines and human tumor xenograft models. These include hydrazine-derivatized peptides that function as substrate mimetics, polyamines and the small molecule inhibitors Namolin and GSK354. However, more detailed in vitro and in vivo studies are required before these compounds are ready to be tested clinically.

Inhibitors of JmjC family demethylases

Jumonji domain containing αKG-dependent dioxygenases can demethylate mono-, di- or tri-methylated lysines by a hydroxylation pathway involving a reactive Fe(IV) intermediate. These demethylases contain Tudor and plant homology domains (PHD) that are responsible for recognition and specificity of particular histone lysine residues. KDM2A and 2B demethylate H3K36me2, and KDM2B also demethylates H3K4me3. KDM2B is reported to be significantly overexpressed in pancreatic ductal adenocarcinoma to repress development genes and senescence mechanisms as well as regulate the TRAIL response in glioblastoma cells. The KDM3 family consists of KDM3A, KDM3B, and JMJD1C. KDM3A and 3B remove repressive H3K9me1/me2 marks. In addition, KDM3A has been reported to promote chemo-resistance by demethylating p53 leading to increased chromatin binding by p53, and depletion of KDM3A reactivates p53 to increase expression of proapoptotic genes in breast and ovarian cancer cell lines. The KDM4 family consists of four members that recognize H3K9me2/3, H3K36me2/3, and H1.4K26me3 as substrates. Multiple reports indicate that KDM4A, 4B and/or 4C are overexpressed by translocations or gene amplifications in various solid tumors and hematologic malignancies where they may alter the function of steroid receptor transcription factors. The KDM5 family members demethylate H3K4me2 and H3K4me3 and are often found amplified in cancers to activate oncogene expression and cause therapy resistance. KDM6A and KDM6B act on the H3K27me3 repressive mark, and inactivating mutations or deletions of KDM6A are among the commonest lesions in epigenetic regulators across all cancer, being most commonly inactivated in bladder cancer. KDM6A has been reported to regulate the cell cycle in an RB-dependent mechanism. Loss of KDM6B has been reported in the progression of pancreatic cancer and is associated with a more aggressive phenotype. KDM7A, 7B, and 7C act on the H3K9me2/1, H3K27me2/1, and H4K20 me1 repressive marks. Genetic alterations that elevate expression of KDM7B were reported in several cancers including NSCLC and esophageal carcinoma and KDM7C is an epigenetic activator of p53 located in a colorectal predisposition locus. KDM8 has been reported to demethylate H3K36me2 and be required for regulation of cyclin A1 expression and cell cycle progression.

Because of the large number of JmjC-domain containing demethylases involved in cancer and the availability of structural and mechanistic information, the development and testing of JmjC KDMs is a promising field of cancer therapy development. However, despite the large number of KDM inhibitors developed to date, in general these compounds demonstrate a lack of specificity and have not progressed beyond preclinical studies. One of the first types of JmjC KDM inhibitors discovered were the *N*-oxalyl amino acid-based JmjC KDM inhibitors such as *N*-oxalylglycine (NOG) a closely related analogue of αKG. However, NOG is a broad spectrum inhibitor that inhibits almost all tested JmjC KDMs. In addition, analogs of NOG such as pyridine-2,4-dicarboxylic acid (2,4-PDCA) were also reported to inhibit most JmjC KDMs. Several other efficient KDM inhibitors have been identified recently using high-throughput screening strategies such as GSK-J1 and its prodrug GSK-J4 which inhibit KDM6A and 6B, but these also display lesser activity against KDM5A and 5B. In preclinical studies GSK-J4 was reported to increase H3K27 methylation and demonstrated potent antitumor effects both in cell lines and animal models of glioma. In addition, a potent and selective inhibitor of KDM5B, EPT1013182, discovered by high throughput screening also has antiproliferative effects on cell lines and multiple myeloma xenograft models. Interestingly, several groups have coupled chemical features of KDM1 family inhibitors with JmjC domain family inhibitors to yield compounds that effectively inhibit KDMs with little or no activity against other αKG enzymes lacking KDM

activity. Taken together these reports suggest that although inhibition of KDM activity is a promising therapeutic strategy, significant technical hurdles remain before development of specific inhibitors that will be clinically useful is achieved.

Anti-Cancer Strategies That Target Epigenetic Reader Molecules

The Role of Epigenetic Mark Readers in Cancer

Many proteins that regulate transcription or chromatin structure bind to distinct nucleosome modifications and act as epigenetic mark readers. A variety of specific domains of reader proteins allows recognition of distinct covalent modifications on lysine residues and even distinguish between specific mark complexities such as mono- versus di-methylation. Examples of protein domains commonly found in reader proteins include the plant homology domain (PHD) and the proline–tryptophan–tryptophan–proline (PWWP) domain that bind to methylated histones, bromodomains that bind to acetylated lysines and the YEATS domain that preferentially binds crotonylated histone lysine residues. Reader complexes play an instructive role in transcription by directing the activity of chromatin remodeling complexes and regulating the rate of transcription to maintain homeostasis. For example, bromodomain containing proteins have been found to act as transcription factors (BRD1–4), transcriptional repressors (BRD7), transcriptional co-activators (TAF1), SWI/SNF ATPases (SMARCA2A/B and SMARCA4), methyltransferases (ASH1L), and chromatin remodelers (CECR2, FALZ).

Mutations and translocations that alter reader proteins frequently may occur in cancer and can significantly deregulate gene expression. One of the best characterized family of epigenetic readers in cancer is the bromodomain and extra-terminal (BET) family that includes BRD2, BRD3, and BRD4. These proteins share a conserved structural element consisting of two bromodomains that recognize acetylated lysine on the N-terminal tails of histones H3 and H4. Importantly, BRD4 associates with the positive transcription elongation factor (P-TEFb) protein that activates RNA polymerase II to promote transcription elongation at paused sites. BRD4 is enriched at transcription start sites, enhancer and super enhancer regions, and BRD4 promotes expression of many transcription factors that are known to have roles in cancer development and progression such as Myc. BRD4 was also reported to recruit the histone methyltransferase NSD2 to the estrogen receptor alpha (ERα) gene to increase ER expression in breast cancer cells. Increased BRD4 expression was reported in melanoma tissues and hepatocellular carcinoma where it is associated with poor prognosis. In addition, the majority of patients that develop the rare lethal midline carcinoma have chromosomal rearrangements that create a fusion protein between BRD3 or BRD4 and the NUT protein. The BRD4-Nut fusion is localized in distinct subnuclear speckles in cancer cells, recruits HATs and sequesters cofactors normally associated with activated, acetylated chromatin away from normal gene targets, blocking expression of differentiation associated genes and maintaining cancer cells in an undifferentiated state of self-renewal. Treatment of BRD-NUT expressing cell lines with a small molecular inhibitor of BET domain acetyl lysine binding led to a reactivation of gene expression and induction of differentiation in these cells. This finding helped stimulated the development of pharmacological approaches to inhibit BRD4 and other BET domain proteins.

BET Family Inhibitors as Anticancer Therapy

Pharmacological inhibitors of BET family proteins that block bromodomain binding to acetylated lysine have been studied extensively and some of these agents are currently in clinical trials as anticancer agents (**Table 3**). These BET inhibitors displace BRD4 from gene regulatory regions to inhibit gene expression. Thus, the antitumor efficacy of BET family inhibitors is particularly promising for those cancers with increased expression of oncogenic transcription factors such as c-Myc. The prototypical BET inhibitor is JQ1 that was designed to target BRD4 and displace it from chromatin. JQ1 has been used extensively in preclinical studies where it has been demonstrated to block proliferation and induce apoptosis of many cancer cell lines. JQ1 can downregulate the expression of Myc and its entire transcription program in multiple myeloma cells and displace BRD4 from the promoter of the IL7 receptor gene to promote cell death in B-ALL cell lines. In ER+ tamoxifen-resistant breast cancer cell lines JQ1 suppressed ERα and cMyc expression and restored sensitivity to the mTOR inhibitor everolimus. Furthermore, JQ1 induced apoptosis specifically in KRAS mutant NSCLC cell lines and androgen receptor positive prostate cancer cell lines. JQ1 treatment downregulated NF-κB gene expression in diffuse large B cell lymphoma (DLBCL) cells and also blocked the binding of BRD4 to Ac-Lys310 of NF-kB causing ubiquitin-mediated protein degradation. In addition, JQ1 has been reported to sensitize AML cell lines to p53 mediated cell death. While JQ1 never succeeded in clinical trials due to its short half-life, preclinical results using it demonstrated the promise of BET inhibition as anticancer therapy and provided a template for further drug development.

Another compound used in preclinical studies to demonstrate the promise of BET family inhibitors as anticancer agents is I-BET151 (GSK1210151A) which inhibits BRD3 and BRD4. I-BET151 has been reported to induce apoptosis and cell cycle arrest in mixed-lineage leukemia (MLL) cell lines via decreased BCL-2, MYC, and CDK6 gene expression. In addition, it has been reported to have potent anti-myeloma activity not only through transcriptional repression of MYC but also by upregulation of HEXIM1 expression, an inhibitor of P-TEFb. Furthermore, I-BET151 has been reported to be effective inducing cell death of NPM1-mutated AML cell lines and demonstrated BIM-dependent apoptosis and cell cycle arrest in melanoma cells. Significantly, I-BET151 was reported to arrest cell cycle progression and inhibit proliferation even in myeloproliferative neoplasms and glioblastomas driven by constitutively active JAK2 kinase.

Recently, more effective bioavailable BET inhibitors have been developed and about 20 early phase clinical trials are currently open to evaluate various 2nd generation BET inhibitors on patients with cancer (**Table 3**). Among those being tested clinically,

Table 3 Select therapies targeting BET family epigenetic mark readers

Drug	*Target*	*Cancer type*	*Clinical status*
Single agent therapy			
OTX015 (MK-8628)	BRD2, 3, 4	Breast, NMC, B cell lymphoid, MDS, NSCLC, GBM, AML, DLBCL: advanced solid tumors	NCT01713582 NCT02259114 NCT02296476 NCT02698189 NCT01713582
TEN-010	BET domain	AML, MDS, solid tumors, NMC	NCT02308761 NCT01987362
I-BET762 (GSK525762)	BET domain	Prostate, MM, refractory hematological malignancies, NMC	NCT01587703 NCT01943851
GS-5829	BET domain	DLBCL, T cell lymphoma, solid tumors, ER+ breast cancer	NCT02392611 NCT02983604
PLX51107	BET domain	Solid tumors, lymphoma, AML, MDS	NCT02683395
ZEN003694	BET domain	Metastatic CRPC	NCT02705469 NCT02711956
CPI-0610	BET domain	Lymphoma, MM, acute leukemia, MDS, myelofibrosis	NCT01949883 NCT02157636 NCT02158858
Combination therapy			
GSK525762/trametinib	BET + MEK1/2 inhibitor	Solid tumors	NCT03266159
GSK525762/fulvestrant	BET + ER inhibitor	Estrogen receptor positive (ER+) breast cancer	NCT02964507
ZEN003694/enzalutamide	BET + nonsteroidal antiandrogen	CRPC	NCT02711956
OTX015/azacitidine	BET + DNMT inhibitor	AML and B cell lymphoma	NCT02303782
RO6870810/daratumumab	BET + CD38 inhibitor	MM	NCT03068351

Abbreviations: *AML*, Acute myelogenous leukemia; *CRPC*, Castration resistant prostate cancer; *DLBCL*, Diffuse large B-cell lymphoma; *ER*, Estrogen receptor; *MDS*, myelodysplastic syndrome; *MM*, Multiple myeloma; *NMC*, Nut midline carcinoma.

OTX015 (also named MK-8628) is a broad spectrum BRD (BRD2/3/4) inhibitor that has been investigated for treatment of AML and solid tumors such as glioblastoma multiforme (GBM). In addition, OTX015 targets genes associated with NF-κB and JAK-STAT signaling pathways that are significant participants in the initiation and progression of cancer. In preclinical studies, OTX015 promoted cell cycle arrest and apoptosis in acute leukemia cell lines and had a stronger anti-proliferative effect than JQ1 to inhibit tumor growth in GBM mouse models. A Phase 1 clinical trial examining OTX015 in solid tumor patients was recently completed (NCT02259114) but results have not yet been reported, and another Phase 1 trial for patients with hematologic malignancies is currently underway (NCT02698189). In addition, GSK525762 (also named I-BET762), is in a Phase 1 trial for patients with relapsed refractory hematologic malignancies (NCT01943851), a Phase 1 trial for patients with solid tumors (NCT01587703) and a Phase 2 trial that tests BET inhibition in combination with the MAPK inhibitor Trametinib in patients with solid tumors (NCT03266159). In vitro, GSK525762 disrupted the expression of inflammatory genes in activated macrophages. Another BET inhibitor currently being tested is CPI-0610 that is in Phase 1 or 2 clinical trials for patients with lymphoma (NCT01949883), multiple myeloma (NCT02157636), acute leukemias and MDS (NCT02158858), as well as nerve sheath tumors (NCT02986919). CPI-0610 is a BET family inhibitor that decreases MYC gene expression and inhibits growth of MV-4-11 cells in mice. Other BET inhibitors currently being evaluated in clinical trials include FT1101 for hematologic malignancies (NCT02543879), RO6870810 (also named TEN-010) for multiple myeloma (NCT03068351) and ZEN003694 in combination with the hormone therapy enzalutamide in patients with metastatic castration-resistant prostate cancer (NCT02711956).

While the mechanism for many of the BET inhibitors being evaluated clinically has not been fully characterized, these agents are expected to decrease oncogene expression that may restore cell sensitivity to anticancer therapies. For instance, if resistance is mediated by the upregulation of specific transcription factors, BET inhibitors may reduce their expression. In support of this, ER positive breast cancer resistance to the mTOR inhibitor everolimus is usually mediated by c-Myc and treatment with the BET inhibitor OTX015 leads to downregulation of MYC expression and restoration of cell sensitivity to everolimus in breast cancer cell lines. Similarly, other studies have reported that treatment with BET inhibitors can reverse tamoxifen resistance in ER-positive tumors or increase the potency of an ER antagonist (fulvestrant) when cells are also treated with BET inhibitor. Furthermore, BET inhibitors can restore sensitivity to lapatinib, a tyrosine kinase inhibitor of EGFR and HER2, via reprograming of the kinome and activation of ERBB2/ERBB3 receptors in breast cancer cell lines. These results suggest that BET inhibitors are not only promising anti-tumor agents on their own but combination therapies may be useful to overcome therapy resistance caused by activation of alternative gene expression or signaling pathways. Recent findings by many laboratories have demonstrated that BET inhibitors can synergize with other anti-cancer agents such kinase inhibitors, DNMT inhibitors, BCL2 inhibitors and HDAC inhibitors to promote cell death of various hematologic and solid tumor cell lines. These multiple reports highlight the potential of using a BET inhibition strategy for frontline cancer therapy.

Additional Reader Domain Inhibitors in Preclinical Studies

Along with the widely studied BET inhibitors, a handful of inhibitors targeting methyl lysine reader domains such as PHD or Royal family domains (Chromodomains, Tudor, Agenet, PWWP, and MBT) have been identified and tested in preclinical studies. These compounds typically target the amino acid pocket that forms the "aromatic cage" of the reader domain which is responsible for binding to methylated lysine. For instance, MS37452 and UNC3866 are small molecule chromodomain inhibitors that block methyl lysine interaction with CBX7, a chromodomain protein associated with growth advantage and aggressive cancers. In prostate cancer cell lines, MS37452 reduced binding of CBX7 to the INK4A/ARF locus and de-repressed gene expression while UNC3866 inhibited prostate cancer cell line proliferation. Similarly, UNC2170 is a compound identified to bind at the interface of two Tudor domains of the p53BP1 dimer to block chromatin binding, and in cultured splenocytes UNC2170 significantly reduced class switch recombination. While results such as these are promising, relatively few inhibitors directed towards blocking methyl lysine reader domains have been identified and none have progressed to clinical trials of cancer patients. A significant technical hurdle remains to identify compounds that selectively target a single member among a family of structurally similar reader proteins.

Prospective Vision

It is now widely accepted that dysregulation of the epigenetic state plays a significant role in cancer development but our ability to target the epigenome therapeutically is still in its early stages. While many epigenetic therapies have reached clinical trials only a few have been approved for patient use. Novel drugs directed against many epigenetic regulatory mechanisms such as histone methylation/demethylation have been difficult to develop. Perhaps the most promising emergent cancer therapies that target epigenetic mechanisms are those being used in combination with immunotherapy. However, a caveat against combining epigenetic and immunotherapy may be that epigenetic therapies can be immunosuppressive or even promote a malignant phenotype in some contexts. For instance Aza has been reported to increase development of T-reg cells while treatment with EZH2 inhibitors can decrease CD4+ helper T-cells. These changes in T-cell populations may prevent effective immunotherapies based on T-cell mediated tumor destruction. Furthermore, epigenetic therapies may inadvertently promote T cell exhaustion which has been associated with nonresponse or relapse after treatment with checkpoint inhibitors.

It is likely that a survey of DNA methylation state and histone modifications in a specific patient tumor may be helpful to determine the context specific responses to therapy. For instance only specific epigenetically defined subgroups of patients may benefit from a particular epigenetic therapy. In support of this, recent studies suggest that methylation of certain CpG islands predict response to chemotherapy in AML and methylation of the *MGMT* promoter is prognostic for temozolomide response in glioblastoma. In addition, altering the balance of epigenetic regulator activity in the cell may have unexpected or detrimental consequences. For instance, epigenetic regulators such as EZH2 and DNMT3A have been reported to have both oncogenic and tumor suppressor functions depending on the context. Thus, only specific epigenetically defined subgroups of patients may benefit from particular combinations of epigenetic treatment strategies. However, work to define specific epigenetic signatures that are predictive of therapy response is still in its early stages.

See also: Chromatin Dynamics in Cancer: Epigenetic Parameters and Cellular Fate. Mutations in DNA Methyltransferases and Demethylases. Mutations in Chromatin Remodeling Factors. Mutations in Histone Lysine Methyltransferases and Demethylases. Mod Squad: Altered Histone Modifications in Cancer.

Further Reading

An in depth review of combination therapies that include both epigenetic inhibitors and immunotherapies: Chiappinelli, K.B., Zahnow, C.A., Ahuja, N., Baylin, S.B., 2016. Combining epigenetic and immune therapy to combat cancer. Cancer Research 76 (7), 1683–1689. https://doi.org/10.1158/0008-5472.CAN-15-2125.

The landmark report of an inhibitor (JQ1) directed against an epigenetic reader: Filippakopoulos, P., et al., 2010. Selective inhibition of BET bromodomains. Nature 468, 1067–1073.

Clinical trial demonstrating that DNMTi and HDACi are an effective therapeutic strategy in lung cancer: R.A. Juergens, J. Wrangle, F.P. Vendetti, S.C. Murphy, M. Zhao, B. Coleman, R. Sebree, K. Rodgers, C.M. Hooker, N. Franco, B. Lee, S. Tsai, I. E. Delgado, M. A. Rudek, S. A. Belinsky, J.G. Herman, S. B. Baylin, M. V. Brock, and C. M. Rudin. Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. Cancer Discovery December 1, 2011 (1) (7) 598–607. https://doi.org/10.1158/2159-8290.CD-11-0214.

Approval of azacitidine for myelodysplastic syndrome: Kaminskas, E., et al., 2005. Approval summary: Azacitidine for treatment of myelodysplastic syndrome subtypes. Clinical Cancer Research 11, 3604–3608.

Combination therapy of Tazemetostat and CHOP demonstrates synergy between EZH2 chromatin remodeling and glucocorticoid signaling: Knutson, S.K., Warholic, N.M., Johnston, L.D., Klaus, C.R., Wigle, T.J., Iwanowicz, D., et al., 2014. Synergistic anti-tumor activity of EZH2 inhibitors and glucocorticoid receptor agonists in models of germinal center non-Hodgkin lymphomas. PLoS One 9 (12), e111840. https://doi.org/10.1371/journal.pone.0111840.

Discovery of the first histone lysine demethylase: Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., Shi, Y., 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119 (7), 941–953. https://doi.org/10.1016/j.cell.2004.12.012. PMID 15620353.

Relevant Websites

https://Clinicaltrials.gov—U.S. national library of medicine clinicaltrial.gov database of privately and publically funded clinical studies conducted around the world.
https://www.cancer.gov—U.S. National Institute of Health, National Cancer Institute website.
https://ctep.cancer.gov—National Cancer Institute, Cancer Therapy Evaluation Program website.

Epithelium to Mesenchyme Transition

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Glossary

Cancer stem cell (CSC) Rare subset of cells within a tumor that could self-renew and give rise to many cell types that constitute a tumor.

Circulating tumor cell (CTC) A cancer cell shed by primary tumor mass into the blood circulatory system.

Disseminated tumor cell (DTC) CTC that have left the circulatory system and survived after infiltrating a distant site.

Epigenetics DNA or histone modification of chromatin structure that modulate accessibility of transcription machinery to gene loci to regulate gene expression levels without a change of DNA sequence.

miRNA Non-coding RNA of 21–25 nucleotides in length which repress the expression of its target(s).

Transforming growth factor β (TGFβ) A multifunctional cytokine that regulate cell growth, differentiation and apoptosis in any cell types. It is also a potent inducer of EMT.

Tumor microenvironment The tumor milieu, including tumor cells, stromal cells, immune cells, and ECM.

Introduction

There are two main cell types, epithelial and mesenchymal, that are described based on the cell shape and cellular organization. Epithelial cells form sheets of contiguous cells connected by specialized cell-to-cell and cell-to-substratum adhesion contacts. These cell-to-cell adhesive structures include adherens junctions, tight junctions, gap junctions, and desmosomes. They ensure close lateral connections between adjacent epithelial cells. They are polarized along the apical-basal axis and attached firmly to the basal lamina, which is part of the extracellular matrix (ECM). Mesenchymal cells present disorganized adhesive structures and exhibit spindle-like morphology. They form weak contacts with neighboring cells and do not have the same apical-basal polarity as epithelial cells. Instead, they have front-rear polarity that allows cell migration. Furthermore, they secrete ECM-degrading enzymes, and these properties allow their invasion through the basement membrane and surrounding tissues.

Epithelial–mesenchymal transition (EMT) occurs during embryogenesis and development, as well as in several pathologic conditions, such as tissue fibrosis and cancer. EMT was first described in the 1960s by development biologist Elizabeth Hay in the formation of primitive streak in chick embryo model. EMT is a differentiation switch, where epithelial cells dedifferentiate into motile mesenchymal cells. It is a vital program during embryogenesis for the formation of mesoderm. Newly formed mesenchymal cells are able to travel long distances to differentiate into various cell types. After migration and homing in to new sites, mesenchymal cells revert back to epithelial cells for tissue and organ development in the reverse process, mesenchymal–epithelial transition (MET). The ability of a cell to change phenotype, between EMT and MET processes, reflects a high degree of cellular plasticity.

There are several molecular mechanisms involved in the initiation and progression of EMT. These mechanisms include the induction and activation of transcription factors, suppression of expression of specific intercellular adhesion complexes, reorganization of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs (miRNA). Upon undergoing EMT, cells acquire migratory and invasive properties that allow them to migrate through the ECM. Finally, it is worth to mention that cancer cells may pass through EMT to differing extents; some cells will retain some epithelial traits as they acquire some mesenchymal ones (i.e., partial EMT), and other cells will lose all their epithelial characteristics and become fully mesenchymal (Fig. 1). Furthermore, EMT attributes tumor cells with properties associated with cell survival, resistance to anoikis and senescence, evasion of immunosurveillance, gain of cancer stem cell (CSC)-like features and therapeutic resistance.

Several studies now provide direct evidence that an EMT process occurs in carcinomas. In cancer, the epithelial tumor cells become more invasive after undergoing EMT and access the circulatory system through intravasation, resulting in dissemination of cancer cells to distal loci away from the primary tumor. Consequent metastatic colonization of secondary sites by cancer cells is thought to involve the MET process, based on the observation that metastases re-express the E-cadherin protein and are epithelial-like, resembling the primary tumor. This reversibility in gene expression suggests that transcriptional regulation rather than irreversible genetic loss may confer a selective advantage for cancer cell progression. However, there is also evidence that tumor invasion can occur in the absence of EMT. Some studies show that expression of podoplanin promotes an alternative pathway of tumor cell invasion in the absence of EMT. There are several lines of evidence suggesting that some invasive carcinomas have not undergone a complete transition to a mesenchymal phenotype or even lack signs of EMT, and that invasive carcinomas do not invade adjacent connective tissue as individual mesenchymal-like cells. Instead, these carcinoma cells invade as multicellular aggregates or clusters. In fact, it has been described that hepatocytes can migrate as cohorts in response to transforming growth factor β (TGFβ) stimuli.

 https://doi.org/10.1016/B978-0-12-801238-3.65024-9

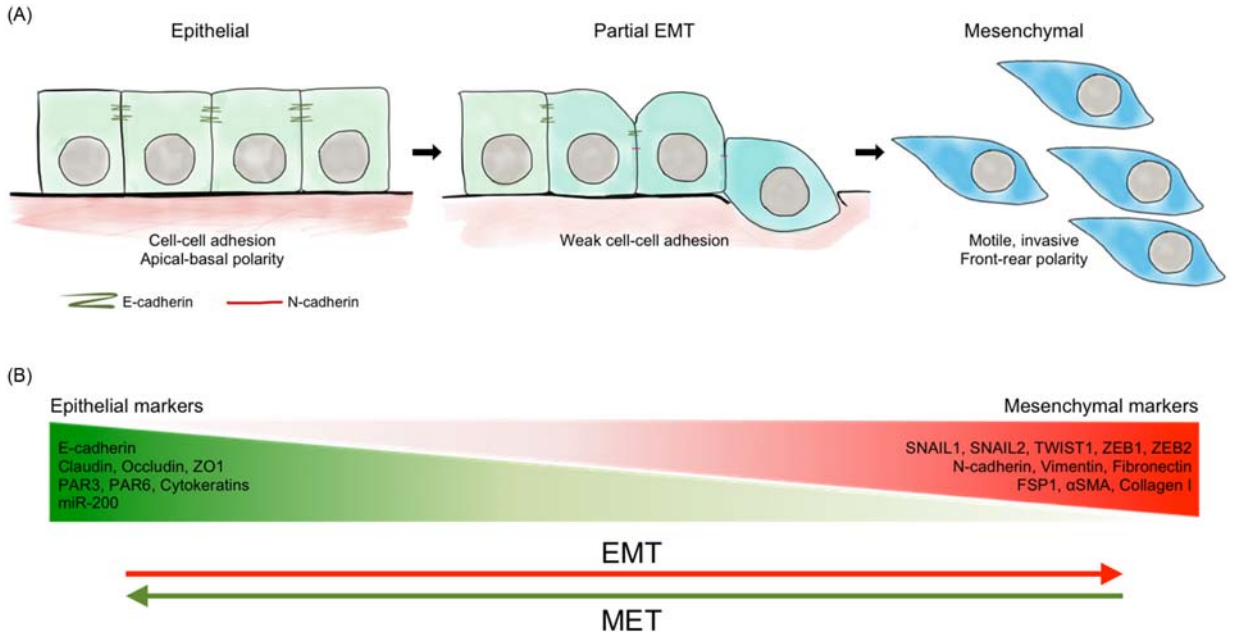

Fig. 1 Morphological changes during EMT. (A) EMT involves the disassembly of cell–cell junctions, loss of apical-basal polarity, upregulation of mesenchymal proteins, cytoskeletal reorganization, degradation and remodeling of the ECM, and thereby allowing migration and invasion. (B) Expression gradients of epithelial (*green*) and mesenchymal (*red*) markers during EMT and MET.

EMT Characteristics

Change in expression of cadherins, or cadherin switch, is a classical EMT mark. E-cadherin, a cell adhesion molecule that is important for the maintenance of epithelial integrity, is expressed in epithelial cells. Its expression is decreased during EMT in embryonic development, tissue fibrosis, and cancer. Its downregulation represents a determining step in destabilizing the epithelial architecture and is often associated with tumor aggressiveness and poor clinical outcome. Other epithelial markers include occludin, claudin, desmoplakin, and cytokeratin. The cadherin switch from E-cadherin to N-cadherin, which is expressed in mesenchymal cells, fibroblasts, cancer cells, and neural tissue, has often been used to monitor the progress of EMT during embryonic development and cancer progression. Typical mesenchymal markers expressed in cells that have undergone EMT are: (1) fibroblast-specific protein 1 (FSP1; also known as S100A4 and MTS-1) is a member of the family of Ca^{2+}-binding S100 proteins, which is detected in cells that have suffered EMT in cancer and fibrogenesis; in the same line of evidence FSP1 itself facilitates EMT in adult epithelial cells and cancer cells. (2) Vimentin is commonly used to identify cells undergoing EMT in cancers. (3) Alpha-smooth muscle actin (αSMA) is also expressed in mesenchymal cells after an EMT process. Other mesenchymal markers that are upregulated during an EMT process include fibronectin and matrix metalloproteinase (MMP).

EMT Classification

The EMT program also operates in other pathological settings, which involves inflammatory responses. Based on the biological context, EMT can be classified into three subtypes. Developmental EMTs are type 1 (not associated with inflammation); type 2 EMT is associated with tissue regeneration, such as wound healing, tissue repair and organ fibrosis; and type 3 EMT contributes towards cancer progression and metastasis. EMT confers tumor cells with motility, survival, stemness properties and therapeutic resistance.

Type 1 EMT is associated with implantation, embryo formation, and organ development to generate diverse cell types that share mesenchymal phenotypes and biomarkers; it generates cells with mesenchymal phenotype to create new tissue(s) with diverse functions. During development, EMT plays a critical role in generating the first set of mesenchymal cells, which are known as the primary mesenchyme. Subsequently, as tissue expands and specifications emerge, primary mesenchyme gives rise to secondary epithelia via MET. The EMT associated with gastrulation is dependent on and orchestrated by canonical Wnt signaling, TGFβ superfamily proteins, notably Nodal and Vg1. Their deficiencies can lead to mesodermal defects due to a dysfunctional EMT process; Wnts also cooperate with FGF receptors to help regulate EMT associated with gastrulation. In the absence of the EMT process, gastrulation cannot occur, and therefore, the development of the embryo does not progress past the blastula stage.

Type 2 EMT is associated with wound healing, tissue regeneration, and organ fibrosis; EMT in this context begins as part of a repair-associated event to generate fibroblasts to reconstruct and repair tissue following trauma and/or inflammatory injury. This process ceases once repair is achieved and inflammation is attenuated. However, in the case of organ fibrosis, type 2 EMT can continue to respond to ongoing inflammation, leading eventually to organ destruction. Organ fibrosis, which occurs in

a number of epithelial tissues, is mediated by inflammatory cells and fibroblasts that release a variety of inflammatory signals as well as components of a complex ECM that includes collagens, laminins, elastin, and tenacin; such EMTs are found to be associated with fibrosis occurring in kidney, intestine, liver, and lung. Cells that have undergone a type 2 EMT process express the mesenchymal marker FSP1 and αSMA, but concomitantly continue to have epithelial-specific morphology and molecular markers, such as cytokeratins and E-cadherin. Such cells are likely to represent intermediate stages of EMT, the behavior of these cells provided one of the first indications that epithelial cells under inflammatory stresses can advance to various extents through an EMT, creating the notion of "partial EMTs." TGFβ has proved to be an inducer of EMT, promoting type 2 EMT; interestingly, BMP7 functions as an endogenous inhibitor of TGFβ-induced EMT; systemic administration of recombinant BMP7 to mice with severe fibrosis resulted in reversal of EMT and repair of damaged epithelial structures, with repopulation of healthy epithelial cells and restoration of organ function. Different cell types in the liver have been shown to undergo an EMT process: cholangiocytes from rats with biliary fibrosis co-expressed epithelial and mesenchymal markers; and hepatocytes undergo EMT both in in vitro and in vivo-induced fibrosis. Moreover, three different fate-mapping studies have provided data showing that hepatocytes, hepatic stellate cells or oval cells might be contributing to the fibrotic process in certain types of adult liver injury through an EMT/MET mechanism.

Type 3 EMT occurs in carcinoma cells that have already suffered genetic and epigenetic alterations, which make them more sensitive to EMT-inducing signals originated from the tumor-associated stroma. The EMT process does not mean a lineage change for these cells, but a mechanism of transition and to provide epithelial tumor cells the ability of movement, invasion, and metastasis; these cells invade and metastasize via the circulation, and once they find themselves in distant tissue, they form secondary tumors exhibiting an epithelial phenotype, through a MET process.

EMT Induction

Signaling Networks Regulating EMT

EMT can be triggered in a tissue-specific manner by several extracellular stimuli. There are many growth factors and cytokines implicated in activating the EMT program, such as TGFβ, Wnt, Notch, Hedgehog, epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and hepatocyte growth factor (HGF). These diverse signal transduction pathways, influenced by microenvironmental factors such as hypoxia and inflammation, cross-talk to activate downstream effectors such as ERK, MAPK, PI3K, β-catenin, NFκB, SRC, integrin, and RhoGTPases pathways. These signaling networks converge to orchestrate EMT transcription factors (EMT-TFs) which establish the EMT program by regulating epithelial and mesenchymal genes (**Fig. 2**).

TGFβ is a major inducer of EMT in embryogenesis and cancer progression by regulating a plethora of genes controlling cell motility, invasion, actin cytoskeleton, and ECM. TGFβ ligand binding leads to formation of TGFβ type I/II receptors (TβRI/TβRII) heterotetrameric receptor complex. TβRI is activated and phosphorylates SMAD2/3, which in turn translocate with SMAD4 to the nucleus. In the nucleus, the SMAD complexes associate with other cofactors to regulate expression of target genes (**Fig. 2**). Heart valve morphogenesis in chicken involves TGFβ signaling. While the TGFβ2 member is required to initiate EMT, it is TGFβ3 that is accredited with driving EMT-invasion of endocardial cells into the cardiac cushion to establish the septa and valves. Moreover, TGFβ3 is required for cleft palate formation in mice. Transgenic mice with aberrant TGFβ1 expression in keratinocytes subjected to a chemical carcinogenesis treatment, secreted endogenous TGFβ3 to accelerate EMT and development of highly invasive spindle cell carcinomas, indicating that TGFβ drives EMT during cancer progression in vivo. TGFβ plays a role in dissolution of tight junctions and disruption of cell polarity, through protein interactions with occludin and PAR6, respectively. Moreover, TGFβ mediates EMT via chromatin protein high mobility group A2 (HMGA2) to induce SNAIL1, SNAIL2, TWIST1 and repression of inhibitor of differentiation 2 (ID2). In other words, TGFβ directly regulate majority of the EMT-TFs, underpinning the potency of this cytokine in EMT.

A key component of epithelial junction complex is β-catenin, which is part of the protein complex that connects E-cadherin to the actin cytoskeleton at the adherens junctions. In response to Wnt ligands, glycogen synthase kinase 3β (GSK3β) is inhibited, allowing β-catenin translocation from the cell membrane to the nucleus, where it can activate TCF/LEF transcription factors to induce EMT. Interestingly, SNAIL1 interacts with β-catenin and stimulates its transcriptional activity. TGFβ plays a role to disrupt cell polarity during EMT. TβRI is found in complex with PAR6 and occludin at epithelial tight junctions. The activation of TGFβ signaling pathway leads to phosphorylation of PAR6 by TβRII, and subsequently, leads to RhoA degradation and dissolution of tight junctions. Furthermore, Notch signaling has also been shown to contribute to EMT in both tumor progression and cardiac development. In addition, integrin signaling facilitates EMT, and various integrins are expressed on both epithelial and mesenchymal cells. As a result, integrins in general have limited utility as generalized biomarkers for EMT. Kidney fibrosis is associated with increased α5 integrin expression. Moreover, high α5 integrin expression also correlates with EMT and metastatic potential in melanoma cells, suggesting that α5 integrin plays a role in EMT.

EMT-Transcription Factors

The well-studied and classic EMT-TFs include the zinc finger SNAIL superfamily (SNAIL1 and SNAIL2/Slug), ZEB family members (ZEB1 and ZEB2/SIP1), and basic helix-loop-helix transcription factors (TWIST and E47). These factors have the common characteristic of repressing the expression of E-cadherin, a suppressor of invasion during carcinoma progression. EMT-TFs can be divided into two groups depending on their effects on the *CDH1* (gene encoding E-cadherin) promoter. SNAIL1/2, ZEB1/2, E47, and KLF8

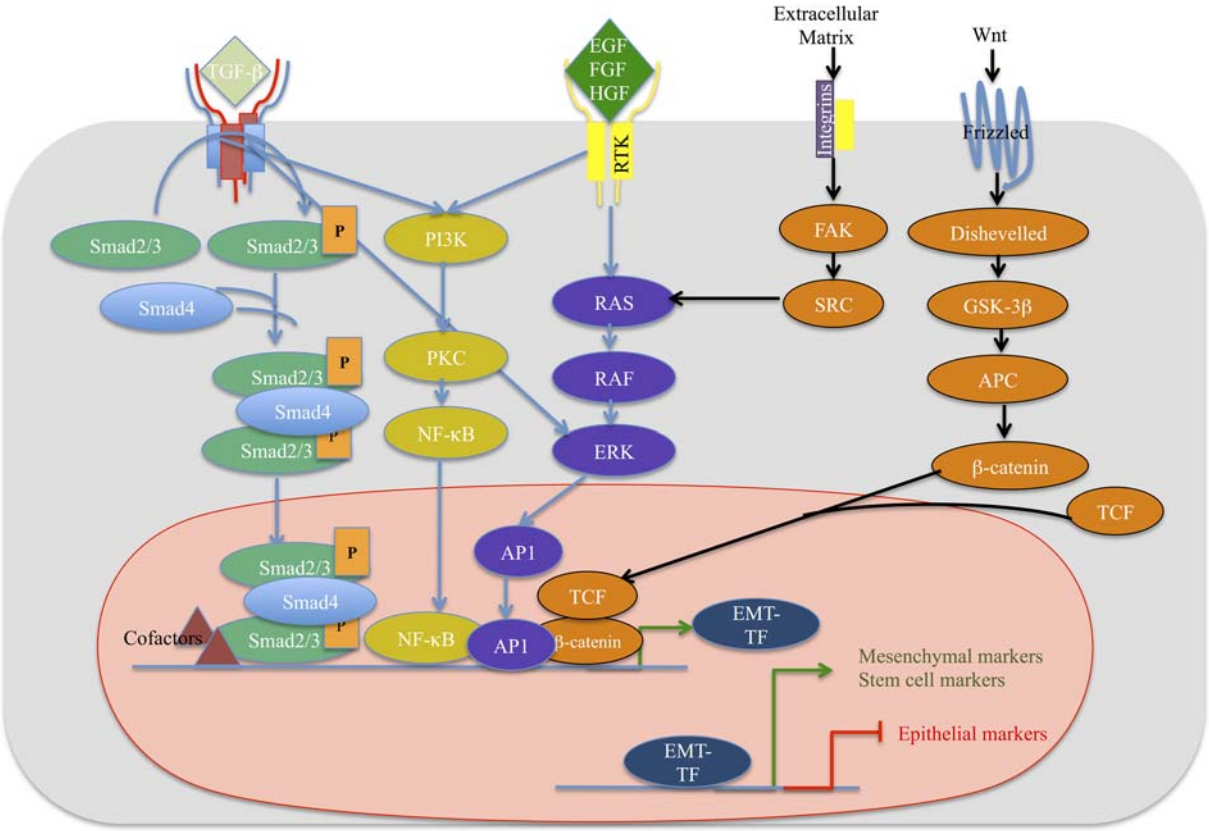

Fig. 2 Signaling pathways promoting epithelial–mesenchymal transition. Hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) signal via receptor tyrosine kinases (RTKs) to activate the signaling cascade RAS-RAF-MAPK or the PI3K pathway. The extracellular matrix, via integrins, transduces the SRC-STAT pathway. TGFβ signals via its serine/threonine kinase receptors to activate the canonical SMAD pathway or the non-canonical pathways via PI3K and MAPK. The Wnt ligands signal via Frizzled receptors to activate β-catenin and the transcription factors TCF/LEF. All these pathways modulate gene expressions that are involve in the activation of the EMT program.

factors bind to and repress the activity of the *CDH1* promoter, whereas factors such as TWIST1, Goosecoid, E2.2, and FoxC2 repress E-cadherin transcription indirectly and induce a mesenchymal gene program. In certain contexts, other transcription factors also have critical roles, such as HMGA2, PRRX1, and SOX4. Interestingly, these transcription factors regulate each other enabling positive feedback loops acting as master regulators of the EMT program and empowering the induction of EMT. The classic EMT program involves the repression of genes related to the epithelial phenotype such as E-cadherin, tight junctions proteins and cytokeratins, while inducing the expression of genes related to the mesenchymal phenotype such as N-cadherin, fibronectin, and vimentin (Fig. 1B).

The role of SNAIL1 in EMT is evident as SNAIL1 deletions in mice are embryonic lethal because they fail to complete gastrulation. SNAIL1 directly represses the expression of *CDH1* gene by binding to the *E*-box element with the consensus 5′-CANNTG-3′ on the promoter and recruits transcriptional corepressors SIN3A and HDAC complexes. SNAIL2, ZEB1, and ZEB2 use similar mechanisms to repress *CDH1* gene. Additionally, SNAIL proteins repress a variety of genes involved in maintaining epithelial structure and function, including tight junction genes encoding claudin and occludin, as well as genes important for apicobasal polarity, such as CRUMBS3. SNAIL1 upregulation also results in decreased expression of a subset of cytokeratins, that is, cytokeratin 17, 18, 19, and 20, thus affecting the epithelial cytoskeletal organization. While repressing epithelial gene expression, SNAIL1 proteins activate the expression of the fibronectin, vitronectin and N-cadherin, the extracellular matrix proteins collagen type III and V, and proteins involved in migration and invasion, such as RhoB, plasminogen activator inhibitor-1 and MMP family which help to degrade the basal membrane.

ZEB1- or ZEB2-knockouts in mice are not viable, which indicate that the two ZEBs are not able to compensate for each other's function despite being structurally similar. In addition to repressing E-cadherin, ZEB2 directly represses the expression of the tight junction proteins claudin 4 and ZO3, ZEB2 also suppresses the expression of the desmosome protein plakophilin 2; while it induces the expression of vimentin, N-cadherin, and MMP2; consequently, ZEB proteins promote cell migration and induce invasion.

TWIST1 proteins are essential for proper gastrulation, mesoderm formation and neural crest migration during development. *Twist1*-null mice die shortly after gastrulation due to defects in neural tube closure and malformation of the cranium and limbs. Ectopic expression of TWIST1 induces EMT of Madin-Darby canine kidney cells (MDCK) cells by repressing expression of

E-cadherin, α-catenin, and promoting expression of vimentin and N-cadherin. TWIST1 upregulates the expression of N-cadherin and AKT2, promoting migration and invasion. TWIST1 is essential for invasion and lung metastasis as silencing of TWIST1 suppressed lung metastases in a mouse mammary tumor model. TWIST1 also upregulates PDGF receptor α (PDGFRα), which participates in invadopodia formation that facilitates metastasis. High expression of TWIST1 and PDGFRα correlated with poor survival outcome in breast cancer patients.

Epigenetic and miRNA Regulation in EMT

How do these EMT-TFs repress gene expression? In order to make some of the changes in expression more stable and to provide long-term regulation, EMT-TFs cooperate with many other proteins that can control several layers of epigenetic regulation: (a) DNA methylation, (b) histone modifications, and (c) RNA interference. Each of these processes requires a tightly regulated machinery.

Epigenetics is the heredity of a phenotype that is the result of alterations on the chromatin and not due to genetic changes in the DNA nucleotide sequence. There are two main epigenetic mechanisms—histone modification and DNA methylation. DNA methylation is a stable mark which is usually linked to gene repression. A methyl group is covalently attached to cytosine in a CpG dinucleotide sequence by DNA methyltransferases (DNMTs). This process can be reversed by the ten–eleven translocation oxidases (TET). CpG islands are clusters of CpG sites that are sometimes found near active gene promoters. Methylation of such CpG islands lead to stable gene silencing. For example, both SNAIL1 and ZEB1 associate with DNMT1 to methylate the *CDH1* promoter, and thereby inactivate the E-cadherin gene, which is a common event in multiple carcinomas including breast, bladder, lung, liver, and prostate.

Histone posttranslational modifications by methylation, acetylation, ubiquitinylation, phosphorylation and sumoylation can alter histone–DNA interactions, and thereby, affecting chromatin structure and DNA processes like transcription, replication or repair. Through these modifications the chromatin can shift from active state, in which lysine 4 on histone H3 (H3K4) is methylated or lysine 9 on histone H3 (H3K9) is acetylated; to intermediate poised states, which are bivalently marked by trimethylation of H3K4 or lysine 27 on histone H3 (H3K27); and to a stably repressed chromatin with H3K9 or H3K27 trimethylation marks; and vice versa. The bivalent state of certain promoters in embryonic stem cells, for example, bears both trimethylation marks on lysine 4 and 27 of histone H3 (i.e., H3K4me3 and H3K27me3) to allow prompt response to specific signaling cues in transcribing their genes. The *ZEB1* promoter in some epithelial cancer cells is maintained with a bivalent H3K4me3 and H3K27me3 configuration, poised to become activated rapidly upon TGFβ signaling. The induction of ZEB1 expression commits cancer cells to the EMT program, with a gain of migratory mesenchymal phenotype and stem cell-like properties. Thus, the bivalent epigenetic modification of EMT-related genes could be a possible mechanism for epithelial plasticity observed in cancer cells.

SNAIL1 represses *E*-cadherin expression by specific binding to the latter's E-box at the proximal promoter region of *CDH1* gene. SNAIL1 recruits and interacts with various epigenetic regulators like histone methyltransferases, G9a and suppressor of variegation 3–9 homolog 1 (SUV39H1); polycomb repressive complex 2 (PRC2) which consists of the enhancer of zeste homolog 2 (EZH2) and suppressor of zeste 12 homolog (SUZ12) subunits; co-repressor SIN3A; histone deacetylases (HDACs); and lysine-specific demethylase 1 (LSD1). These proteins, together, coordinate histone modifications, that is, methylation, demethylation, and/or deacetylation, of H3K4, H3K9, and H3K27. TWIST1 induces the expression of B lymphoma Mo-MLV insertion region 1 homolog (BMI1), a component of polycomb repressive complex 1 (PRC1). TWIST and BMI bind to *E*-box sequences and cooperate to repress the expression of E-cadherin and the cell cycle inhibitor p16 (*CDKN2A*). This cooperation involves recruitment of PRC2, which then trimethylates H3K27 at the *CDH1* and *CDKN2A* gene promoters. ZEB1 also interacts with the SWI/SNF chromatin remodeling protein BRG1 to inhibit E-cadherin expression.

MicroRNAs (miRNAs) are a subclass of non-coding regulatory RNAs spanning 21–25 nucleotides in length. MiRNA are involved in post-transcriptional control of gene expression, they repress the translation of their target mRNA or promote the degradation of such mRNAs by binding to the 3′ untranslated region. MiRNAs have been shown to regulate many biological processes such as embryonic development, stem cell function, angiogenesis and cancer progression, and their biogenesis is regulated by different signaling pathways. Intriguingly, each miRNA can regulate more than one target gene and pathway. They can be classified as oncogenic miRNA (*oncomir)* or tumor suppressor miRNA. Tumor suppressor miRNA expressions are usually downregulated in cancer. In contrast, the expressions of oncomirs are overexpressed in EMT.

The EMT program is regulated by miRNA networks that target EMT-TFs, and as well as, the epithelial and mesenchymal genes. One of the best-characterized tumor suppressor miRNAs in EMT is the miR-200 family which downregulates the expression of ZEB1 and ZEB2 proteins. Forced expression of miR-200 family members in mesenchymal cells led to MET and high E-cadherin expression. On the other hand, the ZEB proteins repress the expression of miR-200 during TGFβ-induced EMT, establishing a double negative feedback loop. Interestingly, miR-200 can also act as tumor promoter because it targets *Sec23a* which is involved in the anterograde transport of metastasis suppressor proteins from the endoplasmic reticulum to the Golgi apparatus, such as insulin-like growth factor binding protein 4 (IGFBP4) and tubulointerstitial nephritis antigen-like 1 (TINAGL1). Therefore, miR-200 has a dichotomous function in metastasis as it blocks EMT and invasion (by repressing ZEB1/2), but promotes colonization of tumor cells via MET at distal sites by targeting the cancer cell secretome, avoiding the secretion of tumor suppressor proteins. SNAIL1 and miR-34 are another example of a double feedback loop. The miR-34 family attenuates the expression of SNAIL1, which in turn represses the expression of miR-34. Other tumor suppressor miRNA that inhibit EMT are: miR-124 which directly target of SNAIL2

in gastric cancer cells; miR-720 and miR-300 target TWIST1 expression in breast cancer cells and head and neck squamous cell carcinoma, respectively.

TGFβ upregulates miR-155 during EMT to disrupt tight junctions by targeting RhoA. In addition, miR-155 targets differentiation transcription factor C/EBPβ which activates epithelial genes like *CDH1* and *CAR*. High miR-155 expression correlates with C/EBPβ loss in triple-negative breast cancer, which are often negative for E-cadherin expression. MiR-373 drives EMT and metastasis by suppressing thioredoxin-interacting protein (TXNIP) pathway, which in turn promotes the upregulation of HIF1α and TWIST proteins. The miR-221/222 cluster was first identified as a basal-like breast cancer-specific miRNA and promotes EMT by affecting several targets like ERα, Dicer and trichorhinophalangeal syndrome type 1 protein (TRPS1). Downregulating TRPS1 relieves ZEB2 repression, and thereby, allowing ZEB2 to repress *CDH1*. Furthermore, miR-9 directly suppresses E-cadherin mRNA, leading to increased cell motility and micrometastasis; whereas miR-9 inhibition resulted in reduced metastasis formation by highly malignant breast cancer cells.

Long noncoding RNAs (lncRNA) consist of RNA transcripts longer than 200 bp, and have been implicated in epigenetic regulation of genes in relation to cancer progression and metastasis. TGFβ induces a few lncRNAs during EMT, one of which is Hotair. Hotair binds and guides PRC2 to repress its gene targets during the EMT process and maintenance of cancer stem cells. Similarly, Malat1 lncRNA associates with PRC2 subunit, SUZ12, to regulate EMT genes, and knockdown of Malat1 inhibits tumor metastasis. The H19 lncRNA is upregulated in a feed-forward mechanism by SNAIL2, that is induced by TGFβ during EMT. H19 and SNAIL2, together with miR-675, repress E-cadherin expression and promote metastasis in vivo. Recently, ZEB1 antisense RNA1 (ZEB1-AS1) and ZEB2 antisense RNA1 (ZEB2-AS1) were identified as non-coding transcripts in the promoters of ZEB1 and ZEB2, respectively. ZEB1-AS1 and ZEB2-AS1 positively regulate ZEB1 and ZEB2 expression, respectively, and promote tumor progression.

Collectively, EMT-TFs guide chromatin-modifying proteins and non-coding RNAs, with specificity, in order to amplify their effects on gene expression facilitating the transcription and epigenetic regulation of epithelial and mesenchymal genes.

EMT Generates Stem Cell-Like Properties

The concept of cancer stem cells (CSCs) defines the existence of a subpopulation of cells in a tumor capable of seeding new tumors, which are more resistant to conventional chemo- and radiotherapies. CSCs resemble normal stem cells in that they are able to self-renew, and generate new CSCs and progenies that differentiate into more differentiated and less tumorigenic cells, the non-CSCs, which are also described as the bulk tumor.

CSCs were initially isolated from acute myeloid leukemia based on cell-surface marker ($CD34^{+}/CD38^{-}$) expression. Based on similar principle, CSCs have been identified and isolated from several solid malignancies such as breast, brain, colon, and pancreatic cancer. They are defined for having the ability to form spheres in vitro from very few cells, and more importantly, the ability to seed new tumors in host mice when very few cells are injected (100–1000 cells per mouse).

Interestingly, in the last decade a link between the EMT program and the acquisition of CSC properties has been elucidated. The induction of EMT by overexpression of TWIST1, SNAIL1, ZEB1, or TGFβ treatment, in human mammary epithelial (HMLE) cells or in immortalized breast epithelial cell line MCF10A, results in the enrichment of $CD44^{+}/CD24^{-/low}$ population, which is associated with both human breast CSCs and normal mammary epithelial stem cells. Moreover, cells generated by an EMT process acquired the ability to form mammospheres and enhanced the ability to seed tumors in mice, an attribute of mammary stem cells. Another example, is that the metastatic breast cancer cell line to the lung, SUM1315, requires Wnt signaling for maintenance of the dedifferentiated epithelial phenotype consistent with EMT and cancer cell self-renewal. Wnt signaling maintains the expression of $CD44^{high}/CD24^{low}$, and the ability of these cells to metastasize through the expression of SNAIL2 and TWIST1. Furthermore, the sine oculis homeobox homolog (SIX1), expressed during early embryogenesis for the development of numerous organs, is silent in most adult tissues. SIX1 induces EMT and promotes stem/progenitor cell phenotype in the mouse mammary gland and in SIX1-driven mammary tumors. TGF-β has also been described to induce EMT, concomitant with acquisition of CSC traits in hepatocellular carcinoma.

The ability of EMT to confer CSC properties indicates that the activation of EMT in non-CSCs allows them to convert to CSCs through a dedifferentiation process. Therefore, in theory, CSCs can differentiate via MET into non-CSCs. Based on these assumptions, together with the reversibility of EMT and MET observed in carcinoma cells, suggest that cancer cells have a lot of plasticity.

However, under certain conditions, the link between EMT and stemness becomes uncoupled. PRRX1, an EMT inducer, needs to be downregulated in order for MET to occur at the metastatic site. Breast cancer cells decrease PRRX1 levels, with concomitant increased proliferation and gain of stemness properties. Similarly, it has been shown that EMT can suppress stem cell-like properties in prostate and bladder cancer models.

Tumor Microenvironment

The tumor microenvironment (TME) has a fundamental role in the contribution towards the malignancy of cancer progression. Tumor cells recruit nearby non-cancerous cells and modify ECM to support their growth and survival. The TME harbors a myriad of immune and inflammatory cells, fibroblasts, endothelial cells, ECM components and soluble factors, such as cytokines and

chemokines, growth factors and proteinases. The interactions between the TME and tumor cells add another layer of complexity, as not only is EMT subjected to dynamic regulation by the TME, the EMT program could influence changes in the TME to promote metastasis.

Inflammation in the Tumor Microenvironment

Chronic inflammation is associated with tumorigenesis, due to the infiltration of immune cells that are recruited by tumor cells into the TME to elicit proinflammatory responses to support tumor growth, progression, and metastasis. Tumor-infiltrating inflammatory cells, which include tumor-associated macrophages (TAMs), mast cells, neutrophils, T- and B-cells, produce an ample source of growth factors, chemokines and cytokines. Regulatory loops occur between EMT induction and inflammation. TAMs are one of the most potent EMT inducers as they secrete cytokines like TGFβ, tumor necrosis factor α (TNFα), interleukin 6 (IL6), and interleukin 8 (IL8). TAMs are often found in abundance in the TME and are associated with poor prognosis. TAMs produce TGFβ to sustain the EMT program in tumor cells to maintain the latter mesenchymal phenotype. TNFα activates the NFkB signaling pathway to stabilize SNAIL1. IL6 is one of the most abundant cytokine in cancer and has been shown to drive EMT in MCF7 breast adenocarcinoma cells with upregulation of SNAIL1 and TWIST1 and concomitant downregulation of E-cadherin. In a xenograft model where injected cancer cells with ectopic expression of IL6 generated tumors with EMT phenotype, that is, loss of E-cadherin and gain of vimentin expression, and increased lung metastasis. In a paracrine or autocrine manner, IL8 can trigger EMT in carcinoma cells. EMT-TFs regulate the expression of proinflammatory factors, which are capable of triggering EMT. Chemokine IL8 expression is regulated by EMT-TFs, SNAIL1 and TWIST, in cancer cells and secreted to recruit other immune cells. Recently, ZEB1 has also been reported to promote an inflammatory phenotype in several breast cancer cells, by binding to IL1β, IL6, and IL8 gene promoters and inducing their expressions, which results with increased recruitment of myeloid-derived suppressor cells (MDSCs) to the tumors formed by the breast cancer cell line 4T1 overexpressing ZEB1. Little is known about how tumor cells break out of latency to reinitiate metastatic outgrowth. A recent study suggests that a synergistic role by the ZEB1 and inflammation (possibly mediated by neutrophils) for tumor cells to escape dormancy to permit metastasis in a mouse model.

Cancer-Associated Fibroblasts

One key component of the TME are the cancer-associated fibroblasts (CAFs), they are a major player in tumorigenesis, regulating cell proliferation, angiogenesis, EMT, and metastatic processes. The origin of CAFs remains unclear, and presumably from: (1) resident fibroblast and host mesenchymal cells; or (2) myofibroblasts, which are activated fibroblasts that are identifiable by expression of αSMA and often found at inflammatory sites, as well as in the stroma surrounding high-grade carcinomas. Tumor cells produce TGFβ to stimulate CAFs to secrete an enormous supply of cytokines and growth factors (most notably TGFβ), MMPs, and ECM components, thus, underlying the importance of CAFs.

Interestingly, CAFs exhibit different biological properties compared to fibroblasts from adjacent non-neoplastic tissues. CAFs, not normal fibroblasts, could activate EMT, tumor-initiation and invasive processes in cancer cells. Furthermore, a subtype of CAFs that express FSP1 were found to have higher tumor-promoting potential than other subtypes. CAFs are often observed at invasive tumor fronts in carcinomas, an indicator of active paracrine signaling between CAFs and invasive tumor cells in sustaining EMT and ECM remodeling. In addition to TGFβ, CAFs produce FGF, PDGF and HGF to promote cancer cell proliferation. CAFs are known to deposit ECM and modulate its matrix composition. Lysyl oxidase (LOX), an enzyme which crosslinks collagen, is secreted by CAFs to drive matrix stiffening to regulate EMT and facilitate tumor dissemination and metastasis. An increasing number of studies have described the induction of EMT-TFs expression in CAFs which is required for CAFs activity to support paracrine signaling of cancer cells in a loop of feed-forwards. CAFs upregulate SNAIL1 and TWIST1 expressions to promote matrix rigidity. Furthermore, CAFs activity is dependent on SNAIL1 to promote tumor progression in vivo.

Hypoxia

Tumor hypoxia contributes to tumor aggressiveness and is associated with poor clinical outcome in cancer patients. Under hypoxic conditions, transcription factors hypoxia-inducible factors 1 and 2 (HIF1; HIF2) become stabilized and drive the transcription of many hypoxia-response genes which are involved in cell proliferation, survival, angiogenesis, EMT, and metastasis. Furthermore HIF is essential for CSC maintenance. HIF activates the EMT program by regulating the transcription of EMT-TFs, such as SNAIL1, SNAIL2, ZEB1, ZEB2, and TWIST1. For example, HIF has been shown to bind directly to *TWIST1* promoter to induce its expression and promote metastasis. Hypoxia-induced TWIST1 induces BMI1, a PRC1 component that maintains self-renewal, and together they promote EMT and tumor initiation. Blocking EMT by silencing TWIST1 attenuates self-renewal capacity in hypoxia-induced CSCs.

The Intermediate EMT State

EMT is not a "all-or-none" unidirectional process and not necessary entails a full mesenchymal phenotype. Observations from development, wound healing, fibrosis and cancer progression studies, have described the existence of an intermediate hybrid state (or partial EMT), where cells have both epithelial and mesenchymal features (Fig. 1A). Complete EMT with mesenchymal markers

have been observed in carcinosarcomas, whereas most other tumors often display a mix of epithelial and mesenchymal phenotypes. Co-expression of epithelial markers, cytokeratin 8 and cytokeratin 18, together with mesenchymal marker vimentin, in invasive breast carcinoma correlates with aggressiveness, metastatic potential and poor prognosis.

Cells do not need to acquire the expression of a mesenchymal marker to achieve an intermediate state. Epithelial cells remodel their cell adhesion complexes to gradually destabilize cell polarity and weaken cell–cell contact. A transcription factor OVOL has been identified from a mathematical model, and shown to maintain partial EMT status by modulating ZEB1/miR-200 and SNAIL1/miR-34 axes. Knocking down OVOL induces EMT. Conversely, OVOL overexpression leads to MET.

Interestingly, cells in the intermediate state, also gained stemness properties. CSCs from various breast cancer subtypes coexpressing both epithelial and mesenchymal signatures had increased plasticity, compared to the mesenchymal-like CSCs; and higher self-renewal capacity, compared to the epithelial-like CSCs. In line with this, hybrid EMT tumor cells isolated from a prostate cancer mouse model had higher sphere formation and tumor-initiating capacity than those with mesenchymal phenotype.

Cells undergoing incomplete EMT retain some epithelial properties with the concomitant acquisition of migratory traits. This allows them to coordinate their movements collectively while maintaining cell–cell adhesions. Collective migration have been observed at tumor fronts of invasive carcinomas, where invasive "leader" cells activate the EMT program for motility, and the "follower" cells express E-cadherin to sustain the cohesion among the latter in the tumor bulk.

Circulating tumor cells (CTCs) isolated from breast cancer patients typically express both epithelial and mesenchymal markers and tend to migrate in clusters. Clustered CTCs are 50-fold more metastatic than individually migrating CTCs. Furthermore, the heterogeneity within the clusters that have disseminated could account for the polyclonal nature of metastatic outgrowth. CTC clusters can be cloaked by platelets, which provides an ample source of TGFβ. These CTC clusters are well-protected from anoikis during circulation and easily extravasate through blood vessels at distant sites. Given their high plasticity, CTCs could switch between EMT and MET without being dependent on external signaling cues.

EMT–MET Dynamics in Progression of Metastasis

Metastasis is the leading cause for majority of cancer-associated deaths. It is a complex, multistep progression of malignant cancer cells spreading from the primary tumor to form a new tumor at a distal secondary site. The metastatic cascade begins with local invasion of tumor cells into surrounding tissue and intravasation into the circulatory system. These CTCs have to survive the sheer forces and immune cells encountered in transit through the blood stream. Surviving CTCs extravasate out of the vasculature and infiltrate distant tissues to seed micrometastases and initiate outgrowth into overt macrometastases.

The acquisition of invasive phenotype in carcinoma cells and metastatic dissemination have been linked to EMT. Disseminated tumor cells (DTCs) are CTCs that survived the blood circulatory passage have to undergo MET to establish secondary metastases, drawing parallels with embryogenesis when migrating embryonic cells shed their mesenchymal characteristics via MET to generate new tissues. This has been met with skepticism by pathologists, as EMT could not observed histopathologically in patient samples. Neighboring stromal cells display similar mesenchymal markers, makes them indistinguishable from cancer cells undergoing EMT by histological or molecular methods. Metastatic lesions at distal sites showed epithelial characteristics that resemble the primary tumor, and not mesenchymal if ascribed to activation of EMT program. However, the cells at tumor invasive fronts often exhibit signs of EMT activation. Reduced or loss of E-cadherin expression in cancers correlates with metastasis and poor prognosis, although E-cadherin loss is not sufficient to induce complete EMT. The EMT and MET processes are highly plastic and transient, thus, are hard to follow in time and space in human cancers. In the past decade, advances in imaging techniques, animal models and CTC analyses have amass strong data for the relevance of EMT in cancer progression.

Expression of EMT-TFs are often found upregulated in many tumors, including breast, colon, gastric, lung, pancreatic, and prostate cancers. SNAIL1 directly represses the *CDH1* gene and drives EMT in cancer cell lines and in vivo models. Detection of SNAIL1 was also found in tumor samples from breast cancer patients and correlates with metastasis and recurrence. The expression of SNAIL1 is transient as deletion of SNAIL1 in primary breast tumor resulted in decreased DTCs and metastases; on the other hand, a continuous overexpression of SNAIL1 has also been shown to reduce metastases despite an increase in DTCs. TWIST1 is required for invasion and metastasis, though not necessary for tumor initiation, in mouse mammary tumor model. However, the inactivation of TWIST1 expression is needed for the MET process to occur for the formation of macrometastasis in mouse skin cancer model. Similarly in a breast cancer cells, another EMT-TF, PRRX1, is required to be silent at distant site for overt metastasis. Furthermore, the miR-200 family, which negatively regulate ZEB1 and ZEB2, induces MET to promote metastatic colonization and associates with poor prognosis in breast cancer. SNAIL1 and TWIST1 have been shown to reduce cell proliferation, therefore, the downregulation of EMT-TFs during MET allows tumor cells to revert back to the epithelial phenotype and grow the secondary tumors. To put it simply, a constant activation of the EMT program would inhibit metastasis, and its reversibility, that is MET, would be essential for colonization. The above, not only attest that EMT and stemness need to be uncoupled, but also suggest an important role for EMT–MET dynamics in primary tumor invasion, dissemination and metastatic colonization.

Is EMT a Prerequisite for Metastasis?

Two mouse lineage-tracing models of breast cancer and pancreatic ductal adenocarcinoma (PDAC) have challenged the notion of the role of EMT program in metastasis progression. In a PyMT breast-to-lung metastasis model, FSP1 was used as a marker to

monitor cells that have undergone EMT and found that FSP1-negative (non-EMT) tumor cells formed metastasis in the lung. In a PDAC model which used αSMA as an EMT readout, deletion of SNAIL1 or TWIST1 in the primary tumor did not block tumor dissemination or formation of metastasis. Interestingly, the cancer cells in the above two studies were found to have increased chemoresistance after EMT, contributing to the aggressiveness of the disease. This raises the question whether if EMT is indeed a requirement for metastatic capability in carcinomas.

However, the use of FSP1 and αSMA as mesenchymal markers reflects the terminal mesenchymal status in tumor cells, and preclude those aggressive cells that are in the EMT/MET intermediary state (partial EMT) in the lineage-tracing systems used in these studies. EMT-TFs, not only cooperatively target common genes, but they regulate each other's expression. A recent study performed in the same PDAC model showed that ZEB1 depletion suppressed stemness, colonization, invasion, and metastasis. The deletion of one EMT-TF could allow compensatory effects by the upregulation of other EMT-TFs to drive metastasis. In a separate study based also on the PyMT breast cancer model supports the role of EMT and plasticity in vivo. Intravital microscopy was utilized to identify a rare pool of tumor cells spontaneously undergoing EMT, without any modifications of EMT-TFs expression levels, became motile, disseminate, and finally reverting to an epithelial state upon arrival at metastatic site. This indicates that the EMT program does not operate in a stereotypical mode and more studies are needed to determine whether EMT is critical for metastatic progression in other tumors and under various conditions.

Therapeutic Resistance

The contribution of EMT program to metastasis remains debatable, as mentioned above, but solid evidence from many research groups concur that the EMT program confers therapeutic resistance. This has been observed in many cancer cell lines and in vivo mouse models. However, the exact mechanisms in gaining therapeutic resistance remains unclear.

EMT contributes to immunosuppression in tumors. SNAIL1-induced EMT in melanoma cells confers resistance to immunotherapy by inducing immunosuppression. SNAIL1^{+} melanoma cells secrete TGFβ and thrombospondin into the tumor milieu, leading to an increase of regulatory T cells population in the TME. In line with this, mesenchymal breast cancer cells of a mouse breast cancer model elevate their expression of programmed cell death 1 ligand 1 (PD-L1) to evade recognition by cytotoxic T cells, and increased their production of thrombospondin. Due to the increased presence of regulatory T cells, this protects the other epithelial cancer cells with low PD-L1 protein levels from the cytotoxic T cells.

CSCs are known to have drug resistance properties due to their ability to mediate drug efflux. Breast cancer cells overexpressing SNAIL1, TWIST1 or FOXC2, have an increased expression of ATP-binding cassette (ABC) transporters, which play a role in the development of multidrug resistance. In addition, several binding sites for EMT-TFs were identified on the promoters of ABC transporters. Hence, these EMT-cells showed a higher doxorubicin resistance compared with to the control cells.

Chronic exposure to TGFβ, not only induced EMT, but also allows mammary epithelial cells to escape apoptosis. Moreover, TGFβ treatment allow cells to resist ultraviolet-light-induced apoptosis. EMT induced by EGFR signaling has been linked to tamoxifen resistance and increased invasiveness of MCF7 breast cancer cells. In addition to regulating genes involved with migration and invasion, SNAIL1 regulates genes required for cell survival, which are frequently intertwined with EMT during embryonic development or in pathological conditions. SNAIL1 inhibits cell proliferation through suppression of cyclin D proteins, and protects against apoptotic signals induced by serum-withdrawal, pro-apoptotic proteins or DNA damage, by regulating the expression of caspases and BCL-XL. Additionally, overexpression of SNAIL1 and SNAIL2 generates cells with stem cell-like phenotype and promotes chemoresistance against doxorubicin, paclitaxel and radiotherapy in several cancer cells, including breast and ovarian cancer. Similarly, TWIST1 inhibit apoptosis and have pro-survival functions in many cancer cells. TWIST1 directly upregulate AKT2 expression, promoting survival and chemoresistance to paclitaxel in breast cancer cells. TWIST1 and ZEB1 protect cells from oncogene-induced senescence by downregulating cyclin-dependent kinase inhibitors, $p16^{Ink4a}$ and $p21^{Cip1}$.

There is an association of elevated resistance towards chemotherapy in cells that have undergone a partial EMT, compared to their epithelial counterparts. In the clinic, the cell populations that survived and found in patients receiving chemotherapy had the mesenchymal phenotype. Chemotherapy had enriched for cells with CD44^{+}/CD24^{-} in these patients, with increased mesenchymal markers and claudin-low signatures, insinuating that the EMT program had been activated.

Conclusion

EMT is an important and complex process in embryonic development that is recapitulated during cancer progression. In the past several decades, there has been an enormous progress in understanding the signaling pathways and molecular mechanisms that contribute towards EMT. The EMT program could bestow many capabilities, such as migratory and invasiveness, pro-survival, stemness and immunosuppression—all of which are favorable functions for any cancer cell to usurp and initiate metastasis.

There has been much interest in the link between EMT and CSC, and the debate of EMT contribution to the metastasis. EMT is not a dichotomous switch between epithelial or mesenchymal entities, but shifts through a spectrum of intermediate states, that is, partial EMT. This helps to reconcile the notion that cancer cells utilize the EMT program for invasion and dissemination, whereas MET helps establish the metastatic outgrowth. Therefore, it would seem that targeting EMT would be an ideal strategy to block

invasion and dissemination. This could have dire consequences as it could promote colonization of DTCs, via MET, and reinstate rapid proliferative activity of the metastatic cells. Cells in the partial EMT state acquire stemness properties, as it has been demonstrated that they have higher self-renewal and tumor-initiating capacity than their mesenchymal counterparts. The partial EMT state is also associated with both de novo and adaptive drug resistance and higher malignancy than cells in complete mesenchymal state. Therefore, they could be regarded as CSCs with high plasticity with the flexibility to shift through the spectrum between EMT and MET. Hence, it would be vital to find a set of markers to identify and target these partial EMT cells as a strategy for therapeutics development.

Further Reading

Jolly, M.K., Boareto, M., Huang, B., Jia, D., Lu, M., et al., 2015. Implications of the hybrid epithelial/mesenchymal phenotype in metastasis. Frontiers in Oncology 5, 155.
Marcucci, F., Stassi, G., De Maria, R., 2016. Epithelial–mesenchymal transition: A new target in anticancer drug discovery. Nature Reviews Drug Discovery 15, 311–325.
Moustakas, A., Heldin, C.-H., 2016. Mechanisms of TGFβ-induced epithelial–mesenchymal transition. Journal of Clinical Medicine 5, 63.
Nieto, M.A., Huang, R.Y.-J., Jackson, R.A., Thiery, J.P., 2016. EMT: 2016. Cell 166, 21–45.
Skrypek, N., Groossens, S., De Smedt, E., 2017. Epithelial-to-mesenchymal transition: Epigenetic reprogramming driving cellular plasticity. Trends in Genetics 33, 943–959. https://doi.org/10.1016/j.tig.2017.08.004.
Shibue, T., Weinberg, R.A., 2017. EMT, CSCs, and drug resistance: The mechanistic link and clinical implications. Nature Reviews. Clinical Oncology 14, 611–629.
Tan, E.-J., Olsson, A.-K., Moustakas, A., 2015. Reprogramming during epithelial to mesenchymal transition under the control of TGFβ. Cell Adhesion and Migration 9, 233–246.

Esophageal Cancer: Diagnosis and Treatment

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Abbreviations

18F-FDG 18-Fludeoxyglucose
BMI Body mass index
CT Computed tomography
EAC Esophageal adenocarcinoma
EMR Endoscopic mucosal resection
ER Endoscopic resection
ESCC Esophageal squamous cell carcinoma
ESD Endoscopic submucosal dissection
EUS Endoscopic ultrasound
FISH Fluorescence in situ hybridization
FNA Fine-needle aspiration
GERD Gastro-esophageal reflux disease
HER2 Human epidermal growth factor 2
IARC International Agency for Research on Cancer
ICD-O International Classification of Diseases for Oncology
IHC Immunohistochemistry
LOH Loss of heterozygosity
MIE Minimally invasive esophagectomy
NCCN US National Comprehensive Cancer Network
pCR Pathological complete response
PET Positron emission tomography
TCGA The Cancer Genome Atlas
Tis Carcinoma in situ
TRG Tumor regression grade

Definition and Classification

Malignant neoplasms of the esophagus comprise mainly two entities: squamous cell carcinoma and adenocarcinoma.

Squamous cell carcinoma of the esophagus (ESCC; ICD-O code: 8070/3; also called epidermoid carcinoma, squamous carcinoma, and squamous cell epithelioma) is a malignant epithelial tumor with squamous-cell differentiation, microscopically characterized by keratinocyte-like cells with intercellular bridges and/or keratinization.

Adenocarcinoma of the esophagus (EAC; ICD-O code: 8140/3) is a malignant epithelial tumor with glandular differentiation. These tumors arise predominantly from columnar ("Barrett") mucosa in the lower third of the esophagus. In rare cases, EAC originates from heterotopic gastric mucosa in the upper esophagus, or from mucosal and submucosal glands.

SCCs are more common in the upper and middle third of the esophagus, whereas EACs usually originate in the lower third of the esophagus (distal esophagus), often involving the esophagogastric (gastroesophageal) junction. The appropriate demarcation between gastric and esophageal adenocarcinoma as well as the classification of adenocarcinomas spanning over the esophagogastric junction and the utility of treating them as distinct entities are a subject of debate.

Presentation and Diagnosis

Despite important epidemiological and biological differences between the two major histological types of esophageal cancer, their symptoms and diagnostic workup are similar.

The most common presenting symptom is solid food dysphagia, followed by liquid food dysphagia. The latter usually occurs when esophageal lumen diameter is under 13 mm and indicates locally advanced disease. Dysphagia and tumor-related anorexia lead to weight loss which is observed in about half of the cases. Patients may also experience bleeding from the tumor leading to iron deficiency anemia. Other symptoms include regurgitation, epigastric or retrosternal pain, hoarseness due to invasion of the

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recurrent laryngeal nerve, and respiratory symptoms (persistent cough and recurrent pneumonia). Pain in the bones indicates metastatic disease.

As early-stage disease does not give any apparent symptoms, most patients present with advanced disease. Superficial ESCC may sometimes be associated with a tingling sensation and is therefore occasionally detected at endoscopy of the upper gastrointestinal tract. Barret's esophagus, the precursor lesion for EAC, gives symptoms in about 10% of patients (the same as those of the gastro-esophageal reflux disease (GERD)). Physical findings other than cachexia and palpable supraclavicular lymph nodes are rare.

Endoscopic examination is the standard technique used to determine the presence and exact localization of esophageal neoplasia. High-resolution and narrow-band endoscopic imaging may be used to enhance visualization (i.e. resolution of the surface mucosa) and improve cancer detection. Endoscopy can also be improved by using Lugol's iodine dye (chromoendoscopy) to identify early cancers. Multiple biopsies are taken during endoscopy for histological evaluation. In early stage disease, endoscopic resection (ER) of focal nodules should be performed for pathological evaluation of the tumor differentiation and the depth of invasion. This is particularly important in case of Barret's-associated high-grade dysplasia and squamous cell dysplasia. Cytological brushings and washings are usually not adequate for initial diagnosis.

Any complaint of dysphagia in an adult should always prompt an endoscopy to help rule out the presence of esophageal cancer. Endoscopy may be followed by a barium swallow study which is very sensitive in detecting strictures and intraluminal masses. However, as it does not allow for staging or biopsy, it is no longer often used, unless to study distal anatomy in obstructive tumors which are inaccessible to endoscopy. Endoscopy is also used for surveillance of high-risk individuals, for example those with Barrett's esophagus or a familial history of esophageal cancer.

Computed tomography (CT) of the chest and abdomen is usually the first diagnostic workup procedure conducted for staging. It allows to exclude metastases to the liver and the lungs and may be helpful in determining invasion to adjacent structures. Positron emission tomography (PET) scanning is also useful, in particular to detect occult distant lymph node metastases and bone spread, and it becomes standard in esophageal cancer staging. Endoscopic ultrasound (EUS) has been shown to be the most accurate in assessing tumor depth and it allows for lymph node sampling. EUS is recommended to improve the accuracy of staging in patients with no evidence of unresectable metastatic disease. However, PET using the radiolabeled glucose analog fluorine F 18-fludeoxyglucose (18F-FDG) is more sensitive than CT or EUS in detection of distant metastases. EUS-guided fine-needle aspiration (FNA) biopsy provides a greater accuracy than EUS alone for the evaluation of lymph node metastasis.

Laparoscopy and thoracoscopy are also used in some centers for esophageal cancer staging. Laparoscopy allows to assess subdiaphragmatic, peritoneal, liver, and lymph node metastases with a high accuracy, whereas thoracoscopy can spare radical resections in patients with intrathoracic dissemination. Bronchoscopy is indicated for cancers of the middle and upper third of the thoracic esophagus (tumor at or above carina) in nonmetastatic patients to help exclude invasion of the trachea or bronchi.

The macroscopic appearance of ESCC varies according to the depth of invasion and it may be polypoid, flat, or ulcerated. ESCC spreads both horizontally and vertically. Tumors in which invasion is restricted to the mucosa and submucosa are often referred to as "superficial," independently of the presence of regional lymph node metastasis.

Early AECs are often contiguous with the squamous epithelium. Typical salmon-pink mucosa of Barret's esophagus may be evident adjacent to the tumor. At the time of diagnosis, most tumors are advanced, with deep infiltration of the esophageal wall. A majority of advanced adenocarcinomas are flat and ulcerated, while about one third have a polypoid or fungating appearance. AEC may occasionally arise independently of Barret's mucosa from ectopic gastric glands (mostly ulcerated tumors) and esophageal glands (usually polypoid). These tumors are also found in the upper and middle third part of the esophagus where ESCC is more common.

Epidemiology and Risk Factors

Burden

Esophageal cancer is the eighth most common cancer worldwide, with estimated 456,000 new cases in 2012, and the sixth most common cause of cancer-related death (400,000 deaths in 2012). There are striking geographical variations in the prevalence of the two histological subtypes. Still, taking the two subtypes together, about 80% of cases occur in less developed regions. Esophageal cancer incidence rates worldwide in men are more than double those in women (male:female ratio 2.4:1). Geographical variations in the incidence (up to 20-fold) are observed for both sexes, with the highest rates in Asia, south-east Africa, and Northern France (Fig. 1). The incidence increases with age in all countries. Esophageal cancer patients have a very poor survival, with an overall mortality to incidence ratio of 0.88 and 5-year survivals for all patients below 20% even in developed countries with high standards of medical care.

The most common type worldwide is ESCC (almost 90% of all esophageal cancer cases). However, EAC prevails in the United States and Western European countries. The incidence of ESCC is particularly high in the so-called esophageal cancer belt which stretches from northern China through central Asia to northern Iran. The overall worldwide incidence of esophageal cancer is rising. 725,582 new cases are predicted to occur in the year 2030 (643,095 of them in less developed regions). EAC is predominant in white males in whom its incidence has been steeply rising. However, it is gradually rising also in men of other ethnicities and in females in Western countries, while ESCC becomes less frequent. This "histological shift" merely reflects lifestyle changes.

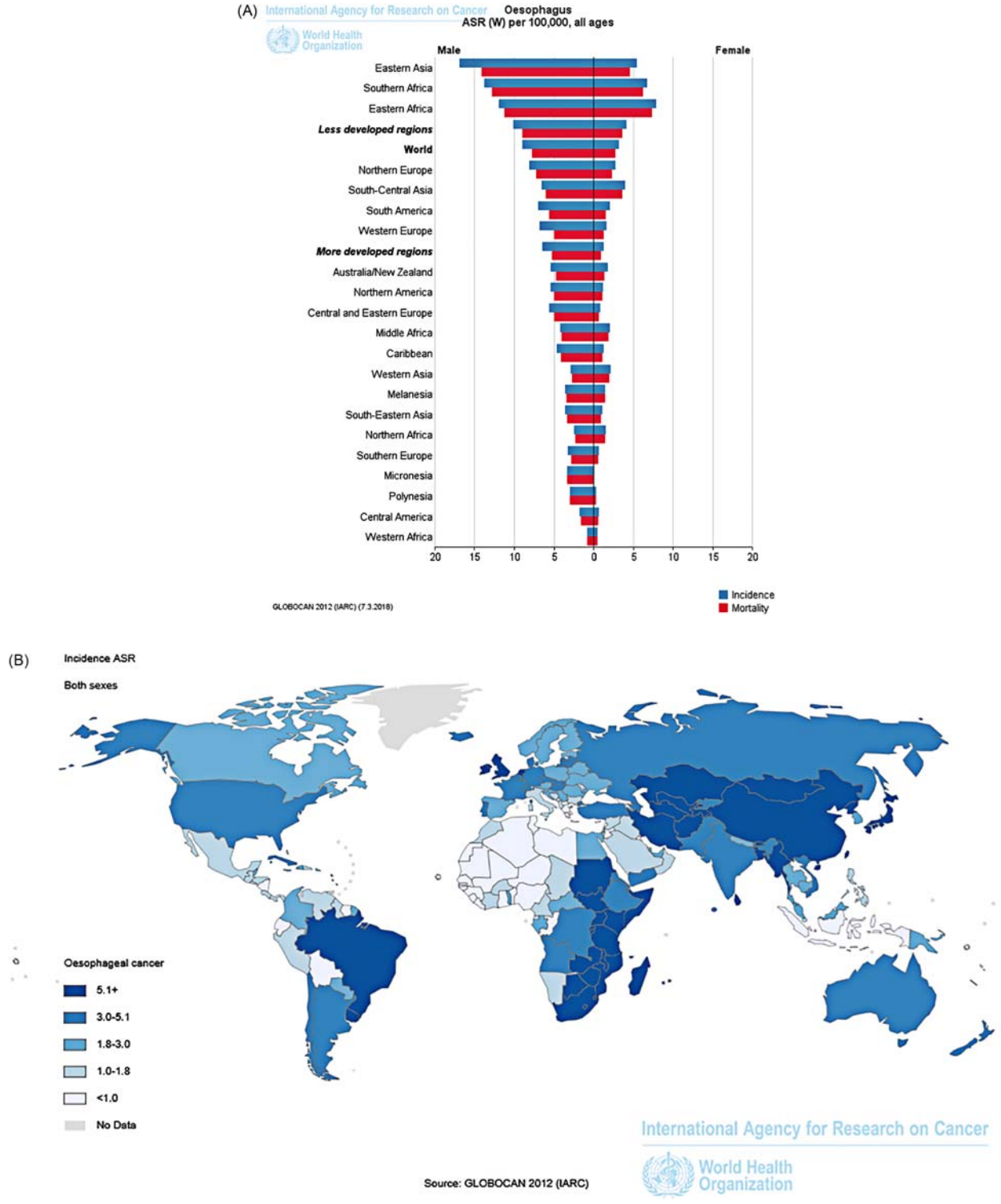

Fig. 1 Incidence and mortality of esophageal cancer worldwide. (A) Age-standardized incidence and mortality rates (ASR) by gender and geographical area. (B) Incidence distribution worldwide. From Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D and Bray, F. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC Cancer Base No. 11 [Internet]*. Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr, accessed on February 20, 2018.

Etiology and Risk Factors

The etiological factors differ between the two histological types of esophageal cancer. The major risk factor for developing ESCC is tobacco smoking, followed by consumption of alcohol and hot beverages. A clear dose-response relationship has been shown between tobacco smoking (duration and quantity) and ESCC risk, while smoking cessation reduces the risk of developing the

disease. Also tobacco chewing is associated with an increased ESCC risk, which may explain a high incidence of this cancer type in India and Southern Africa. Many tobacco carcinogens have been incriminated as mutagens, including PAH and nitrosamines. The deleterious effect of alcohol on the esophageal mucosa is mostly mediated by acetaldehyde, secondary to oxidation by the oral microbiota and salivary products. Alcohol and tobacco have a synergistic effect on the risk of developing ESCC and, taken together, they account for over 90% of ESCC cases in Europe and North America. In the same line, some variants of genes involved in detoxification processes, such as alcohol dehydrogenase, may increase the susceptibility of squamous epithelium to environmental carcinogens, thus contributing to an increased ESCC risk. Drinking very hot beverages, like mate in Brazil and Argentina or hot tea in Iran, contributes to ESCC carcinogenesis by inducing chronic thermal injury.

A low intake of fruit and vegetables as well as specific regional marginal micronutrient deficiencies (e.g., vitamin A and E) are also associated with an increased risk of ESCC. In the same line, Plummer–Vinson syndrome, a sideropenic dysphagia caused by deficiency in iron, riboflavin, and other vitamins, is associated with a higher risk of developing ESCC. Low fruit and vegetable consumption as well as high red meat consumption are also associated with an increased risk of EAC.

Tobacco smoking is also a risk factor for EAC but the association is moderately strong. High body mass index (BMI) and obesity, especially central/visceral obesity increase the risk of EAC but not that of ESCC. The major condition predisposing to EAC, however, is the gastro-esophageal reflux disease (GERD) with associated Barrett's esophagus. In GERD patients, the squamous epithelium of the esophagus is damaged and replaced by metaplastic, columnar, or glandular epithelium predisposed to malignancy. Patients with Barret's esophagus have a 30–60-fold higher risk to develop EAC than the general population.

Some studies have suggested that infection with high-risk Human Papillomaviruses may be associated with the development of ESCC. However, the data had been inconsistent and the results of a recent study by The Cancer Genome Atlas (TCGA) Research Network have rejected this hypothesis.

ESCC may also develop in relation with some hereditary conditions. In particular, Howel–Evans syndrome (tylosis), a very rare autosomal dominant disorder caused by a germline mutation in the *RHBDF2* gene, resulting in palmar and plantar hyperkeratosis, is associated with a 90% cumulative risk of developing ESCC by 70 years of age. Overall, nearly 40% of individuals with tylosis develop ESCC during their lifetime.

In contrast to ESCC, genetics contributes to about one-third of the risk to develop EAC, and approximately 7% of Barret's esophagus and EAC cases may be familial. A number of susceptibility loci have been identified, in particular in genes whose products are involved in the embryonic development of the esophagus and in regulating inflammatory response. The established risk factors for developing esophageal cancer are listed in Table 1.

Pathology and Genetics

Consistent with different etiologies, ESCC and EAC develop through different pathways and have distinct molecular profiles. A recent TCGA study has shown that ESCCs share more genetic features with squamous cell carcinomas of the head and neck than with EACs which, in turn, strongly resemble the chromosomally unstable variant of gastric carcinoma.

ESCC develops from basal cell hyperplasia and dysplasia (from low to high grade) which progresses to carcinoma in situ (Tis) and invasive carcinoma. Dysregulation of *TP53* and other genes encoding cell cycle regulators, such as cyclin-dependent

Table 1 Risk factors for esophageal cancer

Carcinogenic agents classified by "IARC monographs on the evaluation of carcinogenic risks to humans (volumes 1–120)"
Agents of sufficient evidence for esophageal carcinogenicity in humans
Acetaldehyde associated with consumption of alcoholic beverages (ESCC)
Alcoholic beverages (ESCC)
Betel quid with and without tobacco (ESCC)
Smokeless tobacco and tobacco smoking (strong association with ESCC, moderate with EAC)
X-radiation, gamma-radiation
Agents of limited evidence for esophageal carcinogenicity in humans
Dry cleaning
Traditional Asian pickled vegetables (ESCC)
Rubber production industry
Very hot beverages (ESCC)
Other carcinogenic agents and lifestyle risk factors
High BMI and obesity (EAC)
Low fruit and vegetable, and high red meat consumption (EAC)
Medical conditions and hereditary factors
Gastro-esophageal reflux disease (GERD; EAC)
Barret's esophagus (EAC)
Howel–Evans syndrome (tylosis; ESCC)
Plummer–Vinson syndrome (ESCC)

BMI, body mass index; *EAC*, esophageal adenocarcinoma; *ESCC*, esophageal squamous cell carcinoma.

kinase inhibitor 2A (CDKN2A) and retinoblastoma-associated protein (RB; encoded by the *RB1* gene), are early events in ESCC development and can already be detected in precursor lesions. Abnormal p53 expression has been shown in normal esophageal tissue adjacent to dysplasia or ESCC, and increased levels of CDKN2A and RB have been associated with a stepwise progression from inflammation to cancer in esophageal lesions. In a recent TCGA study, ESCC showed a higher prevalence of C>A substitutions (associated with tobacco smoking and chewing) and of the APOBEC mutational signature than EAC.

EAC usually develops from Barret's mucosa, which is an adaptive response to recurrent injury of the squamous mucosa by acid and bile which damage esophageal mucosa by inducing the formation of reactive oxygen species and nitric oxide. These species lead to DNA damage with a characteristic mutational signature of A>C transversions. These transversions are commonly found both in Barrett's esophagus and EAC, meaning that DNA damage contributes to the disease pathogenesis at early precancer stages. Two most probable mechanisms of Barret's mucosa progression to EAC have emerged, with loss of p53 function being an early event in both cases. The first mechanism involves a stepwise loss of tumor suppressor genes, such as *CDKN2A* and *TP53*, as well as mutations in *SMAD4* and the disruption of chromatin modeling pathway, but without major amplifications in the genome. The second one involves acquisition of large-scale chromosomal instability that is associated with aneuploidy following loss of p53 function. Indeed, loss of heterozygosity (LOH) of 17p (which contains *TP53*) in Barrett's esophagus has been associated with the development of aneuploidy and with an increased potential for malignant progression.

Overall, *TP53* and *CDKN2A* are the two genes that are most frequently altered in both ESCC and EAC. Inactivation of these two tumor suppressors is an early event in tumor progression for both tumor types and can be detected in a large proportion of precancerous lesions. Alterations in numerous other genes have also been identified, with different proportions between ESCC and EAC (**Table 2**). Accumulation of all these alterations results in a substantial deregulation of multiple cellular pathways. It also opens the door to screening and early detection strategies based on molecular analyses as well as to molecular therapies targeting specifically the altered genes and pathways (**Fig. 2**). Interestingly, a recent TCGA study has identified three molecular subtypes of ESCC correlated with some clinical parameters.

Table 2 Gene loci that are most frequently altered in esophageal carcinoma

Pathway	*Gene (locus)*	*Alteration type*	*Prevalence*
Cell-cycle control	*TP53* (17p13.1)	Point mutations, small insertion or deletions, and LOH of 17p	Inactivation occurs early in both EAC and ESCC development (alterations frequently detectable in precursor lesions)
	CDKN2A (9p21.3)	Deletions, epigenetic silencing (hypermethylation), and infrequent point mutations	76% of both ESCC and EAC cases; mostly deletions in ESCC, hypermethylation most common in EAC (detectable in Barrett's mucosa)
	CCND1 (11q13.3)	Amplifications	57% of ESCC and 15% of EAC cases
	CDK6 (7q21.2)	Amplifications	16% of ESCC and 14% of EAC cases
	CCNE1 (19q12)	Amplifications	4% of ESCC and 14% of EAC cases
	RB1 (13q14.2)	Deletions	9% of ESCCs, not found in EAC
	MYC (8q24.21)	Amplifications	23% of ESCC and 32% of EAC cases
Cell differentiation	*GATA4* (8p23.1)	Amplifications	1% of ESCC and 19% of EAC cases
	GATA6 (18q11.2)	Amplifications	3% of ESCC and 21% of EAC cases
	NOTCH1 (9q34.3)	Mutations	4% of EAC and 17% of ESCC cases
	TP63 (3q28)	Amplifications	48% of ESCC and 11% of EAC cases
	SOX2 (3q26.33)	Amplifications	
Hippo–YAP pathway (organ size control)	*VGLL4* (3p25.3-p25.2)	Chromosomal deletions encompassing the locus	ESCC
Autophagy regulation	*ATG7* (3p25.3)	Chromosomal deletions encompassing the locus	ESCC
Chromatin remodeling	*ARID1A* (1p36.11)	Deletions and truncating mutations	1% of ESCC and 8% of EAC cases
	KDM6A (Xp11.3)	Deletions and rare truncating mutations	19% of ESCC and 4% of EAC cases
ERBB/P13K signaling (cell metabolism)	*ERBB2* (HER-2; 17q12)	Amplifications and point mutations	3% of ESCC (amplifications only) and 32% of EAC cases
	VEGFA (6p21.1)	Amplifications	3% of ESCC and 28% of EAC cases
	EGFR (7p11.2)	Amplifications and rare point mutations	19% of ESCC and 15% of EAC cases
	KRAS (12p12.1)	Mostly amplifications	7% of ESCC and 14% of EAC cases
	PIK3CA (3q26.32)	Point mutations	13% of ESCC and 3% of EAC cases
	FGFR1 (8p11.23)	Amplifications	12% of ESCC and 4% of EAC cases
Embryonic development	*SMAD4* (18q21.2)	Deletions	8% of ESCC and 24% of EAC cases

EAC, esophageal adenocarcinoma; *ESCC*, esophageal squamous cell carcinoma; *LOH*, loss of heterozygosity.

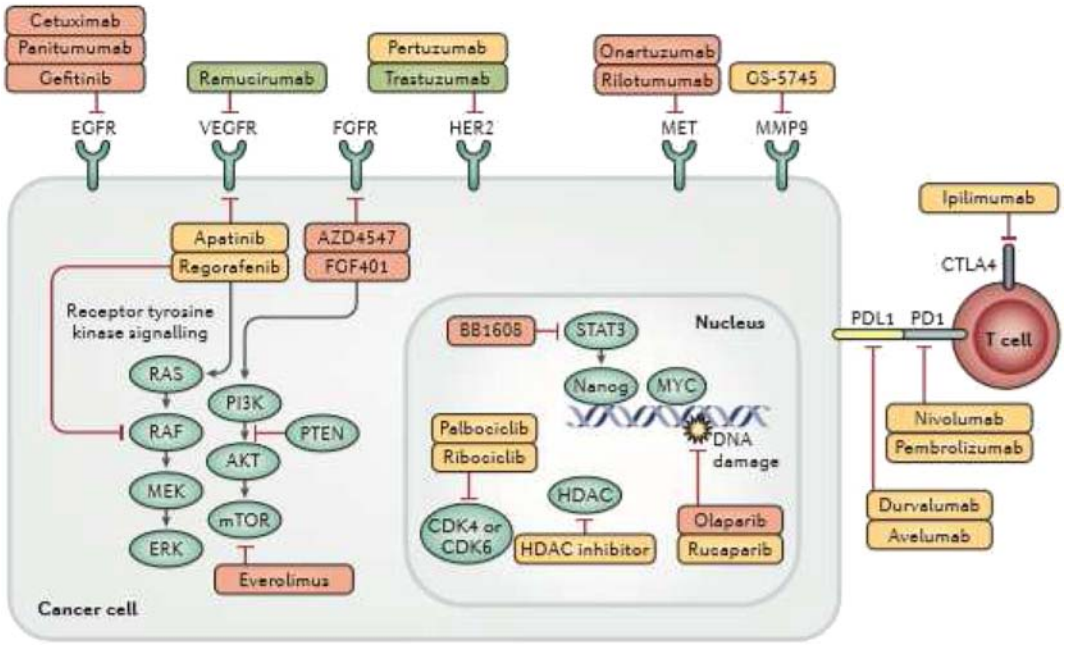

Fig. 2 Potential drug targets in esophageal cancer. Drugs shown in red have been tested in patients with esophageal cancer, without success. Drugs highlighted in yellow are currently being evaluated or could be evaluated on the basis of emerging data on active pathways in esophageal cancer. Regorafenib, apatinib, and nivolumab have improved the overall survival of patients with gastric cancer in randomized trials. At the time of publication, the only drugs that have achieved a survival advantage in patients with esophageal cancer in randomized trials with a control group are trastuzumab and ramucirumab (*in green*). CDK, cyclin-dependent kinase; CTLA4, cytotoxic T lymphocyte protein 4; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor 2; MEK, MAPK/ERK kinase; MMP9, matrix metalloproteinase 9; mTOR, mechanistic target of rapamycin; PD1, programmed cell death protein 1; PDL1, PD1 ligand 1; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue; STAT3, signal transducer and activator of transcription 3; VEGFR, vascular endothelial growth factor receptor. Reprinted with permission from: Smyth, E.C. et al. (2017). Oesophageal cancer. *Nature Reviews Disease Primers* **3**, 17048. https://doi.org/10.1038/nrdp.2017.48.

Biomarkers

Precursor lesions which predispose to esophageal cancer are well known and quite well described in molecular terms. However, screening for esophageal cancer is based on invasive endoscopy and identifying reliable noninvasive biomarkers which would allow to identify premalignant lesions that are likely to progress to malignant tumors remains an unmet need. Similarly, no diagnostic biomarkers exist other than standard techniques used to evaluate histological samples.

Generally, esophageal cancer is associated with poor survival rates. Complete cure is only possible in early-stage disease, with tumor stage, the presence and depth of invasion, lymph node involvement and the presence of metastases being adverse prognostic factors for both ESCC and EAC. In patients undergoing preoperative therapy, assessing pathological complete response (pCR) and histopathological tumor regression grade (TRG) following this treatment provides the most reliable prognostic information for both tumor types.

Recent molecular profiling studies have identified a number of potential molecular therapeutic targets for treatment of esophageal cancers (Fig. 2). Determining the molecular subtype of the tumor to predict its responsiveness to particular treatments is certainly the future of esophageal cancer management. However, as of now, few molecular analyses have been introduced into clinical practice. Among those, testing for the expression of human epidermal growth factor 2 (HER2) in EAC patients has long been standard practice. The prognostic significance of HER2 overexpression in esophageal cancer is not very clear, even though it has been shown to be correlated with tumor invasion and lymph node metastasis, and thus associated with poorer survival. However, HER2-positive tumors may be responsive to treatment with trastuzumab (an anti-HER2 antibody). Therefore, evaluating HER2 expression is recommended for advanced and metastatic tumors which cannot be cured by surgical resection. This is usually done by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). An IHC score of 3+ or 2+ with additional evidence of HER2 amplification by FISH is indicative of a possible trastuzumab treatment benefit.

Management and Therapy

Management of esophageal cancer depends on the tumor stage and localization as well as on the patient's performance status.

Early-stage tumors confined to the mucosa in patients with no evidence of metastasis may be treated by endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD), frequently combined with radiofrequency ablation, especially in patients with Barrett's mucosa. Both these procedures are associated with substantially lower complication rates than surgical resection while giving similar survival rates. For advanced and node-positive disease, a multimodality approach employing chemotherapy with radiation in the preoperative setting (trimodality therapy) is favored.

Surgical resection of the esophagus (esophagectomy) remains an important component of the treatment. However, it is associated with considerable morbidity and changes in postoperative life quality. Therefore, a careful selection of patients who meet the criteria for resection is essential to minimize the risk of futile surgery in patients with incurable disease. This includes assessing whether patients are medically fit (able to tolerate general anesthesia and a major thoracic or abdominal surgery) and staging the tumor. Laboratory studies include complete blood count (CBC) and comprehensive metabolic panel (CMP). Liver function studies should be performed in patients who abuse alcohol. Evaluating nutritional status is particularly important, and pretreatment nutritional support should be considered for all patients with significant dysphagia and weight loss. Enteral nutrition is usually the best option. According to the guidelines of the US National Comprehensive Cancer Network (NCCN), surgery should be considered for all physiologically fit patients with localized, resectable thoracic esophageal tumors more than 5 cm from cricopharyngeus as well as intra-abdominal esophageal or esophagogastric junction tumors, whereas cervical and cervicothoracic tumors less than 5 cm from cricopharyngeus should be treated with definitive chemoradiation. Lymph node dissection (lymphadenectomy) should also be performed. However, the optimal number of lymph nodes that should be removed depending on the treatment regimen is not clear.

The optimal surgical approach for radical resection of esophageal cancer is not defined and depends pretty much on the preference of the patient as well as on the experience and preferences of the surgeon. The two most common techniques are transhiatal esophagectomy and transthoracic esophagectomy. Recently, minimally invasive approaches have emerged as alternatives to open esophagectomy. These may be entirely minimally invasive procedures, or hybrid operations combining laparoscopy with open thoracotomy, or thoracoscopy with open laparotomy. Minimally invasive esophagectomy (MIE) offers potential advantages of smaller incisions, decreased intraoperative blood loss, fewer postoperative complications, and shorter hospital stays. However, the ability to obtain negative surgical margins, the adequacy of lymph node dissection, and long-term outcomes following MIE have not been fully assessed. Moreover, the optimal location of anastomosis is a subject of debate but a recent randomized trial has shown cervical and thoracic anastomoses to be equally safe when performed in a standardized way. Gastric conduit is preferred by a majority of surgeons for esophageal reconstruction.

Surgery alone is associated with poor survival rates even in patients in whom complete tumor resection is possible (5-year survival rates of 15%–20%). Therefore, combined regimens are usually applied. Most commonly, patients with resectable tumors are treated with preoperative chemoradiation, even though this approach remains investigational. So far, carboplatin and paclitaxel seem to be the best combination to this effect, in particular in EAC patients. Induction chemotherapy prior to preoperative chemoradiation may also provide important benefit to some patients. However, it has not yet been evaluated in phase III randomized trials.

Postoperative treatment depends on histology, surgical margins and nodal status. NCCN recommend no further treatment for ESCC patients with no residual disease at surgical margins (R0 resection). For EAC patients, perioperative chemotherapy is recommended in all cases of R0 resection, while postoperative fluoropyrimidine-based chemoradiation is recommended for most R0 patients who have not received a preoperative therapy.

Definitive chemoradiation is the preferred first-line treatment for patients who are either medically unfit for surgery or have advanced unresectable tumors, with fluoropyrimidine- or taxane-based regimens being the recommended options. For patients who are unable to tolerate chemotherapy or chemoradiation, palliative radiotherapy and best supportive care are the remaining options.

Palliating the obstructed esophagus and relieving the symptoms of dysphagia are particularly important for the quality of life of the patients. A log-term palliation of partially obstructed esophagus and associated dysphagia may be obtained by placing an expandable metallic stent or by radiation therapy (if the patient has disseminated disease or is not a candidate for surgery). Alternative methods of relieving dysphagia have been reported, including laser therapy and electrocoagulation to destroy intraluminal tumor. In case of complete esophageal obstruction in patients with no clinical evidence of systemic metastasis, palliative surgery (surgical excision of the tumor with mobilization of the stomach to replace the esophagus) is the most popular solution.

Much hope for treatment of esophageal cancer is placed in targeted molecular therapies (**Fig. 2**). Metastatic AECs which overexpress HER2-neu may be responsive to trastuzumab, an anti-HER2 monoclonal antibody. Combining trastuzumab treatment with cisplatin-fluoropirymidine chemotherapy has been shown to improve overall survival in gastric and esophagogastric junction cancer patients. As AEC has recently been shown to be molecularly similar to these cancers, the current consensus is to follow the same HER2-positivity criteria to select AEC patients for trastuzumab treatment which—in combination with cisplatin and fluoropyrimidine—is now considered to be the preferred first-line treatment for patients with HER2-overexpressing advanced or metastatic AEC. Similarly, ramucirumab, an antibody against vascular endothelial growth factor receptor (VEGF), may provide some benefit to AEC patients. Ramucimurab has been shown to improve overall survival in patients with gastric and esophagogastric junction cancer. It has been approved by the US Food and Drug Administration (FDA) for treatment of adenocarcinomas originating at these sites and refractory to or progressing after first-line treatment with platinum- or fluoropyrimidine-based chemotherapy, either as a single agent or in combination with paclitaxel. However, its utility for treating EAC patients awaits clinical trial evaluation. A novel epidermal growth factor receptor (EGFR) inhibitor, nimotuzumab, has shown promising results for treatment of locally

advanced EAC in combination with standard chemoradiotherapy in a phase II trial. Studies on immune checkpoint inhibitors, like pembrolizumab or nivolumab (PD1 inhibitors), have also given encouraging results. Finally, some new approaches, such as peptide vaccines and T-cell immunotherapies, may potentially offer some benefits in the treatment of this so-far extremely fatal cancer.

See also: Esophageal Cancer: Pathology and Genetics.

Further Reading

Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), 2010. WHO Classification of tumors of the digestive system, 4th edn. International Agency for Research on Cancer, Lyon, France.

Cancer Genome Atlas Research Network, 2017. Integrated genomic characterization of oesophageal carcinoma. Nature 541 (7636), 169–175.

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.

Cox, S.J., et al., 2017. Update on neoadjuvant regimens for patients with operable oesophageal/gastrooesophageal junction adenocarcinomas and squamous cell carcinomas. Current Oncology Reports 19 (1), 7.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Oesophageal and Oesophagogastric Junction Cancers. Version 4.2017. October 13, 2017: https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Smyth, E.C., et al., 2017. Oesophageal cancer. Nature Reviews Disease Primers 3, 17048.

Esophageal Cancer: Pathology and Genetics

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Glossary

Adenocarcinoma A malignant epithelial tumor with glandular differentiation.

Barrett's esophagus An acquired condition, in which a segment of normal squamous esophageal epithelium is replaced by a metaplastic columnar epithelium.

Dysplasia An unequivocal neoplastic lesion strictly limited to the epithelium (synonymous: intraepithelial neoplasia).

Squamous cell carcinoma A malignant epithelial tumor with squamous cell differentiation, microscopically characterized by keratinocyte-like cells with intercellular bridges and/or keratinization.

Definition and General Features

This group of neoplasms comprises all malignant tumors of the esophagus. There are two major histological types with entirely different risk factors, squamous cell carcinoma (SCC) and adenocarcinoma. All other tumor types are uncommon, including neuroendocrine neoplasms, mesenchymal tumors, lymphomas and secondary tumors; due to their rarity, they will not be presented in this chapter, which is only devoted to esophageal SCC and adenocarcinoma. The current World Health Organization (WHO) classification of esophageal tumors (fourth edition, 2010) is shown in Table 1, and the current TNM system of esophageal tumors (eighth edition, 2017) is shown in Table 2.

Esophageal cancer is the eighth most common cancer overall. In 2012, including SCC and adenocarcinoma, worldwide there are estimated to have been 456,000 new cases (3.2% of all incident cancer cases). About 80% of cases occur in less developed regions. The incidence in men is more than double that in women (male to female ratio 2.4:1). In both sexes, a striking feature is a major variability across the regions of the world, with 2012 incidence rates (per 100,000 population) ranging from 0.8 case in Western Africa to 17.0 cases in Eastern Asia in men, and from 0.2 case in Micronesia/Polynesia to 7.8 cases in Eastern Africa in women. The incidence of esophageal cancer is increasing. However, some features differ between the two major histological types, with a declining incidence for SCC and a major increase for adenocarcinoma.

Esophageal cancer is the sixth most common cause of cancer death, with an estimated 400,000 deaths in 2012 (4.9% of all cancer deaths).

In both histological types, recent advances have been made in endoscopy, allowing detection of early cancers and precancerous lesions. Although still a disease with dismal prognosis in most patients, advances in therapeutics including endoscopic resection, surgery, radiotherapy, chemotherapy, and targeted therapies have led to substantial improvements in clinical management and outcome. Recent basic research studies have improved knowledge in cellular and molecular mechanisms leading to esophageal malignant transformation.

Squamous Cell Carcinoma

Definition

The WHO defines esophageal SCC as a malignant primary epithelial tumor of the esophagus with squamous cell differentiation, microscopically characterized by keratinocyte-like cells with intercellular bridges and/or keratinization.

Burden, Epidemiology

Globally, the incidence of esophageal SCC is threefold higher in men than women, and approximately 80% of cases occur in developing countries. The incidence increases with age and peaks in the seventh decade. There is a hugely different frequency in various locations of the world and certain ethnic groups, with gradients of incidence between regions located in the same geographic area. The incidence is high in East Asia, Eastern and Southern Africa, and some parts of Europe. On the contrary, the incidence is low in North America and other parts of Europe. Although still the predominant type of esophageal cancer, the incidence of SCC is on the decline, both in Western countries and in Asia. In the United States, the incidence is higher in African Americans than in European Americans, and the rate for both groups declined during the past decades.

Risk Factors

The known risk factors for esophageal SCC are summarized in Table 3. Although the etiology of this tumor type is unknown, it is well established that tobacco smoking and alcohol drinking play a major role, but still several other factors have been recognized as

 https://doi.org/10.1016/B978-0-12-801238-3.65075-4

Table 1 WHO classification of tumors of the esophagus (fourth Edition, 2010)

Epithelial tumors
Premalignant lesions
Squamous
Intraepithelial neoplasia (dysplasia), low grade
Intraepithelial neoplasia (dysplasia), high grade
Glandular
Dysplasia (intraepithelial neoplasia), low grade
Dysplasia (intraepithelial neoplasia), high grade
Carcinoma
Squamous cell carcinoma
Adenocarcinoma
Adenoid cystic carcinoma
Adenosquamous carcinoma
Basaloid squamous cell carcinoma
Mucoepidermoid carcinoma
Spindle cell (squamous) carcinoma
Verrucous (squamous) carcinoma
Undifferentiated carcinoma
Neuroendocrine neoplasms
Neuroendocrine tumor (NET)
NET G1 (carcinoid)
NET G2
Neuroendocrine carcinoma (NEC)
Large cell NEC
Small cell NEC
Mixed adenoneuroendocrine carcinoma
Mesenchymal tumors
Granular cell tumor
Hemangioma
Leiomyoma
Lipoma
Gastrointestinal stromal tumor
Kaposi sarcoma
Leiomyosarcoma
Melanoma
Rhabdomyosarcoma
Synovial sarcoma
Lymphomas
Secondary tumors

Table 2 TNM classification of carcinoma of the esophagus (eighth Edition, 2017)

T—Primary tumor
T1: Tumor invades lamina propria, muscularis mucosa, or submucosa
T1a: Tumor invades lamina propria or muscularis mucosa
T1b: Tumor invades submucosa
T2: Tumor invades muscularis propria
T3: Tumor invades adventitia
T4: Tumor invades adjacent structures
T4a: Tumor invades pleura, pericardium, azygos vein, diaphragm, or peritoneum
T4b: Tumor invades other adjacent structures such as aorta, vertebral body, or trachea
N—Regional lymph nodes
NX: Regional lymph nodes cannot be assessed
N0: No regional lymph node metastasis
N1: Metastasis in 1 to 2 regional lymph nodes
N2: Metastasis in 3 to 6 regional lymph nodes
N3: Metastasis in 7 or more regional lymph nodes
M—Distant metastasis
M0: No distant metastasis
M1:Distant metastasis

Table 3 Risk factors for esophageal squamous cell carcinoma

Tobacco smoking
Alcohol consumption
Tobacco and alcohol: independent effects, but synergistic interaction
Thermal irritation by hot beverages and food
Physical irritation due to loss of teeth
Dietary factors
Low intake of fruits and vegetables
Carcinogens. e.g., nitrosamines, polyaromatic hydrocarbures
Betel quid chewing
Extremely high salt intake
High-risk HPV (16 and 18) *suspect, still not proven*
Ionizing radiation
Plummer Vinson syndrome
Achalasia
Celiac disease

potentially responsible, that may differ between geographical areas and may act synergistically. These factors include nutritional/dietary factors, and probably genetic factors. In low incidence regions the use of alcohol and tobacco plays a major role, with a strong male predominance, while in high incidence areas, nutritional factors are on the lead, with a male to female ratio close to 1.

Alcohol

Alcohol consumption is a major risk factor for esophageal SCC. Ingested alcohol is absorbed from the upper GI tract and transported to the liver where it is metabolized to acetaldehyde by alcohol dehydrogenase 1B (ADH1B). Acetaldehyde is then detoxified to acetic acid by ALDH2. The high amount of acetaldehyde in certain types of alcoholic beverages may explain why these beverages incur a higher risk of SCC. Numerous studies support the mutagenic and carcinogenic effects of acetaldehyde. Among the mechanisms that have emerged from experimental and epidemiological studies, the formation of DNA adducts and ALDH2 polymorphisms could account for the higher incidence of this tumor type in Asian versus Western countries.

Tobacco

Tobacco smoking is another major risk factor for esophageal SCC. The relative risk in heavy smokers is four to eight times that observed in nonsmokers. A dose-risk relationship exists for smoking duration and average consumption. Stopping smoking reduces the risk, with a relative risk approaching that of never smokers 10–20 years after cessation. Chewing tobacco-containing products is an important risk factor in some parts of the world. Tobacco smoke contains carcinogenic substances such as nitrosamines, which are directly in contact with the esophageal epithelium. Tobacco smoke also contains the carcinogen acetaldehyde, which could explain the synergistic effect of both carcinogens (alcohol and tobacco) in people with some specific polymorphisms in ADH1B and ALDH2.

Taken together, tobacco smoking and alcohol consumption account for more than 90% of cases of esophageal SCC in Western Europe and North America. However, this proportion is lower in developing countries, especially in regions of Asia and South America with a very high risk, suggesting other risk factors in these areas.

Dietary/nutritional factors

The risk of esophageal SCC is reduced by a diet rich in fruit and vegetables, and increased by a high intake in meat. Deficiencies of various micronutrients are common in regions with a high incidence of SCC, including selenium, zinc, calcium, vitamin A, and vitamin C among others. However, large chemoprevention trials and meta-analyses have failed to firmly demonstrate their responsibility. In high-risk areas of China, eating of pickled vegetables increases the risk of esophageal SCC. There is also an association between SCC and consumption of hot foods and drinks. Contamination of food by fungi is frequent in high-incidence regions of China, and may play a role.

Other risk factors

Numerous studies have focused on the role of human papilloma viruses (HPV) in esophageal squamous carcinogenesis. However, due to the marked differences in the association between esophageal SCC and HPV infection, a role of this infection is still considered as unproven.

Other associated conditions include ionizing radiation, caustic esophagitis, achalasia, esophageal diverticula, and Plummer-Vinson syndrome. This latter syndrome can be secondary to celiac disease induced anemia, which may explain the increased risk observed in celiac patients.

Precancerous Lesions

As in many epithelial cancers, the development of esophageal SCC is a multistep process, going from normal epithelium to invasive carcinoma through dysplasia (or intraepithelial neoplasia, a synonymous term, favored in the Far East).

Before the development of morphologically recognizable dysplasia, a stage of chronic esophagitis with basal cell hyperplasia has been described, especially in populations with a high incidence of esophageal SCC. This lesion manifests as white patches on endoscopy, which do not stain with lugol iodine. However, the precise role of this lesion in the development of esophageal SCC is still debated in the literature.

Dysplasia is defined as an unequivocal neoplastic lesion strictly limited to the epithelium (intraepithelial). This definition applies both in squamous and glandular epithelium.

Squamous dysplasia is asymptomatic, and on endoscopy it has a wide variety of aspects, from normal mucosa to erythematous or plaque-like lesions. The endoscopic diagnosis is sensitized by the application of lugol iodine solution. Endoscopic screening is not applicable in the general population. In high-risk areas, balloon cytology has been proposed as a screening test. In low-risk areas, endoscopic screening can be proposed to selected high-risk populations, such as patients with head and neck SCC.

Histologically, as in other epithelium, squamous dysplasia shows both architectural and cytological abnormalities. Architectural abnormalities include cellular disorganization with a disorderly proliferation of abnormal cells. Cytological abnormalities include hyperchromasia, increased mitoses, and an increased nucleus-cytoplasm ratio. A two grades classification is recommended, in which low-grade dysplasia shows abnormalities confined to the lower half of the epithelium and high-grade dysplasia has abnormal cells involving the upper half of the epithelium (**Fig. 1**). Carcinoma in situ constitutes the upper end of the spectrum of dysplastic lesions, and is difficult to distinguish from high grade dysplasia. Dysplastic cells may extend laterally in a Pagetoid fashion or deeply into the ducts of the submucosal esophageal glands.

The most frequent and crucial differential diagnosis is a regenerative process secondary to inflammation, due to reflux in most cases. The only immunohistochemical marker that can help distinguishing dysplasia from regeneration is p53 protein, as p53 abnormal expression is observed in approximately 80% of high-grade dysplasia and cancer. In case of severe inflammation, the diagnosis of dysplasia has to be made with caution. In such cases, a diagnosis of epithelium "indefinite for dysplasia" may be appropriate.

Macroscopy

Esophageal SCC is localized most often in the middle third, then in the lower and upper third, respectively. The gross appearance differs between superficial (early) cancers limited to the mucosa and submucosa, and advanced cancers. The Japanese classification of superficial cancers (type 0) comprises three types: a protruding type (0-I), a flat type (0-II) subclassified in 0-IIa (slightly elevated), 0-IIB (completely flat), 0-IIc (slightly depressed), and an ulcerated type (0-III). Advanced SCC may be exophytic, ulcerating or infiltrating, with often a mixture of these different aspects. In patients treated preoperatively with radio- and/or chemotherapy, the tumor may show a wide spectrum of changes. In case of complete response, this results to an esophageal scar with frequent partially re-epithelialized ulcer.

Microscopy

By definition esophageal SCC is a squamous neoplasm that penetrates the epithelial basement membrane and invades at least the lamina propria, and often deeper layers of the esophageal wall. In most cases the tumor shows the classical features of squamous carcinoma, with varying grades of differentiation, from well-differentiated mature keratinizing neoplasms to undifferentiated tumors. The WHO classification grades esophageal SCC in four groups of differentiation: well differentiated carcinoma with prominent keratinization showing squamous pearl formation (**Fig. 2**)—moderately differentiated, the most common histological type

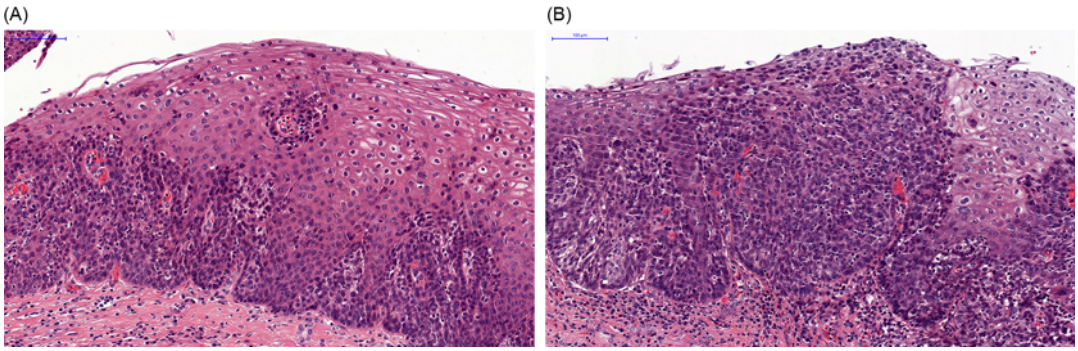

Fig. 1 Esophageal squamous dysplasia. In low-grade dysplasia, abnormal cells remain in the lower half of the epithelium (A). In high-grade dysplasia, the entire thickness of the epithelium is composed of neoplastic cells (B).

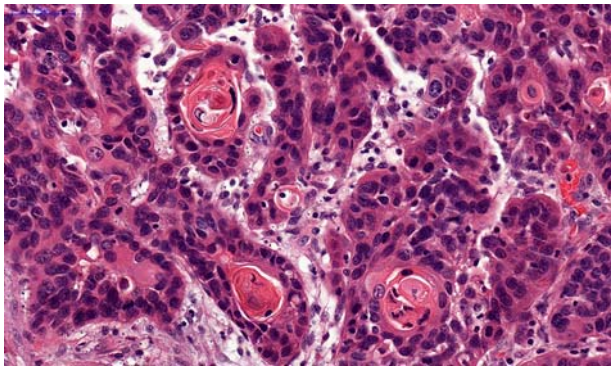

Fig. 2 Well-differentiated squamous cell carcinoma of the esophagus, with a trabecular proliferation of squamous cells showing prominent keratin pearl formation.

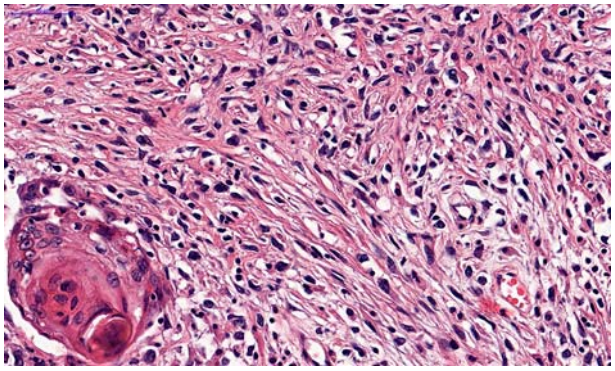

Fig. 3 Sarcomatoid variant of squamous cell carcinoma of the esophagus, with prominent "sarcomatous" cells and a focal epithelial squamous component on the lower left.

with variable signs of squamous differentiation but usually lacking pearl formation—poorly differentiated with sheets of basal-like cells and occasional parakeratotic cells—undifferentiated with no features of squamous lineage, confirmed by immunohistochemistry that also shows negative neuroendocrine markers. Variation of differentiation in different parts of the tumor is a common feature, and even focal adenocarcinomatous differentiation can be seen in a proportion of cases of the lower third of the esophagus. However, such cases are considered as SCC in terms of grading, treatment, and prognosis.

Three histological variants are recognized in the WHO classification:

- Verrucous carcinoma is extremely rare, and usually presents as a large papillary or warty tumor, often localized in the upper third of the esophagus. On histological examination, it is a highly differentiated tumor, which may be difficult to diagnose as a malignant neoplasm on a biopsy. This tumor has a slow local growth with locoregional invasion but rare metastases.
- Spindle cell carcinoma, also called sarcomatoid carcinoma, is an uncommon type of esophageal SCC. Macroscopically it is a large polypoid mass, often located in the middle and lower esophagus. On histological examination, it is a biphasic tumor, with prominent pleomorphic spindle and stellate cells and an epithelial squamous component that may be focal, localized in tiny zones at the base of the polypoid mass (Fig. 3). Hematogenous spread is common in this tumor type, but the overall prognosis is similar to that of classical SCC.
- Basaloid SCC is a relatively uncommon variant of squamous cancer, similar to tumors that occur more commonly in the upper aerodigestive tract. It is more common in elderly men, and presents in most cases as an advanced lesions of the middle or lower third of the esophagus. On histology, it is composed of basaloid cells arranged in solid or in cribriform lobules (Fig. 4). Areas of conventional SCC are often present. Basaloid carcinoma has to be distinguished from adenoid cystic carcinoma, which is less aggressive.

Molecular Pathology and Genetics

Molecular pathology

Several recent studies have used high-throughput next generation sequencing (NGS) to characterize molecular features of esophageal cancer. They confirm that, similarly to epidemiological and histopathological features, molecular alterations clearly

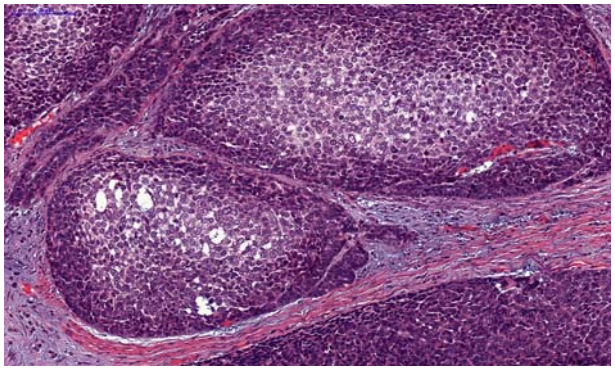

Fig. 4 Basaloid variant of squamous cell carcinoma of the esophagus composed of basaloid cells arranged in large lobules.

differentiate esophageal SCC from esophageal adenocarcinoma. The former resembles SCC from other organs (lung, head and neck…) more than it does esophageal adenocarcinoma.

Whole exome sequencing and whole genome sequencing analyses of esophageal SCC have confirmed the frequency of mutations in several genes: *TP53*, *CCND1*, *CDKN2A*, *FHIT*, *NOTHCH1*, *PIK3A*, *KMT2D*, *NFE2L2* among others. Mutation in the *TP53* gene is the most frequent event, present in 70%–80% of the cases, followed by amplification of *CCND1* in 20%–40%, and inactivation of *CDKN2A*. *TP53* pattern of mutation differs from that observed in lung SCC and other tobacco-associated cancers.

Precancerous lesions (dysplasia—intraepithelial neoplasia) show the same repertoire and frequency of mutations and copy number alterations that invasive SCC, suggesting that these events occur early during the transformation of esophageal squamous epithelium. However, basal cell hyperplasia, frequently observed in high-risk areas, harbors few somatic alterations compared to intraepithelial neoplasia and SCC, which suggests that it is an adaptive nonneoplastic response.

The major signaling pathways dysregulated in esophageal SCC include the Notch pathway, cell adhesion, PIK3CA pathway, *TP53*, Hedgehog pathway, chromatin remodeling, and the MAPK pathway. An APOBEC signature is predominant in ESCC individuals in China. However, differences in pathways involved are observed between geographical populations, suggesting a role for environmental factors, such as tobacco smoking or chewing. Limited molecular data are available regarding HPV, but they do not support an important etiological role for HPV in esophageal SCC.

Genetics

Tylosis esophageal cancer (TOC) is the only hereditary disease with a strong susceptibility to esophageal SCC. This autosomal dominant syndrome is characterized by palmoplantar keratoderma, oral and esophageal leukoplakia, and esophageal SCC. The causative gene is *RHBDF2*, coding for a rhomboid protease involved in EGFR signaling.

Polymorphisms in enzymes involved in alcohol and tobacco metabolism are responsible for increased susceptibility to these carcinogens. Among these enzymes, *ALDH2*2* variant allele increases the risk of esophageal SCC in alcoholics, and some CYP450s and GSTM1 variants increase the risk in heavy smokers.

Staging and Grading

As in many tumor types, grading of esophageal SCC is based on mitotic activity, nuclear atypia, and more importantly degree of squamous differentiation. Those differentiation criteria have been described on resection specimens, but they can also be used on endoscopic biopsies. In heterogeneous tumors, the overall grade is assigned based on the foci with the highest grade within the specimen. The grading can be more difficult in patients operated after neoadjuvant chemotherapy or chemoradiotherapy, in which the residual tumor cells may be dispersed as single, atypical cells within the esophageal wall. In such cases, the cancer may be upstaged inaccurately to poorly differentiated carcinoma.

The natural history of esophageal SCC goes from a "superficial" cancer, limited to the mucosa or submucosa, with or without lymph node metastases, to deep esophageal cancer. After invading the esophageal wall, locally advanced lesions may involve adjacent structures such as the trachea, aorta and pericardium. The risk of lymph node metastasis increases with the depth of invasion, but is already as high as 30%–40% in tumors that invade the submucosa.

The TNM (tumor–node–metastasis) staging system proposed by the UICC–AJCC (American Joint Committee on Cancer) has been revised recently, resulting in the eighth edition, with only minimal changes in the esophagus compared to the previous version. The staging criteria are presented in **Table 2**. Although the prognosis differs between SCC and adenocarcinoma, the TNM categories are identical in both tumor types. Those TNM categories allow determining the anatomic pathologic stage groups (from stage IA to stage IVB), identical in SCC and adenocarcinomas. The TNM system also provides pathological prognostic groups that take into account tumor grade and location in the esophagus in supplement to TNM categories, and differ between SCC and adenocarcinoma (**Fig. 5**).

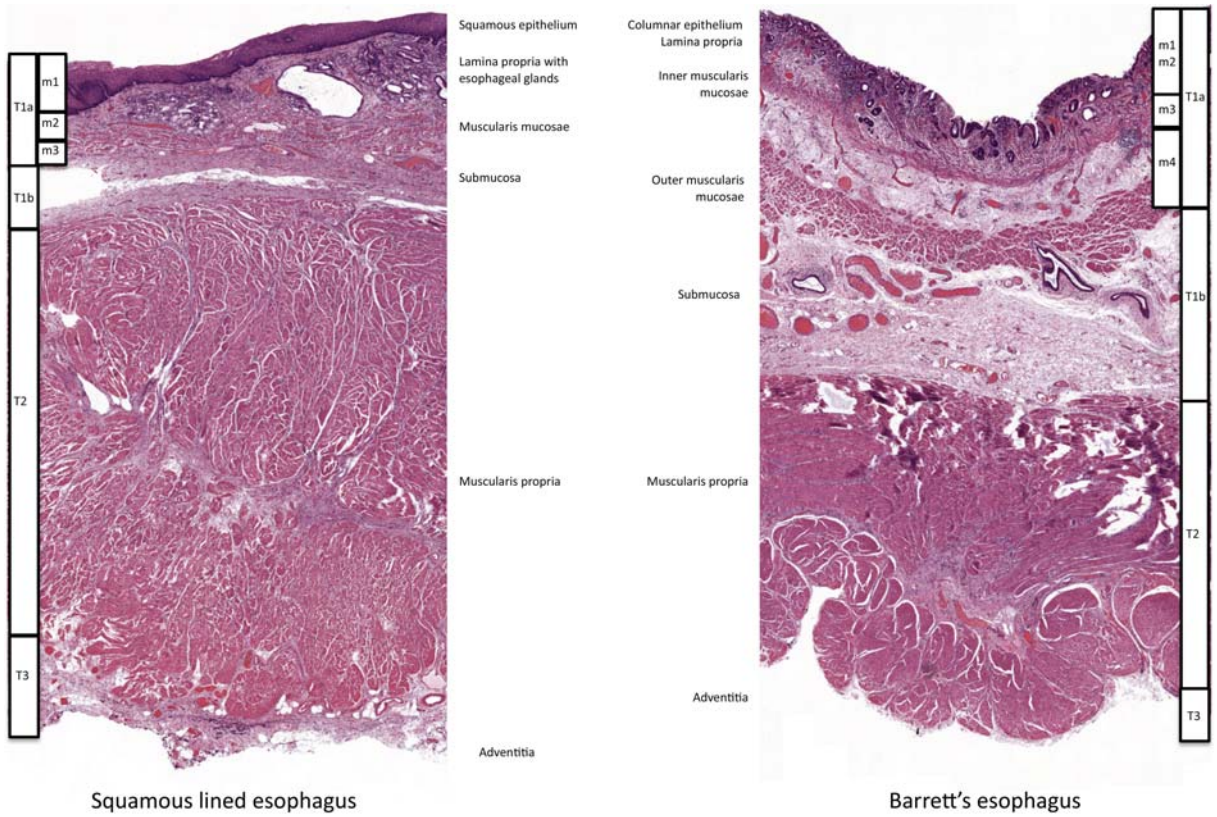

Fig. 5 T stages of esophageal carcinoma in the TNM classification (eighth edition), comparing squamous cell carcinomas and adenocarcinomas developed in Barrett's esophagus.

In superficial SCC, the intraepithelial (Tis) and mucosal (T1a) cancers can be divided into m1 (epithelium—Tis), m2 (lamina propria T1a), or m3 (muscularis mucosae T1a), and the submucosal cancers (T1b) into cases invading the inner (sm1), middle (sm2), and outer (sm3) thirds of the submucosa. In early tumors treated with endoscopic resection, it is recommended to estimate the submucosal invasion by the depth of vertical infiltration, ideally limited to 200 μm or less from the muscularis mucosae.

Neoadjuvant therapy induces major changes that can render the ypTNM staging more difficult. The residual cancer is admixed with fibrosis and elastosis. These changes have to be estimated semiquantitatively. The TRG (tumor regression grade) described by Mandard et al. is still the most widely used system, classifying cancers from TRG5 (absence of regressive changes) to TRG1 (absence of residual cancer).

Prognostic and Predictive Biomarkers

The overall prognosis for esophageal SCC is poor, with a global 5-year survival rate at about 10%. Besides pathological and molecular factors, the prognosis is influenced by age and gender, nutritional status; together with the stage of the tumor disease, these criteria are decisive to decide the therapeutic scheme in individual patients. The squamous phenotype of the tumor is by itself a negative prognostic factor, as the prognosis of SCC is less favorable than that of adenocarcinoma, with higher postoperative mortality, and more frequent lymph node metastasis even in early-stage tumors.

Morphological factors

The most important prognostic factor is tumor stage. The depth of invasion and the presence of nodal or distant metastasis are independent predictors of survival. Any lymph node involvement indicates poor prognosis, even in tumors limited to the esophageal wall (T1b or T2). The prognostic influence of TNM stage is demonstrated both in patients operated without (pTNM stage) or with (ypTNM stage) neoadjuvant treatment.

Additional prognostic factors recommended by the AJCC include:

- Differentiation (four grades), although there is no consensus on its prognostic influence.
- Tumor site.
- Lymphatic and/or vascular invasion.

- Assessment of the surgical margin (R category in the TNM system), a positive microscopic margin (R1) being correlated with the risk of locoregional recurrence.

Molecular factors

A number of studies have indicated a prognostic influence of various molecular alterations in esophageal SCC, including *TP53*, cyclin D1, cyclin B1, EGFR, HER2, TGFa, VEGF, E-cadherin. Until now, none of these factors is used in clinical practice, neither for therapeutic decision making nor for prognostic evaluation. However, it might be that results of large-scale genomic studies of esophageal SCC will provide indications of the efficacy of new-targeted anticancerous drugs that are currently tested in many tumor types, including esophageal cancer.

There is no biomarker that is either available for early detection of cancer or for prediction of the efficacy of neoadjuvant or adjuvant therapy.

Esophageal Adenocarcinoma

Definition

Esophageal adenocarcinoma is a malignant epithelial tumor of the esophagus with glandular differentiation. In the vast majority of cases it develops from acquired columnar metaplasia of the esophagus, the so-called Barrett's esophagus (BE). Much more rarely, adenocarcinoma originates from heterotopic gastric mucosa in the upper esophagus, or from mucosal and submucosal esophageal glands.

Burden, Epidemiology

The most striking feature of esophageal adenocarcinoma is its rapid increase in frequency in many Western countries, including Europe, North America, and Australia. Esophageal adenocarcinoma is now the main histological type of esophageal cancer in the West, with more than 18,000 cases in 2014 in the United States. However, in 2012, there were approximately 52,000 incident cases of esophageal adenocarcinoma worldwide, to be compared to 400,000 cases of SCC. Therefore, the major type of esophageal cancer remains SCC, due to its very high frequency in Asia and some parts of Africa. In the United States and several European countries, it is estimated that the incidence of esophageal adenocarcinoma doubled between the early 1970s and late 1980s, and continued to increase at a rate of about 5% per year, although the increase seemed to have slowed down to 2.6% per year from year 2000 onwards. Esophageal adenocarcinoma is most common in industrialized countries with populations of predominantly European ancestry. The countries with the highest incidence are the United Kingdom, Australia, the Netherlands, and the United States. Cases of esophageal adenocarcinoma are rare in Asia and Africa. In the United States, the incidence is higher in non-Hispanic whites, followed by Hispanic whites, American Indian natives, Blacks, and lowest in Asian.

This increase in esophageal adenocarcinoma is paralleled by rising rates of adenocarcinoma of the esophago-gastric junction and of the proximal stomach. Interestingly, esophageal adenocarcinoma and adenocarcinoma of the esophago-gastric junction share most epidemiological characteristics, including a high male: female ratio (between 4:1 and 7:1), and a high incidence among Whites and groups with higher socioeconomic status. As for most epithelial cancers, esophageal adenocarcinoma incidence increases with age.

Risk Factors

Gastro-esophageal reflux disease and Barrett's esophagus

Gastro-esophageal reflux disease (GERD) is the most important risk factor for esophageal adenocarcinoma. The relative risk of adenocarcinoma in persons with heartburn for at least 30 years is 6.2-fold higher than in persons without heartburn (Table 4).

Table 4 Risk factors for esophageal adenocarcinoma

Gastro-esophageal reflux disease (GERD)
Barrett's esophagus
Tobacco smoking
H. pylori (protective role)
Overweight and obesity
Dietary factors
High intake of vegetables (protective role)
Medications use
Anticholinergic drugs
Aspirin and NSAIDS (protective)

In predisposed people, GERD induces erosive esophagitis, followed by an abnormal healing process resulting in Barrett's epithelium, a metaplastic, so-called "specialized" epithelium. The relative risk of esophageal adenocarcinoma in patients with BE is in the order of 30–60. However, BE progresses to adenocarcinoma in only a small percentage of patients, at a rate of approximately 0.12% to 0.60% per year, resulting in a lifetime risk of adenocarcinoma in patients with BE between 2.7% and 10%. Factors predisposing for the development of BE and esophageal adenocarcinoma include increased duration of esophageal exposure to refluxed gastric and duodenal content (a "mixed" reflux).

Tobacco smoking

Tobacco smoking is a well-established moderately strong risk factor for esophageal adenocarcinoma, with ever smoking conferring a doubled risk of adenocarcinoma compared with never smoking. There is dose–response association between pack-years of smoking and adenocarcinoma risk. Although smoking cessation appears to reduce the risk of adenocarcinoma, this risk in former smokers does not return to the level observed in never-smokers.

Alcohol consumption

Although alcohol is a risk factor for the development of many cancers, including esophageal SCC, the association does not apply to esophageal adenocarcinoma. It is conceivable that individuals with GERD reduce alcohol consumption.

Overweight and obesity

Increasing body mass index has been consistently associated with increased risk of esophageal adenocarcinoma in a linear exposure-response pattern. In meta-analyses, the risk of adenocarcinoma was increased 2.4–2.8-fold among obese individuals, compared with individuals of normal weight. The increasing prevalence of obesity in Western populations may partially account for the increasing incidence of esophageal adenocarcinoma. The mechanism behind this association is not fully elucidated. This may be mechanical through intra-abdominal obesity, which might increase intra-abdominal pressure, promoting GERD and BE. Obesity, as a systemic condition, may also increase esophageal adenocarcinoma risk through inflammatory and metabolic alterations.

Dietary habits

Although many observational studies have associated dietary habits with the risk of esophageal adenocarcinoma, these findings have not been supported by subsequent randomized trials of dietary supplementation. Therefore, at present, for none of the dietary aspects evidence of an association with an increased risk of adenocarcinoma is judged strong, whereas only for vegetable intake limited evidence suggests a relation to a reduced risk of esophageal adenocarcinoma.

Helicobacter pylori

Infection with *H. pylori* appears to protect against esophageal adenocarcinoma. Observational studies have reported a 40%–60% reduced risk associated with *H. pylori* infection, especially with CagA positive strains. The mechanism of this reduction might include reduced volume and acidity of gastric juice, because of *H. pylori* induced atrophic gastritis. It has been proposed that the decreasing prevalence of *H. pylori* infection in Western populations may have contributed to the increasing incidence of esophageal adenocarcinoma.

Medications

The use of certain medications has been associated with an increased or decreased risk of esophageal adenocarcinoma. Medications that relax the lower esophageal sphincter—particularly anticholinergics, have been associated with an increased risk of esophageal adenocarcinoma. On the contrary, use of aspirin and nonsteroidal antiinflammatory drugs is associated with a decreased risk of adenocarcinoma, particularly when used daily and for a long duration. However, it is considered that there is insufficient evidence to use these drugs in esophageal adenocarcinoma prevention.

Precancerous Lesions

Barrett's esophagus

BE is an acquired condition, in which a segment of normal squamous esophageal epithelium is replaced by metaplastic columnar epithelium. It occurs secondary to GERD, and affects approximately 1%–5% of the general population. However, it is clinically silent in the majority of patients. Currently, the definition of BE requires both endoscopic and histological criteria. On endoscopy, there must be visible columnar mucosa proximally to the gastro-esophageal junction, extending more (long segment BE) or less (short segment BE) than 3 cm. The Prague C and M criteria are used to designate the circumferential extent (C) and the maximum extent (M) of Barrett metaplasia.

Histologically, biopsies taken from the endoscopically visualized columnar mucosa must show metaplastic glandular epithelium (Fig. 6). In the United States, the presence of intestinal metaplasia with goblet cells is mandatory, while in the United Kingdom the definition is broader, needing only a columnar epithelium on histology, which can be either cardia- or intestinal-type, or both. The Barrett epithelium usually contains a mixture of gastric foveolar and intestinal cells. Intestinal cells include goblet cells, "intermediate" columnar cells, endocrine cells, and less frequently Paneth cells. Intermediate cells have features of both absorptive and mucus secreting cells. A villiform architecture is common at the surface. In the deeper part of the mucosa, glands may be intestinal,

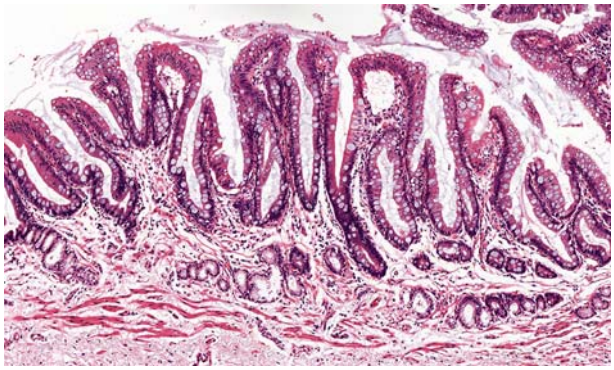

Fig. 6 Metaplastic intestinal Barrett's mucosa, with intermediate columnar cells and numerous goblet cells. Note the villiform pattern, and the clear pyloric type glands in the deeper part of the mucosa.

cardiac, oxyntic or a mixture of these types. The lamina propria shows variable numbers of inflammatory cells. The muscularis mucosa is almost always duplicated in Barrett's mucosa, an important feature to be aware of for correct staging of early adenocarcinoma developed in BE.

As intestinal differentiation is the pivotal criterion to diagnose Barrett epithelium, there has been much debate on the techniques that have to be used on mucosal biopsies. Indeed, goblet cells are usually easily identified on standard HE stained sections. Goblet cells contain acidic mucin, strongly stained with Alcian blue, making their recognition easier. However, there may be dilated pseudogoblet intermediate cells that also stain positively with Alcian blue. Another difficult diagnostic issue is to distinguish "ultrashort" segment BE from intestinal metaplasia of the gastric cardia. Some histological features may help to confirm the esophageal origin of the mucosa, including the presence of submucosal esophageal glands, squamous islands surrounded by columnar epithelium, hybrid glands showing both intestinal (in their upper part) and gastric (in their lower part) differentiation, multilayered epithelium, and duplicated muscularis mucosa. However, these features are inconstant, especially in superficial biopsy samples. In addition, numerous immunohistochemical markers have been proposed to ascertain a diagnosis of Barrett epithelium, including cytokeratins (CK7/CK20 pattern), Das-1, MUC2, CDX2. However, at present there is no recognized biomarker able to distinguish Barrett epithelium from intestinal metaplasia of the cardia.

Dysplasia—Intraepithelial neoplasia

As the major risk of patients with BE is to develop an esophageal adenocarcinoma, there has been considerable interest in defining a subgroup of high-risk patients in whom effective surveillance can be undertaken. Adenocarcinoma develops in BE through a classical multistep process with progression via dysplasia/intra-epithelial neoplasia toward invasive neoplasia. The risk of adenocarcinoma increases parallel to the severity of the dysplastic changes. At present, the morphologic identification of dysplasia in endoscopic mucosal biopsies is the standard method of detecting patients at increased risk for cancer and is used to delineate this population. The most widely used system to classify dysplastic changes in BE is the Vienna classification (**Table 5**), adapted from the Riddell's classification of dysplasia in inflammatory bowel disease; this classification can be used in every location of early type of columnar neoplasm of the tubal gut, including BE. On endoscopy, dysplasia is either undetectable, or visible as elevated, flat or depressed lesions. Endoscopic screening consists of systematic quadrant biopsies every other centimeter plus biopsies on every suspicious lesion, the so-called "Seattle protocol."

As for other epithelia, the diagnosis of dysplasia in BE relies on both cytological and architectural changes (**Fig. 7**). Architectural changes include glandular distortion and crowding. Papillary extensions into gland lumina may be present, and villiform configuration of the mucosal surface can be observed. Cytological changes include nuclear alterations such as variation in size and shape,

Table 5 Vienna classification of neoplasms of the gastrointestinal tract, including Barrett's neoplasia, with clinical implications

	Terminology in Vienna classification	*Clinical consequences in patients with Barrett's esophagus*
Category 1	Negative for dysplasia	Follow-up or no surveillance
Category 2	Indefinite for dysplasia	Follow-up. Reinforce medical treatment
Category 3	Low-grade dysplasia	Endoscopic treatment or reinforced follow-up
Category 4	4.1 High-grade dysplasia	Endoscopic or surgical treatment
	4.2 Noninvasive carcinoma (carcinoma in situ)	
	4.3 Suspicion of invasive carcinoma	
	4.4 Intramucosal carcinoma	
Category 5	Invasive neoplasia	*Surgical resection*
	Submucosal carcinoma or beyond	

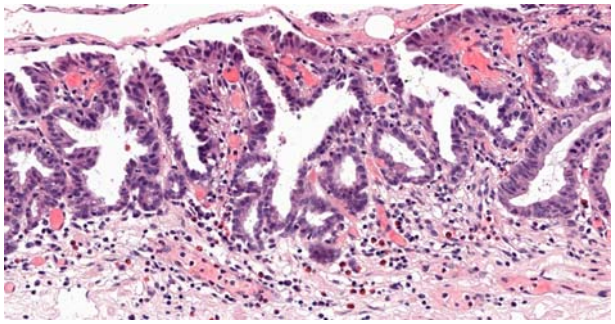

Fig. 7 High-grade dysplasia in Barrett's mucosa, with both cytologic and architectural abnormalities.

nuclear and/or nucleolar enlargement, increased nuclear-to-cytoplasmic ratio, hyperchromasia, and increased number of abnormal mitoses. In Barrett mucosa negative for dysplasia, although there may be reactive changes with crowded nuclei and numerous mitoses, the changes remain confined to the deeper parts of the glands. Dysplasia typically involves the surface of the mucosa. However, there are cases showing features of dysplasia that are confined to the deeper part of the mucosa, with a surface still appearing mature. Those changes are designed as crypt dysplasia with surface maturation, and are associated with an increased frequency of dysplasia or adenocarcinoma elsewhere in BE. In typical cases of dysplasia, the cytological changes involve the surface epithelium. Compared to low-grade dysplasia, in high-grade dysplasia cytologic atypia is more pronounced, and architectural changes are present, with branching of crypts and intraglandular epithelial budding, forming in the most severe cases a cribriform pattern. In such cases, it may be difficult to distinguish high-grade dysplasia (Vienna category 4.1) from intramucosal carcinoma (Vienna category 4.4), and one can make a diagnosis of high-grade dysplasia with suspicion of invasive carcinoma (Vienna category 4.3). The distinction between high-grade dysplasia and carcinoma in situ (Vienna category 4.2) is more theoretical than usable in practice. The category indefinite for dysplasia (Vienna category 2) can be used when histological features do not allow to make a distinction between reactive and neoplastic changes, especially when ulceration and inflammation are present. It does not correspond to a specific intercalated lesion between nondysplastic and dysplastic mucosa, but motivates the clinician to close endoscopic surveillance, with re-biopsy after proton pump inhibitor therapy.

Histological subtypes of dysplasia in BE have been described, that do not result in variation in surveillance protocols, but that can induce diagnostic difficulties. Adenomatous or intestinal dysplasia is the most common type. Dysplastic cylindrical cells have basophilic cytoplasm, and atypical, hyperchromatic and enlarged nuclei, with irregular nuclear membranes, prominent nucleoli, increased nuclear to cytoplasmic ratio, increased mitotic figures, marked nuclear pseudostratification and loss of nuclear polarity. Goblet cells and Paneth cells are scarce or completely absent. Foveolar (nonadenomatous) dysplasia is characterized by glands lined by cuboidal to columnar cells with pale clear to light eosinophilic cytoplasm, and round to oval nuclei. Mitoses are scarce and nuclear pseudostratification mild. Foveolar type dysplasia commonly expresses MUC5AC but is negative for markers of intestinal differentiation (MUC2, CDX2 and villin). An even less frequent serrated type of dysplasia has been described.

The diagnosis of dysplasia in BE remains a difficult issue, with major consequences for the patient. There is important interobserver variability, especially at the lower end of the spectrum (indefinite for dysplasia and low-grade dysplasia). For this reason, considerable attempts have been made to find markers able either to confirm a diagnosis of dysplasia, or to indicate independently an increased risk of malignant progression. In BE the situation is similar to that in squamous esophagus, with p53 protein as the only marker usable in daily practice. Abnormal expression of p53 occurs in a minority of low-grade dysplasia cases, and in the vast majority of cases of high-grade dysplasia and adenocarcinoma. Cases of low-grade dysplasia with p53 overexpression have a high risk of rapid progression to a high-grade or malignant lesion. Interestingly, in addition to this classical pattern of p53 overexpression, a p53 "absent" pattern can be observed, with a very high risk of rapid evolution to adenocarcinoma. These different patterns result from different *TP53* gene mutations, the most frequent molecular alteration in Barrett neoplastic mucosa.

Macroscopy

A large majority of adenocarcinomas arise in BE. Therefore, most cases are located in the distal part of the esophagus. However, in case of very long segment BE, the tumor can be situated more proximal, in rare cases even in the upper third of the esophagus. As for any carcinoma developing in the tubal gut, the tumor can present as a polypoid or fungating mass, a flat or depressed lesion, or an ulcerative and infiltrating lesion. Contrarily to what is observed in *linitis plastica* of the stomach, a diffusely infiltrative pattern is very uncommon. In typical cases, adjacent to the tumor, the salmon-pink Barrett mucosa may be evident, especially in early carcinomas. However, in large tumors, the metaplastic mucosa may be replaced entirely by the tumor. The tumor is usually limited to the esophagus, but large tumors of the distal part of BE can cross the gastroesophageal junction, becoming indistinguishable from gastric cardia adenocarcinoma (Siewert types I and II). Adenocarcinomas at an early stage can be difficult to recognize, almost invisible in some cases, or appearing as sessile flat or depressed lesions. Multifocal adenocarcinomas may be seen. When patients have surgical resection of their cancer after strong tumor response to neoadjuvant radio-chemotherapy, the lesion may appear as an

ulcerated and/or indurated scar, or can even entirely disappear. In such cases, the whole tumor bed has to be sampled for histological confirmation of the tumor response.

Rare adenocarcinomas do not develop from BE, but from heterotopic gastric patches and native esophageal glands. They display predominantly ulcerated and fungating gross features in the upper and middle esophagus.

Microscopy

Microscopic features of esophageal adenocarcinoma are similar to those of gastric adenocarcinoma. The majority of cases show a tubular or papillary pattern, corresponding to the intestinal type in Lauren's classification of gastric adenocarcinoma. Well or moderately differentiated tumors with glandular structures in more than 50% of the tumor are predominant (Fig. 8). Extremely well differentiated tumors can be very difficult to distinguish from high-grade dysplasia, especially on endoscopic samples of early neoplastic lesions. Mucinous adenocarcinomas with tumor cells floating in mucinous pools in more than 50% of the tumor can be seen (Fig. 9). Poorly differentiated infiltrative "signet ring cell" adenocarcinomas (Lauren's diffuse type of gastric cancer) are less common than in the stomach. Mixed cases showing both intestinal and diffuse histology can be seen occasionally. Pagetoid spread of malignant glandular cells into the surrounding squamous epithelium can be seen in poorly differentiated adenocarcinomas.

Tumors examined after neoadjuvant radio-chemotherapy often show major changes, with clusters of residual pleomorphic tumor cells embedded in dense fibrosis, and acellular pools of mucin. The amount of residual tumor tissue must be evaluated semi-quantitatively, as it has been shown to be a prognostic factor. However, there is no universal consensus for a particular grading system to evaluate this response.

Some histological variants of esophageal adenocarcinoma have been described:

- Adenosquamous carcinomas have discrete elements of adenocarcinoma and SCC, and mucoepidermoid carcinomas have an intimate mixture of these two components. Both types have a poor prognosis.
- Adenoid cystic carcinoma is also infrequent, and is believed to arise from esophageal glands. Its morphology is identical to the classical salivary gland tumor (Fig. 10). They can be confused with basaloid SCC, a much more aggressive tumor.
- Very rare cases of medullary carcinoma can be seen, with microsatellite instability involved in their development.
- Choriocarcinoma of the esophagus is a very uncommon tumor. It presents as a large fungating lesion with necrosis and hemorrhage. Syncitiotrophoblastic tissue and cytotrophoblastic tissue are present in the tumor. The prognosis is very poor.

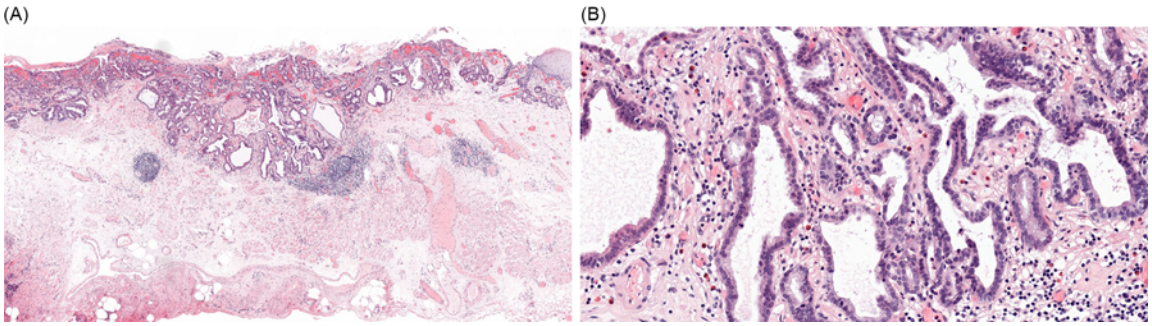

Fig. 8 Well-differentiated adenocarcinoma developed in a short segment Barrett's esophagus. The lesion has been removed by mucosectomy, and invades the mucosa between the two layers of muscularis mucosae (A). On high power, the tubulopapillary pattern can be seen (B).

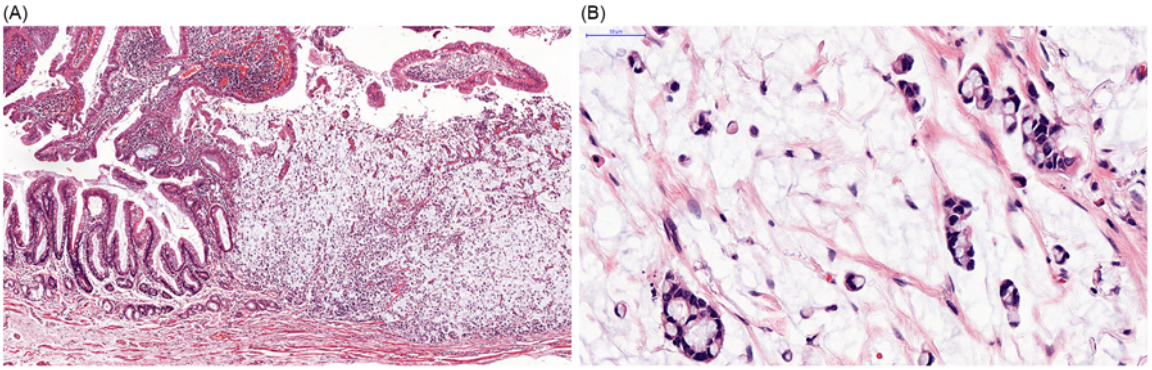

Fig. 9 Mucinous adenocarcinoma developed in Barrett's esophagus (A). On higher power, mucinous pools containing mucin-secreting cells are visible (B).

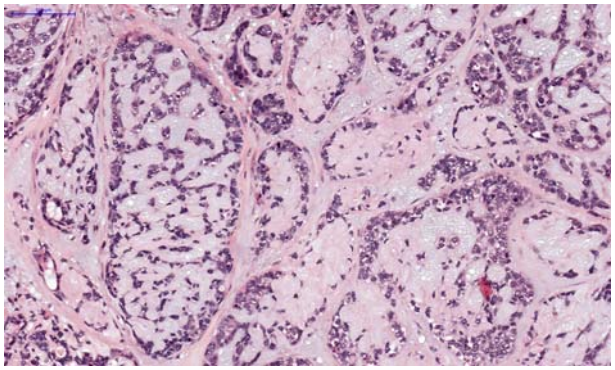

Fig. 10 Adenoid cystic adenocarcinoma of the esophagus, with a characteristic architectural pattern, similar to that observed in salivary glands.

- Poorly differentiated and undifferentiated carcinomas must be distinguished from small cell neuroendocrine carcinoma. In this latter type, there may be focal squamous or glandular differentiation. The diagnosis depends on the expression of neuroendocrine markers (chromogranin A, synaptophysin, CD56).

Molecular Pathology and Genetics

Molecular pathology

It has been known for a long time that BE shows increasing chromosomal instability as it progresses toward adenocarcinoma. Early founding studies, using DNA cytometry, fluorescent in situ hybridization, array comparative genomic hybridization, and *TP53* analyses, have been confirmed by more recent large scale genomic investigations, including those performed by international consortia such as TCGA (The Cancer Genome Atlas) and ICGC (International Cancer Genome Consortium). These studies have demonstrated that most esophageal adenocarcinomas have massive genomic alterations, including high frequency of mutations and chromosomal alterations. Different molecular signatures have been described in esophageal adenocarcinoma, with potential therapeutic relevance. In large whole-genome sequencing analyses, three distinct molecular subtypes have been identified: a BRCA signature with defects in homologous recombination pathway, T > G mutational pattern with a high mutational load and neoantigen burden, C > A/T mutational pattern with evidence of an aging imprint.

TP53 has been known for a long time as the only gene with a very high (70%–80%) rate of mutation in esophageal adenocarcinoma (70%–80%), and this has been confirmed by more recent analyses. Other less commonly altered genes include *CDKN2A* (encoding p16), *SMAD4*, *ARID1A*. High-level amplification of genes encoding receptors to tyrosine kinases are observed in subgroups of cancers, including amplifications of *ERBB2* (*HER2*), *EGFR*, *MET*, *FGFR*.

Among these various molecular alterations, only *TP53* gene mutation and the ensuing p53 protein modified expression (overexpression or more rarely complete loss of expression) can be used to strengthen a diagnosis of dysplasia, as this event occurs in few cases of low-grade dysplasia, and in high-grade dysplasia at the same frequency as in adenocarcinomas.

Genetics

Approximately 7% of cases of BE or esophageal adenocarcinoma occur within families. These familial cases develop at an earlier age than sporadic cases. It is considered that BE and esophageal adenocarcinoma have sizable heritable components, estimated around 35% and 25%, respectively. This heritability is conferred by a combination of many genetic factors that each increase risk by a small amount. Candidate gene studies and more recently genome wide association studies (GWAS) have associated loci with BE and EAC risk (*CRTC1*, *BARX1*, *FOXF1*, *FOXP1*, *GDF7*, *TBX5*). Almost all discovered genetic variants have been associated with both BE and esophageal adenocarcinoma, confirming the neoplastic sequence from Barrett's metaplasia to adenocarcinoma. Even if the massive increase in esophageal adenocarcinoma in Western countries cannot be explained by rapid changes in the population's genetic make-up, genetic predisposition might play a role in the variation of incidence of esophageal adenocarcinoma in different geographic areas.

Staging and Grading

Contrarily to esophageal SCC and to other digestive adenocarcinomas (gastric, colorectal), there is no precisely defined WHO grading system of differentiation for esophageal adenocarcinoma. However, the same rules can probably be applied as those used for gastric cancer. Most esophageal adenocarcinomas are well or moderately differentiated. Glandular structures predominate in well-differentiated cancers, are only slightly formed in poorly differentiated cancers, and are absent in undifferentiated cancers. On endoscopic biopsies, well-differentiated adenocarcinomas may be difficult to recognize as invasive and to distinguish from high-grade dysplasia. Similarly to many other carcinomas, the grading is more difficult when patients have surgical resection after neoadjuvant therapy.

The natural history goes from superficial cancer limited to the mucosa or submucosa to deeply infiltrative cancers. After invasion of the esophageal wall, adenocarcinomas behave in the same manner than SCC, extending into the adventitial tissue, and then into adjacent organs. Metastases appear in para-esophageal and paracardial lymph nodes, and then into lymph nodes of the lesser curvature of the stomach and celiac nodes.

The TNM classification used for esophageal SCC is applicable to adenocarcinoma, with identical categories of staging. However, the TNM prognostic groups slightly differ between SCC and adenocarcinoma, taking into account not only the tumor stage but also the tumor grade and location in the esophagus.

Importantly, among superficial cancers, tumors infiltrating between the inner and outer muscularis mucosae of BE remain intramucosal (T1a) although they have already a slightly increased frequency of angio-invasion and lymph node metastasis, compared to those limited to the inner muscularis mucosae. This peculiarity renders the subclassification of superficial cancers a little bit more complex in adenocarcinoma than in SCC. In particular, among intramucosal adenocarcinomas (T1a), a fourth m4 category has been added by some authors to designate adenocarcinomas invading between the two muscularis mucosae layers (Fig. 5).

Prognostic and Predictive Biomarkers

Although the prognosis of esophageal adenocarcinoma is slightly better than that of SCC, it is still a disease with a poor prognosis, with overall survival of 20% at 5 years.

Morphological factors

Tumor stage remains the major prognostic factor, with 5 years survival rates decreasing from more than 80%–90% in superficial cancers to 10%–20% for those that invade the muscularis propria. Metastasis to regional lymph nodes is the other powerful indicator. Histological type and grade are not considered as independent prognostic factors, although mucinous and signet ring cell carcinomas are associated with lower survival rates.

As for esophageal SCC, there is no consensus regarding the system that has to be applied for the evaluation of tumor regression after neoadjuvant therapy of esophageal adenocarcinoma. However, it is admitted that complete pathological response and absence of lymph node metastasis after neoadjuvant therapy are associated with good prognosis.

Molecular factors

The only molecular marker with recognized prognostic influence in esophageal adenocarcinoma is HER2 protein overexpression, due to HER2 gene amplification. HER2 expression is associated with lower tumor grade, fewer lymph node metastases, and improved survival. Moreover, HER2 overexpression renders the patient eligible to receive anti-HER2 therapy, a targeted therapy shown to improve survival in patients with HER2 positive tumors, both gastric adenocarcinomas and esophageal adenocarcinomas. Although the expression profile and mutational status of several other proteins and genes has been shown in some studies to affect prognosis in esophageal adenocarcinoma, including EGFR, VEGF, COX-2, none of these markers is recommended in routine practice for prognostic evaluation.

See also: Esophageal Cancer: Diagnosis and Treatment.

Further Reading

Castro, C., Peleteiro, B., Lunet, N., 2017. Modifiable factors and esophageal cancer: A systematic review of published meta-analyses. Journal of Gastroenterology 53, 37–51.

Coleman, H.G., Xie, S.H., Lagergren, J., 2017. The epidemiology of esophageal adenocarcinoma. Gastroenterology 154, 390–405.

Contino, G., Vaughan, T.L., Whiteman, D., Fitzgerald, R.C., 2017. The evolving genomic landscape of Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 153, 657–673.

Fléjou, J.F., Odze, R.D., Montgomery, E., et al., 2010. Adenocarcinoma of the esophagus. In: Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), WHO classification of tumors of the digestive system, 4th edn. International Agency for Research on Cancer, Lyon, pp. 25–31.

Montgomery, E., Field, J.K., Boffetta, P., et al., 2010. Squamous cell carcinoma of the esophagus. In: Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), WHO classification of tumors of the digestive system, 4th edn. International Agency for Research on Cancer, Lyon, pp. 18–24.

Ohashi, S., Miyamoto, S., Kikuchi, O., et al., 2015. Recent advances from basic and clinical studies of esophageal squamous cell carcinoma. Gastroenterology 149, 1700–1715.

Quante, M., Graham, T.A., Jansen, M., 2017. Insights into the pathophysiology of esophageal adenocarcinoma. Gastroenterology 154, 406–420.

Reid, B.J., Paulson, T.G., Xi, L., 2015. Genetic insights in Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 149, 1142–1152.

Rice, T.W., Ishwaran, H., Hofstetter, W.L., 2016. Recommendations for pathologic staging (pTNM) of cancer of the esophagus and esophagogastric junction for the 8th edition AJCC/UICC staging manuals. Diseases of the Esophagus 29, 897–905.

Rice, T.W., Kelsen, D., Blackstone, E.H., et al., 2017. Esophagus and esophagogastric junction. In: Amin, M.B., Edge, S., Green, F., et al. (Eds.), AJCC Cancer staging manual, 8th edn. Springer, Chicago, pp. 185–202.

Robertson, E.V., Jankowski, J.A., 2008. Genetics of gastroesophageal cancer: Paradigms, paradoxes, and prognostic utility. American Journal of Gastroenterology 103, 443–449.

Rubenstein, J.H., Shaheen, N.J., 2015. Epidemiology, diagnosis, and management of esophageal adenocarcinoma. Gastroenterology 149, 302–317.

Secrier, M., Li, X., de Silva, N., et al., 2016. Mutational signatures in esophageal adenocarcinoma define etiologically distinct subgroups with therapeutic relevance. Nature Genetics 48, 1131–1141.
The Cancer Genome Atlas Research Network, 2017. Integrated genomic characterization of oesophageal carcinoma. Nature 541, 169–175.
Yamamoto, Y., Wang, X., Bertrand, D., 2016. Mutational spectrum of Barrett's stem cells suggests paths to initiation of a precancerous lesion. Nature Communications 7, 10380. https://doi.org/10.1038/ncomms10380.

Relevant Websites

https://cancerstaging.org/—American Joint Committee on Cancer.
http://www.iarc.fr/—International Agency for Research on Cancer.
http://www.who.int/en/—World Health Organization.

Eye and Orbit Cancer: Pathology and Genetics

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Abbreviations

ACC Adenoid cystic carcinoma
ALT/WDL Atypical lipomatous tumor/well-differentiated liposarcoma
AJCC/TNM American Joint Committee on Cancer/Tumor Node and Metastasis
BCC Basal cell carcinoma
BAP1 BRCA1-associated protein-1
CGH Comparative genomic hybridisation
CNS Central nervous system
CNSL Central nervous system lymphoma
CSF Cerebrospinal fluid
CT Computed tomography
DLBCL Diffuse large B-cell lymphoma
DLBCL ABC Activated B-cell type
EBV Epstein-Barr virus
EMZL Extranodal marginal zone B-cell lymphoma
FISH Fluorescent in situ hybridization
FL Follicular lymphoma
FLIPI-2 Follicular Lymphoma International Prognostic Index-2
FNCLCC French Fédération Nationale des Centres de Lutte Contre le Cancer
GEP Gene expression profiling
HIV Human Immunodeficiency virus
HPV Human papilloma virus
IgH-PCR Immunoglobulin heavy chain polymerase chain reaction
LOH Loss of heterozygosity
MCC Merkel cell carcinoma
MCL Mantle cell lymphoma
MCPyV Merkel cell polyomavirus
MLPA Multiplex ligation-dependent probe amplification
MSA Microsatellite analysis
MYB Myeloblastosis gene marker
NHL Non-Hodgkin lymphoma
OAL Ocular adnexal lymphoma
PLAG1 Pleomorphic adenoma gene marker 1
RB Retinoblastoma
RMS Rhabdomyosarcoma
SCC Squamous cell carcinoma
SNPs Single nucleotide polymorphisms
UM Uveal melanoma
UV Ultraviolet
UVA Ultraviolet A
UVB Ultraviolet B
VHL Von Hippel Lindau
VRL Vitreoretinal lymphoma

Introduction

Although ophthalmic pathology specimens are rarely encountered by general pathologists, when it happens there tends to be a general sense of anxiety surrounding them. Hence, we have included a range of neoplasms that occur in and around the eye and which are regularly encountered by the ophthalmic pathologist, in an attempt to allay these fears. Biopsies from various ocular adnexal and intraocular sites, including incisional and excisional, ranging from tiny biopsies to local tumor resections, enucleation or orbital exenteration are received frequently for histopathological and molecular analysis. Like all specialist pathologists, ophthalmic pathologists are being sent samples of diminishing size with the challenge of obtaining as much (if not more!) information with respect to morphology, immunohistochemistry and molecular genetics from them. In this article, the basic aspects of each neoplasm—epidemiology, microscopical features, immunophenotype, genotype and typical clinical behaviour—are discussed. All ophthalmic malignancies described are staged according to the 8th edition of the AJCC TNM Staging system. Ophthalmic lymphomas are also staged using the Ann Arbor system. Carcinomas (except Merkel cell carcinoma) and retinoblastoma are graded into four categories based on its morphology and differentiation. Sarcomas are graded into three grades according to French Federation of Cancer Centers Sarcoma Group (FNCLCC). Similar to skin melanomas, no grading system is applied for to the conjunctival or intraocular (uveal) melanomas.

Tumors of the Eyelid

Basal Cell Carcinoma

Definition: Basal cell carcinoma (BCC) of the eyelid is a primary malignant epithelial tumor that originates by neoplastic transformation of the basal cells of the epidermis, which proliferate and invade into the underlying dermis.

Burden: BCC is the most common cancer in the world, with 80% occurring in the head and neck region, of which 20% involve the eyelids. BCCs represent 90% of malignant eyelid tumors. Of the eyelid BCCs, 50% arise on the lower lid, 30% in the medial canthus, 15% on the upper lid, and 5% in the lateral canthus. The typical age of onset is 60–80 years. There is a slight male preponderance. The incidence of BCC is higher in more equatorial latitudes. High incidence of BCC and a larger BCC size are two features that are associated with patients living in areas of socioeconomic deprivation.

Risk factors: The most important known risk factor of BCC is the intermittent intense exposure to ultraviolet (UV) radiation. Short-wavelength UVB radiation (290–320 nm, sunburn rays) plays a more important role in BCC formation than long-wavelength UVA radiation (320–400 nm, tanning rays). Other risk factors include sunbed use, family history of skin cancers, immunosuppression, previous radiotherapy, and chronic exposure to toxic substances.

Pathology: The histological types of BCC are solid (**Fig. 1**), nodular, nodular-ulcerative, morpheaform, infiltrative, cystic, nodular-cystic, basosquamous, and plexiform (adenoid pattern). The nodular and superficial types of BCC are the most common in the eyelid. The morpheaform, infiltrating and basosquamous subtypes of BCC are rare (only 5%–7% of all cutaneous BCCs). BCC usually presents as a single lesion with ulceration, or central scarring and peripheral keratosis, with even deep infiltration. BCCs with orbital invasion or aggressive histology occur more frequently in the medial canthus (53%–56%) compared to the lower eyelid (20%–35%), upper eyelid (4%–7%) or lateral canthus (3%–18%).

Molecular pathology and genetics: Mutations in the *TP53* tumor-suppressor gene induced by UV radiation have been found in about 50% of BCC cases. *Ptch-1* mutations promote the development of eyelid BCC. Long-term exposure to UV radiation may induce *Ptch-1* mutations and thus promote the development of BCC. The morpheaform type of BCC shows a high expression of Bcl-2 and moderate levels of proliferation markers (e.g., Ki-67) in a histological and immunohistochemical study of eyelid BCCs. The telomere length is shortened in BCC samples compared with normal tissues.

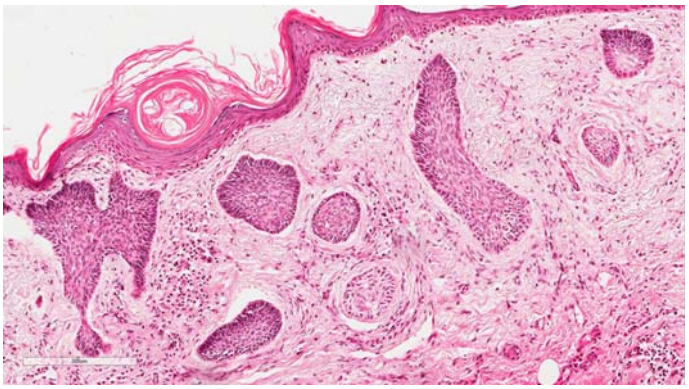

Fig. 1 BCC. Small nodules of basaloid cells infiltrate the dermis. Peripheral palisading and clefts are seen between tumor cells and the dermis. (H&E 10×).

Microenvironment including immune response: BCCs are typically characterized by a desmoplastic reaction with little inflammation, with the exception of those cases with central or extensive areas of ulceration, which can induce an acute inflammation.

Staging and grading: The Staging system used is the 8th edition of the AJCC TNM Staging system.

Grading of Eyelid BCCs: In four grades from well- to undifferentiated.

Prognostic parameters: The prognosis of a completely excised nodular BCC is excellent. Incomplete primary resection of an eyelid BCC is the main risk factor for recurrence. Incomplete resection is significantly associated with medial canthus location and morpheaform type of BCC.

Sebaceous Carcinoma

Definition: Sebaceous carcinoma of the eyelid is generally considered the second most common neoplasm of this anatomical region after BCC. Sebaceous carcinoma usually originates from the tarsus of the upper eyelid and less commonly from the lower lid, the Zeiss gland, or the sebaceous glands of the caruncle with secondary involvement of the conjunctival epithelium by pagetoid invasion.

Burden: Its incidence is about 5% of all malignant eyelid tumors. Mean age at diagnosis ranges from 57 to 72 years.

Risk factors: Advanced age, Asian race, and female gender. Muir–Torre syndrome, a form of the rare Lynch syndrome, is associated with sebaceous carcinomas.

Pathology: The pre-invasive form of sebaceous carcinoma is "in situ" disease. Three histopathologic patterns are seen in sebaceous carcinoma in situ: bowenoid, pagetoid, and papillary (**Fig. 2**). The bowenoid type is characterized by replacement of the epithelium by large, pleomorphic cells with hyperchromatic nuclei and prominent mitotic activity. The pagetoid type is characterized by scattered individual or aggregates of large pleomorphic tumor cells with a vacuolated foamy cytoplasm and nuclei with dense chromatin within the epidermis or conjunctival epithelium. The papillary pattern is less common and exhibits papillary projections with areas of confluent tumor cells.

Invasive sebaceous carcinoma is characterized by infiltrating lobules of tumor cells with vacuolated foamy cytoplasm extending into the underlying dermis or conjunctiva stroma. Tumor cell nuclei are hyperchromatic, pleomorphic with prominent nucleoli and atypical mitoses. Some tumors also grow in a comedocarcinoma-like pattern with large lobules having central necrosis. The tumor lacks peripheral palisading unlike BCC. Other sebaceous carcinomas can have a squamous-like appearance with areas of keratinization, and therefore can be mistaken for squamous carcinoma.

Molecular pathology and genetics: Dysregulation of several pathways have been identified in sebaceous carcinoma: *MAPK* and *JAK/STAT*, *NF-κB*, *PTEN* as well as *TGF-β*. UV radiation independent *p53* mutations have been found in a high proportion of sebaceous carcinomas. Sebaceous carcinoma associated with Muir–Torre syndrome shows microsatellite instability and loss of expression of DNA mismatch repair genes MLH1, MSH2, and MSH6, which are usually not detected in sporadic sebaceous carcinoma.

Microenvironment including immune response: Sebaceous carcinomas are typically characterized by a distinct lack of an inflammatory response.

Staging and grading: The Staging system used is the 8th edition of the AJCC TNM Staging system, and is the same as used for BCC as described above. Similarly, the same grading system as for BCC is used for sebaceous carcinoma.

Prognostic and predictive biomarkers: Sebaceous carcinomas with increasing AJCC tumor stage, pagetoid invasion of the overlying epithelia, poor differentiation, and multifocal origin, including orbital infiltration as well as vascular involvement, are associated with worse prognoses.

Merkel Cell Carcinoma

Definition: A rare aggressive neuroendocrine tumor of the skin. It also occurs rarely in the eyelid.

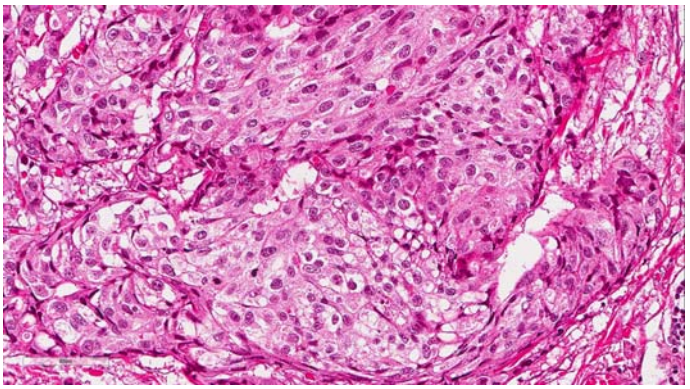

Fig. 2 Sebaceous carcinoma. Well -differentiated sebaceous carcinoma. Tumor cells show large nuclei with prominent nucleoli. The cytoplasm is vacuolated. (H&E 20×).

Burden: Merkel cell carcinoma (MCC) mostly affect the head and neck region, followed by the trunk and the limbs. Its incidence varies; it is higher in Australia (1.6 per 100.000) than in the United States (0.44 per 100.000). Immunocompromised patients with T-cell dysfunction are more likely to be affected by MCC. Eyelid MCC mostly occurs in the upper lid. The median age at presentation is 77 years (range, 58–91 years), and 52% are women.

Risk factors: Fair skin, chronic sun exposure, chronic immune suppression, and advanced age.

Pathology: Infiltration of the dermis by monotonous tumor cells with large nuclei and scant cytoplasm (**Fig. 3**). Frequent apoptotic bodies and mitotic figures are also present. Immunohistochemical stains can be needed to differentiate this tumor from other skin tumors and small cell carcinoma. MCC is positive for CK20 (perinuclear dot staining pattern) and EMA. Neuroendocrine stains are positive including synaptophysin, chromogranin, and CD56. TTF-1 is negative in this tumor unlike small cell carcinoma.

Molecular pathology and genetics: MCC development has been linked to Merkel cell polyomavirus (MCPyV) and UV radiation. Integration of the viral genome into the host-genome and mutation of the large T (LT) antigen eventually leads to uncontrolled proliferation. Exposure to UV radiation leads to mutation involving *p53* and *Rb* genes. In both cases (MCPyV-positive and -negative) MCC exhibit nuclear accumulation of oncogenic transcription factors such as *NFAT*, phosphorylated *CREB*, and phosphorylated *STAT3*.

Microenvironment including immune response: A subset of MCCs express PD-1 on tumor-infiltrating lymphocytes and express PD-L1 on tumor cells, which suggests an endogenous tumor-reactive immune response.

Staging: The Staging system used is the 8th edition of the AJCC TNM Staging system (Merkel cell carcinoma staging system).

Grading: There is no recommended histologic grading system at this time.

Prognostic and predictive biomarkers: MCC has a mortality rate of 30%. Compared with MCCs occurring in other locations, MCCs of the eyelid appear to be associated with a better prognosis, which may be related to earlier detection. They are still associated with significant rate of metastasis and recurrences.

Tumors of the Conjunctiva

Squamous Cell Carcinoma

Definition: Squamous cell carcinoma (SCC) of the conjunctiva and caruncle is a malignant epithelial tumor with squamous differentiation and can be keratinising or non-keratinising.

Burden: There is significant geographical variation in the incidence of conjunctival SCC. Worldwide, the mean age-standardized incidence is 0.18 per year per 100,000 for males and 0.08 per year per 100,000 for females. Worldwide, a high incidence has been demonstrated in HIV-positive and in other immunosuppressed populations. A higher incidence is also seen in xeroderma pigmentosum.

Risk factors: UV radiation remains the strongest risk factor. It is the only risk factor where a dose–response relationship has been established. Impaired immune surveillance clearly plays a role in the development of conjunctival SCC, and it unites risk factors such as UV (which suppresses cell-mediated immunity) and immunosuppression (HIV, post-transplant). Reduced immunity potentiates the effect of oncogenic viruses such as HPV. In Africa, the combination of low latitude, HIV and/or HPV infection explains the relatively high incidence of conjunctival SCC.

Pathology: Both pre-invasive and invasive forms of conjunctival SCC are recognized. In SCC in situ, dysplasia involves the full thickness of conjunctival epithelium; however, the basement membrane is not breached. It can be seen in flat or papillary lesions. Invasive SCC breaches the epithelial basement membrane of the conjunctiva and extends into the subepithelial stroma (**Fig. 4**). Both SCC in situ and invasive SCC can occasionally involve pterygia.

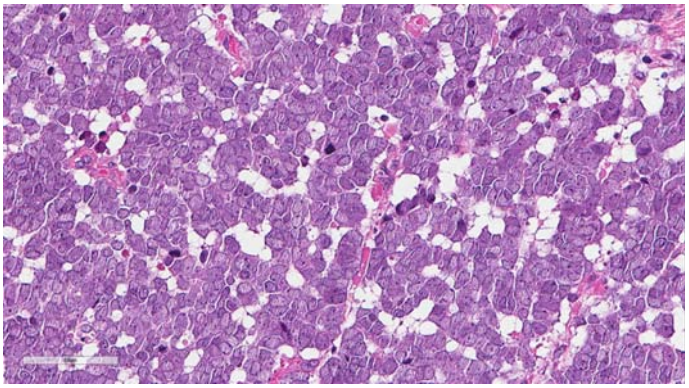

Fig. 3 Merkel cell carcinoma. Sheets of monotonous tumor cells with large nuclei and little cytoplasm. Numerous mitotic figures and apoptotic bodies are present. (H&E 40 ×).

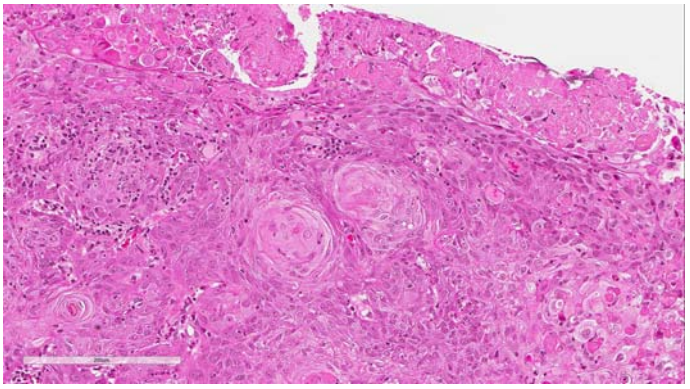

Fig. 4 SCC. The epithelium is eroded. The conjunctival stroma is infiltrated by nests of keratin-forming malignant squamous cells showing abundant eosinophilic cytoplasm and intracellular bridges. (H&E 20 ×).

Molecular pathology and genetics: UV-induced mutations in the p53 gene is the only consistent genetic abnormality identified to date. Xeroderma pigmentosum. No other genetic susceptibilities have been demonstrated to date.

Microenvironment including immune response: SCC typically induces a desmoplastic reaction with varied scattering of T-cells at the edge of the invasion front.

Staging: The Staging system used is the 8th edition of the AJCC TNM Staging system.

Grading: Grading of conjunctival SCC is based on degree of differentiation. Well-differentiated SCC produce keratin, have abundant eosinophilic cytoplasm, readily identifiable intercellular bridges, low mitotic counts and minimal nuclear pleomorphism. Moderately differentiated SCCs show variable keratinisation. Compared to well-differentiated SCCs, they are mitotically more active and show increased nuclear pleomorphism. Poorly differentiated invasive SCC can have basaloid or spindled morphology, with scanty cytoplasm, and necrosis is often present.

Prognostic and predictive biomarkers: SCC of the conjunctiva and caruncle is considered a disease of low virulence but high rate of recurrence (12%–40%). Prognosis for invasive SCC is favorable if treated at an early stage by local surgical excision with adjuvant cryotherapy or topical chemotherapy. Recurrence is most likely in those SCC of stage T3 or T4 with positive surgical margins or with untreated in situ disease. Orbital exenteration may be required for advanced stage disease. Recurrences can occur >5 years after primary excision, particularly in cases where there is poor differentiation. The risk of metastasis and death is considered very low; however, the data are sparse.

Conjunctival Melanoma

Definition: A malignant tumor arising from conjunctival melanocytes.

Burden: Conjunctival melanoma represents 5% of all ocular melanomas. The annual incidence ranges from 0.1 to 0.7 per million population. Geographic variation follows racial composition of populations. Increasing incidence has been shown in several populations over the last 50 years. Roughly 10% of conjunctival melanomas arise in or involve the caruncle. The average age at diagnosis is 50–60 years. They are rare in children, and there is no gender predilection.

Risk factors: Evidence linking chronic UV light exposure to conjunctival melanoma is accumulating, but is less robust than for cutaneous melanoma. No other environmental cause is established.

Pathology: Conjunctiva melanomas may arise from melanocytic intraepithelial neoplasia (about 65%), pre-existing nevus, or de novo (Fig. 5A). These may be present adjacent to the main invasive lesion. Invasive conjunctival melanomas consist of varying combinations of atypical spindle cells, polyhedral cells, and epithelioid cells. These are usually contained within ill-defined nests within the conjunctival stroma (Fig. 5B). Large epithelioid cells are characterized by nuclei with conspicuous nucleoli, and quite often stain with cell cycle markers, such as cyclinD1 and Ki-67. Spindle-shaped melanoma cells tend to be less pigmented. Clusters of so-called "balloon" cells with clear or vacuolated cytoplasm are occasionally encountered. Invasion of capillaries and lymphatics within the stroma can be seen. It is often difficult to judge the surgical margins, particularly in tangential sections of small biopsies. MelanA immunohistochemical staining may aid this.

Molecular pathology and genetics: There is no known genetic susceptibility. Between 30% and 50% of conjunctival melanomas have *BRAF* mutations, and nearly 20% have *NRAS* mutations, similar to the proportions found with cutaneous melanoma. Other less common mutations involve *TERT* and *KIT*.

Microenvironment including immune response: Conjunctival melanomas are typically surrounded by a dense chronic inflammatory infiltrate, composed predominantly of T-cells. Melanophages are typically interspersed within the tumor cells.

Staging and grading: Melanomas are not graded. The Staging system used is the 8th edition of the AJCC TNM Staging system.

Prognostic and predictive biomarkers: Depending on the case series, the 10-year melanoma-related mortality is 25%–30%. Tumor-related death has been associated with de novo origin, nonlimbal location, large T size, orbital invasion, as well as nodular growth and multi-centric origin. Nonlimbal location or thickness over 2 mm have been proposed as indication for considering a sentinel

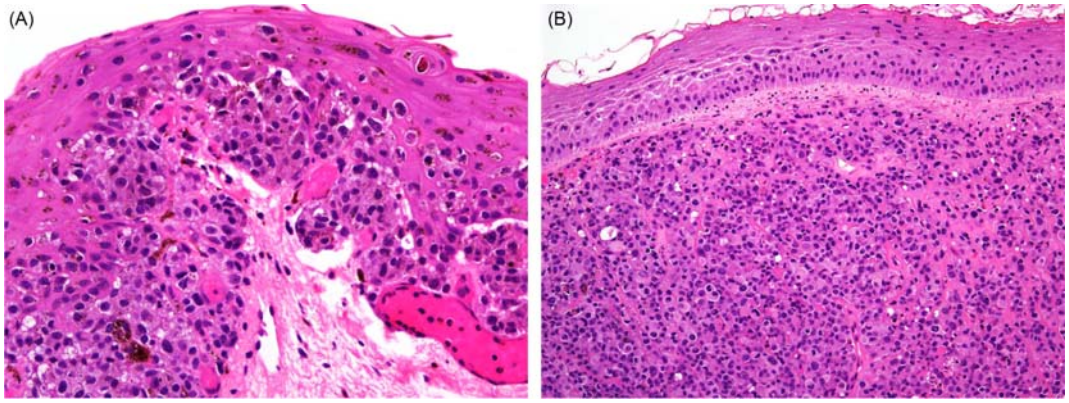

Fig. 5 Conjunctival melanoma. (A) Conjunctival biopsy demonstrating melanoma in situ. (B) Invasive conjunctival melanoma of epithelioid cell type with extensive subepithelial infiltration. (H&E 20 ×).

lymph node biopsy. Determination of the presence of *BRAF*-mutations at first diagnosis may aid the patient management should they develop metastases. Incisional biopsy and excision without adjuvant therapy (e.g., plaque or topical chemotherapy) have also been correlated with poor outcome.

Conjunctival Lymphoma

Definition: Primary conjunctival lymphomas are extranodal monoclonal proliferations of atypical lymphocytes arising in the bulbar, palpebral, forniceal or caruncular conjunctiva. Secondary conjunctival lymphomas are manifestations of systemic non-Hodgkin lymphoma (NHL) in the conjunctiva.

Burden: Primary conjunctival lymphomas represent 25%–30% of ocular adnexal lymphomas (OAL) which constitute 2% of all extranodal lymphomas. They occur more commonly in middle-aged and elderly individuals, although they may occasionally occur in young adults. Low-grade primary conjunctival lymphomas tend to occur in the sixth decade, whereas high-grade tumors affect those >70 years of age. Females are slightly more commonly affected than males. The incidence of conjunctival NHL has increased at up to 3% per annum from 1980 to 2005. This may be biased due to their increased recognition rather than subsuming them under the diagnosis of "pseudotumour." Secondary conjunctival lymphomas occur more commonly in males, with the most common subtype being mantle cell lymphomas (MCL).

Risk factors: Proposed risk factors for conjunctival lymphoma development include: autoimmune disease, infection by *Chlamydia psittaci*, *Helicobacter pylori* or HIV-infection.

Pathology: Most primary conjunctival lymphomas are non-Hodgkin lymphoma (NHL) of B-cell type (98%) whereas T-cell NHL are exceptionally rare (2%). The three most common primary conjunctival NHL types are extranodal marginal zone lymphoma (EMZL) (60%) (Fig. 6), follicular lymphoma (FL) (10%), and diffuse large B-cell lymphoma (DLBCL) (9%).

The most common "secondary" conjunctival NHLs include MCL, plasmacytomas and T-cell lymphomas. Hodgkin lymphoma are exceptionally rare. See reviews for the morphological, immunophenotypical and genomic features of each of these tumors.

Molecular pathology and genetics: Conjunctival EMZL are characterized by the following translocations: t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), and t(3;14)(p13;q32), resulting in uncontrolled activation of the NF-κB signaling pathway.

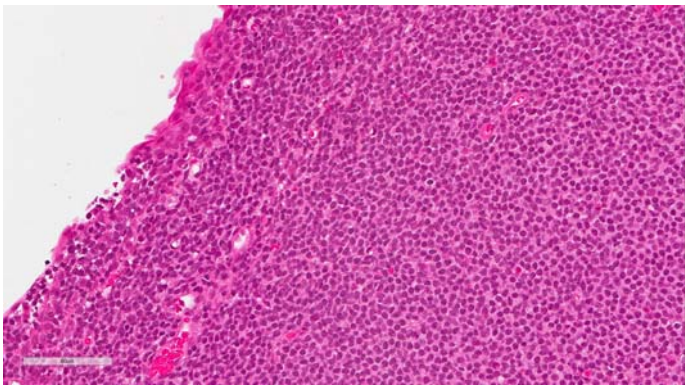

Fig. 6 Extranodal marginal zone lymphoma. The conjuctival stroma shows monotonous proliferation of small B lymphocytes. Lymphoepithelial lesions are seen in the epithelium. (H&E 20 ×).

Conjunctival FL show the pathognomic translocation t(14;18)(q32;q21), involving the *BCL2* gene rearrangement, resulting in apoptosis arrest in the neoplastic B-cells.

Most conjunctival DLBCL demonstrate immunophenotypic and molecular features of the activated B cell (ABC)-like subtype. Multiple oncogenic alterations and dysregulating signal transduction pathways have been documented in DLBCL, including mutations of *MYD88*, with recurrent targets of genetic lesions being *NF-κB*, particularly in the less curable ABC-like subtype.

Microenvironment including immune response: The microenvironment of conjunctival lymphomas is dependent on the lymphoma subtype. Conjunctival FL can be surrounded by regions of sclerosis but this tends not to be as extensive as that seen in nodal FL.

Staging and grading: Staging according to the Ann Arbor staging system as well as the 8th edition of the TNM/AJCC system.

Grading: Is assigned only to follicular lymphoma and DLBCL.

Prognostic and predictive biomarkers: Prognosis of conjunctival and caruncular lymphomas is dependent on lymphoma subtype (EMZL vs. non-EMZL) and clinical stage of disease (Stage IE vs. >Stage IIE; <T2 vs. ≥T3). Lymphoma-related survival (5-year) depends on subtype: conjunctival EMZL and FL patients with Stage IE have a favorable prognosis (82% and 72%, respectively), whereas DLBCL and MCL carry a poor prognosis (33% and 8%, respectively).

Age and gender are reliable predictors of disease-specific survival for EMZL and FL, respectively, as are Ann Arbor and TNM stages of disease for EMZL, FL and DLBCL, respectively. The tumor cell growth fraction (using Ki-67) is also proposed to be predictive. Conjunctival T-cell NHL is associated with a poor prognosis, with 50% of patients having progression or recurrence within the first year of diagnosis.

Tumors of the Uveal Tract

Melanoma

Definition: Uveal melanoma (UM) is a malignant tumor occurring predominantly in the choroid (90%) with remainder being confined to the ciliary body and the iris.

Burden: The overall incidence of UM is 6–7 per million per year. It increases with age from about 2.5 per million between the ages of 15 and 44 years to 25 per million after the age of 65 years. The tumor is extremely rare in children. There is no significant sex difference.

Risk factors: UM is much more common in Caucasians than in Africans or Asians. Epidemiological studies suggest that UM is 2–3 times more common in blue/gray than in brown eyes. The etiology of UM is unknown; however, there are known associations with UM. These include: ocular melanocytosis (increased population of melanocytes within the uvea and episclera), oculodermal melanocytosis (naevus of Ota, which also involves the periocular skin and meninges), simple and dysplastic cutaneous naevi and cutaneous melanomas, and neurofibromatosis type 1.

Rare reports of families with an excess of UM cases have been published. UM is part of the autosomal dominantly inherited "*BAP1* (BRCA1-associated protein-1) familial cancer syndrome." Affected patients, who have germline mutations in *BAP1* develop mesotheliomas and unusual benign atypical melanocytic skin tumors as well as UM.

Pathology: UM can be either dome- or mushroom shaped lesions, the latter due to perforation of Bruch's membrane (Fig. 7A). They consist predominantly of tumor cells with some admixed reactive cells usually representing a smaller proportion of the tumor's cellular content. According to the modified Callender classification, UM consist of spindle B cells and/or of epithelioid cells (Fig. 7B and C). The former are long and fusiform with a small oval nucleus and a prominent nucleolus. Epithelioid, which means "epithelial-like," cells are large and round with abundant eosinophilic cytoplasm, usually distinct cell margins, and prominent nucleoli. Variants of epithelioid cells include wildly anaplastic tumor giant cells and relatively uniform small epithelioid cells. Spindle A cells are now regarded as benign and most commonly seen in uveal naevi.

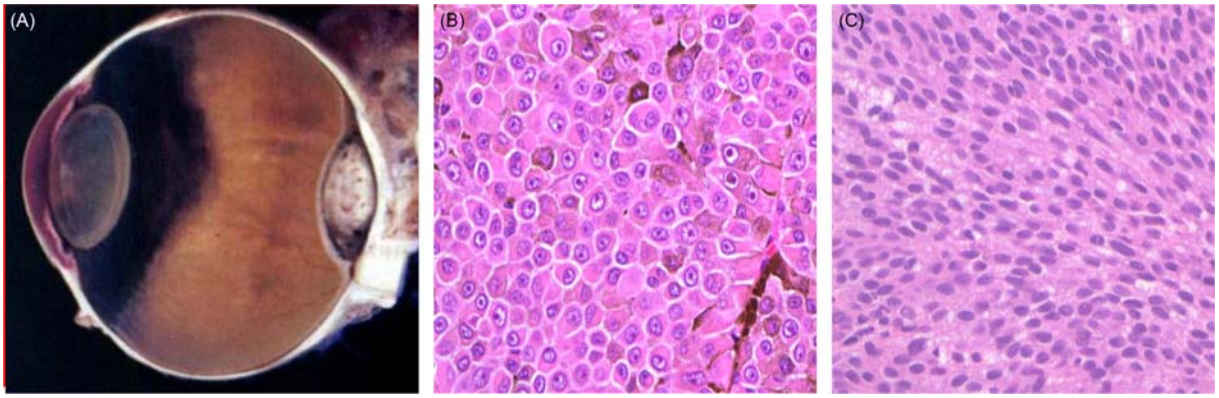

Fig. 7 Uveal melanoma. (A) A dome-shaped posterior choroidal melanoma. (B) Epithelioid cells vary in their proportions within the tumor, are usually nuclear BAP1 negative, and as such are strongly predictive of the metastatic potential of the UM (H&E 20×). (C) Spindle cell UM tend to be associated with disomy 3 and nuclear BAP1 positive tumors, but not always.

Notable histological features include:

(a) Pigmentation of the melanoma cells—this can vary considerably between tumors and within tumors.
(b) Lymphocytic and macrophage infiltration, which can be prominent and has been associated with a poorer prognosis.
(c) Connective tissue or extracellular matrix patterns: nine different types have been identified, of which the "closed (or back-to-back) loops" are associated with a poor prognosis.
(d) Necrosis—varying degrees of necrosis may be found in UM. It tends to be more prominent in rapidly growing choroidal melanomas at the equator of the globe, or in tumors that have undergone brachytherapy. Aggregates of melanophages are typically in the necrotic areas. Total infarction of the tumor may result in destruction of adjacent tissues, including the sclera, possibly resulting in extraocular spread. The necrosis may lead to a "cystic"-like appearance to the tumor on ultrasound biomicroscopy.
(e) Extraocular melanoma extension. The size of this should be noted, as this implications for the risk of recurrence and metastasis.

Positive immunohistochemistry with S-100P, Melan A or HMB45 can aid confirmation of the diagnosis in most cases, but are not really necessary for routine diagnostics. Cell proliferation status can be gauged by markers such as Ki-67, PC-10, and PHH3 (Ser10). Nuclear BAP1 protein expression using immunohistochemistry corresponds closely with BAP1 mutational status, and indirectly with chromosome 3 status.

Molecular pathology and genetics: As above, the familial *BAP1 syndrome* is associated with UM. *GNAQ* and *GNA11* mutations are also found in uveal naevi and in most UM regardless of their tumor stage, chromosomal constellation or other outcome predictors (see below). These mutations appear to be necessary but not sufficient for complete malignant transformation to UM. These data suggest that *GNAQ* and *GNA11* mutations are early events in the molecular pathogenesis of UM.

It has been known for almost 20 years that UM show specific chromosomal alterations, which are quite distinct from melanomas at other sites, particularly those of the skin. The most striking abnormality in UM is the complete or partial loss of chromosome 3. Other common genetic abnormalities of UM include loss on 1p, 6q, 8, and 9p as well as gain on 1q, 6p, and 8q. These alterations were initially identified by standard karyotypic analyses. They have subsequently been confirmed by several groups using differing technologies, including: fluorescence in situ hybridization (FISH); comparative genomic hybridisation (CGH); spectral karyotyping; microsatellite analysis (MSA); multiplex ligation-dependent probe amplification (MLPA), and single nucleotide polymorphisms (SNPs).

Recent advances in the molecular characterization of UM using multiple and complex molecular techniques have identified additional molecular changes that contribute to the development and progression of this disease. These include alterations in the following genes with decreasing frequency: *BAP1*, *SF3B1*, *EIFAX1*, *PLCB4*, *CYSLTR2*, *TP53BP1*, *CSMD1*, *TTC28*, *DLK2*, and *KTN1*. The biological significance of all of these findings is not yet clear. Other genetic changes of relevance to metastatic progression include the amplification of *c-MYC* on chromosome 8q.

Microenvironment including immune response: UM are typically characterized by varying infiltrates of T-cells and macrophages: increased densities of both are associated with poor prognoses. Following proton beam therapy, there is often an increased infiltration of macrophages and interestingly plasma cells.

Staging and grading: Melanomas are not graded. The Staging system used is the 8th edition of the AJCC TNM Staging system.

Prognostic and predictive biomarkers: The above-mentioned chromosomal alterations in primary UM are clinically relevant because of their correlation with the risk of metastatic death. Chromosome 3 loss is associated with a reduction of the 5-year survival probability from approximately 100% to 50%. Similarly, chromosome 8 gains and loss of chromosome 1 significantly correlate with reduced survival. Both chromosome 3 loss and polysomy 8q are also associated with other poor prognostic factors, including increasing tumor basal diameter, ciliary body involvement, presence of epithelioid cells, high mitotic count, and closed connective tissue loops. Conversely, gains in chromosome 6p correlate with a good prognosis, suggesting this aberration may have a functionally protective effect. A PCR-based 12-gene assay based on gene expression profiling (GEP), divides UM into two "classes" on the basis of an mRNA expression signature: class 1 and class 2. Class 1 UM often show 6p and 8q gain. Class 2 UM tend to show more aneuploidy with 1p loss, 3 loss, 8p loss, and 8q gain. Increasingly prognostic testing is being performed on small intraocular biopsies. Essential for precise prognostication is: (a) morphological examination of the biopsy; and (b) the integration of relevant clinical, histological and genetic factors, resulting in an individualized curve allowing for improved downstream personalized care (e.g. https://mpcetoolsforhealth.liverpool.ac.uk/matsoap/lumpo3cr_v5.htm).

Tumors of the Retina

Retinoblastoma

Definition: A malignant neoplasm of the neurosensory retina, affecting young children. Hereditary cases are typically bilateral with multiple tumor foci in one or two eyes and, more rarely, intracranially at midline.

Burden: Retinoblastoma (RB) is the most common primary intraocular malignant tumor in infants and young children, and is thought to be the most common primary malignant intraocular neoplasm overall. It accounts for approximately 3% of all cancers occurring in young children. The incidence of RB is reported as 1 in 16,000–18,000 live births with approximately 7000–8000 new cases worldwide yearly. The incidence of retinoblastoma appears to be higher in Africa, India, and among children of Native American descent.

Risk factors: Germline and somatic mutations in the *RB1* gene or rarely somatic amplification of *MYCN*.

Pathology: Histopathologically, retinoblastoma is a "small round blue cell tumour," composed of primitive neuroblastic cells with basophilic nuclei and scant cytoplasm (Fig. 8). Numerous mitotic figures and apoptotic cells are present. Retinoblastoma cells tend to outgrow their blood supply and undergo necrosis. This typically occurs when the cells have grown 90–110 μm away from a nutrient vessel. A characteristic pattern of basophilic "sleeves" and "cuffs" of viable basophilic tumor cells separated by sheets of necrotic tumor results. Foci of dystrophic calcification typically develop within the necrotic foci.

Varying degrees of retinal differentiation are evident in retinoblastoma as "Homer Wright" and "Flexner–Wintersteiner" rosettes and fleurettes, which represent photoreceptor differentiation. "Homer Wright" rosettes represent neuroblastic differentiation, and appear as tangle of neurofilaments ringed by nuclei. They also occur in other tumors such as neuroblastoma. "Flexner–Wintersteiner" rosettes are a characteristic feature of retinoblastoma, but do occur in some other neoplasms, such as medulloepithelioma. Flexner–Wintersteiner rosettes represent early retinal differentiation and have a central lumen that corresponds to the subretinal space. Rosettes are more common in retinoblastomas that are enucleated from younger children. Tumors typically become less well-differentiated as the age of the patient increases.

Foci of photoreceptor differentiation called "fleurettes" are found histopathologically in at least 15%–20% of retinoblastomas. Photoreceptor differentiation occurs in areas of viable tumor that appear more eosinophilic than typical retinoblastoma. A retinal neoplasm composed entirely of photoreceptor differentiation is termed a retinoma (clinically) or retinocytoma (histopathologically), and is considered to be a benign, often transient, precursor of retinoblastoma. Foci of photoreceptor differentiation are found in the basal portions of endophytic retinoblastomas, supporting the notion they may be precursor lesions. Photoreceptor differentiation appears as bouquet-like aggregates of eosinophilic structures (fleurettes, from fleur-de-lis) that correspond to the inner segments of photoreceptors, and often are aligned along segments of neoplastic external limiting membrane.

Varying degrees of anaplasia have been described in retinoblastoma, and it has been suggested that severe anaplasia is a risk factor for metastasis, although this requires validation. Severely anaplastic tumors are poorly differentiated and have cells with large, very pleomorphic nuclei that are angular, rhomboid or fusiform in shape. Cell wrapping and numerous mitotic figures also are present.

Retinoblastoma arises from the retina and initially invades the vitreous cavity and subretinal space. Ultimately, the tumor may infiltrate Bruch's membrane and invade the choroid, gaining access to vessels that serve as a route for distant haematogenous metastasis. Retinoblastoma also readily invades the optic nerve. The tumor may travel along the nerve to the brain or be dispersed in the cerebrospinal fluid. Massive posterior uveal invasion and retrolaminar optic nerve invasion are high-risk features and indicate to the clinicians that adjuvant chemotherapy is required. Invasion of anterior segment structures also is thought to increase the risk of metastasis.

On immunohistochemistry, retinoblastoma stains for neuron specific enolase and synaptophysin, with these stains being of help to confirm the presence of optic nerve invasion when routine histopathology is equivocal.

Molecular pathology and genetics: Mutations in the *RB1* gene are responsible for almost all cases of retinoblastoma. The tumor suppressor gene *RB1* located on chromosome 13, and follows the Knudson's two hit hypothesis for development of tumors. Approximately 40% of Rb cases are caused by heterozygous de novo or inherited germline mutations in the *RB1* gene. Offspring of affected individuals have a 50% chance of inheriting the pathogenic variant. The inheritance pattern is autosomal dominant. In 60% of cases retinoblastoma is sporadic and unilateral, and the pathogenic variant of *RB1* occurs only within retinal cells. To develop retinoma or retinoblastoma both alleles must have the pathogenic variant. Two percent of unilateral patients have the *MYCN* oncogene amplified (RB1+/+ MYCNA) in the tumors.

Microenvironment including immune response: Very little inflammatory infiltrate is associated with intraocular retinoblastoma.

Staging: The Staging according to the AJCC 8th edition.

Grading:

GX: Grade cannot be assessed.

G1: Tumor with areas of retinoma (fleurettes or neuronal differentiation).

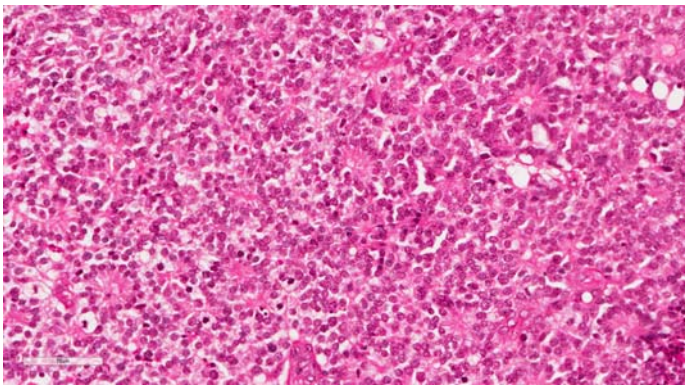

Fig. 8 Retinoblastoma. Primitive neuroblastic cells with small basophilic nuclei and scant cytoplasm, forming Homer-Wright and Flexner–Wintersteiner rosettes (H&E 20×).

G2: Tumor with many rosettes (Flexner–Wintersteiner or Homer Wright).

G3: Tumor with occasional rosettes (Flexner–Wintersteiner or Homer Wright).

G4: Tumor with poorly differentiated cells without rosettes and/or with extensive areas (more than half of tumor) of anaplasia.

Prognostic and predictive biomarkers: The most powerful feature predictive of successful treatment of retinblastoma is early diagnosis when the tumor is small and entirely contained within the eye and has not invaded any of the ocular coats, especially in sporadic tumors. The cure rate is >95% in such cases.

Retinal Haemangioblastoma

Definition: Retinal hemangioblastoma is a tumor derived from developmentally arrested angioblasts, generally due to a mutation in the *VHL* gene.

Burden: Von Hippel–Lindau (VHL) disease occurs in 1/36,000 live births per year. VHL has a remarkable penetrance of over 90% by 65 years of age. Of all VHL patients, 49%–85% develop retinal hemangioblastomas, making this ocular sign the most common presentation of the disease. VHL-associated retinal hemangioblastomas usually develop at a mean age of 25 years.

Risk factors: VHL which is an autosomal dominant inherited syndrome. It is caused by a germline alteration of the *VHL* gene, a tumor suppressor gene located on chromosome 3p25.5. Retinal hemangioblastomas are often the most common and earliest presentation of VHL disease.

Pathology: Hemangioblastomas are composed of capillary sized vascular channels surrounding stromal cells. The stromal cells characteristically have a prominently vacuolated cytoplasm. In addition, small clusters of angiomesenchymal cells, tumourlet cells, have been described in ocular and cerebellar hemangioblastomas. The stromal cells and the "tumourlet" cells may express CD133, a marker present on hematopoietic, endothelial, and neural progenitor cells. Stromal cells are the cells that harbor the genetic alterations in the *VHL* gene. These alterations are not present in the vascular or glial components.

Molecular pathology and genetics: In VHL disease there is a germline defect in one copy of the gene and with random loss of the normal allele (loss of heterozygosity, LOH) patients are predisposed to develop hemangioblastomas. Presumably, in sporadic cases there is somatic loss of both normal copies of the *VHL* gene, resulting in a similar but solitary tumor.

Microenvironment including immune response: Surrounding the vascular channels are stromal cells with vacuolation. There are limited inflammatory infiltrates within this stroma.

Staging and grading: Not applicable.

Prognostic and predictive biomarkers: The type of germline mutation present in the *VHL* gene has an effect on the disease phenotype. Deletions and truncating mutations are associated with a Type I phenotype, characterized by renal cell carcinoma and haemangioblastoma but not pheochromocytomas. Type II phenotype with renal cell carcinoma and pheochromocytoma is associated with missense mutations. Patients with complete deletions of the *VHL* gene are also much less likely to develop retinal hemangioblastomas (9%), than patients with partial deletions, simple missense, and nonsense mutations (45%).

The prognosis for vision is good in patients with VHL. Overall, 88% of patients will retain 20/20 or better vision in at least one eye. Laser or cryotherapy is effective treatment for small to medium sized intraocular hemangioblastomas that do not involve the optic nerve. Increasingly, surgical resection of unresponsive and large retinal hemangioblastomas is being employed with good visual outcomes. Treatment of hemangioblastomas involving the optic nerve remains challenging. The prognosis related to systemic morbidity and overall survival in VHL is dependent on the treatment of the associated cerebellar haemangioblastoma and renal cell carcinoma.

Vitreoretinal Lymphoma

Definition: A high-grade malignant lymphoproliferative disease in which atypical lymphocytes infiltrate the retina and vitreous in the absence of lymph node or visceral involvement. It is frequently accompanied by involvement of the central nervous system.

Burden: The incidence of primary vitreoretinal lymphoma (PVRL) is difficult to estimate because of its rarity and the effect of improvement in diagnostic techniques employed. The incidence is reported to have steadily increased from 0.23–0.48 per million population from 1990 to 2010. A slight predominance of female patients is reported.

Risk factors: Based on overlap with primary central nervous system lymphoma (PCNSL), HIV and Epstein-Barr virus (EBV) infections are predisposing factors for the development of PVRL. No other environmental risk factors are known.

Pathology: Most PVRL are diffuse large B cell lymphomas (DLBCL) of ABC type, according to the WHO lymphoma classification. The atypical lymphoid cells are generally medium-to-large cells with a condensed chromatin pattern and prominent nucleoli (**Fig. 9**). The nucleus often is irregular in shape. The neoplastic lymphocytes are usually admixed with reactive inflammatory cells, such as T-cells and macrophages. In some cases, the presence of small, mature T-lymphocytes overwhelms and can mask the neoplastic B-cells. PVRL cells are typically friable with numerous apoptotic bodies, thus vitreous samples often contain abundant lytic cell remnants or ghosts, often within macrophages. Such cell ghosts are uncommon in other diseases associated with vitreous infiltrates, so their presence is highly suggestive of PVRL. The neoplastic B-cells of PVRL have the following profile: CD79a+, CD20+, PAX5+, BCL-2+, BCL-6+, MUM1+, IgM+, and a high cell proliferation.

Molecular pathology and genetics: Monoclonal expansion of B lymphocytes can be assessed using immunoglobulin heavy chain (IgH) polymerase chain reaction (PCR). IgH-PCR can be performed on fluid and tissue samples (fresh-frozen or fixed), and is

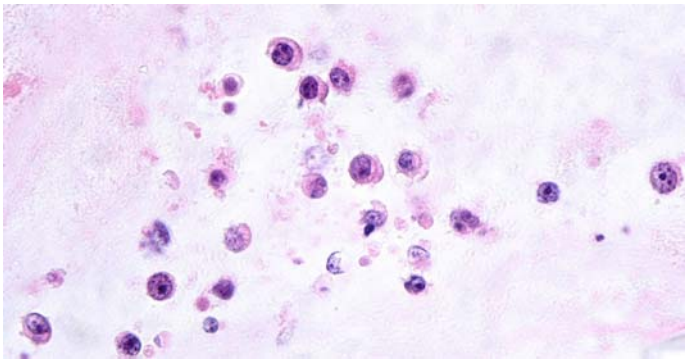

Fig. 9 Vitreoretinal lymphoma. Scattered lymphoid blasts in a cytoblock of a vitrectomy sample (H&E 20×).

very useful in PVRL diagnosis as an adjunctive test to cytomorphology and immunophenotyping. Recently, evaluation for the *MYD88* mutation has confirmed its presence in 70% of cases of PVRL.

Microenvironment including immune response: Typically PVRL contain admixed macrophages and T-cells, which can make the diagnosis difficult, particularly as the neoplastic B-cells are quite fragile.

Staging: PVRL can be staged using the Lugano Classification (Modified Ann Arbor Classification) for Hodgkin and Non- Hodgkin Lymphoma.

Prognostic and predictive biomarkers: The prognosis of PVRL patients is generally poor: it is dependent on the presence and/or subsequent development of CNSL. In patients diagnosed with PVRL, CNS involvement is present in 15% of patients at first diagnosis. Secondary CNSL has been reported to develop in a high percentage of PVRL patients during follow-up. To date, there are no predictive factors for the development of CNS involvement. Steady improvement in the survival of PNCSL patients has been observed with varying chemotherapeutic regimens. At present, the median overall survival is close to 5 years.

Tumors of the Lacrimal Gland and Orbit

Adenoid Cystic Carcinoma (ACC)

Definition: A rare aggressive epithelial malignant neoplasm of the lacrimal gland and other glandular tissues, most commonly the salivary glands, characterized by malignancy of modified myoepithelial and ductal (luminal) cells.

Burden: ACC represents around 20% of epithelial lacrimal gland tumors and is the commonest malignant tumor accounting for approximately half or even more of malignant epithelial lacrimal gland lesions. ACC is generally known to have a wide age range (12–75 years) and a bimodal age distribution with a peak in adulthood around the age of 40 and a smaller peak in teenage years.

Risk factors: Not known.

Pathology: ACC is a non-encapsulated tumor, which infiltrates the surrounding orbital soft tissue and includes proliferating modified myoepithelial cells as well as luminal ductal differentiated cells. Depending on the cellular composition and cytomorphological features, the tumor shows three growth patterns seen individually or together in variable combinations (Fig. 10).

The cribriform pattern is the commonest, described as "Swiss cheese" because of the formation of cystic structures where the connective tissue stroma is filled with basophilic amorphous glycosaminoglycans and/or eosinophilic hyalinized basal lamina.

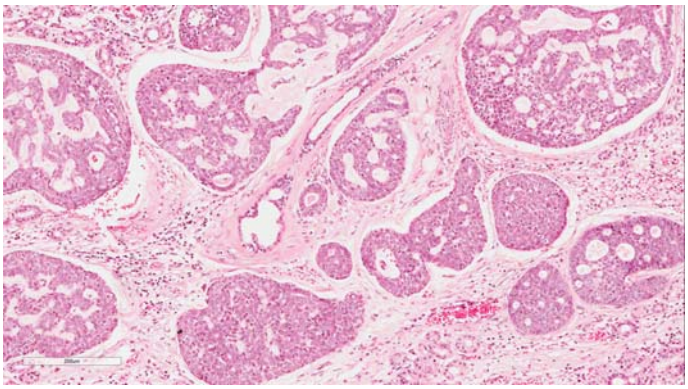

Fig. 10 Adenoid cystic carcinoma. Cribriform ("Swiss cheese") growth pattern of myoepithelial cells as well as luminal ductal cells. The stroma is filled with basophilic amorphous material. (H&E 20×).

The tubular pattern shows small glandular structures while the solid pattern shows predominantly proliferating basaloid myoepithelial cells with little glycosaminoglycan deposits or cyst-like basal lamina glandular spaces. In most series, the solid pattern is the least common. This pattern can be associated with higher numbers of mitotic figures which may explain its worse prognosis. Histopathological confirmation of perineural and bone invasion is essential. Tumor extent and margins are also important to document histopathologically. On immunohistochemistry, most ACC are MYB (myeloblastosis gene marker) positive and all are PLAG1 (pleomorphic adenoma gene marker 1) negative, in contrast to pleomorphic adenoma. High-grade transformation, previously known as dedifferentiation, may occur.

Molecular pathology and genetics: Loss of chromosome 6q is a frequent finding in ACC. This may be associated with t(6;9)(q22–23; p23–24) resulting in a *MYB-NFIB* fusion gene. This may be associated with t(6;9)(q22–23; p23–24) resulting in a MYB-NFIB fusion gene. *KRAS*, *NRAS* and *MET* mutations were reported in lacrimal gland epithelial neoplasms with highest frequency (44%) in ACC.

Microenvironment including immune response: ACC are typically surrounded by a variable desmoplastic reaction, but have very limited inflammatory infiltrates around them.

Staging: Staging according to AJCC TNM 8th edition.

Grading: In four grades from well differentiated to undifferentiated.

Prognostic and predictive biomarkers: Age at diagnosis, completeness of surgical resection and tumor stage are important determinants of disease specific survival in epithelial lacrimal gland tumors in general.

Prognostic factors in ACC include: Tumor histologic features (solid pattern being associated with a worse prognosis, perineural invasion being associated with local recurrence and skull invasion, bone invasion being associated with a fatal outcome in large tumors), tumor size (with the cut-off size of 2.0 cm in greatest dimension for T1 according to the 8th edition of AJCC), and tumor stage (recurrence, metastasis are significantly more common while survival is significantly worse in patients with T3 = 2.5–5.0 cm according to the 6th edition of the AJCC, which is approximately equivalent to T2 = > 2.0–4.0 cm in the 8th edition).

Lacrimal Gland Lymphomas

Definition: Primary lacrimal gland lymphoma is a malignant proliferation of lymphocytes arising in the tissue without evidence of concurrent systemic lymphoma. Secondary lacrimal gland lymphoma occurs in patients as part of disseminated NHL.

Burden: Lacrimal gland lymphomas are rare, representing ~9% of all ocular adnexal lymphomas, which in turn constitute 2% of all extranodal lymphomas.

Risk factors: They occur more commonly in elderly females, particularly those with Sjögren's syndrome. No racial predilection.

Pathology: Most lacrimal gland NHL are of B-cell type and are usually low-grade, although high grade transformation can occur. The most common subtype is the extranodal marginal zone B-cell lymphomas (EMZL), followed by FL and DLBCL. Histologically, EMZL show a nodular to diffuse heterogeneous B-cell infiltrate surrounding scattered reactive germinal centres and cause effacement of the normal glandular architecture.

They are variably comprised of atypical small lymphocytes, centrocyte-like cells, monocytoid B-cells, immunoblasts, lymphoplasmacytic cells and plasma cells. Plasmacellular differentiation, including Dutcher bodies, may be striking. Lymphoepithelial lesions, representing infiltration of the ductal and epithelial structures by neoplastic B cells, can be seen. EMZL cells show reactivity for B-cell markers (CD20, CD79a, PAX5), BCL-2, and surface immunoglobulin. They are negative for CD5, CD10, CD23 and Cyclin D1. Cytokeratin highlights epithelial remnants in the lymphoepithelial lesions. Ki-67 growth fraction is low, ~5%.

Molecular pathology and genetics: Molecular testing using IgH-PCR demonstrates clonal rearrangement of the immunoglobulin chains. The genetic profile is dependent on lymphoma subtype.

Microenvironment including immune response: Variable according to lymphoma subtype.

Staging and grading: According to the 8th edition of the AJCC TNM Staging 2018, and also to the Ann Arbor staging system.

Prognostic and predictive biomarkers: Most patients with stage I or II lymphoma are treated with low-dose radiotherapy ± chemotherapy. Patients presenting with stage > III lymphoma were treated with chemotherapy alone. The prognosis of lacrimal gland lymphomas depends on the histologic type and clinical stage; however, the 5-year overall survival is 70%.

Rhabdomyosarcoma

Definition: A malignant neoplasia that arises from pluripotential mesenchymal cells with a predilection to differentiate into skeletal muscle cells. Ocular rhabdomyosarcoma (RMS) may originate from the orbit, conjunctiva, eyelid or, rarely, the uveal tract.

Burden: RMS is the most common primary orbital tumor in childhood accounting for 5% of all childhood malignancies. Based on histopathologic features, RMS is subdivided into embryonal, alveolar and pleomorphic type (see below). The embryonal type is most frequently seen in the first decade of life, accounting for > 90% of published cases of ocular origin. Alveolar RMS is observed in adolescents and young adults. Pleomorphic RMS occurs almost exclusively in adults.

Risk factors: None known.

Pathology: Tumor cells appear in the substantia propia beneath the epithelium as loosely spindle-shaped cells, with hyperchromatic nuclei with indistinct tapered cytoplasmic processes, some with long ribbons of eosinophilic cytoplasm ("tadpole"-like cells), with variable degrees of pleomorphism and proliferative rate (Fig. 11). The botryoid type contains linear condensations of tumor cells underneath the epithelial layer ("cambium layer") and polypoid nodules with abundant, loose, myxoid stroma. Striking

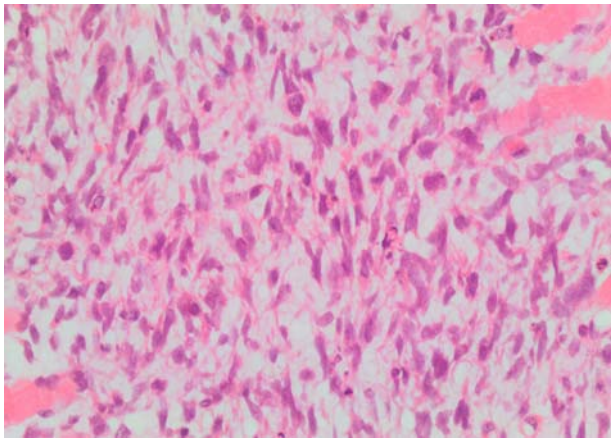

Fig. 11 Rhabdomyosarcoma. Typical "tad-pole" like cells in a botyroid type rhabdomyosarcoma of the orbit (H&E, 40 ×).

anaplasia may be present. Alveolar RMS is a small blue round cell neoplasm, with a worse prognosis. The term "alveolar" refers to a growth pattern in nests with central discohesion, separated by fibrovascular septa.

The presence of specific immunohistochemical markers for muscle correlates with the degree of tumor cell differentiation. Desmin and actin are acquired by developing rhabdomyoblasts. Myoglobin, myosin and creatine kinase correspond to terminal differentiation. Antibodies against MyoD1 and myogenin/Myf4 are sensitive and specific to RMS.

Molecular pathology and genetics: Embryonal RMS is characterized by loss of heterozygosity on the short arm of chromosome 11 (11p15.5), suggesting inactivation of a tumor-suppressor gene. By contrast, the majority of the alveolar RMS have the reciprocal chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14), the former in approximately 70% of patients. The molecular counterpart of this translocation consists of the generation of a chimeric fusion gene involving the *PAX3* gene, located on chromosome 2, and a member of the fork-head family *FOXO1*, located on chromosome 13.

Staging and grading: Staging according to AJCC 8th edition.

Grading: Currently, the preferred system for grading of sarcomas is the one proposed by the French Federation of Cancer Centers Sarcoma Group (FNCLCC). It uses three independent prognostic factors to determine the grade: mitotic activity, necrosis, and degree of differentiation of the primary tumor. Each feature is scored separately and the three scores are added to obtain the grade. Grade 1 is defined as a total score of 2 or 3, grade 2 as a total score of 4 or 5, and grade 3 as a total score of 6–8.

Prognostic and predictive biomarkers: Poor prognostic factors in adults include age, pleomorphic histological subtype and more advanced disease at presentation. The embryonal type has a better prognosis.

Orbital Liposarcoma

Definition: Malignant mesenchymal tumor with lipomatous differentiation.

Burden: Generally, liposarcoma is the most common soft tissue sarcoma in adults. Liposarcoma of the orbit is rare; it can be primary or metastatic in origin. The mean age of presentation is 42.9 years (range 15–77) with slight female preponderance.

Risk factors: Patients with genetic evidence of Li-Fraumeni syndrome harbor a risk to developed liposarcoma including two reported cases of orbital myxoid liposarcoma.

Pathology: Liposarcoma is characterized by the presence of lipoblasts (**Fig. 12**). There are four major liposarcoma subtypes: atypical lipomatous tumor/well-differentiated liposarcoma (which includes the adipocytic, sclerosing, inflammatory and spindle cell variants), de-differentiated liposarcoma; myxoid liposarcoma; and pleomorphic liposarcoma. Myxoid liposarcoma is a low-grade tumor characterized by myxoid stroma containing chicken-wire vascular channels and lipoblasts in variable stages of differentiation. It is reported to be the most common subtype occurring in orbital region. High-grade myxoid liposarcoma (formerly round cell liposarcoma) is defined by the presence of hypercellular areas, exceeding 5% of the lesion. ALT/WDL type is the second most common type occurring in this anatomical region; it is composed of variably sized adipocytes and bands of fibrosis containing spindle cells and hyperchromatic nuclei. The dedifferentiated type has been reported in the orbit, and is composed of high-grade spindle cell component resembling fibrosarcoma in addition to the well-differentiated component. Pleomorphic liposarcoma is a high-grade tumor and is composed of large polymorphic cells with bizarre nuclei. Abundant mitotic figures and areas of necrosis are also present.

Molecular pathology and genetics: Both well differentiated liposarcoma and dedifferentiated liposarcoma harbors cytogenetic aberration in the form of Ring chromosomes and giant markers (12q13–15), and show amplification of *MDM2*, *CDK4*, and *HMGA2*. Myxoid liposarcoma is characterized by two main karyotypic aberrations: >95% of cases carry a specific t(12;16)(q13;p11), which fuses the *DDIT3* (CHOP) gene on 12q13 with the *FUS* (TLS) gene on 16p11. Approx. 5% of myxoid liposarcoma harbor t(12;22)(q13;q22), which fuses *DDIT3* with *EWSR1* on 22q12. Pleomorphic liposarcoma in contrast has complex karyotypic aberrations. p53 mutation occur in 60% of cases and NF1 mutation occur in 5% of cases.

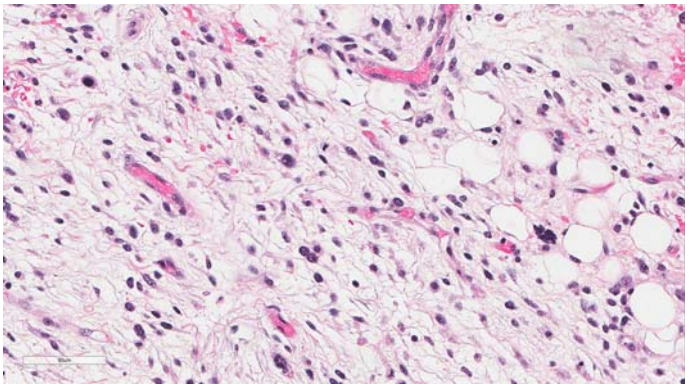

Fig. 12 Liposarcoma. Hyperchromatic spindle cells in a myxoid background. Lipoblasts are seen characterized by variably sized clear cells with scalloped hyperchromatic nuclei (H&E 20×).

Staging and grading: Staging according to AJCC 8th edition.

Grading: See rhabdomyosarcoma.

Prognostic and predictive biomarkers: Prognosis depends on histological type and the size of tumor. Orbital liposarcoma appears to be relatively favorable, likely due to small tumor size and predominantly well-differentiated histology. Plemorphic liposarcoma has a poorer prognosis with evidence of multiple recurrences. Distant metastasis from liposarcoma happens with the lung being the most common site.

See also: Malignant Tumors of the Eye, Conjunctiva, and Orbit: Diagnosis and Therapy.

Further Reading

Andreasen, S., et al., 2017. An update on tumors of the lacrimal gland. Asia-Pacific Journal of Ophthalmology 6 (2), 159–172.

Araujo, I., Coupland, S.E., 2017. Primary vitreoretinal lymphoma—A review. Asia-Pacific Journal of Ophthalmology 6 (3), 283–289.

Arnold, M.A., et al., 2016. Histology, fusion status, and outcome in alveolar rhabdomyosarcoma with low-risk clinical features: A report from the Children's oncology group. Pediatric Blood & Cancer 63 (4), 634–639.

Farquhar, N., Thornton, S., et al., 2017. Patterns of BAP1 protein expression provide insights into the prognostic significance and biology of Uveal melanoma. Journal of Pathology: Clinical Research 4 (1), 26–38.

Findeis-Hosey, J.J., et al., 2016. Von Hippel Lindau disease. Journal of Pediatric Genetics 5 (2), 116–123. https://doi.org/10.1055/s-0036-1579757.

Finger, P.T., Coupland, S.E., 2017. Ophthalmic tumors (on behalf of the Ophthalmic Sites Expert Panel). In: AJCC/TNM Cancer staging manual, 8th edn. Springer, Switzerland, pp. 779–854. Chapters 64–71.

Kenawy, N., et al., 2013. Conjunctival melanoma and melanocytic intra-epithelial neoplasia. Eye (London, England) 27 (2), 142–152. https://doi.org/10.1038/eye.2012.254.

Kirkegaard, M.M., et al., 2016. Conjunctival lymphoma—An international multicenter retrospective study. JAMA Ophthalmology 134 (4), 406–414. https://doi.org/10.1001/jamaophthalmol.2015.6122.

Larsen, A.C., et al., 2015. A retrospective review of conjunctival melanoma: Presentation, treatment, and outcome and an investigation of features associated with BRAF mutations. JAMA Ophthalmology 133 (11), 1295–1303. https://doi.org/10.1001/jamaophthalmol.2015.3200.

Madge, S.N., et al., 2010. Primary orbital liposarcoma. Ophthalmology 117 (3), 606–614.

Robertson, A.G., et al., 2017. Integrative analysis identifies four molecular and clinical subsets in Uveal melanoma. Cancer Cell 32 (2), 204–220.

Saleh, G.M., et al., 2017. Incidence of eyelid basal cell carcinoma in England: 2000–2010. The British Journal of Ophthalmology 101 (2), 209–212.

Schmitz, E.J., et al., 2017. Sebaceous gland carcinoma of the ocular adnexa—Variability in clinical and histological appearance with analysis of immunohistochemical staining patterns. Graefe's Archive for Clinical and Experimental Ophthalmology 255 (11), 2277–2285.

Soliman, S.E., Racher, H., Zhang, C., MacDonald, H., Gallie, B.L., 2017. Genetics and molecular diagnostics in retinoblastoma—An update. Asia-Pacific Journal of Ophthalmology 6 (2), 197–207. https://doi.org/10.22608/APO.201711.

Sun, E.C., et al., 1997. Epidemiology of squamous cell conjunctival cancer. Cancer Epidemiology, Biomarkers & Prevention 6 (2), 73–77.

Financial Burden of Cancer Care

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Glossary

Economic perspective Individual, group, institution, or entity that bear the costs for goods or services, for example, health insurance payers, hospitals, society in general.

Direct medical costs Expenses directly related to the use of medical services and goods, for example, physician fees, acute inpatient care, laboratory and imaging tests, surgery, radiotherapy, chemotherapy.

Direct nonmedical costs Expenses related to the logistics of pursuing healthcare or costs that are not medically-related but result from the use of healthcare services. Examples include transportation costs to medical facilities, childcare cost for parents undergoing cancer treatment, unpaid caregiver time.

Indirect costs Productivity losses that result from cancer or cancer treatment. Examples include morbidity costs from missed work days due to cancer complications or treatment; mortality costs from loss of productivity due to death.

Targeted therapies Oral or intravenous antineoplastic drugs that bind and modify the activity of specific trans-membrane or intracellular proteins or enzymes, leading to downstream intracellular events that cause cell death or cell cycle arrest.

Medicare The publically-funded federal insurance program in the United States Medicare is the largest insurance program in the country and reimburses medical services for all individuals age 65 year or older, or those with permanent disabilities or chronic renal insufficiency. Commercial insurance plans tend to follow the reimbursement policies and fees set by Medicare.

Introduction

The economics of cancer care has become a central topic in the broader debate of healthcare delivery. A simple, undeniable fact justifies the shifting perception of cancer costs as a relatively unimportant matter to a key factor in health policy: cancer costs are globally soaring and threatening the sustainability of healthcare systems.

From a simplistic point of view, the skyrocketing cancer costs represent an unavoidable consequence of the rapid pace of innovation in cancer diagnostics and therapeutics. Those in support of this view argue that the high costs represent society's willingness to pay for innovation in cancer care. From a granular point of view, cancer costs result from complex interactions between multiple factors, including (in)efficiencies of care delivery, obsolete reimbursement models (e.g., fee-for-service), and conflicting interests among stakeholders, including patients, providers, hospital administrators, insurance payers, pharmaceutical industry, and government.

Irrespective of the different opinions, a growing body of evidence supports the following concerns: (1) cancer care costs are increasing faster than the gross domestic product, at least in the United States, threatening the sustainability of healthcare programs; (2) cancer patients are directly experiencing negative consequences of high cancer care costs in the form of financial distress from unaffordable medical bills, a phenomenon known as "financial toxicity"; (3) uncontrolled costs can magnify cancer disparities by creating further barriers in access to care to those who cannot afford expensive treatments. Collectively, these areas of concern indicate the urgent need for multilevel changes in cancer care delivery that emphasize quality, lower costs, and value (i.e., better health outcomes for the unit of money spent).

This article initially provides an overview of the temporal trends in cancer care costs, followed by a dissection of the healthcare components that significantly contribute to cost. Next, a discussion ensues about the market forces that drive up cancer costs, particularly for hospitalizations and oncology drugs. The final section describes several strategies that have the potential to bend the cost curve and increase the value of cancer care.

Although cancer costs are context-specific and vary broadly across healthcare systems, the article focuses mostly on United States cost data for simplicity of scope and greater availability of published literature. The economic perspective will be the United States public or private healthcare payer (e.g., Medicare and commercial insurance plans), unless mentioned differently. Most data refer to direct medical costs as defined in the glossary, unless noted otherwise.

Temporal Trends in Cancer Costs

Total United States spending in cancer has been continuously increasing over the past several decades, from an estimated $27 billion in 1990 to $125 billion in 2010. Although cancer costs have consistently accounted for approximately 5% of total US

Table 1 Time trends in U.S. total cancer direct medical costs, including healthcare payer and patient out-of-pocket expenses

Period [a]	*Expenditures (2014 $U.S. billions)*	*Growth from previous period (%)*
1998–2000	104.5	–
2001–03	118.8	13.7
2004–06	135.5	14.1
2007–09	140.9	4.0
2010–12	143.6	1.9

[a]Data sources: *NHEA*, National Health Expenditure Accounts; *MEPS*, Medical Expenditure Panel Survey; *NNHS*, National Nursing Home Survey; *HCUP*, Healthcare Cost Utilization Project; IMS Institute for Healthcare Informatics National Sales Perspective (IMS Health, Danbury, Connecticut).

Adapted from Lee, J. A., Roehrig, C. S., Butto, E. D. (2016). Cancer care cost trends in the United States: 1998 to 2012. *Cancer* **122**, 1078–1084.

healthcare expenditures, most health economists consider cancer as an increasingly concerning area of spending, given the increasing number of cancer survivors and the rising costs of cancer treatments.

In recent years, total cancer costs have been increasing at a slightly faster pace than the U.S. gross domestic product (GDP). Between 1998 and 2012, total cancer expenditures increased from $104.5 to $143.6 billion, corresponding to an average annual growth of 2.5% (Table 1). In the same period, the United States GDP grew annually at an average of 2.2%. Conservative projections indicate that US cancer costs will continue to rise at a similar pace, from $125 billion in 2010 to $154 billion in 2020, based solely on current demographic trends in cancer incidence and survival (Fig. 1). Assuming a 2% annual increase in cancer treatment costs, United States cancer expenditures will increase to $187 billion in 2020, or a 49% cost hike from 2010.

Cancer costs also represent a significant portion of healthcare expenditures in other developed nations, accounting for 6.3% and 9.3% of total healthcare costs in the European Union (E.U.) and Japan, respectively. In 2009, the European Union spent an estimated €126 billion in cancer, though the figures varied broadly by country, from €184 per capita in Luxembourg, to €16 per capita in Bulgaria. The United States leads in cancer spending across all developed nations, as indicated by a study that estimated the total direct medical costs attributable to cancer to be €212 per person in the United States for the year of 2004, followed by Switzerland, which spent €199 per capita.

Most studies report on direct medical costs to describe national estimates of cancer spending, as the data sources are more readily available for costs related directly to medical care. Recent studies suggest that direct nonmedical costs (e.g., unpaid caregiver time) and indirect costs (i.e., productivity losses) contribute substantially to total cancer costs, justifying the need for more robust methods to estimate expenses other than those directly related to cancer treatment. In 2009, of the estimated €126 billion spent in cancer by the European Union, €51 billion (41%) related to direct medical costs, €23 billion (18%) corresponded to unpaid caregiver time, and €52 billion (41%) accounted for productivity losses, respectively. In the United States, very few studies have reported on unpaid caregiver time or other cancer direct nonmedical costs, and no readily available data informs on costs of productivity losses from cancer morbidity. One study estimated the United States costs attributable to productivity losses from cancer mortality to be $116 billion in 2000.

Collectively, the available evidence overwhelmingly confirms that cancer is a major source of healthcare spending in most countries. Healthcare policy makers are all too aware of the need to closely monitor trends in cancer expenditures at national and regional levels, as well as engage all stakeholders in a debate about strategies to implement affordable and high-quality cancer care. In order to inform discussions about strategies to implement cost-effective cancer care, the following sections will describe the components of cancer care costs in greater detail, as well as the factors driving these costs.

Components of Direct Medical Costs in Cancer Care

Studies have consistently shown that acute inpatient care (hospitalizations) accounts for the largest fraction (nearly 50%) of cancer care spending, followed by antineoplastic drug therapies (15%–30%). Other services (e.g., imaging diagnostics, radiation therapy, hospice) correspond to smaller fractions of cancer expenditures (Table 2).

Brooks et al. estimated Medicare spending in the first 6 months after the diagnosis of advanced breast, prostate, nonsmall cell lung, colorectal, and pancreatic cancers in a sample of over 60,000 beneficiaries (Table 2). Of the total mean cost of $35,122 per patient, acute hospital care accounted for $16,953 (48%), followed by oral and intravenous chemotherapy ($5705; 16%), and outpatient procedures ($2281; 6%). Utilizing data from the Medical Expenditure Panel Survey (MEPS), one study showed that hospitalizations represented 48%–55% of all cancer care expenditures in the United States between 1998 and 2012, followed by professional and clinical services (including chemotherapy and outpatient visits), which accounted for 33%–40%. Cost distributions are similar in the European Union: in 2009, of the €51 billion spent in cancer, hospitalizations accounted for €28.4 billion

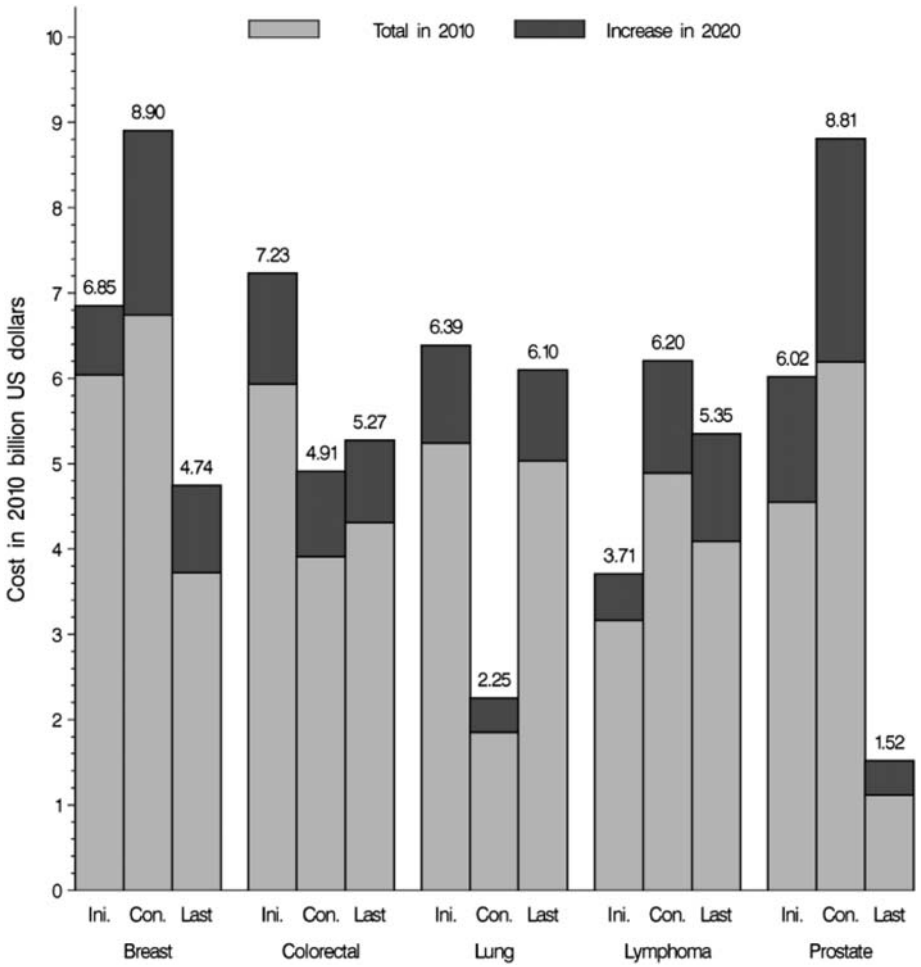

Fig. 1 Projections of National Direct Medical Costs Attributable to Cancer, United States, 2010–20 (2010 $US). *Light gray* areas indicate estimates of national expenditures for cancer care in 2010. *Dark gray* areas show projections of national cancer expenditures under the assumptions of constant incidence, survival, and costs of treatment for major cancer sites. *Ini*, initial year after diagnosis; *Con*, continuing care (i.e., time interval between the first year after diagnosis and last year of life; *Last*: last year of life. Adapted from Mariotto, A. B., Yabroff, K. R., Shao, Y., et al. (2011). Projections of the cost of cancer care in the United States: 2010–2020. *Journal of the National Cancer Institute 103*, 117–128.

Table 2 Average Medicare spending per patient for advanced stage nonsmall cell lung, colorectal, breast, prostate, and pancreas cancer, first 6 months from diagnosis, years 2004–09

Service type	*Mean cost (2011 $US)*	*Percentage of total cost (%)*
Acute hospital care	16,953	48.3
Chemotherapy[a]	5705	16.2
Outpatient procedures[b]	2281	6.5
Imaging[c]	1837	5.2
Radiation therapy	1832	5.2
Hospice	1743	5.0
Home health	870	2.5
Other[d]	3901	11.1
Total	35,122	100.0

Source: 2004–10 Medicare claims linked to surveillance, epidemiology, and end results (SEER) data for 61,838 individuals with common advanced solid tumors.

[a]Intravenous and oral antineoplastic agents.

[b]Outpatient surgery and other outpatient procedures.

[c]X-ray, ultrasound, computed tomography, magnetic resonance imaging, and nuclear medicine.

[d]Home health, outpatient physician fees, laboratory and pathology testing, Medicare part B medications other than antineoplastics, skilled nursing facilities and rehabilitation hospitals, durable medical equipment, other services (e.g., ambulance).

Adapted from Brooks, G. A., Li, L., Uno, H. et al. (2014). Acute hospital care is the chief driver of regional spending variation in Medicare patients with advanced cancer. *Health Affairs* (*Millwood*) **33**, 1793–1800.

(56%), followed by €13.5 billion (27%) spent in antineoplastic drugs, while outpatient and emergency visits accounted for less than 20%.

Although hospitalizations and antineoplastic drugs are responsible for the majority of cancer care costs, spending in other services have been proportionally increasing over time and may become significant contributors in the near future. One example is the use of expensive imaging diagnostic modalities. Between 1999 and 2006, Medicare beneficiaries with lung cancer had a mean annual increase of 36% and 7% in the use of positron emission tomography (PET) and magnetic resonance imaging (MRI), respectively. The increase in utilization of advanced imaging modalities corresponded to a difference in imaging costs per beneficiary of $1482 in 1999, to $3260 in 2006, or an average 9.5% annual escalation, compared with an average 2.6% annual escalation in total costs. No readily available data describe recent trends in radiation therapy costs, but those are likely to increase, given the dissemination of newer and expensive radiotherapy modalities, including stereotactic techniques, proton beam radiation, and tomotherapy.

The following sections focus on costs of hospitalizations and antineoplastic drugs, as those are the two service categories in which the greatest opportunities exist for implementation of strategies that improve the value of cancer care.

Factors Driving Hospitalization Costs

Two groups of factors independently influence hospitalization costs: those associated with higher utilization of acute inpatient care, and those related to variations in the unit price that hospitals charge for inpatient services.

Predictors of Utilization of Acute Inpatient Care

Limited access to outpatient care seems to substantially contribute to higher utilization of inpatient care services, and, by extension, to hospitalization costs. Population-based data from the California Cancer Registry has shown that 71% of 25,032 patients with advanced breast, prostate, colorectal, nonsmall cell lung, and pancreatic cancer underwent at least one hospitalization in the first year after the diagnosis, and 16% had three or more hospitalizations in the same period. Most of the hospital admissions (64%) originated from the emergency department (ED). Several patient and health-system characteristics predicted higher likelihood of re-hospitalizations, including Non-Hispanic Black and Latino race/ethnicity, lower socio–economic status, higher number of comorbidities, for-profit hospital ownership, and lack of outpatient palliative care. In a smaller study, investigators evaluated the medical reasons for hospitalizations in a retrospective cohort of 154 patients treated for gastrointestinal cancers at Dana–Farber Cancer Institute. Of a total of 201 hospitalizations, 53% were due to cancer-related symptoms, followed by 28% attributable to complications from cancer treatment. These studies jointly provide indirect evidence that limited access to outpatient care, which disproportionally affects patients of lower socioeconomic status or from racial/ethnic minority groups, lead to higher utilization of ED and inpatient acute care services.

Aggressive end-of-life (EOL) care leads to higher hospitalization rates and costs, presumably due to cumulative toxicities from consecutive lines of treatment or from poorly controlled cancer symptoms. Although definitions of aggressive EOL care differ across studies, a growing body of evidence consistently demonstrates that patients are more likely to undergo hospitalizations if they receive chemotherapy in the last months of life, do not receive timely hospice services, or do not have access to outpatient palliative care programs. In the study of 154 patients with gastrointestinal cancers treated at Dana–Farber Cancer Institute, investigators classified 39 of the 201 (19%) hospital admissions as avoidable. Patients who received third or subsequent lines of noncurative chemotherapy were significantly more likely to have an avoidable hospitalization compared with patients who received only one line of chemotherapy (36% vs. 23%). In a secondary analysis of a landmark randomized trial comparing early palliative versus standard care in advanced nonsmall cell lung cancer, patients randomized to the early palliative care group had lower hospitalization costs in the last 30 days of life (mean cost difference = $2896 per patient) compared with the control group. In a retrospective matched cohort study of Medicare decedents diagnosed with cancer or other diseases, patients who received hospice care at any time had lower number of hospitalizations, shorter hospital length of stay, and lower likelihood of admissions to the Intensive Care Unit.

Comorbidities and inpatient complications also contribute to higher hospitalization costs for adults with advanced cancer. In a prospective, multicentric cohort study involving 1020 hospitalized patients diagnosed with multiple solid and hematologic malignancies, the mean admission cost was US$10,364 per patient. Mean costs increased by $852, $5289, and $8267 for patients with higher number of comorbidities, or who had minor or major inpatient complications, respectively (**Table 3**). In the same study, consultation with inpatient palliative care was associated with a decrease in mean admission costs of $1506 when no complications occurred, and of $5617 in the setting of a minor or major complication, respectively.

This body of evidence strongly suggests that health systems may reduce the rate and costs of hospitalizations by improving access and coordination of care, symptom management, and by incorporating palliative care services to the inpatient and outpatient settings.

Predictors of Higher Hospital Service Prices

Very few studies have systematically evaluated the degree of variation in prices charged by hospitals for specific inpatient services, but emerging data indicates substantial differences in the dollar amount billed for the same services across healthcare systems. In

Table 3 Factors driving the variation in hospitalization costs in adults with cancer treated in four large US Medical Center (Hospital perspective; $N = 1{,}020$)

Factor	*Average marginal effect (2011 $US)*	*95% Confidence interval*
Patient characteristics		
Age	−53	−99 to −6
Comorbidities	852	550–1153
Admitting diagnosis		
Electrolyte disorder	−4759	−7928 to −1590
In-hospital complications		
Major	8267	4509–12,025
Minor	5289	3480–7097

Results adjusted for other patient characteristics (sex, race, education, insurance status, presence of advance directive, and activities of daily living), and disease characteristics (tumor type, other admitting diagnosis, symptom burden, analgesic use).
Adapted from May, P., Garrido, M. M., Aldridge, M. D. et al. (2017). Prospective cohort study of hospitalized adults with advanced cancer: Associations between complications, comorbidity, and utilization. *Journal of Hospital Medicine* **12**, 407–413.

a retrospective study of claims data from privately insured individuals in the United States between 2007 and 2011, variation in hospital transaction prices was the primary driver of total spending variation across hospital referral regions, after accounting for differences in patient characteristics. The same study revealed that service prices are 15.3% higher for hospitals that hold market monopoly power in their geographic regions, that is, have few or no competitors in the area. This type of data supports the concern that the ongoing merging of large for-profit healthcare systems contribute considerably to total healthcare spending, and, by extension, to cancer costs.

The Growing Costs of Antineoplastic Drugs

The cost of antineoplastic drugs (hereafter, cancer drugs) is the fastest growing of all cancer cost categories. Bach et al. have analyzed temporal trends of monthly acquisition costs for new cancer drugs at the time of their market approval. These costs have exponentially increased from a median of approximately US$100 in the 1970s to more than $10,000 after 2010 (Fig. 2). Medicare spending in cancer drugs has increased from $3 billion in 1997 to $11 billion in 2004, a 267% difference, partly due to increase in drug prices, partly due to higher drug utilization over time. Cancer drugs account for 7 of the 10 most expensive drugs reimbursed by Medicare part B, which is the federal public insurance program responsible for payments of injectable medicines in the United States. Recent studies show that the prices of novel target drugs have been growing at a much faster pace than the overall cancer costs in the United

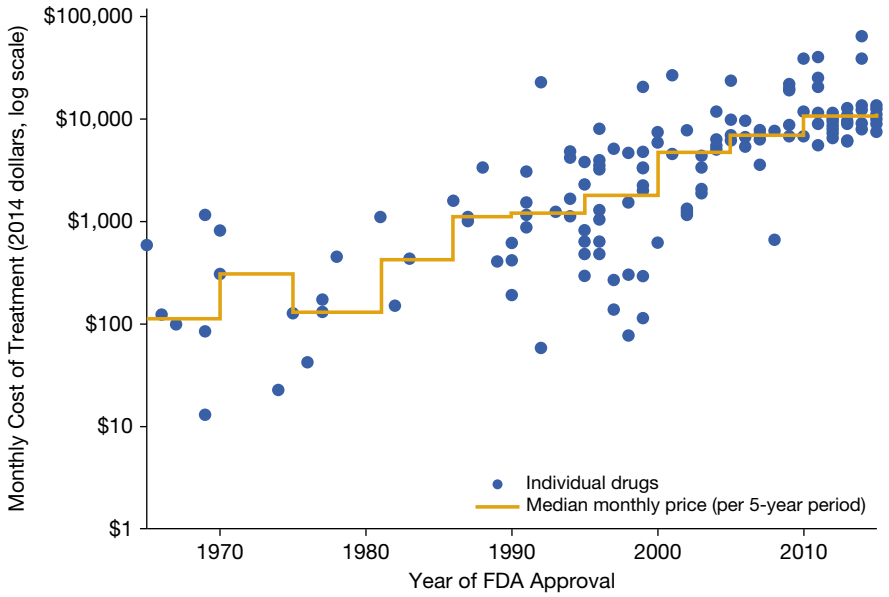

Fig. 2 Temporal trends in monthly drug acquisition costs at the time of drug regulatory approval (1965–2015). Adapted from the American Society of Clinical Oncology, (2016). The State of Cancer Care in America, 2016: A Report by the American Society of Clinical Oncology. *Journal of Oncology Practice* **12**, 339–383.

Table 4 Sources of increase in cancer drug expenditures per beneficiary, from 2001 to 2005 and 2005 to 2010

Source	*Expenditure (2013 $US)*	*%*
Total increase between 2001 and 2005	7765.2	100
Increase in use of targeted therapy drugs	6534.4	84
Increase in launch price of new targeted therapy drugs	432.4	6
Increase in price of targeted therapy drugs after launch	798.4	10
Total increase between 2005 and 2010	6846.5	100
Increase in use of targeted therapy drugs	5091.6	74
Increase in launch price of new targeted therapy drugs	1016.0	15
Increase in price of targeted therapy drugs after launch	738.9	11

Source: Analysis conducted using LifeLink Health Plan Claims Database from January 2001 to September 2011.
Adapted from Shih, Y. C., Smieliauskas, F., Geynisman, D. M. et al. (2015). Trends in the cost and use of targeted cancer therapies for the privately insured nonelderly: 2001 to 2011. *Journal of Clinical Oncology* **33**, 2190–2196.

States, with annual increase rates of 12%–14%. Finally, the prices of cancer drugs have also been rapidly increasing even after their market approval. One study estimated an average annual increase of 11% in the monthly payments for oral target drugs years after approval (Table 4; Fig. 3).

Although one could be tempted to attribute the rapid increase in cancer drug prices to the development of new drugs with innovative mechanisms of action, evidence suggests that this is not necessarily the case. In a metaanalysis of 74 clinical trials that supported the United States regulatory approval of drugs for solid tumors between 2000 and 2015, the median monthly drug acquisition costs (2016 US$) were $10,583, $11,980, $13,320, and $15,240, for chemotherapy, oral target drugs, immunotherapy, and antiangiogenic drugs, respectively ($P = .22$). Of note, this study revealed an annual increase in monthly drug prices of 13%, a finding consistent with other literature reports. In addition, the authors reported a lack of correlation between monthly drug prices with efficacy endpoints reported in the trials, including progression-free and overall survival. This and other studies indicate that high drug prices have little correlation with innovation.

Why Cancer Drugs Are Expensive

The general consensus is that pharmaceutical companies set drug prices based on what "the markets can bear." This practice would be reasonable if it were not for the several distortions that characterize the oncology drug market. These market imperfections are the driving forces of the rapidly escalating costs of cancer drugs, and the misalignment between drug prices and efficacy, as outlined below. For didactic purposes, we classify the drivers of drug costs as those that lead to high acquisition prices, followed by those associated with increased drug utilization.

Factors Associated With High Drug Prices

Pharmaceutical Industry's Monopoly Power

Simply put, patent protection laws allow pharmaceutical companies to behave like monopolies and set very high prices for their drugs. The rationale for patent protection is to give companies the opportunity to obtain a favorable return on the risky investment of developing a drug. In general, patent laws prohibit other companies from manufacturing generic formulations of the same compound, which prevents competition from forcing drug prices down. Pharmaceutical company representatives argue that this monopoly power is necessary and justified given the high risks and costs involved in research and development (R&D), estimated at US$2.6 billion per approved drug if one accounts for the money spent in preclinical and clinical research of agents that fail to receive market approval.

The counterpoints to the Pharma views are several: (1) some health economists challenge the accuracy of reported R&D costs; (2) pharmaceutical companies do not really incur risks if the sales of approved drugs are supposed to compensate for the money spent in the development of failed compounds; and (3) drug prices can be elastic to demand, that is, pharmaceutical companies may decrease prices if clinics are not willing to adopt their high-cost drugs when cheaper alternatives exist. The case of aflibercept (Zaltrap, Sanofi) illustrates the latter point. This drug received US regulatory approval for the treatment of metastatic colorectal cancer in August of 2012. Sanofi set the monthly price of aflibercept at roughly $11,000. In October 2012, clinicians at Memorial Sloan-Kettering Cancer Center publically announced that they would not include aflibercept in their drug formulary after noticing that bevacizumab, a drug of similar class, offered nearly identical efficacy at a monthly cost of roughly $5000. After Memorial's public announcement, Sanofi decreased the price of aflibercept by 50% to allow its inclusion in drug formularies. The aflibercept case indicates that pharmaceutical companies can retain profitability despite large cuts in drug prices, which confirms the notion that monopoly practices contribute to costs that have little correlation with drug efficacy.

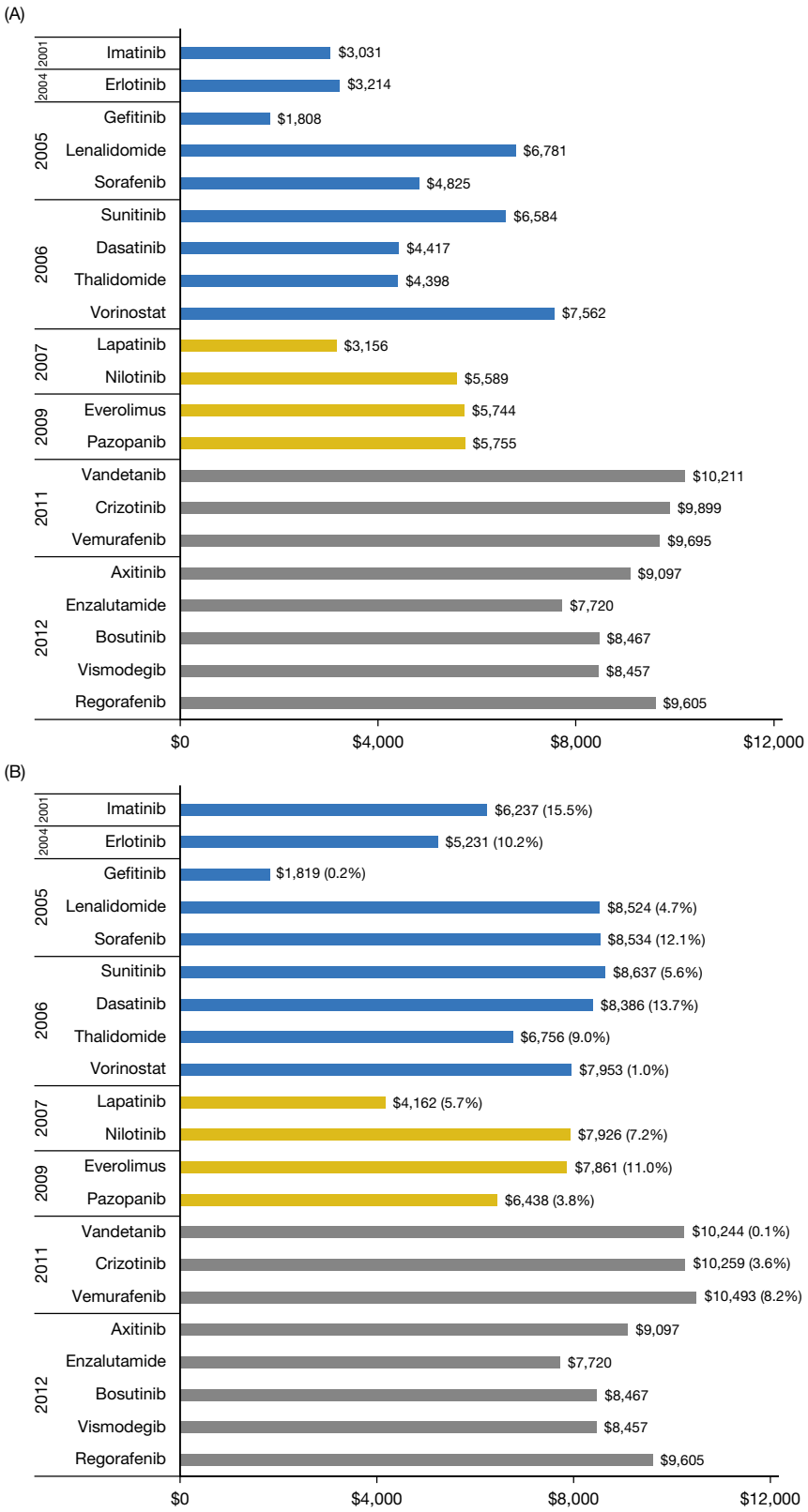

Fig. 3 Per patient per month (PPPM) Medicare costs of oral target therapies at year of launch and in 2012. PPPM costs represent a proxy of actual monthly drug prices paid by Medicare. Panel A shows PPPM costs for oral target drugs at launch year or in 2007 (whichever was later). Panel B shows PPPM costs for the same drugs in 2012, including drugs approved before 2012 and in 2012. Percentages in parentheses indicate the annual rate of increase in PPPM costs. The figure shows that 5 out of the 16 oral drugs approved before 2012 had annual rates of increase above 10%. *Source*: SEER-Medicare, 2007 to 2012. Adapted from Shih, Y. T., Xu, Y., Liu, L., et al. (2017). Rising prices of targeted oral anticancer medications and associated financial burden on Medicare beneficiaries. *Journal of Clinical Oncology*, **35**(22), 2482–2489.

Federal Legislation on Drug Pricing in the United States and Abroad

US federal laws preclude any considerations of costs for regulatory and market approval of drugs. Specifically, the Food and Drug Administration (FDA) appraises the evidence supporting drug efficacy and safety for regulatory approval, but not costs. Federal law also mandates that Medicare covers all drug products approved by the FDA. The Medicare Prescription Drug, Improvement, and Modernization Act of 2003 determined that Medicare reimburses drug costs based on the price set by the manufacturer, without engaging in price negotiations.

In the current format, the United States federal policies on drug pricing give unfair market advantages to the pharmaceutical industry at the expense of patients, payers, and society. Since Medicare has no bargaining power to negotiate the reimbursement for drugs, companies have yet an additional incentive to set high prices to maximize profits regardless of drug efficacy. The influence of legislation on drug prices affects not only Medicare beneficiaries, but also the commercially insured population, since commercial plans tend to adopt similar drug reimbursement schedules paid by Medicare. The ultimate consequence is that the United States legislation significantly contributes to high cancer drug costs.

To quantify the impact of the United States federal legislation on cancer drug costs, investigators have compared drug prices in the United States versus other national health systems that explicitly consider costs for drug regulatory approval. One study showed that the launch prices were 42% higher in the United States compared with the United Kingdom (UK) for 10 top-selling cancer drugs. The British National Institute for Health and Care Excellence (NICE) has developed and implemented a highly transparent and standardized process of appraisal of evidence to recommend the acceptance or rejection of new drug applications for market approval in the United Kingdom. The evidence appraisal requires the conduct of formal cost-effectiveness analysis (CEAs). In order to meet criteria for market approval in the United Kingdom, a given drug cannot exceed preestablished cost thresholds per unit of clinical benefit, usually measured in quality-adjusted life-years (QALYs). The explicit consideration of cost-effectiveness has enabled the U.K. National Health System to cover cancer drugs at lower costs. Additional studies also showed higher cancer drug prices in the United States versus other developed countries, including Norway, Australia, and Canada.

In response to country-specific requirements for drug market approvals, pharmaceutical companies have commonly adopted a strategy of setting lower drug prices where cost regulations exist, and higher prices in countries that do not impose price caps. This global pricing strategy essentially forces the United States healthcare system to subsidize the costs of cancer drugs abroad and contributes to the escalation of cancer costs.

Drug Development Costs

Representatives from the pharmaceutical industry claim that expenditures in drug research and development (R&D) at least in part explain the high prices of approved cancer drugs. A recent study suggests that R&D costs, though considerable, explain only a minor fraction of marketed drug prices. The investigators analyzed the R&D spending and the revenue sales for 10 companies and their respective 10 drugs that received market approval in the United States. The median time and costs (2017 US$) to develop a drug was 7.3 years and $648 million, respectively. With a median time of 4.0 years since approval, the median revenue sales was $1.6 billion, resulting in a $1 billion return on investment per approved drug. For all 10 drugs, the total R&D spending was $7.2 billion, compared with a total of $67.0 billion in revenue sales. The study intentionally did not consider the R&D costs of failed drugs, and concluded that the costs of drug development do not seem to justify the postmarket costs of approved cancer drugs.

Factors Associated With Higher Drug Utilization

Fee-For-Service Reimbursement Models

In the United States, the most common reimbursement policy for injectable drugs follows the fee-for-service (FFS) model, also known as "buy and bill." In FFS, insurers pay a reimbursement fee to clinics whenever physicians prescribe injectable antineoplastic drugs. Clinics derive variable profit margins from administering injectable drugs, which depend on the negotiated drug price with drug manufacturers and the actual amount covered by insurance plans. Since clinics' total revenues increase with higher number of prescribed drugs, this reimbursement model incentivizes physicians to overutilize cancer drugs in general, and to prioritize use of more costly drugs, which contributes to higher spending. The FFS model rewards clinics for higher quantity of drugs delivered, as opposed to better value offered by these drugs.

This perverse incentive became more evident after the implementation of the medicare modernization act (MMA) of 2003, which determined that the reimbursement for injectable drugs would consist of the average sales price (ASP, or the average of sales transactions between drug manufacturers and clinics purchasing the drugs) plus 6%. The goal of the MMA was to reduce drug spending by cutting reimbursement fees. One study reviewed the patterns of chemotherapy prescribing for advanced lung cancer before and after the implementation of the MMA in January 2005, and showed that chemotherapy spending actually increased with the new legislation. The authors explain the findings by a change in prescription patterns from less to more costly agents (e.g., docetaxel as a substitute to paclitaxel), as a way to maximize profit margins from the 6% fee. Clearly, new reimbursement models are necessary to reduce drug spending while preserving treatment efficacy. Such models need to link payments to clinical benefits, as opposed to the quantity or price of drugs prescribed.

Imperfect Information

A perfect competitive market assumes that consumers have adequate knowledge about the features of the goods and services that they purchase. Consumer information influences the demand for goods, which ultimately affects prices. In markets characterized by high degree of consumer information (e.g., food and clothing), prices tend to closely reflect how consumers value goods and services. In markets characterized by inadequate consumer information, prices often exceed what consumers would be willing to pay had they possess appropriate knowledge about the goods. The latter is the case of the oncology drug market. If we consider the patient as the consumer of cancer drugs, a legitimate concern arises about the amount and quality of the information that patients use to make decisions to pursue or forego drug treatments, including data regarding clinical benefits, toxicities, and out-of-pocket costs.

Not only patients may have limited understanding about the benefits and harms of cancer drugs, but their emotions and fears can impact their ability to make rational decisions about their care. Patients frequently make treatment decisions under substantial emotional distress, and often have overly optimistic expectations about the likelihood of benefit. When options are limited and prognosis is poor, fear and despair prompt many cancer patients to pressure their physicians to initiate subsequent lines of chemotherapy despite the minimal likelihood of favorable outcomes and often high risks of harm, which increases utilization of low-value drugs, drug spending, and hospitalization costs. In fact, current evidence shows that second- or third-line chemotherapy lines are not associated with longer overall survival in "real-world" settings (i.e., practice outside clinical trials), but do contribute to cancer spending.

Concerns About High Cancer Drug Costs

The previous sessions characterized cancer drugs as the fastest growing category of cancer-related costs, and provided market-based explanations for this phenomenon. Yet, drug costs account for 15%–30% of cancer spending, which raises the question of whether concerns about drug costs are justifiable or constitute a distraction. The following sessions discuss the reasons why cancer drug costs clearly represent a public health concern.

Low-Value: Marginal Benefits, Sobering Costs, Budget Constraints

A wealth of data demonstrates a lack of correlation between drug efficacy and costs. Despite their soaring prices, most cancer drugs offer modest clinical benefits. In a review of the clinical trials that supported the FDA approval of 71 drugs for a variety of solid tumors between 2002 and 2014, the aggregate median increase in progression-free survival was 2.5 months, and the aggregate median increase in overall survival was 2.1 months (Fig. 4). Of the 71 drug approvals, only 30 (42%) qualify as providing a "clinically meaningful" improvement, as defined by a consensus statement published by the American Society of Clinical Oncology (ASCO). In a review of 20 cancer drugs approved by the FDA between 2009 and 2013, 1-year drug costs ranged from US$59,000 to $157,000. The authors found weak correlations between annual drug costs and improvements in patient outcomes ($R^2 = 0.132$ for progression-free survival; $R^2 = 0.165$ for overall survival). Although some drugs represent true therapeutic breakthroughs (e.g., imatinib in chronic myeloid leukemia), most recently approved agents offer low value for their cost. The adoption of expensive, marginally effective drugs results in waste of scarce healthcare resources that could be spent in other interventions that offer greater benefits to cancer patients or society in general.

If the escalation of cancer drug costs continues, healthcare budgets will eventually approach their limits, and the sustainability of health programs will be at risk. This trend may result in implicit rationing of care. When faced with budget constraints, insurance plans react by imposing coverage restrictions on expensive drugs, increasing copays and deductibles, or increasing premiums. Patients who cannot afford higher co-pays or premiums will have limited access to treatments, a situation that characterizes implicit rationing. Those who oppose the notion of regulation of drug prices avoid the topic of implicit rationing, but at the same time accuse regulatory agencies such as NICE of rationing. If we characterize NICE's processes of drug appraisals as rationing, we should define it as explicit rationing, as the appraisals are at least transparent to the public. Regardless of whether society members choose to ignore or to consider costs in the approval process of new cancer drugs, at some point, decisions will have to be made about who receives treatment and who does not, either implicitly or explicitly.

Is Immunotherapy an Exception?

The emergence of immunotherapy drugs represents the newest paradigm in the treatment of several advanced solid and hematologic cancers. The most popular class of immunotherapy drugs consist of the programmed death 1 (PD1) or programmed death-ligand 1 (PD-L1) checkpoint inhibitors. These are monoclonal antibodies that induce an antitumoral immune response by prompting T-cells to recognize and kill tumor cells. Immune checkpoint inhibitors have gained much attention from the oncology community for their ability to induce durable tumor responses in several advanced solid and hematologic cancers. Although media outlets often portray immune checkpoint inhibitors as miraculous drugs, trial data show that their efficacy is not as impressive. Response rates range from 15% to 40% across diseases, the overall survival gain is usually measured in a few months, and most patients do not derive a survival benefit from these drugs.

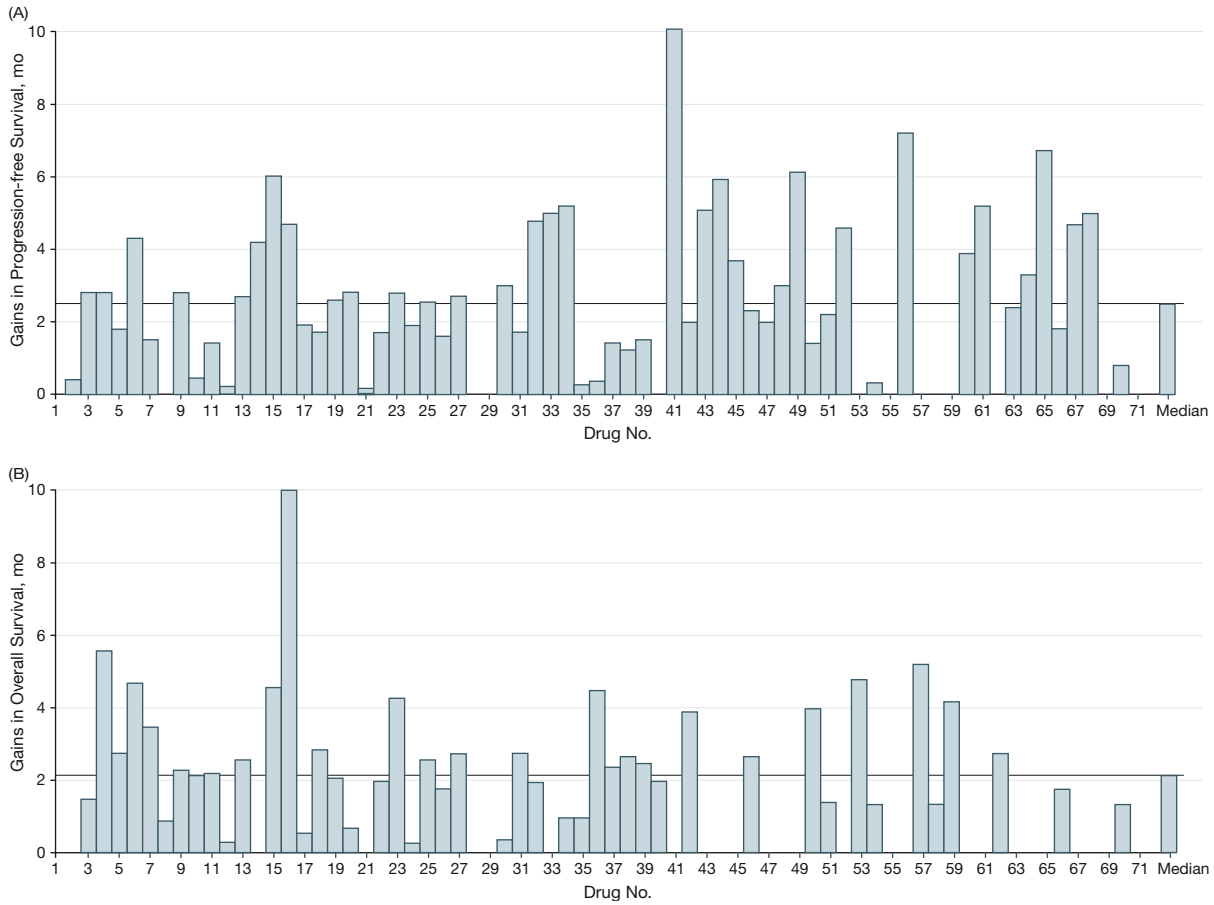

Fig. 4 Progression-free and Overall Survival of 71 Oncology Drugs Approved by the U.S. Food and Drug Administration from 2002 to 2014. The horizontal bars represent median increases in progression-free survival (PFS, panel A) and overall survival (OS, panel B) based on results of clinical trials that led to drug approval by the Food and Drug Administration (FDA). Across the 71 approved drugs, the aggregated gain in PFS and OS were 2.5 and 2.1 months, respectively. Adapted from Fojo, T., Mailankody, S., Lo, A. (2014). Unintended consequences of expensive cancer therapeutics-the pursuit of marginal indications and a me-too mentality that stifles innovation and creativity: The John Conley Lecture. *JAMA Otolaryngology—Head & Neck Surgery* **140**, 1225–1236.

Despite much advertising, PD1 and PD-L1 checkpoint inhibitors bring the same concerns of excessive costs relative to benefits. In the United States, two PD1 (nivolumab, pembrolizumab) and three PD-L1 (atezolizumab, durvalumab, avelumab) checkpoint inhibitors have received FDA approval for the treatment of several cancers. In 2017, the monthly costs for these drugs ranged from US$13.8,000 to $14.9,000, based on average wholesale prices (**Table 5**). Multiple ongoing cost-effectiveness analyses are estimating the value of these drugs, but published studies suggested that checkpoint inhibitors are not cost-effective for the treatment on nonsmall cell lung cancer and head and neck cancers.

Some peculiarities of PD1 and PD-L1 drugs deserve attention when considering the value of checkpoint inhibitors. These drugs are clearly less toxic than conventional chemotherapy. In randomized trials of second-line therapy for metastatic nonsmall cell lung cancer, grade 3 or 4 toxicities occurred in 7%–16% versus 35%–55% of patients treated with checkpoint inhibitors versus docetaxel,

Table 5 Monthly average wholesale price (AWP) for immune checkpoint inhibitors approved in the United States

Drug	*AWP*	*Indication*	*Dose*	*Monthly cost (2017 US$)*
Nivolumab	$3054.18/100 mg	Melanoma	240 mg Q14 days	$14,660.1
Pembrolizumab	$2628.44/50 mg	NSCLC	200 mg Q21 days	$14,018.3
Atezolizumab	$10,344/1200 mg	Urothelial	1200 mg Q21 days	$13,792.0
Durvalumab	$4174.57/500 mg	Urothelial	10 mg/Kg Q14 days	$13,809.5
Avelumab	$1804.8/200 mg	Merkel cell	10 mg/Kg Q14 days	$14,925.7

NSCLC, Nonsmall cell lung cancer; *Q14* days, every fourteen days; *Q21* days, every twenty-one days.

respectively. In order to estimate the value of checkpoint inhibitors, further studies need to fully account for the effect of their low toxicity profile on cost-effectiveness.

Financial Toxicity

In response to rapidly rising healthcare costs in the United States, many insurers have shifted excess costs to patients in the form of higher premiums, deductibles, and copayments. The Kaiser Family Foundation estimates that annual out-of-pocket costs for employer-based health plan premiums and deductibles increased by over 50% between 2008 and 2015 (from $2943 to $5138). Further, an increase in multitiered prescription drug formularies in this same period from 7% to 32% disproportionately affects cancer patients who often require specialty drugs in the highest prescription tier associated with the highest level of cost sharing. In older Medicare beneficiaries, total Medicare spending on noncancer drugs is four times higher than spending on cancer drugs, as might be expected given that other medical conditions (e.g., heart disease, diabetes) are more common in the elderly. However, average annual beneficiary cost share is substantially higher in cancer patients versus others ($7226 vs. $1286). This trend in increased cost sharing coincides with one of the largest economic crises in recent times and a harsh reality that the average family with an income at 250%–400% of the federal poverty level has less than $3000 in liquid financial assets and a recent finding by the pew charitable trust that less than 40% of Americans can come up with $500–$1000 in cash in the case of an emergency.

High cost sharing for cancer drugs and other services in combination with chronicity and intensity of cancer care leading to loss of work and income contributes to a set of circumstances that puts cancer patients and their families at particularly high risk for financial hardship. A growing body of literature had led to the recognition of an emerging side effect of cancer treatment known as "financial toxicity," a term that encompasses a range of financial hardships including difficulty meeting household expenses, debt, bankruptcy, and permanent loss of career and income. A 2011 study by Bernard et al. utilizing data from the Medical Expenditures Panel Survey (MEPS) showed that patients with cancer who were either uninsured or enrolled in private and public health plans consistently reported higher financial burden (defined as $>20\%$ of income spent on healthcare) than individuals with other chronic medical conditions. Another analysis of bankruptcy rates in cancer cases versus matched controls in Washington State showed that individuals with cancer were 2.65 times more likely to file for bankruptcy after diagnosis than matched controls. Several other studies have shown that, across various cancer types, approximately 20%–50% of patients experience significant financial hardship or "toxicity" postdiagnosis including missed work days, disability, inability to cover medical care costs or household expenses, accumulation of debt, and bankruptcy filings. Further, a recent analysis of 2011 MEPS data in approximately 1200 cancer patients showed that approximately 23% experienced "psychological financial hardship" defined as significant anxiety or worry about the costs and affordability of cancer care.

Emerging evidence suggests that financial hardship before and after cancer diagnosis can potentially lead to worse health outcomes including poorer treatment adherence, lower clinical trial enrollment, poorer quality of life, and even worse survival. A recent study reported quality of life scores using multiple measures in patients with lung and colorectal cancer; patients who reported having fewer financial reserves (money in the bank to cover household expenses for 2 months or less) scored significantly lower on all quality of life measures than cancer patients with greater financial reserves. In the previously mentioned bankruptcy study, cancer patients who filed for bankruptcy had a significantly higher risk of death than propensity score matched cancer patients who did not file for bankruptcy (HR 1.79, 95% CI 1.64–1.96). While the exact pathway that leads from poorer financial status or financial hardship to poorer survival is not well elucidated, it is clear that financial toxicity contributes to undesirable outcomes and therefore must be addressed by the oncology community.

Across studies, risk factors for financial toxicity include lower income, younger age, poorer preexisting financial status, advanced stage cancer, cancers requiring more aggressive treatment, and lack of health insurance. Other potential risk factors include role as the primary household income earner and being unmarried, though to potentially a lesser degree. Professional organizations such as the American Society of Clinical Oncology (ASCO) have emphasized the importance of screening patients and families for financial distress and intervening to mitigate financial toxicity in patients who might be at high risk. Unfortunately, few cancer clinics routinely screen for financial distress and, more importantly, even fewer have the capability to prevent financial hardship in those at high risk. Efforts to improve out-of-pocket cost transparency and increase communication between patients and oncologists about cost of care issues are needed. Certainly, addressing the larger problem of value in cancer care and the high price of cancer drugs may help to address financial toxicity. However, novel solutions such as changes in insurance benefit design and provision of financial counseling and navigation services as a routine part of cancer care are also needed.

Disparities in Treatment Access

High drug costs can widen ongoing disparities in access to cancer treatments. Multiple studies have consistently shown that patients of lower social economic status or who lack insurance coverage tend to present with more advanced cancer stages and are less likely to receive standard therapies. Those findings indicate that poverty represents a major access barrier to cancer treatments. New to these investigations is the recent evidence that high drug out-of-pocket costs represent a barrier to treatment initiation in and of itself. The impact of copays on treatment access is particularly relevant for oral cancer drugs. One such example regards to access to imatinib or other similar oral tyrosine kinase inhibitors for the treatment of chronic myeloid leukemia (CML). Imatinib substantially increases overall survival in CML patients, and has become a standard of care for this disease. In a study of Medicare beneficiaries diagnosed with CML, patients initiated imatinib or a similar kinase inhibitor earlier if they had drug cost-sharing subsidies

versus those who did not, suggesting that higher drug copay prevented timely initiation of therapy. Likewise, a cross-sectional survey of 174 cancer patients showed that 28% and 22% of the participants did not fill prescriptions or took fewer doses of oral cancer drugs due to costs, respectively. In this study, lower income was associated with lower adherence to oral medications. Other studies in breast cancer demonstrated an association of higher drug co-pays for tamoxifen and aromatase inhibitors with lower adherence to these medications.

The rising cost of cancer drugs increases the gaps between innovation and access, and accentuates the differences in the treatment patterns of high versus low income patients. Patients who can afford expensive therapies have an increasing number of treatment options, including the latest approved high-cost drugs, while those with limited financial resources can only receive a relatively fixed number of treatments. With the rapid pace of innovation, disparities in treatment access will also intensify on a global scale, as high-income countries will continue to implement cutting edge expensive technologies, while low- and middle-income countries struggle to develop infrastructure for basic cancer treatment, such as centers that offer surgery and radiation. Despite these obvious concerns, more rigorous research and policy changes are in order to address the impact of cancer costs on access to life-prolonging therapies.

Potential Solutions: Focusing on Value

Value is a construct that links the benefits of healthcare interventions to their additional costs. Value-based approaches offer appealing solutions to financially strained healthcare systems, by prioritizing the adoption of interventions that offer higher benefits when costs are similar among alternatives, or interventions that cost less but offer similar benefits compared with the alternatives. The following sessions briefly describe strategies that can improve value in cancer care, including those that focus on better quality care, innovative reimbursement models, and legislation reform.

Better Quality Care

Cancer care pathways

Cancer care pathways consist of software physician-oriented decision tools that provide evidence-based recommendations at the point of treatment decisions through the disease course, from first- to subsequent lines of chemotherapy. Currently available pathways mostly focus on systemic therapies for common advanced solid tumors, although their role is expanding to include early stage cancers. The goal of pathway adoption is to reduce the substantial variation in chemotherapy prescribing practices, thereby reducing costs while maintaining or improving patient outcomes. Several third-party vendors have developed and licensed pathways to clinics, but academic groups and insurance companies have occasionally created their own pathways as well. A recent study from Dana–Farber Cancer Institute showed that adoption of an internally developed pathway decreased the 1-year costs while maintaining overall survival of patients with advanced nonsmall cell lung cancer (**Table 6**). Studies of commercial pathways have shown similar results in early and advanced stage lung and colorectal cancer, respectively. Despite the promising data, concerns have arisen regarding the processes of pathway development and implementation, including issues of transparency about the selection of regimens included in pathways, physicians' restrictions of regimen choices, and the extra administrative burden of reporting pathway adherence. Formal guidelines for pathway implementation, such as the one proposed by the American Society of Clinical Oncology (ASCO), are necessary to ensure that patients have access to high-quality, evidence-based treatments.

Early palliative care

Early integration of palliative care holds great potential to decrease costs, prolong survival, and improve quality of life, as demonstrated in a pivotal randomized trial that assigned patients with advanced nonsmall cell lung cancer to a palliative care team at the time of first-line chemotherapy versus standard of care. Subsequent randomized studies confirmed that early palliative care improved patient outcomes in other advanced solid tumors. These studies suggest that early palliative care can decrease costs particularly by reducing the rates of hospital admissions and emergency room visits, and by facilitating timely transitions to hospice. Inpatient palliative care also decreases hospital costs by avoiding unnecessary escalation of care (e.g., ICU admissions), and by

Table 6 One-Year direct medial costs of before and after implementation of the Dana–Farber Cancer Care Pathways for stage IV Nonsmall cell lung cancer (Hospital perspective; $N = 370$)

Pathways cohort	*Mean cost (2014 US$)*	*95% Confidence interval*
Unadjusted cost		
Prepathway	64,508	53,140–75,876
Postpathway	48,515	41,421–55,608
Adjusted cost		
Prepathway	69,122	33,242–105,001
Postpathway	52,037	25,200–48,849

Adapted from Jackman, D. M., Zhang, Y., Dalby, C., et al. (2017). Cost and survival analysis before and after implementation of Dana–Farber Clinical Pathways for Patients with stage IV non-small-cell lung cancer. *Journal of Oncology Practice* **13**, e346–e352.

shortening the length of admissions. Despite the clear benefits, several challenges prevent widespread adoption of palliative care services, including a shortage of specialists, lack of proper reimbursement, and heterogeneous acceptance of palliative care by oncologists and patients. Future efforts should focus on training a workforce of palliative care providers, and educating patients and physicians about the benefits of early integration of palliative care.

Electronic symptom monitoring

Electronic symptom monitoring is a novel method of following and addressing patients' symptoms through web-based questionnaires. Patients are asked to report their symptoms frequently in between office visits. Symptoms that are intense or worsening prompt electronic messages to the physician's office, allowing providers to timely intervene. A recent randomized study showed that electronic symptom monitoring reduces ED visits and hospitalizations, allows earlier detection of tumor progression and changes in chemotherapy regimens, improves quality of life, and prolongs survival in patients with advanced solid tumors. Economic analyses of electronic symptom monitoring are underway, but this type of intervention will very likely help decrease cancer costs and improve patient outcomes. In Fact, many clinics in the United States and Europe are embracing different versions of electronic symptom reporting tools.

Precision oncology

At least in theory, precision oncology can improve the value of cancer drugs. This concept involves the use of predictive molecular biomarkers that allow the selection of patients who are more likely to benefit from target therapies, while avoiding the unnecessary costs of treating patients that will not benefit. The current data is conflicting to support or refute the hypothesis that precision oncology is cost-effective. Some published cost-effectiveness analyses have shown prohibitive costs per QALY gained for testing lung cancer patients for epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) mutations, and treat those whose tumors harbor these genetic abnormalities with the appropriate target drug. Other studies showed favorable cost-effectiveness ratios ranging from US$30,000 to $40,000 per QALY gained for treating patients with EGFR mutated lung adenocarcinomas with tyrosine kinase inhibitors compared with chemotherapy, but these studies did not account for the money spent in testing several patients to identify one mutation. The value of precision oncology will ultimately depend on several factors, including the performance and prices of multigene testing platforms such as next generation sequencing, the price, and survival benefits of target drugs, and the prevalence of clinically validated biomarkers.

Hospice

Timely referral to hospice decreases cancer costs without impacting on survival. A randomized trial from 1980s showed no differences in overall survival and decreased number of hospitalizations for cancer patients assigned to hospice versus those who did not receive hospice care. In a more recent retrospective study of Medicare beneficiaries with advanced lung cancer, hospice use was associated with a nonstatistically significant survival benefit compared with no hospice use. In a subsequent retrospective matched cohort study of Medicare patients, assignment to hospice decreased total and hospital costs compared with patients who did not receive hospice care. In this study, the decrease in costs was more pronounced with earlier hospice referrals (**Table 7**). Physicians should be aware that a transition to hospice adds value in cancer care, particularly when patients have exhausted treatment options or are experience a decline in performance status. Several barriers may prevent patients from receiving hospice, including the psychological discomfort involved in the discussions of transitions from active therapy to comfort care, and cultural influences on patients' perception of hospice. Oncology providers need appropriate training, psychological support, and reimbursement to appropriately conduct conversations involving hospice care and end-of-life planning.

Decision frameworks

Many organizations have developed decision frameworks to facilitate formal assessments of the value offered by cancer drug regimens, with the hope that summarized information about costs and benefits will encourage stakeholders to make value-based treatment choices. Examples of such efforts include ASCO's Value Framework, the European Society for Medical Oncology (ESMO) Magnitude of Clinical Benefit Scale, and the Institute for Clinical and Economic Review (ICER) Evidence Rating Matrix, among

Table 7 Medicare expenditures at the end of life in hospice and matched nonhospice controls ($N = 3069$)

Hospice use before death	*Mean expenditures per patient, hospice group (2008 US$)*	*Mean expenditures per patient, nonhospice group* [a] *(2008 US$)*	*Adjusted mean difference (2008 US$)*
Last 105 days	22,083	24,644	2561
Last 30 days	10,383	16,814	6431
Last 14 days	5698	10,738	5040
Last 7 days	4806	7457	2651

Data source: Health and Retirement Study; Medicare claims.

[a]Propensity score matched.

Adapted from Kelley, A. S., Deb, P., Du, Q., et al. (2013). Hospice enrollment saves money for Medicare and improves care quality across a number of different lengths-of-stay. *Health Affairs* **32**, 552–561.

others. Frameworks provide various methods and processes that quantify clinical benefits in a transparent manner, while some of these tools also explicitly consider drug costs. Although conceptually sound, prospective studies will be necessary to confirm the impact of value frameworks on treatment patterns, quality of care, and costs. One important limitation of frameworks is that they differ in regards to target audiences and scope, which prevents accurate comparisons of their impact. For example, the ASCO Value Framework targets physicians and patients, whereas ESMO's and ICER's frameworks were designed to inform policy makers and payers.

New Reimbursement Models

Under the pressure to contain costs, healthcare payers are developing and implementing novel treatment reimbursement models that emphasize value. Common to these models is the financial or performance accountability shared by payers, clinics, and drug manufacturers.

Oncology care model

In June 2016, the Centers for Medicare and Medicaid Services launched the Oncology Care Model (OCM) initiative, which consists of payment agreements between physician practices and multiple payers, including Medicare and commercial insurance plans. In OCM, physician practices are accountable for the expenses and performance surrounding predefined episodes of care that involve chemotherapy administration. Clinics that spend less than target expenditures per episode can retain a percentage of the savings if they meet prespecified quality benchmarks for that episode. OCM is an example of performance-based reimbursement models that incentivize physician practices to provide high-quality care at lower costs. Performance-based reimbursement has stirred some controversy, as clinics may have to accept financial losses when expenses exceed the targets or their performance does not meet quality benchmarks, depending on the contents of the payment agreements. Additional time and follow up assessments will be important to determine whether the OCM model actually increases value in cancer care.

Performance-linked reimbursement

Performance-linked reimbursement represents yet another model that links the payments for cancer drugs to the achievement of prespecified clinical outcomes. Different than OCM models, performance-linked reimbursement involves risk-sharing agreements between drug manufacturers and clinics or payers. If a given drug provides the expected benefits in real-world settings, clinics or payers will pay the manufacturer the full drug price. If the drug proves to be less effective than expected, manufacturers offer drug price discounts, rebates, or refunds to the purchaser. One example of this model regards to the use of bortezomib (Velcade, Johnson & Johnson) in patients with multiple myeloma. In 2006, the U.K. National Health System entered a performance-linked agreement with Johnson and Johnson, in which patients would undergo four cycles of bortezomib, followed by an assessment of serum M proteins to determine tumor response. Patients were considered to have had treatment failure if the serum M protein levels declined by less than 50%. For those patients, Johnson & Johnson agreed to refund the expenses for bortezomib, or provide the same amount of drug to other patients at no charge. Although appealing, performance-linked reimbursement requires robust infrastructure for real-world data collection, and considerable negotiations among parties to determine what constitutes the desired outcome measures. Despite these caveats, this model will likely gain traction among drug manufacturers and payers in the near future.

Episode-based payments

Episode-based payment models consist of lump-sum payments made to physicians at the beginning of an episode of care for each patient. Episodes of care are discrete cancer treatment events that span a predictable time period, usually of 6 months (e.g., adjuvant chemotherapy for breast or colorectal cancer). In this model, physicians take financial risk by absorbing the losses when the expenses exceed the amount paid at the beginning of the episode. The rationale is that physicians will prioritize regimens of similar efficacy and lower costs when alternatives exist, and will optimize the management of treatment- and disease-related complications to avoid patients from undergoing costly hospitalizations. A retrospective study evaluated the impact of an Episode-based payment model among volunteer clinics that contracted with United-Healthcare, a large private insurance company in the United States. In a sample of 810 patients with breast, lung, and colorectal cancers, episode-based payments resulted in estimated total savings of US$33.4 million with no effects on overall survival compared with historical controls (**Table 8**). In the Episode-payment patient cohort, chemotherapy utilization and costs were curiously higher than predicted, and most savings seem to have derived from lower

Table 8 Impact of an episode-based payment model on direct medical costs in five medical oncology groups ($N = 810$)

Cost category	*Predicted (2012 US$)*	*Actual (2012 US$)*	*Savings (US$)*
Total	98.1 million	64.8 million	33.4 million
Chemotherapy	7.5 million	20.9 million	−13.4 million

Adapted from Newcomer, L. N., Gould, B., Page, R. D. et al. (2014). Changing physician incentives for affordable, quality cancer care: Results of an episode payment model. *Journal of Oncology Practice* **10**, 322–326.

hospitalization costs. Other insurance groups are implementing episode-payment agreements, but additional data is necessary to inform the applicability of this model to other diseases and episodes of care, as well as the impact on patient survival, quality of life, and satisfaction with care.

Legislation Reform

No matter how impactful are the practice changes and reimbursement models implemented by physician and payers, cancer costs will continue to threaten budgets if drug acquisition prices follow the current escalating trend. Only comprehensive legislation reform can address the current lack of control on drug prices in countries like the United States. New federal policies need to give Medicare and other public insurance programs the autonomy to reject coverage for low-value drugs, and to use bargain power to negotiate prices that reflect value.

The first pillar of legislation reform should be a provision that allows Medicare to decline coverage for drugs based on price. Like in any negotiations, the "walk away" option is crucial to allow buyers and sellers to agree upon prices that are perceived as fair. Without the option to say no, Medicare does not really enter any negotiations, but acts as a price taker. On the same note, provisions need to allow the use of explicit methodologies to evaluate drug value and affordability, including cost-effectiveness analysis and budget impact models. This approach would allow the implementation of value-based drug pricing policies in a publically transparent manner. New policies should also give Medicare the flexibility to consider factors other than cost-effectiveness for drug coverage decisions, including disease severity, the specific characteristics of the patient population, equity issues, and budget constraints.

Legislative changes should promote more competition among manufacturers, instead of less. Competition allows market forces to decrease oncology drug prices. Generic medications and biosimilars need to enter the market sooner, and new laws need to restrict the ability of drug companies to buy extra patent protection time. Likewise, public insurance programs need to be able to import less costly cancer drugs, a strategy that would also create competition among domestic manufacturers and force prices down.

Despite the clear rationale, legislation reform would require strong political motivation from society. The pharmaceutical industry has a vetted interest to maintain current federal laws regarding drug pricing, and industry lobbyists exert powerful political influence that prevent the enactment of any meaningful reform. The prospects of legislative transformation ultimately depend on the leading role of other stakeholders, including healthcare providers, payers, and patient advocates. These groups need to jointly demand major legislative changes from politicians and counterbalance the industry lobby.

Conclusions

The rising costs of cancer care pose a risk to the sustainability of healthcare programs, and this trend will likely continue for the foreseeable future. Hospitalization and oncology drugs account for the majority of cancer expenditures, and result from multiple inefficiencies and misaligned incentives that affect the healthcare system. Stakeholders are developing several strategies to transform cancer care from a high-cost to a high-value enterprise.

See also: Aging and Cancer. Oncology Imaging. Radiation Oncology.

Further Reading

American Society of Clinical Oncology, 2017. American society of clinical oncology position statement on addressing the affordability of cancer drugs. Journal of Oncology Practice/American Society of Clinical Oncology. https://doi.org/10.1200/JOP.2017.027359 (Epub ahead of print).

Bennette, C.S., Richards, C., Sullivan, S.D., Ramsey, S.D., 2016. Steady increase in prices for oral anticancer drugs after market launch suggests a lack of competitive pressure. Health Affairs 35 (5), 805–812. https://doi.org/10.1377/hlthaff.2015.1145.

Brooks, G.A., Li, L., Uno, H., Hassett, M.J., Landon, B.E., et al., 2014. Acute hospital care is the chief driver of regional spending variation in Medicare patients with advanced cancer. Health Affairs 33 (10), 1793–1800. https://doi.org/10.1377/hlthaff.2014.0280.

Fojo, T., Mailankody, S., Lo, A., 2014. Unintended consequences of expensive cancer therapeutics-the pursuit of marginal indications and a me-too mentality that stifles innovation and creativity: The John Conley lecture. JAMA Otolaryngology. Head & Neck Surgery 140 (12), 1225–1236. https://doi.org/10.1001/jamaoto.2014.1570.

Kelley, A.S., Deb, P., Du, Q., Aldridge Carlson, M.D., Morrison, R.S., 2013. Hospice enrollment saves money for Medicare and improves care quality across a number of different lengths-of-stay. Health Affairs 32 (3), 552–561. https://doi.org/10.1377/hlthaff.2012.0851.

Lee, J.A., Roehrig, C.S., Butto, E.D., 2016. Cancer care cost trends in the United States: 1998 to 2012. Cancer 122 (7), 1078–1084. https://doi.org/10.1002/cncr.29883.

Luengo-Fernandez, R., Leal, J., Gray, A., Sullivan, R., 2013. Economic burden of cancer across the European Union: A population-based cost analysis. The Lancet Oncology 14 (12), 1165–1174. https://doi.org/10.1016/S1470-2045(13)70442-X.

Mariotto, A.B., Yabroff, K.R., Shao, Y., Feuer, E.J., Brown, M.L., 2011. Projections of the cost of cancer care in the United States: 2010–2020. Journal of the National Cancer Institute 103 (2), 117–128. https://doi.org/10.1093/jnci/djq495.

Porter, M.E., 2010. What is value in health care? The New England Journal of Medicine 363 (26), 2477–2481. https://doi.org/10.1056/NEJMp1011024.

Prasad, V., De Jesus, K., Mailankody, S., 2017. The high price of anticancer drugs: Origins, implications, barriers, solutions. Nature Reviews. Clinical Oncology 14 (6), 381–390. https://doi.org/10.1038/nrclinonc.2017.31.

Ramsey, S., Blough, D., Kirchhoff, A., Kreizenbeck, K., Fedorenko, C., et al., 2013. Washington state cancer patients found to be at greater risk for bankruptcy than people without a cancer diagnosis. Health Affairs 32 (6), 1143–1152. https://doi.org/10.1377/hlthaff.2012.1263.

Shankaran, V., Ramsey, S., 2015. Addressing the financial burden of cancer treatment: From copay to Can't pay. JAMA Oncology 1 (3), 273–274. https://doi.org/10.1001/jamaoncol.2015.0423.

Shih, Y.T., Xu, Y., Liu, L., Smieliauskas, F., 2017. Rising prices of targeted oral anticancer medications and associated financial burden on medicare beneficiaries. Journal of Clinical Oncology 35 (22), 2482–2489. https://doi.org/10.1200/JCO.2017.72.3742.

Sullivan, R., Peppercorn, J., Sikora, K., Zalcberg, J., Meropol, N.J., et al., 2011. Delivering affordable cancer care in high-income countries. The Lancet Oncology 12 (10), 933–980. https://doi.org/10.1016/S1470-2045(11)70141-3.

Winn, A.N., Keating, N.L., Dusetzina, S.B., 2016. Factors associated with tyrosine kinase inhibitor initiation and adherence among Medicare beneficiaries with chronic myeloid leukemia. Journal of Clinical Oncology 34 (36), 4323–4328. https://doi.org/10.1200/JCO.2016.67.4184.

Relevant Websites

https://www.cms.gov/—Centers for Medicare & Medicaid Services.
https://www.nice.org.uk/—National Institute for Health and Care Excellence (NICE).
https://www.fda.gov/—U.S. Food and Drug Administration.
https://data.worldbank.org/—World Bank.

Gastric Cancer: Pathology and Genetics

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Glossary

Advanced gastric cancer An invasive carcinoma infiltrating into the muscular propria and beyond.

***CDH1* gene** The human *CDH1* gene [MIM#192090] is localized in the long arm of chromosome 16, comprises 16 exons and encodes E-cadherin.

***CTNNA1* gene** The human *CTNNA1* gene [MIM#116805] is localized in the long arm of chromosome 5, comprises 16 exons and encodes α-E-catenin.

Diffuse carcinoma Subtype of gastric carcinoma composed of poorly cohesive neoplastic cells.

Early gastric carcinoma An invasive carcinoma limited to the mucosa or submucosa, regardless of nodal status.

Early onset gastric cancer Early onset gastric cancer (EOGC) is a gastric cancer presenting at the age of 45 or earlier, representing approximately 10% of all patients with stomach cancer.

E-cadherin E-cadherin is a transmembrane calcium-dependent protein that is predominantly expressed at the basolateral membrane of epithelial cells, where its functions are primarily cell–cell adhesion and suppression of invasion.

Epstein–Barr virus Double-strand DNA virus belonging to the herpes virus family (HHV4).

Gastric adenocarcinoma Malignant epithelial neoplasms with glandular differentiation.

Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) GAPPS is a variant of familial adenomatous polyposis (FAP) and is caused by point germline mutations in the promoter 1B of the *APC* gene. It is characterized by fundic gland polyposis, with areas of dysplasia and carries an increased risk of gastric adenocarcinoma.

Gastric carcinoma with lymphoid stroma (GCLS) GCLS is a morphological subtype of GC composed of irregular sheets, trabeculae, poorly developed tubular structures and isolated cells, embedded within a prominent lymphocytic infiltrate, with occasional lymphoid follicles.

Gastric microbiota The whole microbial community within the stomach.

Helicobacter pylori *Helicobacter pylori* are Gram-negative bacteria that colonize gastric mucosa, considered by WHO as a group I carcinogen for gastric cancer.

Hereditary diffuse gastric cancer (HDGC) HDGC is an autosomal dominant cancer susceptibility syndrome characterized by signet ring cell (diffuse) gastric cancer and lobular breast cancer, caused by germline mutations of *CDH1* and *CTNNA1* genes.

Immune inhibitory checkpoint Molecules of the immune system that inhibit the T cell signal by antagonizing costimulatory molecules necessary to the activation of the T cells.

Intestinal carcinoma Subtype of gastric carcinoma with glandular structure.

Tumor immune microenvironment Noncancerous cells and extracellular components constituting the tumor stroma.

Nomenclature

ACRG Asian Cancer Research Group

AJCC American Joint Committee on Cancer

CAF Cancer associated fibroblast

CagA *cag* pathogenicity island-encoded

EBER-ISH EBV-encoded small RNA in situ hybridization

EBV Epstein–Barr virus

EBVaGC Epstein–Barr virus associated gastric cancer

EGJ Esophagogastric junction

EMT Epithelial–mesenchymal transition

FAP Familial adenomatous polyposis

FGP Fundic gland polyps/polyposis

FIGC Familial intestinal gastric cancer

GAPPS Gastric adenocarcinoma and proximal polyposis of the stomach

GC Gastric carcinoma
GCLS Gastric cancer with lymphoid stroma
HDGC Hereditary diffuse gastric cancer
IGCLC International gastric cancer linkage consortium
IHC Immunohistochemistry
ISH In situ hybridization
MSI Macrosatellite instability
MSS Macrosatellite stable
SPEM Spasmolytic polypeptide-expressing metaplasia
TCGA The cancer genome atlas
TAM Tumor associated macrophages
TME Tumor microenvironment
VacA Vacuolating cytotoxin A

Definitions: Types and Subtypes

The vast majority of stomach cancer cases are gastric carcinomas (malignant epithelial neoplasms). Nonepithelial tumors predominantly include lymphomas and mesenchymal tumors. In this article, we will focus on gastric carcinoma (GC).

Most cases of GC are sporadic. Familial clustering is observed in about 10% of the cases. Hereditary GC accounts for a very low percentage of cases (1%–3%), encompassing hereditary diffuse gastric cancer (HDGC) and familial intestinal GC (FIGC). Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) has been recently recognized as a rare variant of familial adenomatous polyposis (FAP) with a distinct gastric phenotype. Furthermore, GC can develop in the setting of other hereditary cancer syndromes (see "**Molecular Pathology and Genetics**" section).

According to the depth of invasion, GC is classified as early and advanced. Early GC refers to an invasive carcinoma that is limited to the mucosa (pT1a) or the mucosa and submucosa (pT1b), with or without lymph-node metastases and regardless of tumor size. This classification is prognostically relevant (see "**Prognostic and Predictive Biomarkers**" section): early GCs have a better prognosis compared to advanced GCs, with a 5-year survival rate of 80%–95%, after surgical resection. By contrast, GCs infiltrating into the *muscularis propria* and beyond are defined as "advanced." *P*atients with advanced GC, if the tumor is unresectable, have a dismal prognosis with an expected survival of few months, even with chemotherapy and best supportive care.

Regarding the anatomic location, tumors arising in the esophagogastric junction (EGJ) are staged as esophageal or GCs, depending on the location of the tumor epicenter with respect to the EGJ. According to the 8th edition of the American Joint Committee on Cancer (AJCC) staging system, EGJ tumors with the epicenter located >2 cm distal to the EGJ should be classified using the stomach cancer system, while EGJ tumors with the epicenter at ≤ 2 cm below the EGJ that straddle the junction should be staged as esophageal tumors.

GCs represent a biologically heterogeneous group of tumors with multifactorial etiologies, encompassing environmental, genetic and molecular factors. They are characterized by broad morphological heterogeneity with respect to the architecture, pattern of growth, cell differentiation, histogenesis and molecular pathogenesis (**Fig. 1**). Several histopathological classifications have been proposed along the years, reflecting the heterogeneity of GC morphology (see "**Pathology**" section). Moreover, the advent of high-throughput molecular technologies has recently led to a better understanding of GC molecular complexity (see "**Molecular Pathology and Genetics**" section).

Burden

GC accounts for about 7% of cancers worldwide (being the fifth most common malignancy in the world, behind cancers of the lung, breast, colo-rectum and prostate) and is the third leading cause of cancer death in both sexes. However, the distribution is uneven, from areas with high incidence (>60 per 100,000 males) such as East Asia, central and eastern Europe and Latin America, to low incidence areas (<15 per 100,000 population) such as North America, Northern Europe and most countries in Africa and South-Eastern Asia. More than 70% of the cases occur in developing countries. Countries with a high incidence of GC, in which there are screening programs of asymptomatic individuals, have a high incidence of EGCs, ranging from 30% to 50% in East Asia (e.g., Japan), with lower figures for the West (16%–24%).

In regions with high incidence of GC, most cases are localized in the antrum and pylorus. GCs of the proximal stomach and the EGJ are more common in low incidence countries (e.g., North America and Europe) and are associated with gastro-esophageal reflux disease (GERD).

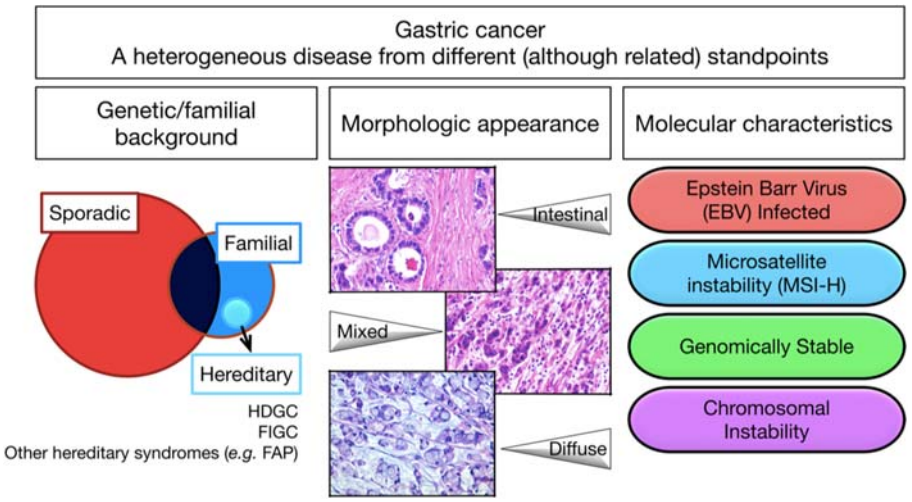

Fig. 1 Gastric cancer is a heterogeneous disease from different standpoints: etiology and pathogenesis, morphology and molecular characteristics. HDGC, hereditary diffuse gastric cancer; FIGC, familial intestinal gastric cancer; FAP, familial adenomatous polyposis.

Notably, EGJ adenocarcinoma shares many epidemiological characteristics with cancers developing in the distal esophagus and proximal stomach, and the incidence is higher in Caucasians, namely in male old patients. The incidence of EGJ adenocarcinoma increased markedly in the second half of the 20th century both in the United States and Europe, in parallel with rising incidence of adenocarcinomas of the lower esophagus.

Time Trends

Worldwide, there has been a steady decline in the incidence and mortality of GC over the last 15 years. However, the absolute incidence rate continues to rise, due to the advancing age of the global population. More specifically, there has been a shift in the proportion of some subtypes: the incidence of "intestinal" carcinoma has decreased mainly in young patients, and the incidence of "diffuse" carcinoma localized to the proximal stomach is increasing.

The decline of incidence has been attributed to the decrease in the prevalence of *Helicobacter pylori* (*H. pylori*) infection, better food refrigeration, reduced intake of preserved foods and salt, and a richer and more varied diet, including a higher intake of fruit and vegetables (see diet in "Risk Factors" section).

Age and Sex Distribution

Age-standardized incidence rates are about twice as high in men compared to women. In general, incidence increases progressively with age in males and females.

Early onset GC (EOGC) is defined as any GC presenting at the age of 45 or earlier and represents approximately 10% of all patients with stomach cancer. In some populations, susceptibility genetic factors and the infection by virulent strains of *H. pylori* may be important for early-onset disease. GC occurrence before the age of 30 years is very rare (1.1%–1.6%) and patients diagnosed before 20 years are exceptional. Many cases display diffuse histology and occur in HDGC families. Several studies point to a pathogenesis of EOGC different from that of sporadic cancers occurring at a later age.

Risk Factors

Gastric carcinogenesis is a multistep and multifactorial process which, in many cases, appears to involve a progression from normal mucosa through chronic gastritis (chronic inflammation of the gastric mucosa), atrophic gastritis (with loss of gastric glands) and intestinal metaplasia (substitution of gastric epithelium by intestinal epithelium) to dysplasia (intraepithelial neoplasia) and carcinoma, a sequence of events that may last several years. This sequence has been designated as the Correa's cascade of multistep gastric carcinogenesis. However, the Correa's model does not explain the carcinogenetic pathway of all types of GC. Actually, a proportion of GCs arises in nonintestinalized mucosa and retains gastric phenotype (and gastric differentiation is also observed in gastric

dysplasia, the ultimate precursor lesion of GC). Another pattern of metaplasia, which is believed to represent an alternative pathway to gastric neoplasia, is spasmolytic polypeptide-expressing metaplasia (SPEM), which expresses trefoil factor family 2 (TFF2) spasmolytic polypeptide and represents the metaplastic replacement of oxyntic glands by mucin secreting antral-like glands. SPEM develops in the gastric body and fundus and is strongly associated with chronic *H. pylori* infection and development of GC. Moreover, as occurs in GAPPS syndrome, gastric dysplasia and GC may arise in fundic gastric polyps. Together, this evidence shows that GC may also arise from the gastric epithelium.

Helicobacter Pylori Infection

Among environmental factors, *H. pylori* infection plays a major role. Almost all noncardiac GCs develop from a background of *H. pylori* infected mucosa. *H. pylori* are Gram-negative, spiral-shaped, microaerophilic bacteria that colonize the gastric mucosa within the mucus layer and also in contact with epithelial cells. In 1994, the WHO categorized *H. pylori* as a group 1 carcinogen for GC, based on results of epidemiological studies that were available at that time, and later confirmed. The bacteria can be identified in gastric mucosa by routine stains, such as hematoxylin–eosin, modified Giemsa and other ancillary methods, such as Warthin–Starry staining and immunohistochemistry (Fig. 2). The infection is commonly acquired during early childhood and persists throughout adult life unless eradicated.

H. pylori virulence factors that appear to influence the pathogenicity of the bacteria, as well the risk of GC development, include the cag pathogenicity island-encoded CagA, and the vacuolating cytotoxin, VacA.

CagA is present in about 60%–70% of strains and is a marker for the presence of the cag pathogenicity island. This pathogenicity island encodes a type IV secretion system that allows the translocation of CagA into the host cells. CagA can activate multiple host signaling pathways leading to cell proliferation, cytoskeletal rearrangements, and disruption of cell–cell junctions. Transgenic expression of CagA was shown to lead to GC in mice. Strains producing the cagA protein that induce a greater degree of inflammation are associated with gastric precancerous lesions and a greater risk of developing cancer of the distal stomach.

The gene encoding VacA is present in all *H. pylori* strains, but is polymorphic and varies significantly in the signal (s)-, the intermediate (i)-, and the mid (m)-regions, each with two major types. Infections with the more virulent vacA s1, i1, and m1 strains increase the risk for peptic ulcer disease and for GC, when compared with the less virulent vacA s2, i2, and m2 strains. The more virulent strains are frequently found associated with higher levels of inflammation, epithelial damage, gastric atrophy and intestinal metaplasia. Although the risk of GC in some countries of Europe and North America has been related to vacA genotype, such relationships have not been observed in eastern Asian countries, suggesting that consequences of the variation in vacuolating activity are dependent on geographical region. In East Asia, most *H. pylori* strains are vacA s1, i1, and m1, and are not associated with any particular clinical outcome.

Corpus-predominant gastritis with multifocal gastric atrophy, and hypo- or achlorhydria is seen in approximately 1% of subjects infected with *H. pylori*.

The acidic pH of the stomach, ranging from 1–3, works as a primary innate defense mechanism and provides an effective barrier to microbial overgrowth. Development of atrophic gastritis, which induces elevation in gastric pH, due to parietal cell loss, may lead

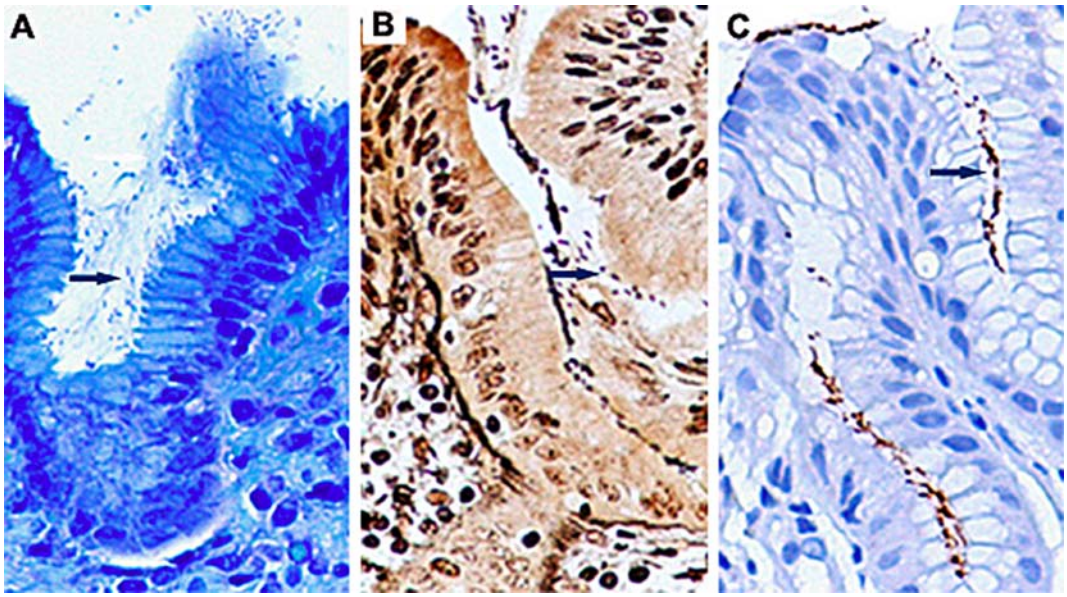

Fig. 2 *Helicobacter pylori* detected in the lumen of gastric glands and adherent to the apical pole of epithelial cells: (A) modified Giemsa, (B) Warthin Starry, (C) immunohistochemistry.

to a shift in the composition of the gastric microbiota (the microbial community within the stomach). This event may contribute to malignant transformation, through maintenance of inflammation, and conversion of nitrates into mutagenic *N*-nitroso compounds. It has been demonstrated that patients with decreased acid secretion caused by *H. pylori*-associated atrophic gastritis, partial gastrectomy, or long-term acid suppression, especially with proton pump-inhibitors, have significant intragastric bacterial overgrowth, increased counts of nitrate-reducing bacteria, and increased nitrite and N-nitrosamine levels. This would in part explain the lack of success of *H. pylori* eradication in preventing GC in patients that have atrophy and intestinal metaplasia at baseline. Recent advancements in molecular techniques and computational analysis have provided evidence that the complex microbiota colonizing the gastric epithelium may influence gastric homeostasis and disease in combination with *H. pylori*. In support of this hypothesis, it was recently demonstrated that the microbial community of GC patients has increased nitrosating functions and genotoxic potential, compared with patients with chronic gastritis.

However, the large majority (>90%) of the individuals do not develop GC, indicating a role for other causative agents and the genetic background (Fig. 3). Host genetic susceptibility to GC involves several genes, each one however, with small effects. In this regard, several genes have been studied, namely those involved in the protection of gastric mucosa against damaging agents, in inflammatory response, in detoxification of carcinogens, in the synthesis and repair of DNA, in folate metabolism, in the regulation of gene expression and in the cell adhesion. Having all these issues in mind, it can be stated that GC (as many other, if not all diseases) is the end product of gene (genetic susceptibility)/environment interaction (Fig. 4).

Epstein–Barr Virus Infection

Epstein–Barr virus (EBV) is a double-strand DNA virus that belongs to the herpes virus family (HHV4) and has been recognized as a distinct pathogenic cause of GC. EBV associated GC (EBVaGC) is considered a distinct subtype with peculiar molecular characteristics (see "Molecular Pathology and Genetics" section) and is defined by monoclonal proliferation of carcinoma cells infected by EBV, as demonstrated by EBV-encoded small RNA (EBER) in situ hybridization (Fig. 5). Distinct latency-associated patterns have been associated with specific malignancies and, in the case of GC, a latency type I or II has been found, in which EBERs, EBNA-1, BARTs, LMP-2A, and BART miRNAs are expressed.

The frequency of EBVaGC varies from 2% to 20%, with a worldwide average of nearly 10%. EBVaGC occurs more frequently in male, younger patients and in the proximal stomach, or in the gastric stump of patients submitted previously to subtotal gastrectomy.

EBVaGC is generally not associated with *H. pylori* infection. In the rare instances of coinfection (*H. pylori* and EBV) EBV appears to potentiate the oncogenic effects of *H. pylori* infection.

EBVaGC may present three histological variants: (1) gastric cancer with lymphoid stroma (see "Pathology" section) (Fig. 5), in up to 50% of cases; (2) GC with Crohn's disease-like lymphoid reaction, characterized by the presence of numerous lymphoid follicles with active germinal centers at the advancing edge of the tumor; and (3) conventional-type GC, with scant lymphocytic infiltrate, in a minority of cases.

EBVaGC has frequently a favorable prognosis, which could be explained, at least in part, by the intense immune response elicited by EBV infection.

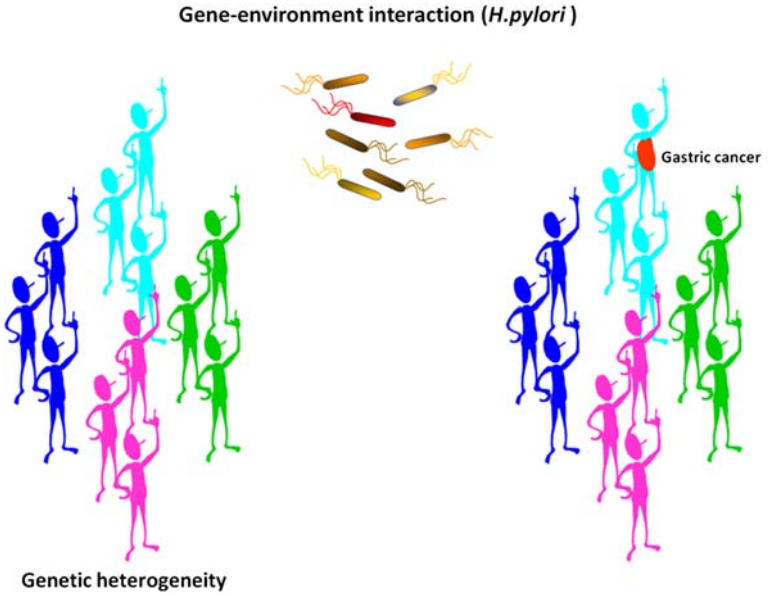

Fig. 3 Among the individuals infected with *Helicobacter pylori*, the large majority (>90%) of the individuals do not develop gastric cancer, indicating a role for other causative agents and the genetic background of the host.

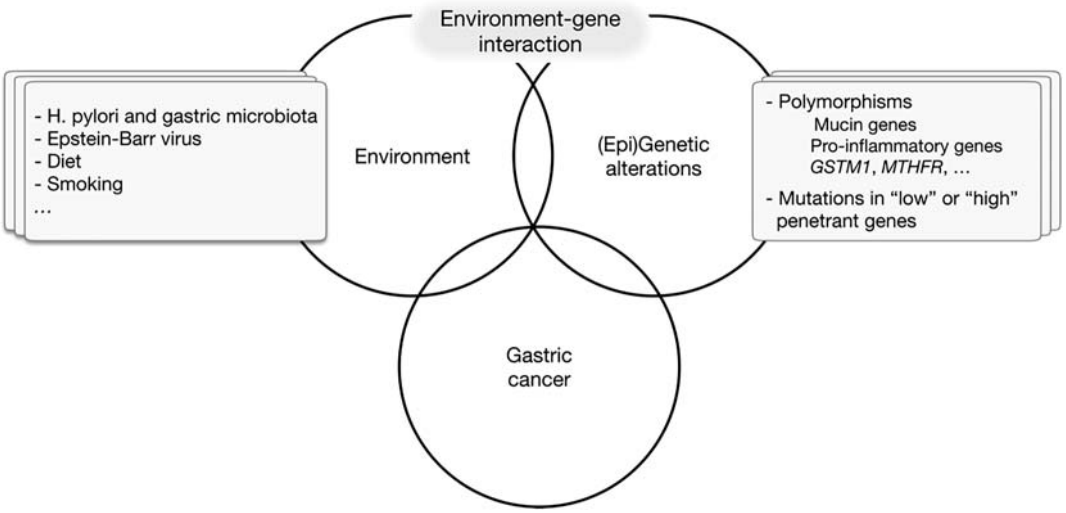

Fig. 4 Gastric cancer (as many other, if not all diseases) is the end product of genetic susceptibility/environment interaction.

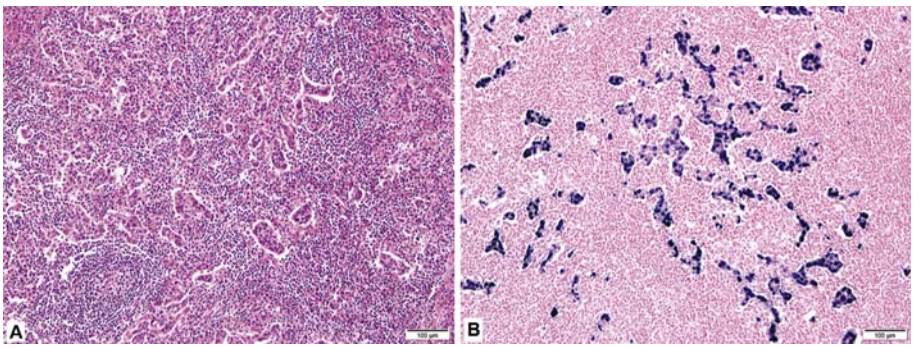

Fig. 5 Gastric cancer with lymphoid stroma (medullary carcinoma, lymphoepithelioma-like carcinoma) is characterized by abundant lymphocytic infiltrate, with scattered lymphoid follicles (A). This morphology is frequently associated to EBV infection, as in this case, demonstrated by EBER-ISH (B).

Diet

Certain dietary habits are associated with an increased risk of GC. These include high intakes of salt-preserved and/or smoked foods and low intakes of fresh fruit and vegetables, particularly in combination with *H. pylori* infection. Intake of all meat, red meat and processed meat is also associated with an increased risk of GC in the distal stomach. The so-called "Mediterranean diet" was shown to be associated with a significant reduction in the risk of incident GC; it is defined as the high consumption of fruit, vegetables, cereals, legumes, nuts and seeds, and seafood, with olive oil as the main fat source, moderate alcohol consumption (particularly red wine), a low-to-moderate consumption of dairy products and a relatively low consumption of red and processed meat.

Smoking

An association has been shown between smoking and stomach cancer that could not be explained by bias or confounding factors. Smoking also potentiates the carcinogenic effect of infection with cagA—positive *H. pylori*.

Genetic Polymorphisms

Polymorphisms of the interleukin 1 β (*IL1B*) gene (initiation and amplification of inflammatory response) and the interleukin 1 receptor antagonist gene (*IL1RN*) (modulation of inflammation) are associated with individual (or familial) susceptibility to carcinogenesis associated with *H. pylori*. It has been shown that in individuals with alleles that predispose to inflammation, infection with *H. pylori* may cause increased production of gastric interleukin 1 β, leading to severe and sustained inflammation that increases the risk of developing GC.

Other clinic-pathologic conditions that are associated with an increased risk of GC encompass autoimmune gastritis, peptic ulcer disease, hypertrophic gastropathies, gastric stump (operated stomach), and gastric polyps.

Precursor Lesions

Gastric dysplasia represents the penultimate stage of the gastric carcinogenesis sequence. It is characterized by cellular atypia, reflective of abnormal differentiation, and disorganized glandular architecture. Determination of the correct diagnosis and grade of dysplasia is critical, because it predicts both the risk of malignant transformation and the risk of metachronous GC.

According to World Health Organization, dysplasia is graded in a two-tier system: low-grade and high-grade dysplasia, on the basis of architectural and cell features. The term "indefinite for dysplasia" (IFD) was coined for epithelia that were neither unequivocally dysplastic nor unequivocally nondysplastic. An alternative designation for dysplasia is intraepithelial neoplasia (IEN).

Low-grade intraepithelial neoplasia/dysplasia shows minimal architectural disarray and only mild to moderate cytological atypia. The nuclei are elongated, polarized and basally located, and mitotic activity is mild to moderate (Fig. 6A). For polypoid lesions, the term "low-grade adenoma" can be used.

High-grade intraepithelial neoplasia/dysplasia displays neoplastic cells that are usually cuboidal instead of columnar, with a high nucleus to cytoplasm ratio, prominent amphophilic nucleoli, more pronounced architectural disarray and numerous mitoses, which can be atypical (Fig. 6B). Increased mitotic activity per se is not pathognomonic of intraepithelial neoplasia/dysplasia, since it is seen in regenerating epithelium as well. Importantly, the nuclei frequently extend toward the luminal half of the cell and nuclear polarity is usually lost. For polypoid lesions, the term "high-grade adenoma" can be used. In general, the diagnosis of high-grade intraepithelial neoplasia/dysplasia is more reproducible than the diagnosis of low-grade lesions. The distinction between high-grade intraepithelial neoplasia/dysplasia and well-differentiated, tubular EGC, can be very challenging, especially in biopsy material, and diagnostic criteria are slightly different for Western and Japanese pathologists. The distinction between high-grade intraepithelial neoplasia/dysplasia and well-differentiated EGC has become of limited clinical interest, since these two conditions could be managed endoscopically.

Further, gastric dysplasia may present an intestinal or gastric phenotype, according to the histomorphological profile. These two phenotypes may be distinguished also on the basis of immunohistochemical expression of lineage-differentiation markers. MUC5AC and TTF1 are markers of surface gastric epithelium (foveolar cells); MUC6 and TTF2 represent markers of mucous neck cells, pyloric glands and Brunner's glands; MUC2, CD10, and CDX2 are intestinal goblet cells differentiation biomarkers. According to their expression, four immunophenotypes may be attributed to dysplastic lesions, both low- and high-grade: intestinal (expressing MUC2, CD10, CDX2), gastric (expressing MUC5AC/TTF1 and/or MUC6/TTF2), hybrid (expressing intestinal and gastric markers) and null phenotype.

Intestinal-type intraepithelial neoplasia/dysplasia generally develops in the setting of chronic atrophic gastritis with intestinal metaplasia and is considered the precursor lesion of intestinal type of GC.

Dysplastic lesions harboring gastric immunophenotype may represent a divergent carcinogenesis model, compared to the conventional Correa's cascade.

Precursor lesions of the diffuse type of GC are not well characterized, except for hereditary diffuse gastric carcinoma (see "Molecular Pathology and Genetics" section).

Pathology

Macroscopy

Endoscopists divide EGCs into three types, on the basis of the endoscopic appearance. These are: protruded (type I), subcategorized into pedunculated (Ip) and sessile (Is); superficial (type II), and excavated (type III). Type II accounts for 80% of EGCs and is further subdivided into elevated type (IIa), flat type (IIb), and depressed type (IIc), the last being the most common.

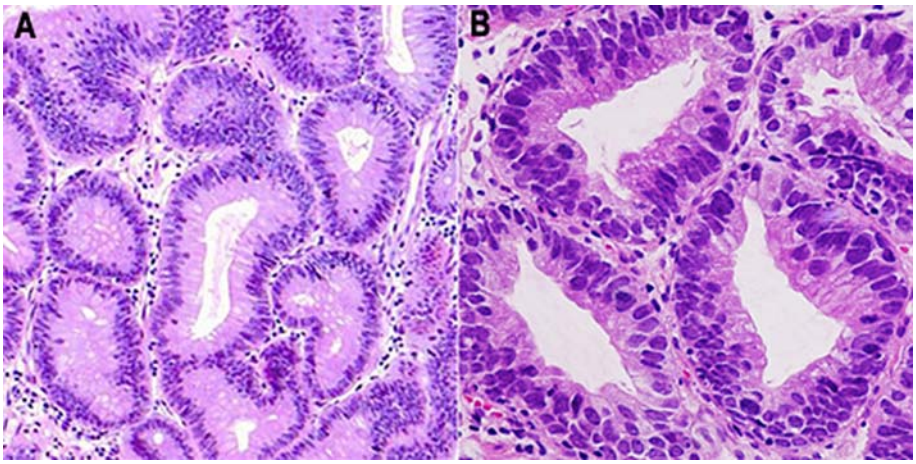

Fig. 6 Gastric dysplasia: (A) low-grade, (B) high-grade.

Advanced gastric cancer

Advanced GC can display various gross appearances. Borrmann's classification remains the most widely used and divides GC into four distinct types: polypoid carcinoma (type I), fungating carcinoma (type II), ulcerated carcinoma (type III), and diffusely infiltrative carcinoma (type IV) (Fig. 7).

Polypoid and fungating tumors typically consist of friable, ulcerated masses that bleed easily and project from a broad base in the gastric lumen. They tend to develop in the body of the stomach, in the region of the greater curvature, posterior wall or fundus.

Ulcerated tumors can differ from benign ulcers by an irregular margin with raised borders and thickened, uneven and indurated surrounding mucosa. The ulcer base is necrotic, shaggy and often nodular. Mucosal folds radiating from the crater are irregular and frequently show club-like thickening and fusion. Malignant ulcers tend to be larger than their benign counterparts. However, many malignant ulcers lack these typical features and, therefore, endoscopic appearance is not a sufficiently reliable guide to diagnosis and should be complemented by systemic biopsies.

Infiltrative tumors may spread superficially in the mucosa and submucosa, giving rise to plaque-like lesions with flattening of the rugal folds. In some cases, superficial ulceration supervenes. Frequently, though, the infiltration involves the entire thickness of the wall, usually over a limited area but sometimes extensively, to produce the so-called linitis plastica or "leather bottle" stomach. In these cases, the wall assumes a stiff consistency due to an extensive desmoplastic response to tumor cells. In such cases, there is usually no visible localized growth. A characteristic feature, at endoscopy, of this tumor type is that, because of the diffuse involvement of the stomach, it fails to inflate, in marked contrast to the normal stomach.

Some GCs may secrete considerable amounts of mucin, which gives the tumor a gelatinous appearance to the naked eye. These are sometimes referred to as mucinous or colloid carcinomas.

Microscopy

GC is very heterogeneous from the morphological standpoint. Several histopathological classifications have been proposed, underlying such heterogeneity. As many carcinomas show morphological heterogeneity, that is, a great variety of different patterns within the same tumor, defining the predominant component could be difficult and some concerns have been raised about accuracy and reproducibility of the current classifications. The most widely accepted and used are the World Health Organization and the Laurén classifications.

Two major types of GC have been described by Laurén—intestinal and diffuse types—which display different clinicopathologic profiles, molecular pathogenesis and biological behavior, often occurring in distinct epidemiologic settings. Intestinal GC occurs predominantly in elderly, male patients; diffuse GC is more common in young, female patients. Both subtypes share environmental risk factors; however, diffuse GC pathogenesis is less well understood and presents a hereditary component.

According to the World Health Organization (WHO), five major types of GC are recognized: tubular, papillary (Fig. 8A), poorly cohesive (with or without signet-ring cells) (Fig. 8B and C), mixed (Fig. 8D), and mucinous (Fig. 8E). Tubular and papillary carcinomas roughly correspond to the Laurén intestinal type and the poorly cohesive carcinomas (encompassing cases constituted, partially or totally, by signet-ring cells) correspond to the diffuse type in Lauren's classification.

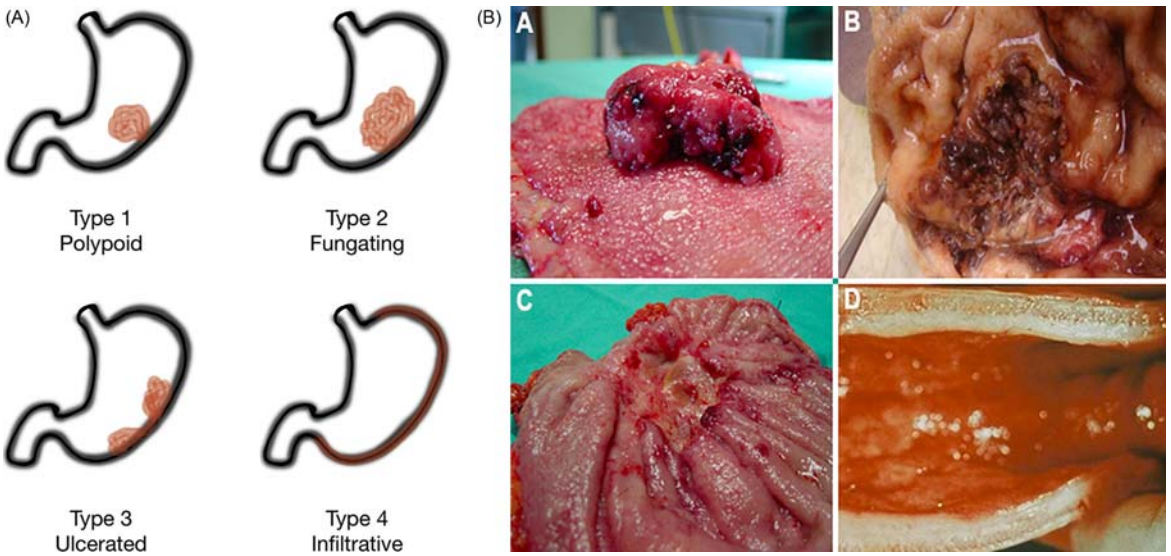

Fig. 7 (A) Growth patterns of advanced gastric cancer according to the Borrmann classification: type 1—polypoid, type 2—fungating, type 3—ulcerated, type 4—infiltrative. (B) Macroscopic appearance of advanced gastric cancer according to the Borrmann classification (surgical specimens): (A) type 1—polypoid, (B) type 2—fungating, (C) type 3—ulcerated, (D) type 4—infiltrative.

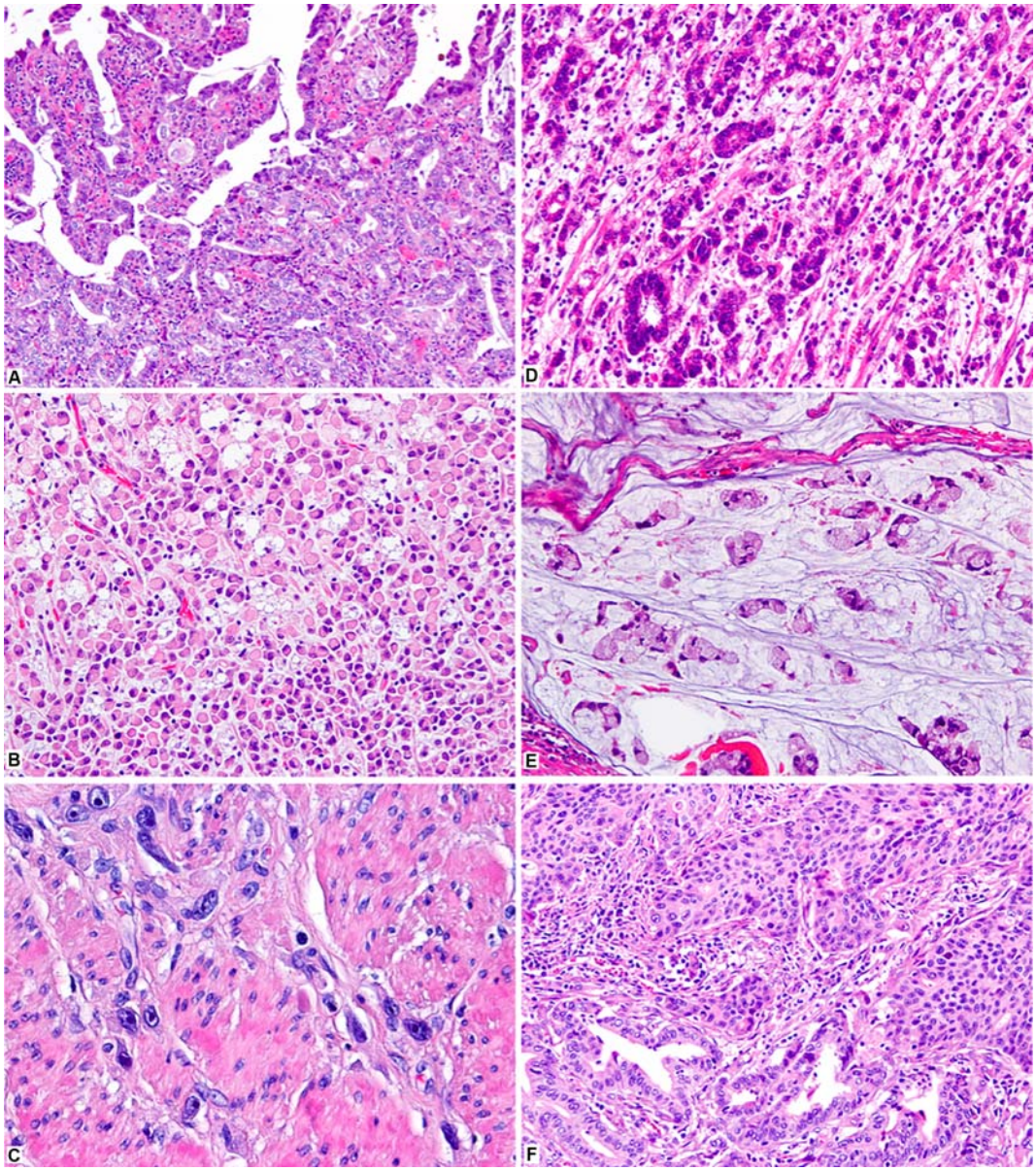

Fig. 8 Gastric carcinoma: (A) Tubulo-papillary (intestinal type), (B) poorly cohesive, with signet-ring cells (diffuse type), (C) poorly cohesive, without signet-ring cells (diffuse type) composed of pleomorphic cells infiltrating the muscularis propria, (D) mixed, (E) mucinous, (F) adenosquamous.

Uncommon histological variants account for about 5% of GCs, encompassing: gastric carcinoma with lymphoid stroma (GCLS, see below) (Fig. 5), adenosquamous (Fig. 8F), squamous cell carcinoma, hepatoid adenocarcinoma, choriocarcinoma, small cell carcinoma, gastric carcinosarcoma, gastroblastoma, micropapillary carcinoma, gastric carcinoma of fundic gland type, parietal cell carcinoma, Paneth cell carcinoma, mucoepidermoid carcinoma, malignant rhabdoid tumor and undifferentiated carcinoma.

GCLS, also described as medullary carcinoma or lymphoepithelioma-like carcinoma (Fig. 5), is composed of irregular sheets, trabeculae, poorly developed tubular structures and isolated cells, embedded within a prominent lymphocytic infiltrate, with occasional lymphoid follicles. The lymphoid infiltrate can be so prominent that immunohistochemical study may be necessary to confirm the epithelial nature of the tumor. GCLS is frequently associated to EBV infection (in over 80% of cases) and a similar morphology can be observed in GC with microsatellite instability. As demonstrated by immunohistochemical studies, cytotoxic (CD8+) T lymphocytes constitute the predominant component of the infiltrate, which also contains B lymphocytes, plasma cells, neutrophils and eosinophils.

Molecular Pathology and Genetics

GC is the result of accumulated genomic damage affecting cellular functions essential for cancer development, the so-called hallmarks of cancer: self-sufficiency in growth signals, escape from antigrowth signals, apoptosis resistance, sustained replicative potential, angiogenesis induction and invasive or metastatic potential. Emerging hallmarks have been recently added to the list: deregulation of cellular energetics, escape from immune destruction, tumor-promoting inflammation and genomic instability/mutations.

Numerous gene mutations, somatic copy number alterations, epigenetic changes, transcriptional changes, etc. have been detected in GC, highlighting the remarkable molecular heterogeneity of this cancer. With the recent advent of next-generation sequencing, several groups have analyzed GC molecular alterations at high resolution, using various high-throughput platforms. These studies have tried to decipher such complex molecular alterations, proposing integrated molecular classification schemes, which attempt to cluster the comprehensive molecular data obtained into clinical and biological homogenous subgroups.

The Gene Expression Profiles From the Singapore-Duke Group

The Singapore-Duke group used high-throughput transcriptomic technologies to identify gene expression signatures with clinical relevance. This study led to the recognition of three subtypes: (1) the mesenchymal subtype, enriched with diffuse GCs and characterized by cancer stem cell and epithelial-to mesenchymal transition (EMT) properties; (2) the proliferative subtype, with high levels of *TP53* mutations, genomic instability, and activation of oncogenic pathways; and (3) the metabolic subtype, overexpressing genes that are normally expressed in gastric mucosa. Proliferative and metabolic subtypes frequently correspond to the Laurén intestinal type. New molecular profiling technologies (e.g., NanoString nCounter technology) have demonstrated the possibility of generating similar accurate molecular information from formalin-fixed and paraffin-embedded GC tissues.

The Four-Tiered Molecular Classification From the Cancer Genome Atlas

The landmark study of GC molecular-based stratification was carried out by The Cancer Genome Atlas (TCGA) research network, which proposed a four-tiered molecular classification (**Fig. 9**) that identifies: (1) Epstein–Barr virus-positive (EBV+) GC, characterized by recurrent PIK3CA mutations, frequent JAK2 and PD-L1 amplification, and a high level of DNA hypermethylation; (2) GC with microsatellite instability (MSI-high) characterized by DNA hypermethylation and *MLH1* silencing; (3) genomically stable GC, associated with a diffuse morphology and recurrent *CDH1* and *RHOA* events; and (4) GC with chromosomal instability (CIN), exhibiting intestinal morphology, a high number of *TP53* mutations, and amplifications of tyrosine kinase receptors (TKR).

The Four-Tiered Molecular Classification From the Asian Cancer Research Group

The Asian Cancer Research Group (ACRG) described four molecular subtypes with distinct prognostic impact: (1) MSI-high tumors, with an intestinal morphology and the best prognosis; (2) MSS/EMT GC, with a diffuse morphology and the worst prognosis; and (3 and 4) MSS adenocarcinomas, with no EMT signature, either *TP53*-active (MSS/*TP53*+) or inactive (MSS/*TP53*−), and with an intermediate prognosis. The MSS/*TP53*− subtype (which roughly corresponds to the proliferative and CIN subtypes) is frequent (36%–50% of GCs) and harbors genomic amplifications of TKR and/or *RAS*, some of which are potential therapeutic targets. The ACRG found that TKR and *RAS* amplifications tended to be mutually exclusive, emphasizing GC intertumor heterogeneity and the importance of investigating molecular alterations for targeted therapy.

As shown in **Table 1**, the three molecular classifications, described above, overlap only partially, highlighting the need for a consensual classification.

In-depth studies combining molecular features with histopathological and immunohistochemical profiles may reveal interesting associations. On this note, several groups have proposed practical algorithms, based on in situ hybridization techniques, that is, EBER-ISH, and immunohistochemistry (IHC), that is, mismatch repair proteins, E-cadherin, and p53 expression, currently available in routine diagnostic practice. In these studies, the authors accomplished to translate different molecular subgroups into specific immunophenotypes with prognostic and predictive significance.

Genetic Predisposition and Hereditary Syndromes

First-degree relatives of patients with GC are almost three times as likely as the general population to develop GC. This may be partly attributable to *H. pylori* infection being common in families and to the potential role of *IL-1* gene polymorphisms. Susceptibility to carcinogens may play a role as well. For example, polymorphisms of genes encoding for glutathione S-transferase enzymes, known to metabolize tobacco-related carcinogens and *N*-acetyltransferase 1, increase the risk of GC development. Evidence of familial clustering is found in about 10% of GCs; among such cases, about 1%–3% GCs are truly hereditary, encompassing hereditary diffuse gastric cancer (HDGC) and familial intestinal gastric cancer (FIGC) syndromes. Despite the growing number of FIGC families, the genetic cause underlying the disease remains to be elucidated. Clinical criteria for the definition of this syndrome vary, based on the incidence of GC in the population to which the family belongs (**Table 2**).

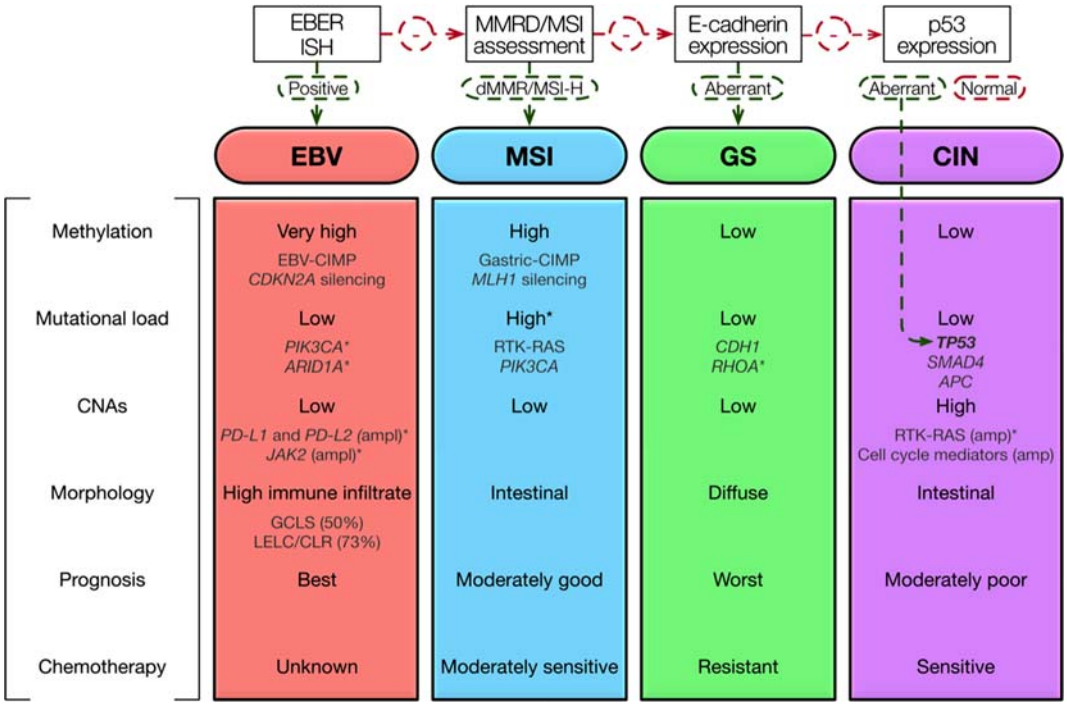

Fig. 9 The molecular classification of gastric cancer proposed by The Cancer Genome Atlas (TCGA) recognizes four molecular subtypes with distinct characteristics. EBER-ISH, EBV-encoded small RNA in situ hybridization; EBV, Epstein–Barr virus; MMRD, mismatch repair deficiency; MSI, microsatellite instability; GS, genomically stable; CIN, chromosomal instability; CIMP, CpG island methylator phenotype; GCLS, gastric cancer with lymphoid stroma; LELC, lymphoepithelioma-like carcinoma; CLR, Crohn-like reaction.

The stomach is also affected by GAPPS syndrome (gastric adenocarcinoma and proximal polyposis of the stomach) that was recently recognized as a rare variant of familial adenomatous polyposis (FAP).

Hereditary diffuse gastric cancer (HDGC)

HDGC (OMIM #137215) is an autosomal dominant cancer syndrome characterized by signet-ring cell (diffuse) GC and lobular breast cancer. The genetic basis of this syndrome was discovered in 1998 by Guilford et al., who studied three Maori kindreds in New Zealand with multigenerational, diffuse GC, in which germline mutations of the *CDH1* gene were identified by linkage analysis and mutation screening. *CDH1* is a tumor suppressor gene, located on chromosome 16q22.1, and encodes epithelial cadherin (E-cadherin), a transmembrane glycoprotein governing the formation of *adherens* junctions and cell–cell adhesion.

In 1999, the International Gastric Cancer Linkage Consortium (IGCLC) defined families with HDGC syndrome, on the basis of clinical criteria. Guidelines for genetic counseling and *CDH1* mutational testing were updated in 2010 and 2015. The current guidelines to select patients for *CDH1* testing are described in **Table 2**.

An alternative genetically based nomenclature has been proposed, in which the term "HDGC" is restricted to families with germline mutations in the *CDH1* gene.

In clinically defined HDGC, *CDH1* mutations are detected in 30%–40% of cases. Most (80%) are pathogenic, truncating mutations. The remainder is missense mutations, which require in silico and in vitro functional studies to elucidate the pathogenicity. In addition to point mutations, *CDH1* germline deletions and promoter methylation have been found, respectively, in about 5% and 1% of HDGC families who tested negatively for point mutations. Another mechanism that has been implicated is germline monoallelic *CDH1* RNA downregulation (allelic imbalance), which is found in about 37% of HDGC families. *CTNNA1* gene, encoding α-E-catenin, which is also involved in intercellular cell adhesion, has been identified as the second gene whose germline alterations cause HDGC. In several familial/hereditary gastric cancer cases, germline mutations were identified in genes associated with other hereditary cancer predisposition syndromes, namely *BRCA2*, *TP53*, *STK11*, *SDHB*, *PRSS1*, *ATM*, *MSR1*, *PALB2*, *INSR*, *FBXO24* and *DOT1L*. *MAP3K6* and *MYD88* genes have been studied in depth, but there is no evidence to consider *MAP3K6* as a GC predisposing genes and the relevance of biallelic MYD88 germline mutations remain to be established. Taking all these data into account, only 16% of HDGC families are negative for germline alterations (**Fig. 10**).

Unlike the somatic *CDH1* mutations, which occur in sporadic GCs and cluster around exons 7 and 8, *CDH1* germline mutations in HDGC families span the whole length of the gene and no hot spots have been identified.

In HDGC, the *CDH1* wild allele can be inactivated by a number of mechanisms. Most frequently, this occurs via promoter hypermethylation (epigenetic modification) and less frequently by loss of heterozygosity (LOH) and *CDH1* mutations. Intragenic deletions in the wild-type allele were also reported.

Table 1 Molecular classifications of GC

Lei Z et al., 2013[a] ($n = 248$)			Mesenchymal Low *TP53* mutations Low E-cadherin mRNA CSC/EMT proprieties mTOR inhibitors	Proliferative High *TP53* mutations Genomic instability Oncogene amplification[d] DNA hypomethylation	Metabolic Low *TP53* mutations Normal gastric mucosa gene expression 5-FU sensitive
			Diffuse GC	Intestinal GC (intestinal phenotype)	Intestinal GC (gastric phenotype)
Bass AJ et al., 2014[b] The Cancer Genome Atlas (TCGA) ($n = 295$)	EBV (9%) EBV-CIMP *CDKN2A* silencing *PIK3A* mutations *PD-L1/2* amplification *JAK2* amplification	MSI (22%) Gastric-CIMP *MLH1* silencing *PIK3A* mutations *HER2/3* mutations *EGFR* mutations	GS (20%) *CDH1* mutations *RHOA* mutations *CLDN18-ARHGAP* fusion (RhoA-GTPase) Diffuse GC	CIN (50%) High *TP53* mutations *TKR-RAS* amplification Amplification of cell-cycle mediators Intestinal GC	
Cristescu R et al., 2015[c] Asian Cancer Research Group (ACRG) ($n = 251$)	EBV+ cases included in MSS/TP53+	MSI (23%) *MLH1* loss Hypermutation (KRAS, ARID1A, PIK3A)	MSS/EMT (15%) *CDH1* loss	MSS/TP53− (inactive) (36%) High *TP53* mutations Genomic instability Oncogene amplification[e]	MSS/TP53+ (active) (26%)
			Worst prognosis Diffuse GC	Intermediate prognosis Intestinal GC	Intermediate prognosis Intestinal GC
		Best prognosis Intestinal GC			

GC, gastric cancer; *5-FU*, fluorouracil; *CSC*, cancer stem cell; *EMT*, epithelial-to-mesenchymal transition; *EBV*, Epstein–Barr virus; *CIMP*, CpG island methylation phenotype; *MSI*, microsatellite instability; *GS*, genomically stable; *CIN*, chromosomal instability; *TKR*, tyrosine kinase receptors; *MSS*, microsatellite stable.

[a]Lei, Z., Tan, I.B., Das, K., et al. (2013). Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. *Gastroenterology* **145**, 554–565.

[b]Cancer Genome Atlas Research Network; Bass, A.J., Thorsson, V., Shmulevich, I., et al. (2014). Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* **513**, 202–209.

[c]Cristescu, R., Lee, J., Nebozhyn, M., et al. (2015). Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. *Nature Medicine* **21**, 449–456.

[d]ERBB2, CCNE1, MYC, and KRAS.

[e]ERBB2, EGFR, CCNE1, CCND1, MDM2, ROBO2, GATA6, and MYC.

Table 2 Clinical criteria, recommended screening and histopathological features of major hereditary syndromes affecting the stomach

Hereditary GC syndrome	*Clinical criteria*	*Recommended genetic testing*	*Histopathological findings*	*Surveillance/risk-reduction surgery*
Hereditary diffuse gastric cancer (HDGC)[a]	- Two or more patients with GC at any age, one confirmed diffuse GC; - Individuals with diffuse GC before the age of 40; - Families with diagnoses of both diffuse GC and lobular breast cancer (one diagnosis before the age of 50) - Gastric polyps restricted to the body and fundus with no evidence of colorectal or duodenal polyposis; - More than 100 polyps carpeting the proximal stomach in the index case or >30 polyps in a first-degree relative;	- *CDH1* mutational analysis; - Search for large *CDH1* rearrangements; - Search for *CDH1* allelic imbalance; - *CTNNA1* mutational analysis	- Diffuse GC and precursor lesions (in situ signet ring cell carcinoma, pagetoid spread of signet ring cells); - Lobular breast cancer	- Prophylactic (risk-reducing) total gastrectomy or annual endoscopic surveillance - Breast magnetic resonance imaging
Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS)[b]	- Predominantly FDPs, some having regions of dysplasia (or a family member with either dysplastic FDPs or GC); - Autosomal dominant pattern of inheritance; - Exclusion of another gastric polyposis syndrome and use of proton-pump inhibitors	*APC* promoter 1B mutational analysis	- Fundic gland polyposis of the stomach with areas of dysplasia; - Hyperplastic polyps of the stomach; - Adenomatous polyps of the stomach; - Mixed polyps with FGP-like, adenomatous and hyperplastic features; - Intestinal-type GC	- Endoscopic surveillance with biopsies, polipectomies or prophylactic (risk-reduction) gastrectomy
Familial intestinal gastric cancer (FIGC)	High incidence countries: - At least three relatives with intestinal GC, one of them a first-degree relative of the other two; - At least two successive generations affected; - In one of the relatives, intestinal GC diagnosed before the age of 50 Low incidence countries: - At least two first/second-degree relatives affected by intestinal GC, one diagnosed before the age of 50; - Three or more relatives with intestinal GC at any age	NA	Intestinal-type GC	NA

GC, gastric cancer; *FGP*, fundic gland polyp; *NA*, not available.

[a]van der Post, R.S., Vogelaar, I.P., Carneiro, F., et al. (2015). Hereditary diffuse gastric cancer: Updated clinical guidelines with an emphasis on germline CDH1 mutation carriers. *Journal of Medical Genetics* **52**(6), 361–374.

[b]Worthley, D.L., Phillips, K.D., Wayte, N., et al. (2012). Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS): A new autosomal dominant syndrome. *Gut* **61**(5), 774–779.

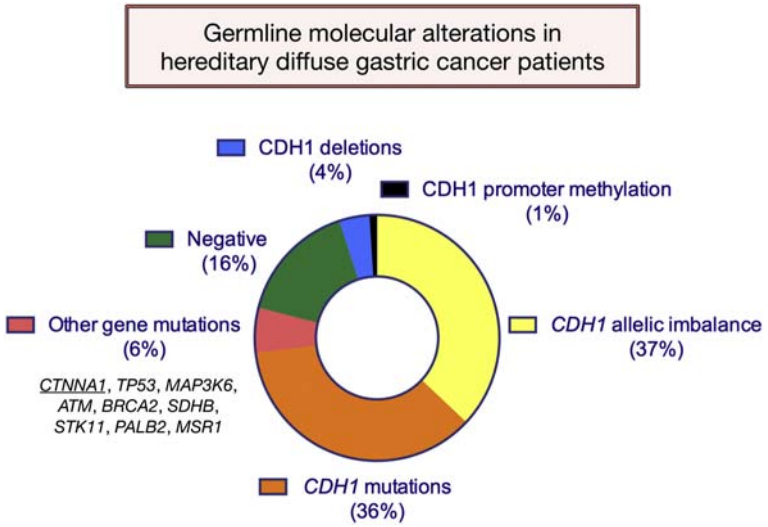

Fig. 10 Germline molecular alterations in hereditary diffuse gastric cancer.

Clinical features of HDGC

The age of onset of clinically significant diffuse GC may be extremely variable (with a range of 14–85 years), even within families. Prophylactic/risk-reduction total gastrectomy (PTG) in early adulthood is the only current curative approach. Indeed, age of onset is unpredictable and, at the time of clinical presentation, affected individuals present with advanced and frequently incurable disease, in >90% of cases. DGC frequently spreads beneath intact gastric mucosa and white light high definition endoscopy, chromoendoscopy, or endoscopic ultrasonography, have low sensitivity for detecting early HDGC.

Because of the great variability in the age of onset, the optimal timing of genetic testing/PTG is still controversial. The age at which to offer genetic testing to at-risk relatives should take into consideration the earliest age of cancer onset in that family. Testing from the late teens or early 20s is favored in families with early onset GC.

Current guidelines recommend that asymptomatic carriers of *CDH1* mutations be offered prophylactic gastrectomy or, for selected groups, annual endoscopic surveillance, as risk-reduction strategies. Surveillance is recommended for individuals aged less than 20 years; for those aged more than 20 years who elect to delay surgery; for those for whom prophylactic gastrectomy (biopsy-negative) is unacceptable but gastrectomy with curative intent (biopsy-positive) is acceptable; and for those with mutations of undetermined significance (e.g., missense). Total gastrectomy is recommended in at-risk family members greater than 20 years of age who have a *CDH1* mutation. In biopsy-positive individuals, a curative total gastrectomy is advised, regardless of age. In women, breast magnetic resonance imaging is advised, starting at 30 years of age.

Histopathology of HDGC

Early-stage HDGC in *CDH1* mutation carriers is characterized by multiple *foci* of invasive (T1a) signet-ring cell (diffuse) carcinoma in the superficial gastric mucosa (Fig. 11), with no nodal metastases. Mapping of the entire gastric mucosa performed in many

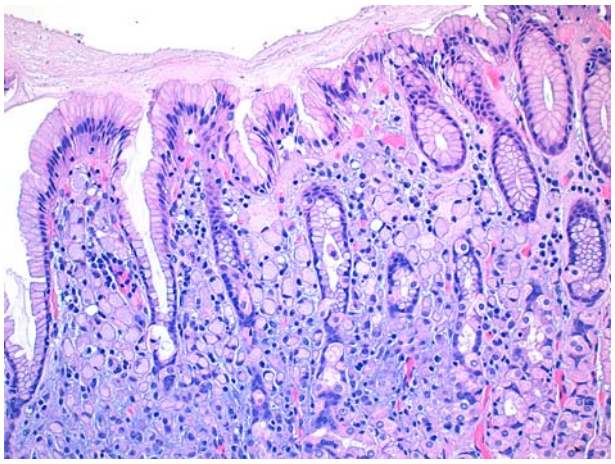

Fig. 11 Intramucosal signet ring cell carcinoma.

stomachs from kindred with different *CDH1* mutations showed that there is wide variation in the number of T1a *foci* observed in these stomachs, both within and between kindreds, ranging from one *focus* to hundreds of tiny *foci*. The cause of this variation in number of *foci* is currently unknown. Discordant results have been published regarding the anatomical localization of the cancer *foci*. Several authors reported a proximal clustering, which is in contrast with previous reports from New Zealand Maori kindred, where most *foci* were found within the body-antral transitional zone and distal stomach. The cause of this variation remains to be clarified, but genetic susceptibility and environmental factors have been suggested as possible contributing factors.

The signet ring cell (diffuse) carcinomas observed in asymptomatic *CDH1* mutation carriers show almost always abnormal staining for E-cadherin, in keeping with a clonal origin of the cancer *foci*, indicating that the second *CDH1* allele has been down-regulated or lost. Aberrant E-cadherin staining patterns include absence of immunoreactivity, reduced membranous expression, "dotted," and cytoplasmic staining, in contrast to the normal membranous, complete pattern of adjacent nonneoplastic gastric mucosa.

Two precursors (Tis) to T1a signet ring cell carcinoma are recognized in HDGC: (i) Pagetoid spread of signet ring cells, below the preserved epithelium of glands and foveolae but within the basal membrane (**Fig. 12**); (ii) in situ signet ring cell carcinoma, corresponding to the presence of signet ring cells within the basal membrane, substituting for normal epithelial cells, generally with hyperchromatic and depolarized nuclei (**Fig. 13**). In cases in which in situ carcinoma is identified adjacent to diffuse-type GC, genetic testing should be considered since this is rarely, if ever, seen in sporadic cases. In these lesions, E-cadherin immunoexpression is reduced or absent. Confirmation of in situ carcinoma by an independent histopathologist with experience in this area is strongly recommended.

The gross and microscopic appearances of advanced ($pT > 1$) HDGCs and sporadic DGC are indistinguishable. Because the neoplastic cells diffusely invade the gastric wall, "*linitis plastica*" is the most common gross phenotype. In contrast to the early,

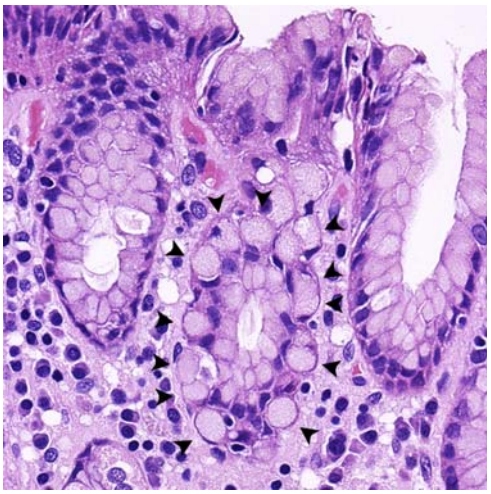

Fig. 12 Pagetoid spread of signet ring cells (arrowheads), below the nonneoplastic foveolar epithelium.

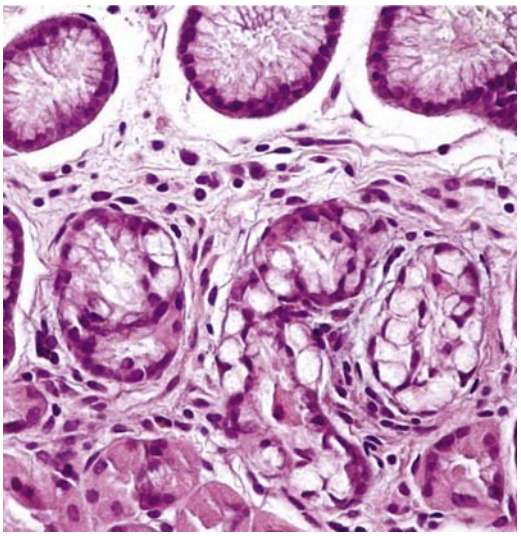

Fig. 13 In situ signet ring cell carcinoma.

"indolent" HDGC phenotype, advanced cases show frequently an aggressive phenotype and are composed of poorly cohesive, pleomorphic cells, sometimes admixed with classic signet ring cells. Glandular/tubular structures, rosettes and mucinous areas can be observed, particularly in foci of lymphovascular invasion and lymph-node metastasis.

The biological events leading to disease progression from early to advanced disease are poorly understood. Humar B et al. suggested that epithelial–mesenchymal transition (EMT) could mediate such progression and demonstrated the activation of c-Src kinase and downstream targets (p-Fak, fibronectin, and Stat-3) in poorly differentiated cells of intramucosal HDGC and more advanced lesions. Still, subsequent studies failed to reproduce such findings. More recently, we found a significant association between "aggressive" morphological features and aberrant (overexpressed) p53 immunoreactivity. These findings are consistent with those observed in murine models, which require inactivation of both *CDH1* and *TP53* genes for DGC development, and indicate that *TP53* may be involved in HDGC progression. Therefore, we suggest that p53 and Ki-67 may serve as biomarkers of prognosis and progression from indolent to widely invasive lesions, and that the finding of an "aggressive" phenotype in screening biopsies of *CDH1* mutation carriers should be taken as a predictive sign of widely invasive carcinoma and require prompt clinical intervention.

GAPPS (gastric adenocarcinoma and proximal polyposis of the stomach)

In 2012, a new hereditary syndrome was identified: "Gastric adenocarcinoma and proximal polyposis of the stomach" (GAPPS). GAPPS is a unique gastric polyposis syndrome with a significant risk of GC, characterized by the autosomal dominant transmission of fundic gland polyposis (FGP), with areas of dysplasia or intestinal-type GC, restricted to the proximal stomach, with no evidence of colorectal or duodenal polyposis or other heritable gastrointestinal cancer syndromes. Nine families have been reported in the literature, so far. Diagnostic criteria for GAPPS syndrome are described in **Table 2**. The syndrome is characterized by incomplete penetrance, with a few elderly obligate carriers having normal endoscopies.

GAPPS syndrome is characterized by florid gastric polyposis. The polyps (>100) are usually <10 mm in diameter and carpet the gastric body and fundus, with relative sparing along the lesser curve of the stomach (**Fig. 14**). The esophagus, gastric antrum, pylorus and duodenum are usually normal. The age of onset of widely invasive GC is variable, ranging from 23 to 75 years (median age of 50 years).

Histopathology is characterized by FGPs, including areas of dysplasia (**Fig. 15**). Hyperplastic, pure adenomatous and mixed polyps with FGP-like, adenomatous and hyperplastic features have also been described. GCs, detected in GAPPS patients, display the features of intestinal-type and mixed GC.

The genetic alteration underlined GAPPS syndrome has been recently identified: point germline mutations in promoter 1B of the *APC (Adenomatous Polyposis Coli)* gene (YY1 binding motif). Therefore, GAPPS syndrome has been interpreted as a variant along the broad phenotypic spectrum of familial adenomatous polyposis (FAP). Genetic testing for point mutations in *APC* promoter 1B is currently advised for patients fulfilling GAPPS diagnostic criteria.

Upper gastrointestinal endoscopy and colonoscopy is advised in all first-degree relatives of the probands.

Endoscopic surveillance with biopsies/polypectomies or prophylactic (risk-reduction) gastrectomy may be considered.

Gastric cancer in other hereditary syndromes

Several inherited cancer predisposition syndromes have been associated with increased risk of GC. These include FAP (*APC* germline mutations), *MUTYH*-associated polyposis, Lynch syndrome (germline mutations in DNA mismatch repair genes), Li–Fraumeni syndrome (*TP53* germline mutations), Peutz–Jeghers syndrome (*STK11* germline mutations), Juvenile Polyposis syndrome (*SMAD4/BMPR1A* germline mutations), hereditary breast and ovarian cancer syndrome (*BRCA1/BRCA2* germline mutations) and Cowden syndrome (*PTEN* germline mutations).

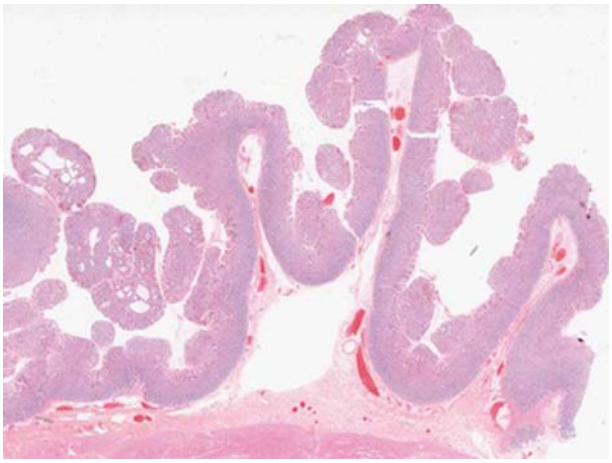

Fig. 14 Fundic gland polyposis in the setting of GAPPS.

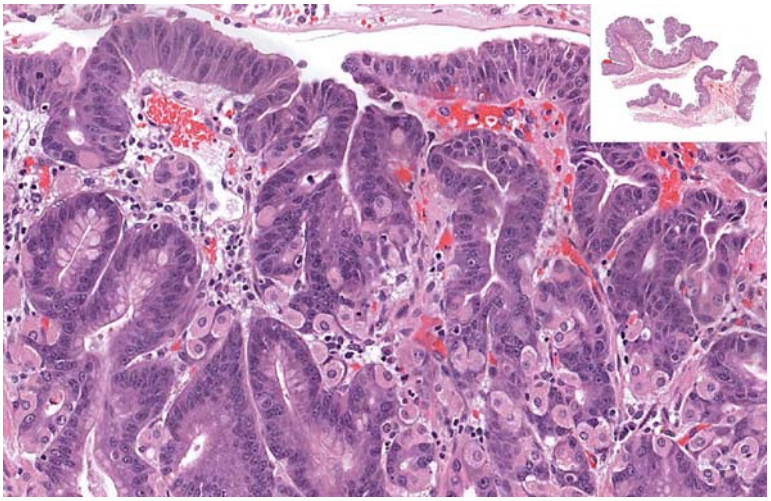

Fig. 15 Dysplasia in a fundic gland polyp from a patient with GAPPS syndrome.

Microenvironment Including Immune Response

The tumor stroma, also referred to as tumor microenvironment (TME) is a complex, dynamic, and heterogeneous tissue, composed of extracellular matrix, vessels, nerves and numerous cell types, including fibroblasts, immune cells and endothelial cells. The inflammatory cells of the TME contribute to its complexity, by supplying also soluble molecules, including growth factors, survival and proangiogenic factors, chemokines, cytokines, metabolites, proteases and other enzymes that may facilitate progression, invasion and metastasis.

Increasing evidence demonstrated the essential role of the interplay between tumor cells and the TME in sustaining tumor cell survival and behavior, tumor growth and progression, contributing to the acquisition of hallmarks traits. In addition, the TME has been associated with response to chemotherapy, affecting drug delivery and efficacy.

Cancer Associated Fibroblasts and Extracellular Matrix

Cancer associated fibroblasts (CAFs) are frequently the most common cell type in TME and play a critical role in regulating the tumor–stroma interactions. Compared to normal fibroblast, CAFs present a cancer-promoting phenotype, with proprieties that resemble the fibroblasts involved in wound healing, and are regulated by protumoral growth and inflammation factors, including, among many others, platelet-derived growth factor (PDGF), TGF-β, and fibroblast growth factor-2 (FGF-2).

Available evidence suggests that an increase in CAF quantity and secretion of matrix components (collagen, fibronectin, etc.) and soluble components (hypoxia inducible factor, VEGF, fibroblast activating protein, metalloproteinases, TGF-β, osteopontin, etc.) is associated to bad prognosis and resistance to chemotherapy, and explain, at least in part, the poor prognosis of diffuse GC patients, whose tumors are frequently characterized by a prominent desmoplastic reaction.

To assess the prognostic and predictive significance of CAFs/extracellular matrix, recent studies have quantified, by morphometric evaluation, the proportion of intra-tumor stroma within GC specimens. A high tumor stroma percentage, defining a "desmoplastic microenvironment," was found to be associated with worse prognosis and rapid tumor progression. By contrast, tumors characterized by high immune infiltrate and low desmoplastic response, were related to early clinical stages and favorable prognosis.

A recent molecular study, based on large transcriptomic data, identify a gene expression program of stromal-related genes ("stromal super-module"), characterized by TGF-β signaling, poor patient survival and high percentages of intratumoral stroma. Such findings suggest that the morphological, morphometric evaluation of tumor stroma may be useful in the daily pathologist's practice, as may serve as a surrogate marker for stromal gene expression and may be predictive of therapies targeting TGF-β signaling.

Tumor Immune Microenvironment

The inflammatory response against tumor has been shown to play an important role in cancer development and progression. Immunosuppression has been considered a new hallmark of cancer. As such, the tumor immune microenvironment, that is, the immune cells that populate the tumor stroma and the soluble factors produced by them, is emerging as prognostic and predictive biomarkers, and, in recent years, many efforts have been made aiming to understand the complex interplay between cancer cells and the host immune system.

The immune context is a double-edge sword in cancer: immunity has not only a host-protective function, by recognizing and protecting tissues from nascent tumor cells ("cancer immune-surveillance"), but also a tumor- promoting action, resulting in tumor

"escape" from immune-surveillance. The tumor cells and the immune cells within the TME can shape the immune response and facilitate tumor cell growth, survival and metastatic potential.

Both tumor-antagonizing and tumor-promoting immune cells can be found in most neoplastic lesions in different proportions, which influence the delicate equilibrium between the phase of tumor elimination or escape. Anti-tumor immune cells include both cells of the innate immune system, for example, dendritic cells, natural killer cells, and cells of the adaptive immune system, for example, tumor-specific CD8+ effector T cells (cytotoxic T lymphocytes), CD4+ helper T cells, and T memory cells.

Tumor associated macrophages (TAM) may present different pattern of differentiation, displaying tumor-suppressing (M1) or tumor-promoting (M2) phenotypes. The M2 macrophages increase immunosuppression and angiogenesis and are associated with unfavorable outcomes. Besides M2 macrophages, a protumorigenic immune infiltrate may be constituted of myeloid derived suppressor cells (MDSCs), neutrophils, regulatory T cells (Tregs) and T helper interleukin (Th17) cells.

The impairment of antigen presentation, for example, alterations of MHC complex, the decrease of costimulating signal pathways of T cells, for example, CD28 interaction with B7-1 (CD80) and B7-2 (CD86), and the aberrant increase of coinhibitory molecules of T cells, for example, CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, are other important factors that may contribute to immune evasion and that may be enhanced by a TME enriched by Tregs.

The systemic inflammatory response is also emerging as having a key role in cancer progression, and may influence the TME composition. Several systemic inflammatory indicators differ between GC patients and healthy controls and have been pointed as independent prognostic factors. Such parameters include preoperative values of neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), plasma fibrinogen, plasma albumin and C-reactive protein, among others.

The deeper knowledge of the tumor immunobiology enabled a direct translation into immunotherapy. Antibodies blocking the interaction between PD-1 and PD-L1 have been used with promising results in several cancer types, including GC. In September 2017 the Food and Drug Administration (FDA) approved the use of pembrolizumab, an anti-PD-1 antibody, for the treatment of patients with recurrent, locally advanced or metastatic, GC/EGJC with PD-L1 expression.

Although the relation between PD-L1 expression and response to PD-1/PD-L1 immune checkpoint inhibitors is not straightforward, PD-L1 expression, evaluated by immunohistochemistry, is the current available biomarker to predict response to immune checkpoint blockade.

A recent study demonstrated that MSI-high solid tumors, regardless of the cancers' tissue of origin, respond better to immune checkpoint blockade, compared to MSS tumors, and the FDA approved pembrolizumab as a treatment also for patients with unresectable or metastatic, MSI-H or mismatch repair deficient solid tumors.

Staging and Grading

Staging

The American Joint Committee on Cancer (AJCC) staging system defines the staging of GC. The most recent edition (Eight Edition) was published in 2017 and is described in the next paragraphs.

The staging of GC encompasses the evaluation of the tumor in the gastric wall (T stage), lymph nodes (N stage) and distant organs (M stage) (**Fig. 16** and **Table 3**). In the gastric wall, GC is staged as T1 (invasion of the mucosa or muscularis mucosae—T1a; or infiltration of the submucosa—T1b), T2 (invasion of the muscularis propria), T3 (invasion of the subserosa) and T4 (penetration of the serosa—T4a; or invasion of adjacent structures—T4b).

Nodal staging is based on the number of metastatic lymph nodes: N0 (no regional lymph-node metastasis), N1 (metastasis in 1–2 regional lymph nodes), N2 (metastasis in 3–6 regional lymph nodes), N3 (metastasis in 7–15 regional lymph nodes—N3a; or in 16 or more regional lymph nodes—N3b). Adequate N staging requires that at least 16 lymph nodes to be examined and, thus, careful examination of surgical resection specimens by pathologists. The ideal number of lymph node to be evaluated is ≥ 30. If the minimum number of lymph nodes is not reached, a pN category should be attributed nevertheless.

For M staging, M0 corresponds to the absence of distant metastasis and M1 to the presence of distant metastasis. It is worthy of note that the designation of pM0 must not be used: if a suspected metastatic site is biopsied and the biopsy is negative, the disease should be classified as cM0. To assign a pM1 category, histopathological diagnosis is required.

Prognostic and Predictive Biomarkers

EGCs have a low incidence of vessel invasion and lymph-node metastasis and a good prognosis (about 90% of patients survive 10 years). If untreated, 63% of EGCs progress to advanced tumors within 5 years. Lymph-node metastasis occurs in 10%–20% of all EGCs: various multivariate analyses have identified (1) submucosal invasion, (2) tumor diameter >3.0–3.5 cm, (3) the presence of vascular invasion, (4) the presence of lymphatic permeation, (5) depressed or ulcerated subtypes and (6) undifferentiated histology as independent risk factors for nodal metastasis. For patients meeting these criteria, endoscopic resection is likely to be an ineffective therapeutic modality and surgery should be considered. However, some authors claim that small intramucosal GCs of undifferentiated histology, measuring <20 mm in size and without lymphovascular invasion have a negligible risk of lymph-node metastasis and could also be considered for endoscopic resection.

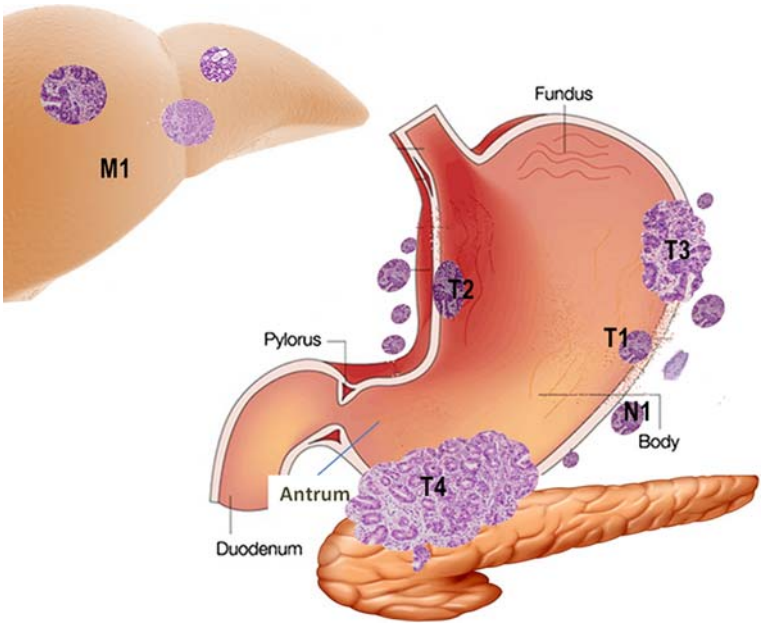

Fig. 16 Staging of gastric carcinoma.

Table 3 TNM classification of gastric carcinoma[a]

T	*Primary tumor*
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria, high-grade dysplasia
T1	Tumor invades lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades lamina propria or muscularis mucosae
T1b	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor penetrates the subserosal connective tissue without invasion of the visceral peritoneum or adjacent structures
T4	Tumor invades the serosa (visceral peritoneum) or adjacent structures
T4a	Tumor invades the serosa (visceral peritoneum)
T4b	Tumor invades adjacent structures/organs
N	*Regional lymph nodes*
Nx	Regional lymph node(s) cannot be assessed
N0	No regional lymph-node metastasis
N1	Metastasis in one or two regional lymph nodes
N2	Metastasis in three to six regional lymph nodes
N3	Metastasis in seven or more regional lymph nodes
N3a	Metastasis in seven to 15 regional lymph nodes
N3b	Metastasis in 16 or more regional lymph nodes
M	*Distant metastasis*
M0	No distant metastasis
M1	Distant metastasis

[a]Ajani, J.A., In, H., Sano, T., et al. (2017). Stomach. In Amin, M.B., Edge, S.B., Greene, F.L., et al. (eds.). *AJCC cancer staging manual*. 8th edn., pp. 203–220. New York: Springer.

In Japan, the 5-year survival for T2 adenocarcinoma is 60%–80% and decreases to 50% for T3 tumors. Lower survival rates have been observed in the West. Female sex and Japanese ethnicity have been associated with a survival advantage. Higher frequency of early-stage carcinomas, accurate staging and surgical expertise has also been associated with improved survival in Japan compared to Western nations.

The stage of GC with special reference to extension to the serosa and lymph nodes (summarized in the TNM staging system) remains the strongest prognostic indicator. Five-year survival is 60%–80% for patients with tumors that invade the *muscularis propria*, but 50% for those with tumors invading the subserosa. At the time of diagnosis most patients with advanced carcinoma already have nodal metastases. Lymphatic and vascular invasion, often seen in advanced cases, carry a poor prognosis. In patients with involvement of 1–6 lymph nodes, the 5-year survival rate is 46%, compared with 30% in patients with more than seven lymph

nodes involved. The extent of the regional lymphadenectomy performed and the quality of lymph-node evaluation are relevant. Patients undergoing a "curative" gastrectomy but limited lymph-node dissection (D1/D0) have an overall 5-year survival of only 23%, versus more than 50% for those undergoing a more aggressive lymphadenectomy (D2).

In resectable cases, complete tumor removal with negative margins (R0) is important. The depth of invasion, the number of positive lymph nodes and postoperative complications are important prognostic factors. After curative resection, recurrence is loco-regional (resection margins, surgical bed and/or regional lymph nodes) in 40% of cases and systemic (liver and peritoneum) in 60% of cases. Whether distal adenocarcinomas have a better prognosis compared with proximal carcinomas is debated. Saito and colleagues reported a 5-year survival rate of 62% in patients with carcinoma of the "cardia" versus 83% for those with carcinoma of the lower third of the stomach. In another series, however, the prognoses were equally grim, with 28% and 29% survival rates, respectively.

Histological Features and Prognosis

The value of histological typing in predicting prognosis is controversial. Whether the prognosis for diffuse carcinoma (Lauren classification) is or is not worse than that for intestinal carcinoma is debated. Recently, it has been suggested that diffuse carcinomas encompass lesions with different prognoses, such as a low-grade desmoplastic subtype (with no or scarce angio-lympho-neuroinvasion) and a high-grade subtype (with anaplastic cells). The prognosis is particularly bad for children and young adults with poorly cohesive carcinoma, for whom diagnosis is often delayed.

Mixed GC displays more aggressive features than "pure" intestinal and diffuse GC, including larger tumor size, deeper invasion, lymphatic invasion, and lymph-node metastases. Interestingly, mixed GC shows a dual metastatic pattern, including hematogenous metastases and peritoneal dissemination with lymph-node metastases, suggesting a cumulative effect of the adverse behaviors of intestinal and diffuse-type GC.

The prognostic significance of histological typing is limited, also because of the intra-tumor heterogeneity of GC, that is, the presence of distinct morphologic patterns in the tumor. Solid, trabecular, tubular and poorly cohesive components frequently coexist in the same tumor. This limitation applies particularly to biopsy specimens.

HER2 Expression/Amplification

HER2 overexpression and/or amplification are found in 7%–38% of EGJC/GC, being slightly more frequent in EGJ tumors. HER2 is a transmembrane TKR of the epidermal growth factor receptor (EGFR) superfamily, which regulates cell proliferation, survival, differentiation, migration, and other cancer-relevant cellular responses.

The prognostic significance of this biomarker is debated, but *HER2* expression/amplification is a predictive biomarker of response to HER2-targeted therapy. In 2010, the phase III ToGA (trastuzumab for GC) trial (ClinicalTrials. gov identifier: NCT01041404) demonstrated a significant improvement in response rate, progression-free survival, and overall survival with the addition of trastuzumab to chemotherapy compared with chemotherapy alone, in prestratified patients with HER2-positive GEJC/GC. Since then, the use of trastuzumab with conventional chemotherapy has been approved by the FDA and the European Medicines Agency (EMEA) as first line therapy in advanced (defined as unresectable loco-regional, recurrent, or metastatic disease) HER2- positive GEJC/GC patients. An HER2-positive status, evaluated by IHC (3+) and/or fluorescent or chromogenic ISH (HER2+/ISH+) (Fig. 17), is predictive of the patient response to Trastuzumab-based treatment.

Compared to breast carcinoma, HER2 expression in GEJC/GC is highly heterogeneous and membrane immunoreactivity is often incomplete. Consequently, a HER2 scoring system specific for EJGC/GCs has been proposed, that is quite different from that of

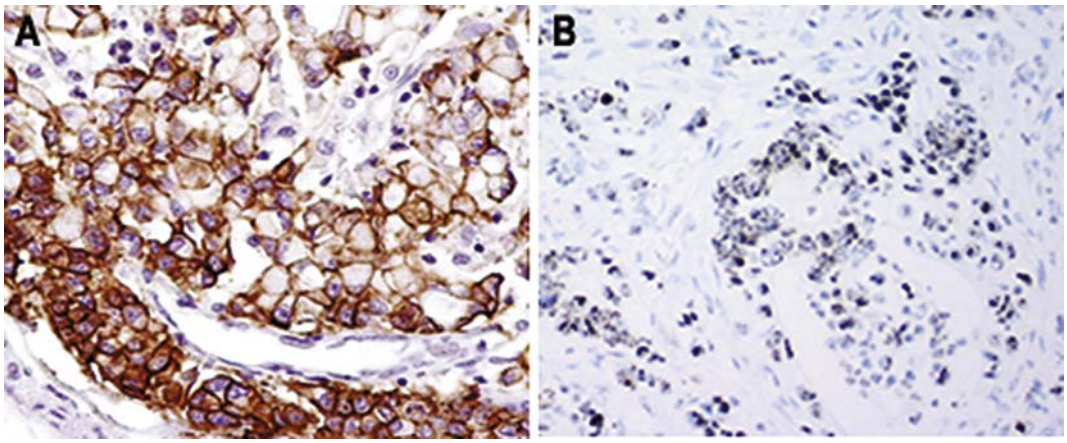

Fig. 17 Expression of HER2: (A) immunohistochemistry, displaying strong immunoreactivity at the cell membrane (IHC 3+), (B) *HER2* gene amplification confirmed by chromogenic in situ hybridization.

Table 4 HER2 testing in gastric cancer[a]

Score	*Criteria for resection specimens*	*Criteria for biopsy specimens*	*Interpretation*
0	No reactivity or membranous reactivity in $<10\%$ of tumor cells	No reactivity in any tumor cells	Negative
1+	Faint (generally visible only at 400× magnification) complete, basolateral, or lateral membrane staining in $\geq 10\%$ of tumor cells	Cluster(s) of at least five tumor cells with faint (generally visible only at 400× magnification) complete, basolateral or lateral membrane staining, irrespective of percentage of tumor cells stained	Negative
2+	Weak to moderate (generally visible only at 100–200× magnification) complete, basolateral, or lateral membrane staining in $\geq 10\%$ of tumor cells	Cluster(s) of at least five tumor cells with weak to moderate (generally visible only at 100–200× magnification) complete, basolateral or lateral membrane staining, irrespective of percentage of tumor cells stained	Equivocal: perform ISH testing
3+	Strong (generally visible at 25–50× magnification) complete, basolateral, or lateral membrane staining in $\geq 10\%$ of tumor cells	Cluster(s) of at least five tumor cells with strong (generally visible at 25–50× magnification) complete, basolateral or lateral membrane staining, irrespective of percentage of tumor cells stained	Positive

ISH, in situ hybridization.

[a]Bartley, A.N., Washington, M.K., Colasacco, C., et al. (2017). HER2 testing and clinical decision making in gastro-esophageal adenocarcinoma: Guideline from the College of American Pathologists, American Society for Clinical Pathology, and the American Society of Clinical Oncology. *Archives of Pathology & Laboratory Medicine* **140**(12), 1345–1363.

breast cancer (Table 4). The College of American Pathologists, American Society for Clinical Pathology, and American Society of Clinical Oncology published in 2016 new guidelines for HER2 testing in EGJC/GC. Among others (see Further Reading), the authors suggest to follow these recommendations: (1) in EJGC/GC patients who are potential candidates for HER2-targeted therapy, the treating clinician should request HER2 testing on tumor tissue, preferably before the initiation of trastuzumab therapy; (2) laboratories/pathologists must specify the antibodies and probes used for the test and ensure that assays are appropriately validated; (3) pathologists should use the Ruschoff/Hofmann method in scoring HER2 IHC and ISH results; (4) pathologists should identify areas of invasive adenocarcinoma and also mark areas with strongest intensity of HER2 expression by IHC in specimens for subsequent ISH scoring when required; (5) HER2 testing on fine-needle aspiration (FNA) specimens (cell blocks) is an acceptable alternative.

Given the issue of intra-tumor heterogeneity of HER2 immunoreactivity, (1) pathologists should select the tissue block with the areas of lowest grade tumor morphology in biopsy and resection specimens. More than one tissue block may be selected if different morphologic patterns are present; (2) testing of multiple biopsy fragments (from a primary or metastatic site), or from the resected primary tumor, is preferred. For biopsy specimens, current recommendations state that, when possible, a minimum of five biopsy specimens and optimally, six to eight, should be obtained.

Other Prognostic/Predictive Biomarkers

Advances in genomic and molecular research have provided large data on molecular biomarkers and genetic tests with promising prognostic and predictive significance. However, despite this rapidly increasing body of evidence, the only predictive markers currently in use for EJGC/GC are HER2 and PD-L1 and no other prognostic factors, besides T, N, M categories, have been recognized for use in the clinical practice. Nevertheless, some recent findings, on molecular biomarkers, are worth of mention.

MSI-H GC is diagnosed more frequently at early stages (Stage I or II) and, among the molecular subtypes defined by TGCA/ARGC, has the best prognosis. A study demonstrated that GC patients with MSI-H or mismatch repair protein deficiency (MMRD) have better survival compared to MSS tumors, when treated with surgery alone. Conversely, MSI-H GC patients have poor survival, when treated with perioperative chemotherapy plus surgery, compared to MSS GC patients. These findings suggest that patients with MSI-H or MMRD may not benefit from perioperative chemotherapy. The study of additional cohorts will be necessary to confirm such data for the application in clinical practice.

MSI-high status and PD-L1 immunoreactivity are predictors of response to monoclonal antibodies directed to immune checkpoint inhibitors (see "**Microenvironment Including Immune Response**" section).

FDA approved Ramucirumab, a recombinant monoclonal antibody that targets the vascular endothelial growth factor receptor 2 (VEGFR2) for use as a single agent for the treatment of patients with advanced or metastatic EJGC/GC. However, no immunohistochemical/molecular biomarker is available to predict response to this targeted therapy.

Further Reading

Ajani, J.A., Jeeyun Lee, J., Sano, T., et al., 2017. Gastric adenocarcinoma. Nature Reviews. Disease Primers 3, 17036.
Ajani, J.A., In, H., Sano, T., et al., 2017. Stomach. In: Amin, M.B., Edge, S.B., Greene, F.L., et al. (Eds.), AJCC cancer staging manual, 8th edn. Springer, New York, pp. 203–220.
Ang, Y.L.E., Yonga, W.P., Tan, P., 2016. Translating gastric cancer genomics into targeted therapies. Critical Reviews in Oncology/Hematology 100, 141–146.

Baraniskin, A., Van Laethem, J.L., Wyrwicz, L., et al., 2017. Clinical relevance of molecular diagnostics in gastrointestinal (GI) cancer: European Society of Digestive Oncology (ESDO) expert discussion and recommendations from the 17th European Society for Medical Oncology (ESMO)/World Congress on Gastrointestinal Cancer, Barcelona. European Journal of Cancer 86, 305–317.

Bartley, A.N., Washington, M.K., Ventura, C.B., et al., 2016. HER2 testing and clinical decision making in gastroesophageal adenocarcinoma: Guideline from the College of American Pathologists, American Society for Clinical Pathology, and American Society of Clinical Oncology. Archives of Pathology & Laboratory Medicine 140 (12), 1345–1363.

Carneiro, F., Charlton, A., Huntsman, D.G., 2010. Hereditary diffuse gastric cancer. In: Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), WHO classification of tumours of the digestive system, 4th edn. IARC Press, Lyon, pp. 59–63.

Ferlay J., Soerjomataram I., Ervik M., et al (2013). GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11. Lyon, International Agency for Research on Cancer. http://globocan.iarc.fr (accessed December 3, 2017).

Gullo, I., Carneiro, F., Oliveira, C., et al., 2017. Heterogeneity in gastric cancer: From pure morphology to molecular classifications. Pathobiology (Epub ahead of print).

Lauwers, G.Y., Carneiro, F., Graham, D.Y., et al., 2010. Gastric carcinoma. In: Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), WHO classification of tumours of the digestive system, 4th edn. IARC Press, Lyon, pp. 48–58.

Oliveira, C., Pinheiro, H., Figueiredo, J., et al., 2015. Familial gastric cancer: Genetic susceptibility, pathology, and implications for management. The Lancet Oncology 16 (2), e60–70.

Shinozaki-Ushiku, A., Kunita, A., Fukayama, M., 2015. Update on Epstein-Barr virus and gastric cancer (review). International Journal of Oncology 46 (4), 1421–1434.

The Cancer Genome Atlas Research Network, Bass, A.J., Thorsson, V., Shmulevich, I., et al., 2014. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 513, 202–509.

van der Post, R.S., Vogelaar, I.P., Carneiro, F., et al., 2015. Hereditary diffuse gastric cancer: Updated clinical guidelines with an emphasis on germline CDH1 mutation carriers. Journal of Medical Genetics 52 (6), 361–374.

van der Post, R.S., Gullo, I., Oliveira, C., et al., 2016. Histopathological, molecular, and genetic profile of hereditary diffuse gastric cancer: Current knowledge and challenges for the future. Advances in Experimental Medicine and Biology 908, 371–391.

Wroblewski, L.E., Peek Jr., R.M., 2016. *Helicobacter pylori*, cancer, and the gastric microbiota. Advances in Experimental Medicine and Biology 908, 393–408.

Genetic Instability

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Glossary

Chromosomal instability Is defined as an increased rate of change in the structure or number of chromosomal segments or whole chromosomes, including amplification, deletion, loss of heterozygosity, translocation, insertion, inversion, and homozygous deletion.

Chromothripsis Is a chromosomal instability phenomenon where hundreds of chromosomal rearrangements occur during one event in a localized region of one or a few chromosomes.

DNA double-strand break Essentially separates the DNA double-helix molecule into two pieces.

Genetic instability Refers to alterations in the DNA of tumor cells, ranging from single nucleotide mutations to changes in chromosomal structures and numbers.

Microsatellite instability Is characterized by the expansion, shortening, deletion or insertion of microsatellites and is often attributed to the impairment of the mismatch repair pathway.

Nucleotide instability Is an increased occurrence of mutations, including base substitutions, deletions and insertions of one or several nucleotides.

Cancer is thought to arise as a result of continuous changes to the genome resulting from random mutation and followed by natural selection applied on the cancerous cell and its progeny by the environment, including by neighboring cancerous cells, infiltrating immune cells, energy and oxygen requirements, exposure to drugs, etc. Early observations of cancer cell division by von Hansemann (1847) and Boveri (1914) indicated that cancer cells were characterized by abnormal hereditary material. Indeed, many cancers contain cells with abnormal chromosome numbers (aneuploidy) or translocations, such as the Philadelphia translocation between chromosomes 9 and 22 in chronic myeloid leukemia. Some propose that cancer is a chromosome rather than a genetic disease due to the frequency that aneuploidy is observed in cancer. Concurrently, the cancer cell genome accumulates several different genetic alterations ranging from single nucleotide variation to insertions or deletions to focal or large scale copy number alterations. It is apparent that some of this variation contributes to a greater degree to the growth and survival advantage observed in cancers; other alterations, termed passenger, may have little function on tumor development. The term genetic instability encapsulates this impressive degree of heterogeneity and the changes to this heterogeneity over time, which is observed in the cancer cell component of the tumor and also in the neighboring cells. This article will review the classification and detection of genetic instability; the cellular and tissue mechanisms for its suppression; environmental factors that induce genetic instability; the relationship between instability, the immune system and cancer progression; and, the emerging approaches to treat a heterogeneous and unstable tumor.

Classification and Detection of Genetic Instability

Genetic instability refers to alterations in the DNA of tumor cells, ranging from single nucleotide mutations to changes in chromosomal structures and numbers. It is important to note that genetic instability refers to changes over time rather than the simple appearance of a mutation or an aneuploid genome. These genetic changes can be divided into the following three categories: nucleotide instability, microsatellite instability, and chromosomal instability (Fig. 1).

Nucleotide Instability

Nucleotide instability is an increased occurrence of mutations, including base substitutions, deletions, and insertions of one or several nucleotides. Replication errors and malfunction in repair pathways, such as base excision repair and nucleotide excision repair, can lead to nucleotide instability. Although nucleotide instability induces small-scale changes in DNA sequence, nucleotide instability can lead to dramatic phenotypic changes, including the development of malignancy, through altered gene expression and changes to the structure or function of the affected gene products. Importantly, nucleotide instability refers to the mutation rate rather than the presence or abundance of mutations in a particular cancer. For example, by analyzing the number of mutations per cell division in human breast cancer cell lines, spontaneous point mutation rates were found to be increased (2.9X to 12X) relative to a nonmalignant mammary epithelial cell line or a colorectal cancer cell line. More recently, single nucleus gene sequencing,

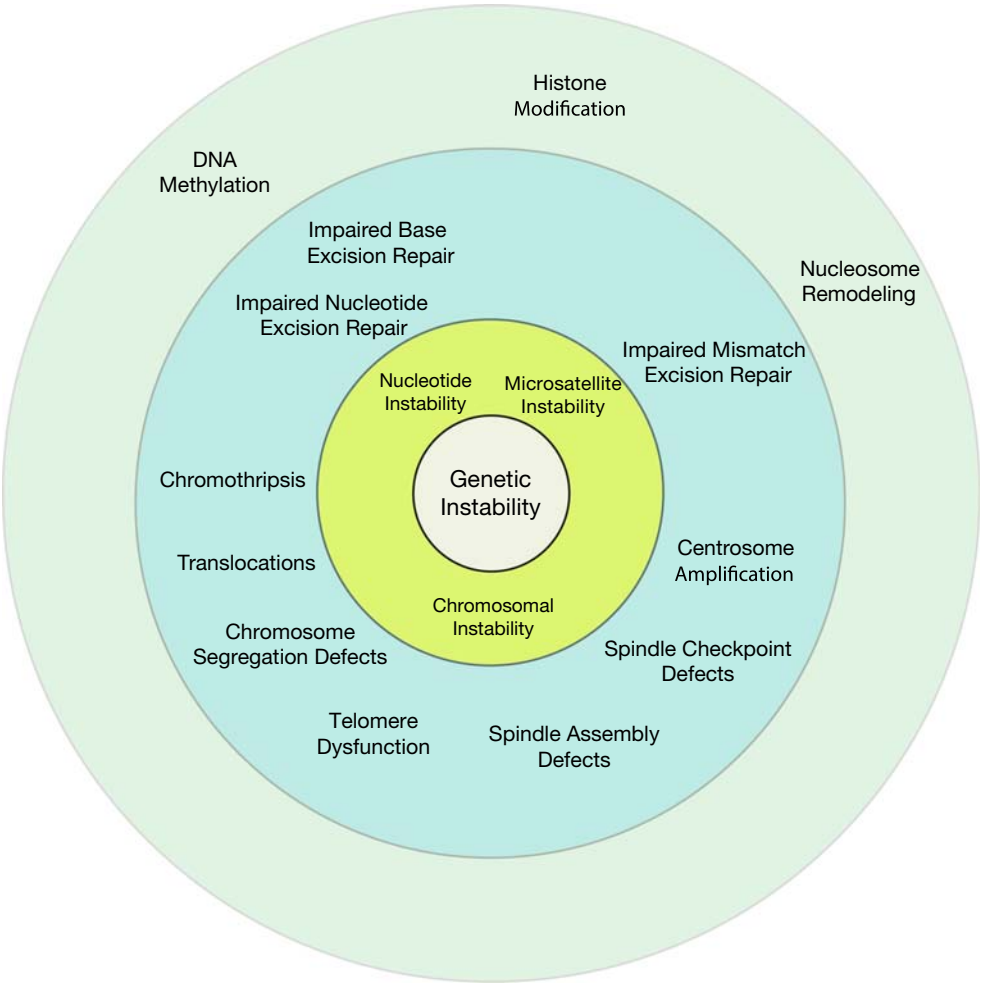

Fig. 1 An overview of the types of genetic instability and the pathways that generate genetic instability. The *yellow inner circle* displays the three main types of genetic instability. The *middle blue circle* displays some processes that result in the three types of genetic instability. The *outermost green circle* represents the epigenetic modifications that affect DNA repair pathways.

in combination with mathematical modeling, found cells comprising a triple negative breast cancer demonstrated an increased mutation rate (13.3X) relative to normal cells and this breast cancer exhibited extraordinary genetic diversity in isolated tumor nuclei.

Microsatellite Instability

Microsatellites, or short tandem repeats, are DNA repeats consisting of one to six base pairs that occur throughout the genome. The distribution of microsatellites in eukaryotic genomes is not random and they display different properties in different genomic regions. Microsatellite instability is characterized by the expansion, shortening, deletion, or insertion of microsatellites and is often attributed to the impairment of the mismatch repair pathway. Changes to microsatellite sequences are thought to occur during DNA replication due to polymerase slippage that is inefficiently mended due to a deficient mismatch repair response. About 15% of colorectal cancers exhibit deficient mismatch repair and microsatellite instability and the inheritance of mutations in mismatch repair genes, such as *MLH1* and *MSH2*, can predispose a hereditary cancer predisposition syndrome (Lynch Syndrome) that associates with an elevated risk to develop nonpolyposis colon cancer.

Chromosomal Instability and Chromothripsis

Chromosomal instability is highly prevalent in human cancers with about 90% of tumors displaying chromosomal abnormalities. Chromosomal instability is defined as an increased rate of change in the structure or number of chromosomal segments or whole chromosomes, including amplification, deletion, loss of heterozygosity, translocation, insertion, inversion, and homozygous deletion. The consequence of chromosomal instability can be severe owning to the large-scale alterations that can affect the expression

of thousands of gene products and, as a result, can dramatically alter cancer cell phenotypes that enable progression or endow intrinsic multidrug resistance. Therefore, many cellular mechanisms are dedicated to the preservation of chromosome stability, including DNA repair pathways, telomere regulation, and checkpoints to ensure mitotic spindle assembly and chromosome segregation.

Chromothripsis is a chromosomal instability phenomenon where hundreds of chromosomal rearrangements occur during one event in a localized region of one or a few chromosomes (Fig. 2). An analysis of 746 cancer cell lines revealed that more than 2%–3% of cancers display massive genomic rearrangements on one chromosome. It is proposed that the massive rearrangements occur from one single chromosome fragmentation event followed by faulty DNA repair that joins the fragments together. DNA sequencing analysis of a patient with chronic lymphocytic leukemia revealed some characteristics of chromothripsis. First, chromothripsis occurs at specific locations of the genome while rearrangement events that characterize conventional genetic instability are assumed to occur randomly across the genome. Second, the copy number at many regions in an individual chromosome changes between one or two copies, and regions with only one copy are not generated simply by deletion but through rearrangements. Third, the breakpoints at the chromosome arm are clustered so that multiple rearrangements take place within a narrow region, and the fragments of the chromosome connected at breakpoints are originally located distal from each other. Since the locations of breakpoints are clustered, it is plausible that chromothripsis occurs during chromosome condensation related to mitosis. The occurrence of chromothripsis is especially high in bone cancers, but the process is not restricted to a specific tumor subtype.

Detection of Genetic Instability

As illustrated above, genetic instability occurs across multiple genetic levels. Thus, methods that measure changes in chromosomes, microsatellites, or nucleotides are adequate to measure a component of genetic instability. Such methods include, but are not limited to, karyotyping, flow cytometry, single nucleotide polymorphism arrays, whole-genome sequencing, and polymerase chain reaction. However, the measurement of the mutation rate or the change of the mutation rate over time can be challenging. Changes to the mutation rate or karyotype can be determined for immortalized cell lines and tumor evolution can be inferred by the examination of multiple sites of the same tumor or the progression of a tumor over time; that is, from diagnosis through minimal residual disease to metastasis or first relapse or second relapse. In addition, a variety of methods are often used to calculate the frequency and extent of genetic changes for static tumor cell populations, which are frequently used as a surrogate to describe genetic instability.

Intratumor heterogeneity is one surrogate marker of genetic instability. Recent developments in the field of single-cell sequencing now resolve cell-to-cell genetic heterogeneity at a base-pair level within a tumor and through tumor evolution. That is, comparative analysis of temporally and spatially distinct regions in a single tumor has revealed clonal dynamics and intratumor heterogeneity. Single-nucleus-sequencing is a method that combines flow-sorting, whole genome amplification and next-generation sequencing to produce genome-wide datasets from single cancer cells. Based on data obtained from this new approach, a punctuated model of chromosome evolution is now postulated, which challenges the model of gradual chromosome evolution. Data from single-nucleus-sequencing imply that chromosome alterations occur at initial stages of carcinogenesis. This method has also uncovered

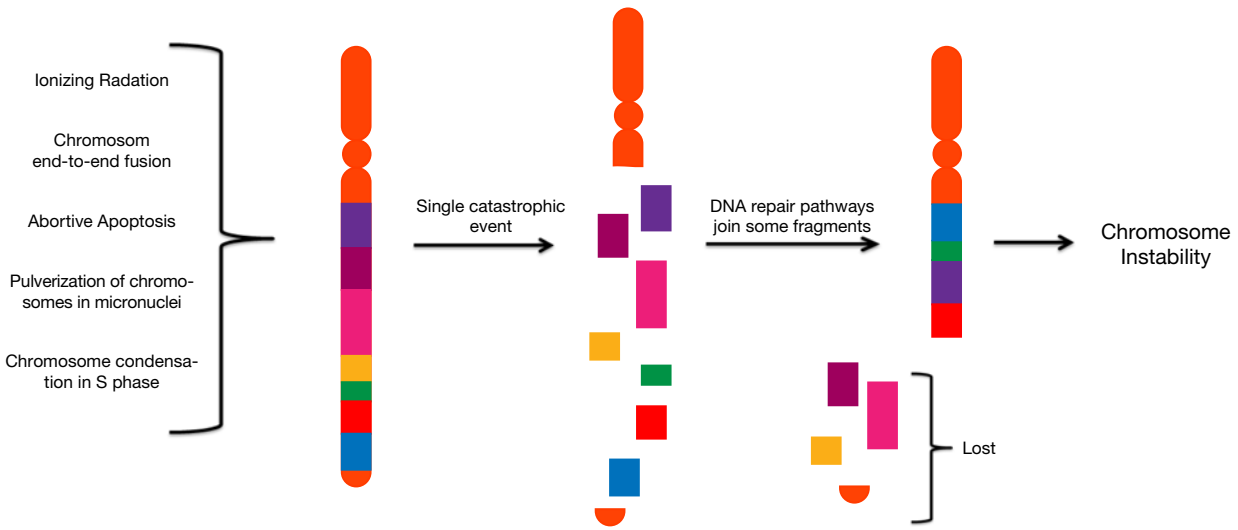

Fig. 2 Chromothripsis leads to chromosomal instability and is often found in aggressive tumor cells. There are several proposed insults that can induce chromothripsis, including ionizing radiation acting on condensed chromosomes, chromosome end-to-end fusion caused by telomere shortening that leads to a double strand break, abortive apoptosis, pulverization of chromosomes in micronuclei, and chromosome condensation that occurs in S phase. These insults may cause a single catastrophic event which fragments specific regions of a chromosome. During the subsequent DNA repair, some of the chromosomal fragments are lost and some are rearranged.

large numbers of subclonal and de novo mutations that were identified to occur at low frequency (< 10%). This illustrates perhaps that no single tumor cell is genetically identical to another in the analyzed primary tumors. Such radical diversity in an evolving tumor context illustrates the remarkable plasticity with which a tumor can respond to a variety of challenges, including those presented by neighboring cancerous cells, infiltrating immune cells, energy and oxygen requirements, and exposure to therapy.

Mechanisms That Suppress Genetic Instability

Genetic instability can occur across many genetic levels, ranging from base-pair changes, to focal or large scale amplifications or deletions, to whole chromosome gains and losses. The cell possesses multiple mechanisms to dampen the development of an unstable genome including: processes that ensure the replication of the genome occurs with high-fidelity; processes that recognize and repair mutations when they arise; and, processes that ensure cell division results in genetically identical daughter cells.

High-Fidelity DNA Replication

The replication of DNA must occur with high fidelity and, therefore, is guarded by many factors. A detailed review of DNA replication is beyond the scope of this article. Briefly, the fidelity of DNA replication depends upon the quality of the template, the selectivity and proofreading of the polymerase, the supply of nucleotides during the process and, finally, the repair of errors by the mismatch repair process. DNA lesions in the template can present challenges to the replicative DNA polymerase and affect the selection of the incoming nucleotide or activate a checkpoint. The MTH1 protein can remove some oxidized dNTPs that may present difficulties to the polymerases. Three DNA polymerases are responsible for eukaryotic DNA replication: DNA polymerase α (Pol α), DNA polymerase δ (Pol δ), and DNA polymerase ε (Pol ε). Theses enzymes individually form a complex with the DNA template and bind a nucleotide and two metal ions in the active site of the enzyme, which induces a conformational change in the polymerase (an induced fit mechanism) that imparts high selectivity to these enzymes (about 1 error per 10^4 to 10^5 nucleotides). Proofreading by DNA Pol δ and Pol ε, which contain an exonuclease activity, and mismatch repair further improves replication fidelity. It is proposed that due to the combination of these processes the error rate for DNA replication is 1 error per 10^9–10^{10} nucleotides.

DNA Repair Pathways

DNA repair pathways are important safeguards to maintain genetic stability and distinct pathways are responsible for the recognition and repair of different types of DNA damage.

Nucleotide excision repair. Nucleotide excision repair can act on a wide range of DNA lesions with different structures, such as lesions caused by ultraviolet radiation, intrastrand crosslinks, chemical adducts, and reactive oxygen species-generated cyclopurines. Nucleotide excision repair occurs during two lesion detection mechanisms: global genome nucleotide excision repair and transcription-coupled nucleotide excision repair. Global genome nucleotide excision repair searches for disturbed base pairing and distortions of nucleotide structures whereas transcription-coupled nucleotide excision repair is triggered by the stalling of RNA polymerase II due to a lesion on the DNA template strand. Deficiency in the process of global genome nucleotide excision repair, such as in Xeroderma pigmentosum (XP), can lead to the accumulation of lesions in the whole genome and result in cancer predisposition. For example, as a consequence of deficient global genome nucleotide excision repair, XP cells are very sensitive to ultraviolet radiation and XP patients have a higher risk of developing skin cancer than normal individuals.

Base excision repair. Reactive oxygen species produced from cellular metabolism are believed to underlie the majority of endogenous DNA damage and inappropriate repair of these oxidative lesions can induce oncogenic mutations. The base excision repair pathway detects and repairs DNA damage caused by oxidation, deamination, and alkylation. Base excision repair is initiated by a DNA glycosylase, which detects and cleaves the targeted base, generating a baseless site (AP site) that is edited by short-patch repair or long-patch repair. Importantly, AP sites are highly prone to mutagenesis and, as a consequence, genetic stability depends upon efficient and appropriate base excision repair.

DNA mismatch repair. A DNA mismatch refers to a non-Watson–Crick nucleotide base pair in double strand DNA. DNA mismatch repair is believed to reduce the amount of DNA errors 100 to 1000-fold. Mismatch repair begins with the detection of mismatched DNA by MutS, an essential protein in mismatch repair that activates downstream repair proteins. With the recruitment of MutL α, strand excision is initiated in a PCNA-, RFC- and ATP-dependent manner. Finally, DNA Pol δ or Pol ε and DNA ligase 1 complete the synthesis of the strand. Deficient mismatch repair is common in colorectal cancers (15%), endometrial cancer (30%), and hereditary ovarian cancer (10% to15%).

Double-strand break repair. A DNA double-strand break essentially separates the DNA double-helix molecule into two pieces. This is frankly more severe than a single strand break wherein the genetic information is still preserved on one intact strand; thus, double-strand breaks can lead to more severe consequences, such as fragmentation and alterations of chromosomes. Double-strand break repair mechanisms can be divided into nonhomologous end-joining and homology-directed repair. Nonhomologous end-joining can occur in all cell cycle phases whereas homology-directed repair requires a homologue and thus only occurs during S- and G2-phases of the cell cycle. Double-strand break repair is a critical tumor suppressive mechanism and deficiencies in these pathways, as the result of mutation to key components such as *BRCA1*, can causes genetic instability and cancer development. For example, female carriers of causal *BRCA1* mutations are more highly prone to develop breast and ovarian cancers and those tumors tend

to display a higher degree of aneuploidy and chromosomal rearrangements, which are potentially indicative of an elevated rate of genetic instability.

Epigenetics and Genetic Instability

While genetic instability refers to the rate of change of alterations encoded in the genome, epigenetic alterations impact not only the expression of molecular regulators of stability but also change the compaction and access to DNA, which directly influences the stability of the genome.

DNA methylation. DNA methylation refers to the attachment of a methyl group to the 5′ carbon of a cytosine ring, usually at CpG dinucleotides, which will alter (often turn off) the transcription of the affected genes. In cancer, the regulation of DNA methylation is impaired and can lead to hypomethylation at repeated sequences throughout the genome and hypermethylation at CpG islands. CpG islands contain 60% of the gene promoters and the hypermethylation of some CpG islands can lead to the reduced expression of tumor-suppressor genes. For example, a hypermethylated promoter region of the mismatch-repair gene *MLH1* causes microsatellite instability. On the other hand, a loss of overall DNA methylation is an epigenetic characteristic of cancer. Hypomethylation of tandem repeats at the centromere can decondense heterochromatin and enable genomic rearrangements. Whereas retrotransposons Alu and LINE-1 elements are silenced by DNA methylation in normal tissues, the overall hypomethylation that characterizes cancer can lead to their elevated activation, which, in the case of LINE-1 elements, can lead to their insertion into the genome and the induction of deletions or inversions.

Histone modification. Histone modifications, such as acetylation and methylation, can either activate or repress gene transcription. Histone acetylation occurs on lysine residues with an N-terminal tail extending from the nucleosome and can induce a chromatin conformation that is more accessible for protein binding. DNA double-strand break repair, for example, is very reliant upon protein access to DNA: histone deacetylase (HDAC) 1 and HDAC2 are recruited and regulate acetylation of histone H3 at sites with DNA damage. Consequently, a deficiency in HDAC1 and HDAC2 will impair double-strand break repair and can induce genetic instability. While histone methylation can either upregulate or suppress transcription depending on specific sites of methylation, SETD2-dependent H3K36 trimethylation regulates DNA mismatch repair while the lysine-specific histone demethylase 5B (KDM5B) enables DNA double-strand break repair. Consequently, cancers with impaired SETD2/HYPB methyltransferase activity may display chromosomal aberrations.

Nucleosome remodeling. The structure of chromatin is dynamic and can have either an "opened" or "closed" conformation, which regulates the accessibility of DNA segments and relies upon the arrangement of nucleosomes. The nucleosome remodeling process is vital to DNA double-strand break repair. Briefly, double-strand break repair requires: (1) the detection of DNA damages in chromatin structure; (2) access to the DNA damage site; (3) a proper organization of nucleosome and DNA for repair process; and, (4) restoration of the chromatin structure after repair. Nucleosome remodeling is needed for each of these processes and it is suggested that, as a consequence, DNA repair is less efficient in heterochromatin, which may lead to the accumulation of mutations in certain heterochromatin regions.

Cell Cycle Checkpoints

DNA damage is induced by many different factors, such as ultraviolet light and oxidizing agents, and these damages can trigger a pause, or checkpoint, in cell cycle progression to allow DNA repair pathways to recognize and repair the damage and prevent the damage from being inherited by progeny cells. The detection and repair of a double-strand break in G1 phase relies upon Ataxia Telangiectasia Mutated (ATM) kinase to phosphorylate and activate Chk2 kinase, which induces a G1-phase checkpoint by stabilizing p53 and, through cyclin-dependent kinase (Cdk) inhibitor p21, blocking cell cycle progression. During S-phase, DNA replication forks stall at genetically fragile sites leading to the binding of replication protein A and the recruitment of Ataxia Telangiectasia and Rad3-related (ATR) kinase, which activates Chk1 to degrade Cdc25A and induce a S-phase checkpoint. ATM and ATR oversee double-strand break repair pathways and can also arrest cells at G2-phase or during the transition from G2-phase into mitosis. Mutations in *ATM* predispose a tumor susceptibility syndrome with heightened risks to develop lymphomas and certain types of solid tumors, including breast cancer.

Chromosomal instability is suppressed by a critical checkpoint during cell division, termed the mitotic spindle assembly checkpoint. Mitosis is a process that ensures the equivalent segregation of the cell's genetic material to two daughter cells. Briefly, this process relies upon the construction of a microtubule-based structure, termed the mitotic spindle, and the coordinated actions of microtubule-associated molecular motor proteins and their adaptors. Microtubules are largely nucleated from two poles, termed centrosomes or spindle poles, as well as near to the chromosomes themselves, termed kinetochore (K)-fibers. These microtubules are aligned and organized into a bipolar structure that contacts each chromosome and forms a bipolar attachment at the kinetochore on each chromosome. A complex of proteins located at the kinetochores monitors microtubule attachment and the establishment of interkinetochore tension, which is a cue that helps fulfill the spindle assembly checkpoint and promotes the transition to anaphase. For example, Aurora kinase B is localized to the inner centromere and will phosphorylate components of kinetochore KNL/Mis12 complex/Ndc80 complex network to destabilize kinetochore-microtubule attachments in the absence of proper interkinetochore tension. Upon bipolar attachment, tension will stretch the kinetochores and separate the outer kinetochore components from Aurora kinase B. Deficiency in the spindle assembly checkpoint pathway, as induced for example through mutation of *BUB1B*, induce a tumor susceptibility disorder termed mosaic variegated aneuploidy.

Centrosome Amplification and Clustering

During cell division, it is vital that each chromosome attains bipolar attachment. However, almost all types of solid tumors display centrosome amplification, which is characterized by an abnormal number or size of centrosomes with, or without, an increased number of centrioles. Centrosome amplification can cause multipolar spindles, which can result in mitotic failure, mitotic death or aneuploidy. For instance, transgenic mice designed to express widespread centrosome amplification, through induced PLK4 overexpression, exhibit an increased tendency to generate lymphomas, squamous cell carcinomas, and sarcomas by 35 weeks of age.

Cancer cells seem to have developed a mechanism to cluster supernumerary centrosomes in order to avoid catastrophic multipolar cell division. Cells with supernumerary centrosomes undergo a MAD2-dependent delay before anaphase and tension on spindle microtubules is proposed to cluster centrosomes to form pseudo-bipolar spindles. Human cancer cells often overexpress proteins involved in the centrosome clustering process, which may permit these cells to bypass potential mitotic catastrophe that may result from recurrent multipolar cell divisions.

Telomere Length

Telomeres are G-rich repetitive sequences (TTAGGG) located at the end of chromosomes that maintain the integrity of replication and genetic stability by protecting the ends of chromosomes from degradation and chromosomal end fusion events. Telomeres are shortened gradually by chromosome end-processing during each cell division and telomere maintenance is required for ongoing cell division. Although telomere shortening prevents transformed cells from proliferating, it can also lead to chromosome breakage and instability. In some cancer types, mutations in *TERT* (encoding the catalytic subunit of telomerase) promoter regions were found to activate transcription suggesting that telomerase activation occurs during tumorigenesis. Moreover, longer telomere lengths may be associated with an increased risk to develop non-Hodgkin lymphoma or chronic lymphocytic leukemia. Telomere crisis can induce fusion of telomeres and lead to dicentric chromosomes, chromosome translocation, regional amplification, and eventually genetic instability.

Environmental Causes of Genetic Instability

In addition to reactive oxygen species produced by cellular metabolism, exogenous agents, such as X-rays, ultraviolet light, and various chemicals, can cause genetic changes that can promote cancer. DNA adducts, resulting from chemical carcinogens or ultraviolet radiation, are sensed by Xeroderma pigmentosum group C (XPC), which initiates the global genome nucleotide excision repair pathway to eliminate damage and maintain genome stability. However, additional precautions that reduce exposure will also dampen the initiation of genetic instability (Fig. 3).

Radiation

Ultraviolet radiation can penetrate tissue and cause biological damage. Ultraviolet radiation induces crosslinks between neighboring pyrimidines creating cyclobutane pyrimidine dimers and (6-4) photoproducts (6-4PPs). However, most organisms possess a very rapid, highly efficient, and safe enzymatic tool for the repair of photolesions in the form of DNA photolyases that, by

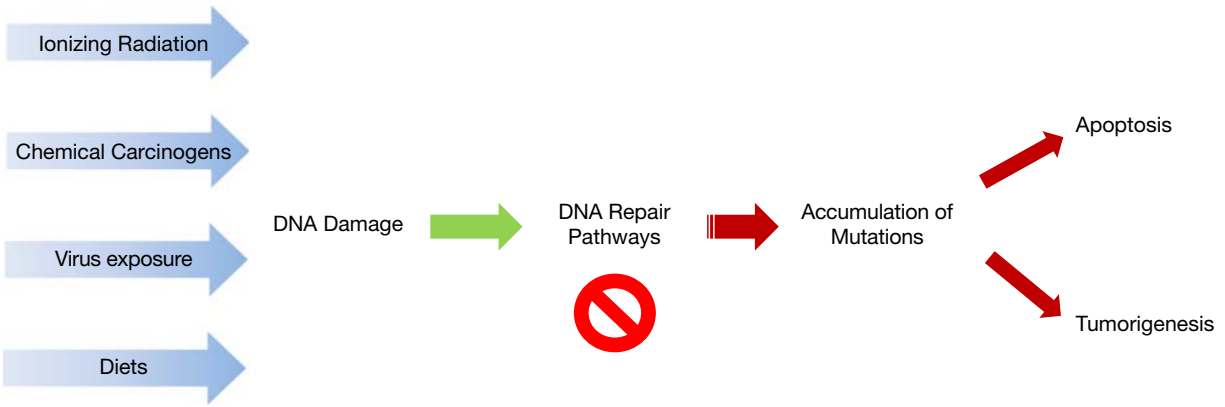

Fig. 3 External causes of genetic instability. Ionizing radiation, chemical carcinogens, exposure to virus and diets are the major mechanisms that cause DNA damage. In the presence of impaired DNA repair pathways, mutations in the DNA can accumulate and lead to programmed cell death or tumorigenesis.

light-driven catalysis, revert cyclobutane pyrimidine dimers or 6-4PPs to pyrimidine monomers without excision of bases or any deoxyribose-phosphate residues. It is believed that skin cancer, the most common cancer, is at least in part the consequence of ultraviolet damage. In melanoma, the majority of mutations are cytosine to thymine (C>T) at adjacent pyrimidine residues, which reflects the generation of thymine dimers after exposure to ultraviolet light. Protective barriers, including shade, ultraviolet-attenuating sunglasses or topical sunscreens, are an effective way to reduce exposure and damage.

Ionizing radiation is suggested to cause 1% to 3% of cancers and exposure to ionizing radiation can arise from cosmic and terrestrial radiation, radon, or medical applications. Like X-rays and gamma rays, ionizing radiation carries enough energy to move electrons from an atom. Ionizing radiation can cause direct DNA damage in tissues, including in the germline, which can induce birth defects and tumorigenesis.

Chemical Carcinogens

Genotoxic chemicals react directly with DNA while nongenotoxic carcinogens interfere with pathways that are critical for DNA repair or chromosome separation. Exposure to either of these classes of chemicals can induce DNA lesions and chromosome rearrangements. Examples of genotoxic chemicals, many of which are present in cigarettes, include polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, aromatic amines, aldehydes, benzene, and 1,3-butadiene. Exposure to PAHs, for example, can give rise to high levels of both 8-OHdG in urine and DNA damage detected by the alkaline comet assay. In addition, high levels of PAH–DNA adducts are seen in tobacco smokers that developed lung cancer. Exposure to benzene, a solvent historically used in printing inks and gasoline, may result in leukemogenesis, including acute myeloid leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, and myelodysplastic syndrome. Exposure to nongenotoxic carcinogens, such as heavy metals, acrylamide, bisphenol A, or benomyl, disrupt DNA repair pathways, DNA damage signaling and chromosome segregation pathways and therefore impair genetic integrity. While it is not possible to absolutely avoid exposure to many of these chemicals, it is critical to limit one's exposure as completely as possible.

Virus

Human papillomaviruses (HPVs) are small DNA tumor viruses that can transform squamous epithelia. HPVs replicate their own genomes by reprogramming the host cell's DNA replication machinery. The high-risk HPV E6 and E7 oncoproteins target negative growth regulatory signaling in the host cell to enable viral genome replication in these postmitotic cells. HPV E6 and E7 oncoproteins disturb the normal regulation of cell division and centrosome duplication, which disrupts chromosomal integrity of the host cell and promotes the proliferation of HPV infected cells. Vaccines against HPV, including HPV-16 and HPV-18, may significantly reduce the prevalence of HPV-induced cancers. In addition, HPV-associated cancers may be combated through the enhancement of host immune responses and more effective antitumor immunity, as these cancers are believed to result in part from immunosuppressive factors. Combinatory therapy, partnering HPV vaccination with immune enhancement, may alter the pro-tumor inflammatory environment and suppress HPV-associated tumorigenesis.

Hepatitis C virus (HCV) infection is highly associated with hepatocellular carcinoma, one of the most common malignancy. HCV replicons can endow host cells with a variety of cancer stem cell-like traits. HCV proteins can also increase the production of reactive oxygen species by enhancing mitogenesis, blocking cell death, or inducing chronic inflammation. The oxidative stress caused by high levels of reactive oxygen species, and the accumulated DNA damage caused by increased cell proliferation and inflammation, may induce genetic instability that acts as the basis for HCV-mediated carcinogenesis.

Diet

Diet is an important environmental factor that influences cancer risk and may modulate genetic stability. There are several mechanisms by which diet can influence genome stability. First, folate plays a key role in several processes related to DNA integrity, such as DNA synthesis and methylation. In vitro studies have shown that folic acid deficiency causes increased uracil incorporation in human lymphocyte DNA. Folate administration reduces DNA uracil incorporation and the presence of chromosome breaks in human cells. A low folate concentration has also been implicated as a potential promoter of tumorigenesis in, for example, colorectal cancer, lung, breast, pancreatic, gastric, esophageal, and prostate malignancies. Second, niacin, or nicotinic acid, is one of the few vitamins that has an important role in DNA synthesis, DNA repair, and cell death. Niacin is required as a substrate for poly(ADP-ribose) polymerase 1 (PARP1), which is involved in DNA repair and telomere length maintenance. The consequence of niacin deficiency is an increase in the number of DNA breaks and in the rate of chromosome damage. Epidemiologic evidence indicates that intake of foods that are naturally rich in vitamin C is associated with reduced risk of various cancers, but the extent to which vitamin C contributes to this effect remains unclear. In general, low dietary intake of folate, nicotinic acid, calcium, vitamin E, retinol, and β-carotene and high intake of pantothenic acid, biotin, and riboflavin are associated with increased genetic instability indicating the importance of a balanced diet to the maintenance of genetic integrity. Finally, a greater understanding of the interconnection between diet, gut microbiota, the immune system, and cancer (including responses to immune-based treatments and immune surveillance) is an emerging and exciting field for future research.

Genetic Instability and Cancer Progression

Many human cancers arise in epithelial tissues and these cancers would be easily treated through surgery were they to remain in their tissues of origin; unfortunately, carcinomas and other human cancers progress and move to other sites in the body, termed metastasis. It is speculated that metastasis accounts for up to 90% of the deaths associated with human cancer. It is also speculated that the process of metastasis involves numerous selective pressures on tumor cells, including: (1) growth at primary site, which involves the escape from antitumor immune responses and intrinsic antiproliferative pathways in their premetastatic niche; (2) invasion of the surrounding parenchyma, lymphatics or blood vasculature; and, (3) survival and proliferation at the metastatic site.

Studies of anatomically distinct metastatic lesions in 29 prostate cancer patients have found close clonal relationships between different metastatic lesions within the same patient, which implies the metastases share a monoclonal origin with the primary tumor. However, a high degree of genetic divergence, based upon radically different patterns of allelic loss, was found between primary prostate tumors and lymph node metastases as well as primary breast tumors and asynchronous metastases. Distinct clonal evolution, as assessed by comparative-genomic hybridization, was also evident in a subset of primary breast or renal cell tumors versus metastases. Moreover, the comparison of mutations present in primary and secondary lobular breast cancers revealed multiple mutations present only in metastases and several other mutations with increased frequency in metastatic sites. Thus, while metastases retain some genetic linkage to primary tumors there is ample evidence for genetic divergence, which may be the result of a heterogeneous primary tumor cell population, generated through genetic instability, overcoming the multiple selective pressures applied through the metastatic process.

The immune system plays a significant role in pruning the growth of primary tumors and maintaining dormancy of metastasized tumor cells. However, tumor variants with reduced immunogenicity will overcome the immune system, which may promote mutated characteristics that escape immunological detection and elimination. This process, termed immunoediting, has three phases: elimination, equilibrium, and escape.

Elimination is the initial phase during which the immune system reacts to and initiates the destruction of transformed cells. When the tumor remodels the local stroma and disrupts the tissue's structure, pro-inflammatory molecules and chemokines produced during this process will recruit innate immune cells to the tumor site. Innate immune cells produce IFN-γ and generate a positive feedback loop that then recruits more immune cells. This inflammation and recruitment of immune cells will lead to apoptosis, angiostasis, and cell cycle inhibition that partially eradicates the tumor. Moreover, dead tumor cells displaying tumor antigens will further activate antitumor responses from the adaptive immune system. Thus, these molecules, IFN-γ, perforin, or NKG2D, and an intact lymphocyte compartment apply a major selection pressure on the tumor cells. During this period of Darwinian selection, many of the original tumor cells will be eliminated, but cells that are genetically unstable and are able to mutate rapidly may withstand immune attack, survive and establish an equilibrium with the immune system.

Tumor cells that survive the elimination phase may equilibrate with the host immune system and undergo the equilibrium phase, wherein the remaining tumor population may be sculpted by the immune system. In particular, lymphocytes and IFN-γ will apply pressures that can amplify genetic instability in the tumor population and enable further adaption, including the promotion of tumor cells with reduced immunogenicity. Such beneficial mutations may occur in tumor cells during the equilibrium phase, which can last for a long time with no obvious sign of tumor development. Eventually, surviving tumor cells that have acquired genetic changes will resume expansion and escape from immunologic detection and elimination. The surviving tumor cells need to escape from both innate and adaptive immune responses. So, tumor cells often escape antitumor immune responses through the production of immunosuppressive cytokines or through immunosuppressive activities of T cells. Interestingly, there is another aspect of the escape phase, termed "reverse immunoediting." Here, the tumor cells may affect immunological response through tumor antigens. That is, the immune system may select for the reduction of strong tumor antigen presented in the context of major histocompatibility complex (MHC) 1 or MHC2, which will favor the development of tumors that are more resistant to future immune responses. In addition, tumor cells actively build an immunosuppressive microenvironment by the generation of cytokines that summon a different population of cells. Taken together, genetic instability in the tumor population enables plasticity in response to the negative selection pressure applied through the immune response, and this plasticity may enable immune suppression and tumor progression.

Cancer Therapies That Target Genetic Instability

Intratumoral heterogeneity enabled by genetic instability presents a great therapeutic challenge given the plasticity with which numerous clones can respond, or evolve, depending upon therapeutic pressures. Alternatively, genetic instability is a phenotype that is likely exclusive to tumor cells and, thus, represents a unique therapeutic target.

Targeting DNA Repair Pathways

Tumors can display a variety of defects in the pathways needed to recognize and repair DNA. These defective repair responses can augment the heterogeneity of the tumor clones but can also increase the tumor cell's susceptibility to inhibition of compensating pathways. That is, a tumor cell with a defective homology-directed repair capacity may be more heavily reliant upon the

nonhomologous end-joining pathway; this is the basis for synthetic lethality approaches to target tumor susceptibilities due to dampened or dysfunctional repair pathways.

BRCA1 and BRCA2 are essential for high-fidelity repair of DNA double-strand breaks through a homology-directed pathway. Cells with deficiencies in BRCA1 or BRCA2 have a decreased rate of homologous recombination and increased sensitivity to ionizing radiation. These observations suggest that tumor cells deficient in BRCA1 or BRCA2 may be more heavily reliant upon PARP1-directed repair and, therefore, susceptible to PARP1 inhibition.

Recent phase 3 trials have been extremely encouraging for the use of PARP inhibitors against *BRCA*-mutated, platinum-sensitive, relapsed ovarian cancer with two companies reporting median progression free survival of approximately 20 months compared to 5.5 months for the control arm. It is proposed that patients with breast cancer may also benefit from PARP inhibition. Although only about 5% of patients carry mutations in *BRCA1* or *BRCA2*, nearly a quarter of breast cancer patients may carry tumor mutational signatures that support the use of these drugs. These encouraging results represent a leap forward following a series of disappointing clinical trial results published in 2011 and 2012. However, acquired resistance to PARP inhibition has been observed in most patients with advanced tumors, which is a common attribute of targeted therapies. Indeed, the PARP inhibitor olaparib may also increase genetic instability in mouse embryonic stem cells leading to translocations and chromosome aberrations by increasing DNA double-strand breaks. With three PARP inhibitors approved for use against ovarian cancer, however, it is an exciting time for the evaluation of combining PARP inhibition with conventional treatments, immunotherapy, or antiangiogenesis treatments for the treatment of genetically unstable and difficult-to-treat, aggressive cancers.

A similar synthetic lethality approach is proposed for the directed inhibition of MutT homologue 1 (MTH1), which regulates the incorporation of oxidized nucleoside triphosphates into DNA or RNA during replication and transcription. The level of reactive oxygen species is generally increased in cancer cells and results in not only more direct damage to DNA but also the incorporation of more damaged deoxynucleoside triphosphates. Consequently, cancer cells may be more heavily reliant upon the action of MTH1 and inhibitors of MTH1 may more effectively damage tumor cells than neighboring normal cells.

Targeting Microsatellite Instability

Hereditary nonpolyposis colorectal cancer is often characterized by high-level microsatellite instability (MSI-H) and an associated increased antigenicity (markers on cell membrane that can bind to antibody). The high antigenicity of MSI-H tumors makes them recognizable by the immune system. In order to overcome host immune defense mechanisms, MSI-H carcinomas express some immune checkpoint molecules to either turn up or down a signal in the immune system and create a localized immunosuppressive environment. For example, tumor cells make proteins to bind to the programmed cell death protein 1 (PD-1) receptor on lymphocytes, which dials down the immune system's response to self and impairs the immune response to the tumor. In this context, checkpoint inhibitors, like pembrolizumab, that inhibit PD-1 on the lymphocytes may release the immune system's ability to target and kill cancer cells. Pembrolizumab has shown an objective response rate of 40% to 50% in patients with progressive mismatch repair-deficient metastatic colorectal cancer compared to 0% in patients with mismatch repair-proficient cancer. This remarkable specificity comes with a cost as these checkpoint inhibitors will have the potential for off-target side effects on the immune system. Moreover, while the blockade of the PD-L1/PD-1 axis is a proven immunologic approach, not all patients will respond to monotherapy. The combination of a checkpoint inhibitor and chemotherapeutic regimens may provide an improved therapeutic index; this combination has been shown to be well tolerated without unexpected toxicities and more clinical trials are ongoing.

Targeting Gene Expression of Cell Cycle Components

As outlined in "**Cell Cycle Checkpoints**" section, cell cycle checkpoints are intended to suppress genetic instability and are often compromised in cancer cells. Moreover, in spite of an unstable genome, tumor cells continue to proliferate without control; thus, a variety of cell cycle regulators are considered potential targets in cancer therapy. There are two types of cell cycle control mechanisms: (1) a cascade of protein phosphorylation that transfers a cell from one stage to the next and, (2) a set of checkpoints that monitor completion of critical events and delay progression to the next stage, when necessary.

Cyclin-dependent kinase (CDK) activity controls many critical events during cell cycle progression. Activation of these kinases generally requires a complex with a second subunit that is only expressed at the appropriate period of the cell cycle; the periodic cyclin subunit associates with its partner kinase to generate an active complex with unique substrate specificity. Regulatory phosphorylation and dephosphorylation control CDK–cyclin complexes and ensure clear transitions between cell cycle stages. Silencing individual cyclins or CDKs, or inhibition of cyclin–CDK kinase activity, in tumor-bearing mice selectively blocks tumor initiation and progression of specific cancer types without having major effects on normal tissues. Various CDK inhibitors (dinaciclib, palbociclib with PD-0325901) have shown encouraging activity against tumors that are known to be characterized by unstable genomes, such as relapsed myeloma or KRAS mutant nonsmall cell lung cancer.

The second type of cell cycle regulation, checkpoint control, is more supervisory. Checkpoint kinase 1 (CHK1) and WEE1 regulate the activation of p53, which inhibits CDKs and arrests the cell cycle. Cancer cells, particularly those with loss of p53 function, often inhibit CHK1 or WEE1 to enable cell cycle progression in the presence of DNA damage; these cancer cells mainly depend on the G2 checkpoint, especially in the presence of DNA damage-inducing drugs. For this reason, inactivation of p53 selectively renders cancer cells sensitive to inhibition of CHK1 or WEE1. For example, a CHK1 inhibitor in combination with radiation is able to kill

p53-defective tumor cells by inhibiting double-strand break repair. An alternate CHK1 inhibitor, alone or in combination with a WEE1 inhibitor, shows activity against solid tumors. Overall, these results support possible clinical trials in the future.

Centrosome Amplification

Centrosome amplification is a recurrent characteristic in tumor cells. Real-time imaging of different cell types in vitro and in vivo indicates that supernumerary centrosomes tend to cluster at the poles of mitotic spindles to induce a pseudo-bipolar spindle and prevent multipolar division. Extra centrosomes assemble in small clusters during prometaphase resulting in two ring-shaped groups at the poles. However, should supernumerary centrosomes fail to cluster, the resulting multipolar spindles often trigger mitotic failure, death or apoptosis. Because supernumerary centrosomes are rare in normal cells, the inhibition of pathways used by tumor cells to cluster centrosomes would specifically target tumor cells with potentially minimal off-target effects. Crenolanib, an inhibitor for platelet-derived growth factor receptor β, inhibits centrosome clustering and induces multipolar divisions with increased cytotoxicity by cofilin-mediated disruption of the cortical actin cytoskeleton for cells with centrosome amplification. A small-molecule screen for inhibitors of centrosome clustering identified a Stat3 inhibitor; Stat3 is a regulator of gene transcription that removes a Stathmin-dependent brake on polo-like kinase 1 activity to promote centrosome amplification. Stat3 depletion and inhibition caused significant inhibition of centrosome clustering and reduced the viability of cancer cell lines and tumors grown in animal models.

Personalized Medicine and Genetic Instability

The US National Cancer Institute defined personalized medicine as "a form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat disease". Such personalized clinical applications include screening, diagnosis, prognosis, prevention and disease-risk reduction, prediction of treatment response, pharmacogenomics and dose adjustments, and the early detection of recurrence and stratification of patients into specific cancer subtypes for more targeted treatment. Many cancer treatments, such as radiotherapy and chemotherapy, are mostly nonselective with strong side effects. In comparison, targeted therapies are hoped to be more specific and the survival rate for some cancers has increased from 0% to 70% due to more developed targeted treatments. In the era of personalized medicine, existing drugs and drugs used for other diseases can be reintroduced for targeted treatments on subgroups or subtypes of tumors.

Individualized treatments are heavily reliant upon accurate measurements of protein, DNA, RNA, and microRNA levels that, in turn, determine the tumor subclasses, prognosis, and treatment responses. Treatment dose and safety in different subtypes of cancer patients can be determined or predicted by genetic variation in the tumor. For example, high-grade serous carcinoma of the ovary displays chromosomal instability that is caused by an impaired homologous recombination repair pathway. Analysis of loss of heterozygosity in these tumors divides patients into different subgroups, which corresponds with different levels of chemotherapy resistance and progression-free survival. Similarly, the level of microsatellite instability in tumors of patients with colon cancer correlates to the efficacy of fluorouracil-based adjuvant chemotherapy.

Genetic instability presents a great challenge to the introduction of targeted therapies and personalized medicine. One of the difficulties is the identification of valid biomarkers for different types of cancers and their subtypes, which is especially difficult for low frequency events. Biomarkers are critical for the development of targeted therapy since they may reveal information of individual response to treatments. An accurate quantification of targeted treatment for one patient not only requires broad sample collection but also needs enormous resources for the analysis, ranging from academic specialists to effective data processing systems. Tumor heterogeneity and selective tumor sampling may provide a biased biomarker analysis and detrimentally affect the application of treatment or the treatment response. Moreover, the need for and generation of high-content data (e.g., whole genome sequencing) and its associated analysis and application in clinical use is still not cost effective. Standardization of testing is also very difficult when many factors need to be considered, including methods of specimen collection and storage, types of specimen, biomarker selection, the platform for experiments and the interpretation of data.

Although intratumor heterogeneity is a challenge to treatment prediction by biomarkers, it is still possible to take advantage of this feature of tumors by giving sequential therapies. Drug resistance is one of the most difficult challenges in cancer therapy development. Often, a population of cancer cells will develop different adaptation mechanisms against the prescribed drug depending on the targeting mechanism and the inherent genetic instability of the tumor. However, there is a cost to tumors cells that develop an adaptation mechanism. That is, tumor cells will need more resources to sustain the development of resistant clones. Thus, there may be a limitation to the number of adaptation mechanisms the tumor population can carry out. It is possible to use this limitation in the design of more effective combinatory targeted treatments.

The evolution of resistance against a specific drug in a tumor population can be modeled for three different types of therapies, including: (1) monotherapy; (2) multidrug therapy where only one adaptation is required to establish resistance; and (3) multidrug therapy that targets different mechanisms. In general, resistance to a drug will increase when the tumor cells are on treatment and will decrease when the tumor cells are off treatment. The tumor cells can become completely resistant to the drugs within on–off cycles. This observation led to the proposal by Cunningham and colleagues of an "evolutionary double blind therapy" that theoretically will use the first line of therapy to drive the tumors to a specific adaptation form which will be targeted by the second line of therapy. Since the tumor cells theoretically invest more resources to adapt to the first drug, the ability of the tumor population to resist the second drug may be reduced and the efficacy of the second line of therapy may be increased. This adaption response may

be informative in another avenue; that is, there is likely an optimal level of instability that affords resistance to cytotoxic therapy. As an example, breast tumors with severe chromosomal instability have a better prognosis than those with moderate chromosomal instability. Thus, drugs that can augment chromosome instability, such as those that decluster centrosomes, may push the tumors into a suboptimal level of instability and make these tumors more vulnerable to cytotoxic therapy.

Perspective Vision

Genetic instability is often associated with cancer and can be indicative of a poor prognosis for some tumor subtypes. But, is genetic instability a consequence of tumor progression or a driving force for tumor evolution? Is genetic instability an early and essential development in tumorigenesis and, if so, what tumor cell-intrinsic or environmental pathways restrict its consequences prior to the observation of an overt tumor? The answers to these questions have still not been completely resolved. Intriguingly, the most common form of genetic instability observed in cancer, aneuploidy, remains in many respects to be the most mysterious. A central goal of ongoing research is to systematically identify the many genetic changes that occur in the cancer cell genome and to understand how these functionally interact to cause cancer cell phenotypes. This effort will provide new insight into the genetic landscape of cancer cells, and the novel driver mutations that are discovered may have a significant impact on future cancer therapy development. In summary, as genetic instability is an enabling attribute of cancer and a modifier of sensitivity and resistance to therapy, our improved understanding of the mechanisms that generate an unstable genome and the pathways that can alter the resulting phenotypes will provide new insights into the origins of cancer subtypes and new directions for their treatment.

See also: Cell Responses to DNA Damage.

Further Reading

Cunningham, J.J., Gatenby, R.A., Brown, J.S., 2011. Evolutionary dynamics in cancer therapy. Molecular Pharmaceutics 8, 2094–2100.
Ferguson, L.R., et al., 2015. Genomic instability in human cancer: Molecular insights and opportunities for therapeutic attack and prevention through diet and nutrition. Seminars in Cancer Biology 35 (Suppl), S5–S24.
Ganai, R.A., Johansson, E., 2016. DNA replication—A matter of Fidelity. Molecular Cell 62 (5), 745–755.
Korbel, J.O., Campbell, P.J., 2013. Criteria for inference of chromothripsis in cancer genomes. Cell 152 (6), 1226–1236.
Krokan, H.E., Bjoras, M., 2013. Base excision repair. Cold Spring Harbor Perspectives in Biology 5 (4), a012583.
Leibowitz, M.L., Zhang, C.Z., Pellman, D., 2015. Chromothripsis: A new mechanism for rapid karyotype evolution. Annual Review of Genetics 49, 183–211.
Lord, C.J., Ashworth, A., 2017. PARP inhibitors: Synthetic lethality in the clinic. Science 355 (6330), 1152–1158.
Price, B.D., D'Andrea, A.D., 2013. Chromatin remodeling at DNA double-strand breaks. Cell 152 (6), 1344–1354.
Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz Jr., L.A., Kinzler, K.W., 2013. Cancer genome landscapes. Science (New York, N.Y.) 339 (6127), 1546–1558.
Wang, Y., Waters, J., Leung, M.L., Unruh, A., Roh, W., Shi, X., Chen, K., Scheet, P., Vattathil, S., Liang, H., Multani, A., Zhang, H., Zhao, R., Michor, F., Meric-Bernstam, F., Navin, N.E., 2014. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature 512, 155–160.

Genome Wide Association Studies (GWAS)

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Glossary

Allele One of several forms of a gene arised through mutation that control a specific characteristic or phenotype. Humans, being diploid organisms, have two alleles at each genetic locus, with one allele inherited from each parent.

Cancer susceptibility variants Pathogenic variants responsible of the predisposition to cancer. Based on their role in biochemical and physiological pathways of tumor cells, cancer susceptibility variants may be common or rare, with high or low penetrance. Usually, variants of genes with a potent effect on apoptosis, proliferation or DNA repair processes have a moderate penetrance and are rare. On the contrary, variants of genes that play a role in molecular and cellular processes controlling the interaction between genetic and environmental components have a low penetrance and are common. Cancer phenotypes associated with high-penetrance susceptibility variants tend to be influenced by the hereditary background, while low-penetrance susceptibility variants could also modify the behavior of hereditary cancer.

Effect size The strength of association between a SNP and risk to develop a specific disease.

Genetic locus A specific position or spot on a chromosome, where the allele is located.

Genetic variant or mutation A change in the DNA sequence of a particular gene with harmful, beneficial, neutral, or uncertain effect on health. It may be inherited as autosomal dominant or recessive trait, or X-linked trait, if the phenotypic effect of the variant is caused when a copy of the altered gene is located on X chromosome.

Genotyping The analysis of the exact sequence of nucleotides, to identify changes in the target sequence compared to the reference sequence that is the most common among genomes in the general population.

Odds ratio (OR) The ratio of the odds of disease for individuals having a specific allele and the odds of disease for individuals who do not have the same allele.

Pathogenic variant or predisposing mutation, or susceptibility variant The genetic alteration that increases the risk to develop deleterious symptoms associated with a certain disease or disorder. Pathogenic variants inherited as autosomal dominant trait that limit life expectancy and reproduction are usually rare in the population (prevalence $<1\%$). Instead, the pathogenic variant inherited as autosomal recessive trait, may be relatively common in the general population (prevalence $>1\%$), because subjects carrying one copy of the altered gene are healthy people without serious disability early in life.

Penetrance The likelihood that in an organism with a particular genotype the corresponding phenotype may or may not be expressed. In particular, the expression of traits with an autosomal dominant pattern of inheritance may be altered if modifier or suppressor genes exist in the rest of the genome or because of a modifying effect of the environment. Thus, penetrance is defined as the percentage of individuals with a given genotype who exhibit the phenotype associated with that genotype. High penetrance would refer to a Mendelian disease.

Polygenic A trait controlled by the interaction of many genes. In contrast to Mendelian disease in which mutations in a single gene cause a specific phenotype and for which the pattern of inheritance is very clear, a polygenic disease is controlled by two or more different genes located at different loci on different chromosomes, each one usually having a relatively small effect.

Risk assessment The quantitative or qualitative assessment of an individual's risk of carrying a certain gene mutation, or developing a particular disorder done by using mathematical or statistical models incorporating selected factors such as personal health history, family medical history and ethnic background.

SNP Single nucleotide polymorphism, a kind of genetic variant characterized by a change of one nucleotide in the sequence of DNA.

Nomenclature

CNV Copy number variations
GWAS Genome-wide association studies
LD Linkage disequilibrium
SNPs Single nucleotide polymorphisms
OR Odds ratio
RR Relative risk

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Introduction

In the last years several progresses in mapping complex traits in humans have revealed that, for prevalent and common diseases, the predominant pattern of hereditability is controlled by the interaction of many loci, with small effect on phenotype if taken individually, in addition to the interactions between inherited genetic and environmental factors.

Advances in both genetic tools, including the mapping of single nucleotide polymorphisms (SNPs), comparative genomic hybridization (CGH) and gene expression microarrays, together with the cross-species approaches, allowed the characterization of loci and genes critical for risk prediction, diagnosis, prevention, and therapy of human diseases.

Moreover, genome-wide association studies (GWAS) have detected—with a strong statistical significance—hundreds of genetic variants associated with a large number of diseases, through the comparison of SNPs in large numbers of affected cases compared to healthy controls.

In particular, GWAS, also known as whole genome association study (WGAS), is an observational study of a genome-wide set of multiple genetic variants in different individuals, able to understand if any variant is associated with a specific trait. As reported above, these studies are focused on the association existing between one or more SNPs and characteristic features of major human diseases (Fig. 1).

In contrast to methods that specifically test a small number of preselected genetic loci, GWAS studies have the capability to investigate the entire genome, using a noncandidate-driven approach, and to identify SNPs and other DNA variants associated with a specific disease. Before the advent of GWAS, linkage and candidate gene studies had limited power to detect common genetic risk factors of diseases. In order to overcome the major limitation of linkage studies, and to improve the resolution owing to the limited number of meiosis within families, the candidate gene studies ignore many causal genomic region or genes because they focus on variants in specific genes that have a priori biological involvement in disease.

Instead, in GWAS, allele frequencies at thousands of polymorphic sites (i.e., SNPs) are compared in a large number of cases versus a similar number of controls. These studies have successfully identified some of the common susceptibility variants for different common diseases and traits, including cancer.

The first GWAS was published in 2005, on patients affected by age-related macular degeneration. Nowadays, it is possible to count over 3000 human GWAS that have examined over 1800 diseases, including several tumors, and thousands SNP association have been discovered.

During last years, many novel associations between genetic variants or chromosomal aberrations and diseases were detected by GWAS, but most of these are not strongly predictive of disease occurrence, due to the low penetrance and limited public health impact, as well as the lack of validated genetic testing. The reason why, for complex traits studied in humans, the identified genetic effect comprises less than half of the estimated trait heritability, is related both to the role of the untested rare variants and the understanding of gene–gene and gene–environment interactions requiring extremely large sample sizes and well-characterized environmental exposures. Interestingly, one might consider genetic tests based on combinations of associated SNPs and evaluate if the inheritance of a panel of SNPs can reveal a higher risk to develop the disease, as often occur for cancer. As sample sizes increase, GWAS will also be able to detect additional SNP associations that have even smaller effect sizes than those observed to date.

Steps of GWAS: From Study Design to Genetic Analysis and Result Interpretation

Around 10 million of the human genome's 3 billion nucleotides are polymorphic SNPs, meaning that they occur in two or more common forms in different subjects. GWAS reveals genetic variations that are correlated with disease, but this does not mean that the variants identified by the studies cause the disease. The idea is that if a genetic variant increases the risk to develop a disease, it should be more frequent among cases than among healthy controls (Fig. 2).

Thus, the genotyping of both cases and control subgroups, could uncover regions of the genome that harbor DNA changes leading to the disease, but the functional role of the identified genetic variants in disease needs to be tested. Common questions in the area of translational medicine are: how do the mutations that underlie GWAS alter the way that genes, protein, cells, or tissue function? How do this knowledge can inform the search for new medicine targeting the molecular basis of the disease? (Fig. 3).

Sequencing of the human genome together with the identification of millions of SNPs, provided the initial foundation for GWAS. In the scientific literature it has been reported that many of these identified SNPs were in linkage disequilibrium (LD) with the common genetic variations.

It is known that, in order to detect with statistical power the expected modest associations (e.g., odds ratio [OR] < 1.5) between a genetic variation and a specific disease, it is needed to involve thousands of subjects and to evaluate hundreds of thousands of SNPs.

Nevertheless, the large sample sizes and initial high cost of SNP arrays motivated the development and use of multistage GWAS designs.

Using a multistage GWAS design, it is possible to discover the strongly associated SNPs in a subset of samples, using the genome-wide SNP array and subsequently validating these SNPs with a less-expensive genotyping platform in the remaining samples (Fig. 1).

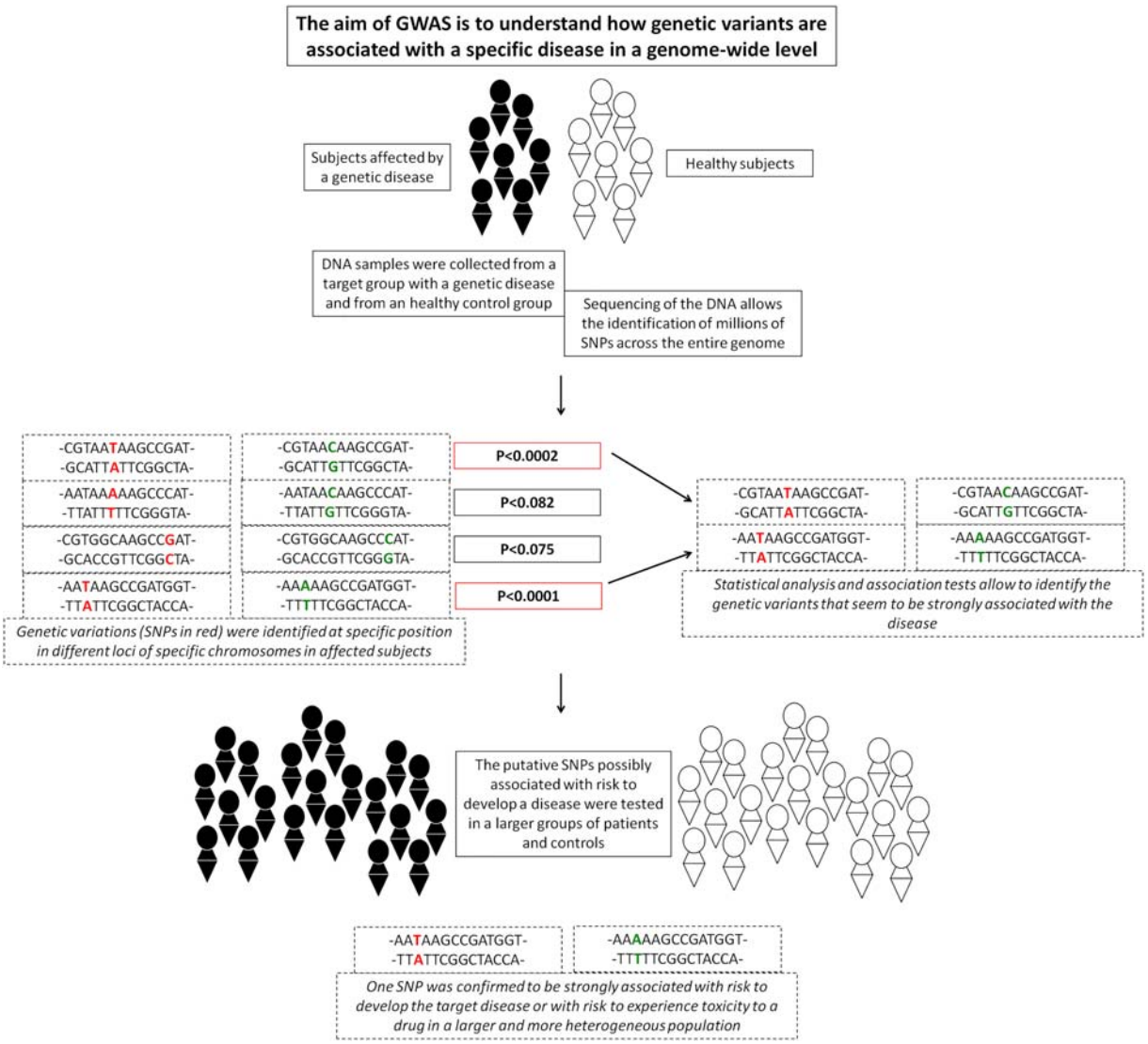

Fig. 1 Steps of genome-wide association study (GWAS). In a multistage GWAS, a large number of individuals affected by a specific type of genetic disease, such as cancer, and a suitable healthy group as a comparison, are selected. In phase I, the entire genome of each subject of a small group of cases and controls are sequenced in order to identify common genetic variants in the form of single nucleotide polymorphisms (SNPs). Stringent statistical methods are used to assess the associations between SNPs and disease. In phase II, the SNPs found to be significantly associated with risk to develop the disease, are tested in a larger group of patients and controls using arrays containing the putative SNPs of interest. The association found with this approach could have clinical utility being possible the use of the biomarker to perform a genetic test to facilitate clinical decision making and improve health outcomes.

Concomitantly with the increasing understanding of the human genome, an enormous number of SNP arrays able to capture an ever-increasing number of variants were developed, permitting to cover a high number of common genetic variations across the human genome.

In the last years, the use of multistage GWAS has permitted to significantly reduce the cost of these analyses. Indeed, while the array prices were initially high, at present, they have steadily decreased, because fewer SNPs are typed in a second stage. Since the data from the first and second stages are combined, analyzed, and penalized for multiple comparisons, the single-stage GWAS has been undertaken.

Today, the decreasing SNP array costs have made multistage GWAS designs less essential. Indeed, genotyping 10,000–20,000 SNPs in a follow-up stage can cost just about as much as a genome-wide SNP array. The newly developed GWAS have permitted to genotype all samples in an initial SNP array, allowing to analyze simultaneously both SNPs and other forms of genetic variation in one study sample (e.g., copy number variants, CNV), but increasing the cost of analysis and encouraging, when possible, the use of multistage design.

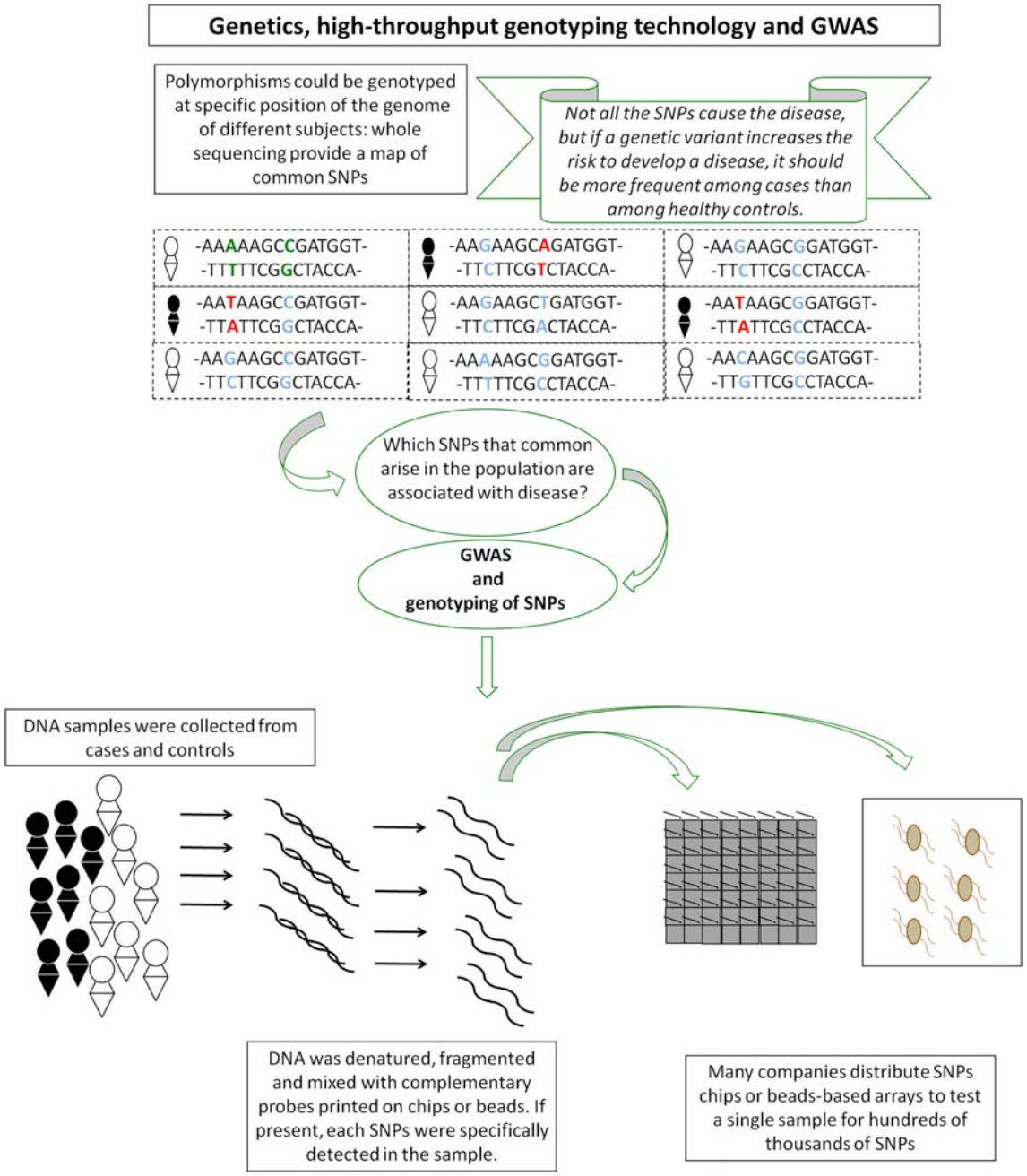

Fig. 2 Genetics, high-throughput genotyping technology and GWAS. GWAS is an "hypothesis-generating" tool. The researchers, through an agnostic genome-based approach and high-throughput genotyping technologies, interrogate the entire human genome to reveal whether SNPs that arise in the population are associated with a specific disease. In this figure, the green colored are the most common nucleotides in the general population, while the others are polymorphic; only those in red represent the genetic variants that increase the risk to develop a disease, and are more frequent among cases colored in black than in controls colored in white. In 2005, as an evolution of the HapMap project, the cost and the resources required to test a DNA sample for the bulk of its common variations became less expensive. The International Hap Map Consortium provides a map of SNPs across the entire human genome that are often inherited together in DNA blocks, called haplotypes. The so called "SNP chips" are cost-effective genotyping arrays produced by several companies that allow the researchers to test a single sample for hundreds of thousands of SNPs.

The primary platforms that have been used for most GWAS are the Affymetrix technology (Santa Clara, CA) that use a chip with short DNA sequence able to detect—by means of hybridization—the specific allele contained in the sample (Fig. 2) and the Illumina system (San Diego, CA) that prints a slightly longer DNA sequence on the surface of beads in order to detect the specific allele with somewhat better specificity.

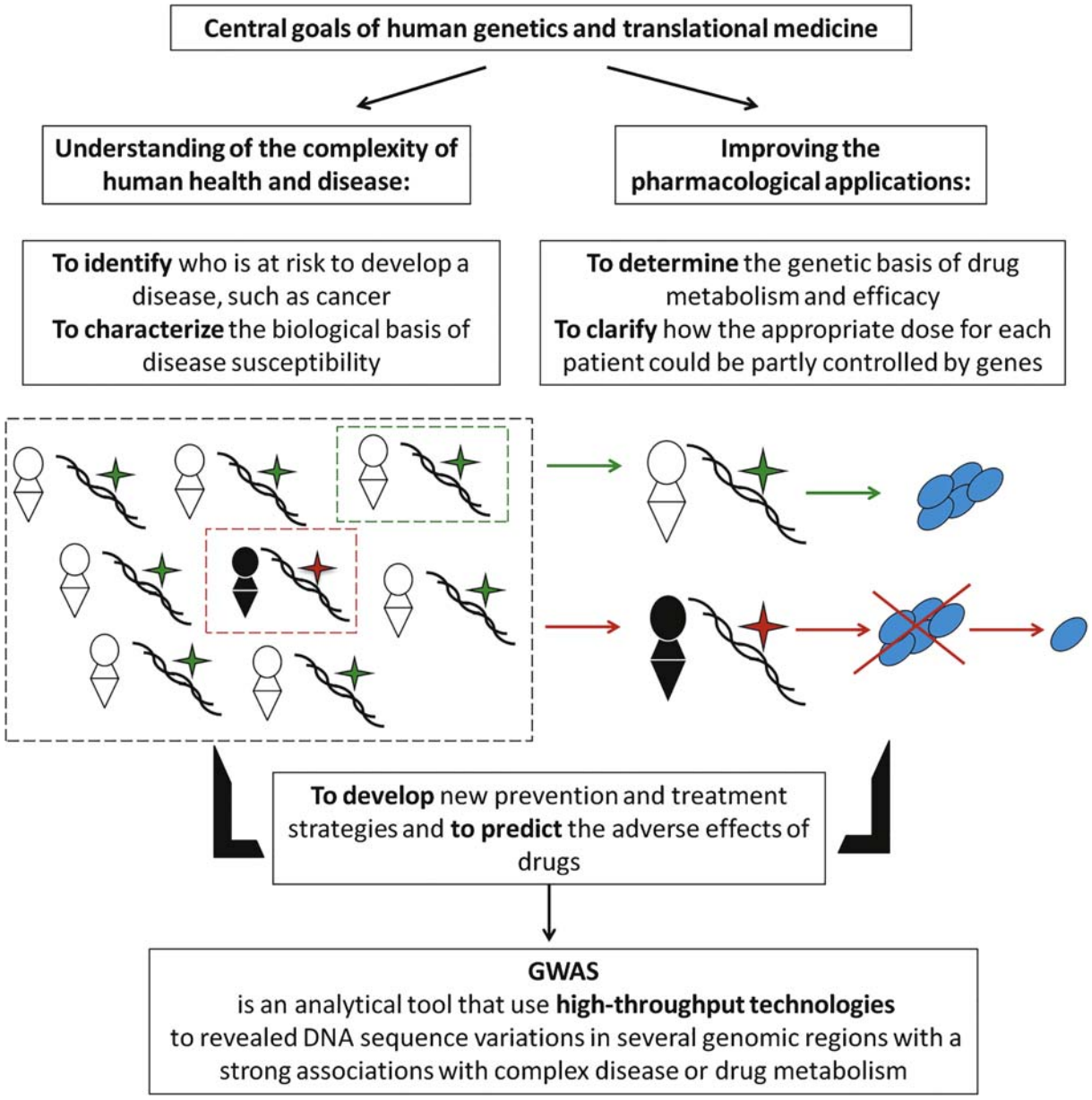

Fig. 3 Central goals of human genetic, translational medicine and the role of GWAS. GWAS plays a central role in human genetic revolution thanks to the identification of robust associations between thousands of genetic variants and hundreds of different traits and diseases. This revolution provides not only an improvement in the understanding of common complex diseases in translational field, but also in clinical and pharmacologic areas where the use of genomic informations contributes to the optimization of patient treatment. In the figure, subjects with genetic variants associated with neither drug toxicity nor pathogenic effects, are represented in white, near a double stranded DNA associated with a green star representing a neutral variant of the DNA sequence. Instead, subjects with a genetic variant associated with an alteration of drug metabolism and toxicity are represented in black, near a double stranded DNA associated with a red star representing the susceptibility variant. The identification of patients carrier of a genetic variant strongly associated with toxicity to a drug (represented as blue circles) allows the clinicians to reduce the dose to minimize the adverse effects to drugs.

The prerequisite to provide a GWAS is to compare two large groups of individuals, comprising selected cases affected by a particular disease and healthy subjects as controls. Generally, all individuals in each group are genotyped for the majority of common known SNPs (Figs. 1 and 2). In order to increase the power to detect associations, it is possible to select those with the extreme traits or symptoms instead to study the entire group of subjects.

Despite study subjects should be representative of their source population and rigorous control selection should be made, in order to reduce the costs of subject recruitment and genotyping, it is possible to use existing genotype informations among controls derived from previous studies and made available to researchers.

Once genotyping is complete, SNPs are subject to a number of quality-control checks and statistical analysis. The relationship between SNPs and disease is generally evaluated trough combinations and interaction tests and haplotypes. For each of analyzed SNPs it is then investigated if the allele frequency is significantly altered between the case and the control group, evaluated by using the OR. Additionally, a *P*-value for the significance of the OR is typically calculated using a simple chi-squared test (the conventional threshold is 5×10^{-8} to be significant in the face of hundreds of thousands to millions of tested SNPs).

The effect size is in inverse relationship with sample size: studies with larger sample size have higher power than those with smaller sample size, being able to detect smaller associations.

Thus, thanks to GWAS, SNPs in genes or genome regions that were not been previously identified as implicated in the initiation and development of the disease, as well as the genetic variants involved in cell adhesion, signal transduction, transport activity, and protein phosphorylation, could be identified.

The GWAS findings are published in the National Human Genome Research Institute's (see the web site of "Catalog of Published Genome-Wide Association Studies").

In the last years, many of the GWAS results have been highly replicated; nevertheless, few of these variants have been described to be associated with disease, although their causative role is not firmly established. A fine mapping and mechanistic studies could determine the pathogenetic implication of GWAS results, even if this could be complicated by the fact that ~30% of the associations detected to date are not even in gene regions, posing a challenge to the understanding of the biological basis of GWAS results, and to the implementation of preventive or therapeutic measures.

Implications and Limitations of GWAS Findings

The findings emerged from GWAS widely increase the interest into a mechanistic work that is aimed at validation of the GWAS data and clarification of the biological basis of disease processes. For example, in order to understand the association between a specific SNP in a given locus with risk to develop a different type of cancer, the in vitro approaches could reveal important molecular and biochemical information.

If of clinical interest, one or more SNPs or a panel of variants, could became part of a genetic test prescribed by a physician or marketed directly to the consumer that pay to have information about variations in the entire genome and genetic counseling about the risk to develop a disease or to benefit from a treatment. Interestingly, in the last years, the pharmacogenetic studies evaluating the genetic basis of drug response, have widely used the GWAS approaches (**Fig. 3**). Indeed, starting from these tests, it is possible to obtain pharmacogenetic information related to the drug and dosage that an individual with a specific pattern of genes involved in drug metabolism should receive.

However, it is important to note that the justification for genetic testing also depends on the existence of effective intervention. Most of these screening tests based on GWAS SNPs may not distinguish between individuals with low and high risk of disease, and, even if some individuals are carriers of a large number of SNPs, their screening in the global population would not be cost-effective.

The use of GWAS have several issues and limitations that should be taken into account for proper quality control and study set-up. Lack of well defined case and control groups, insufficient sample size, control for multiple testing and for population stratification are common problems. In addition, the high number of statistical tests performed have been reported and present a potential for false-positive results.

The Role of GWAS in the Identification of Cancer Susceptibility Genes

Cancer is a highly heterogeneous disease, in terms of time of development and biological properties of each tumor. Among individuals exposed and susceptible to the same carcinogen, tumors do not appear at the same time. The origin and evolution of cancer have been proposed to be the consequence of the combined effects of a number of different alleles and variants, which may control intrinsic (i.e., apoptosis, proliferation, or DNA repair) and/or extrinsic (i.e., stroma, angiogenesis, or endocrine and immune systems) functions of cancer cells.

GWAS have demonstrated that much of heritable risk of most common cancers is polygenic. Thus, every susceptibility variant contributes to a small amount of risk and cancer susceptibility originates from the additive effects of combinations of common low-penetrance variants.

Studies focused on the analysis of linkage in family pedigrees comprising of several members affected by certain cancers allowed the detection of several high-penetrance susceptibility genes: BRCA1 and BRCA2 for breast and ovarian cancers; APC, MLH1, and MSH2 for colorectal cancer; CDKN2a for melanoma. Despite the linkage studies have described candidate loci containing cancer susceptibility genes, often these loci were not statistically significant due to the low number of families affected by each locus and to the regions of interest; it is therefore advisable to replicate and confirm findings in larger populations.

While family studies involve few patients, GWAS involve thousand of patients, thereby significantly increasing density markers. Anyway, these two approaches can be complementary, because the pedigree evaluation, relying on genetic transmission of disease-causing alleles between affected family members, can offer direct and persuasive sign of genetic effects.

As described above, in the last years, multiple GWAS have been reported for each of the major cancers, including breast, prostate, lung, colorectal, pancreatic, gastric, renal, and bladder cancers. In addition, GWAS have been performed for malignant melanoma,

ovarian cancer, glioma, and several hematological malignancies including acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML) and Hodgkin's lymphoma. Additionally, common risk alleles have been identified through GWAS associated with several pediatric solid cancers.

Breast Cancer

Regarding the studies focusing on family members affected by breast cancer, the inherited variations in the two main susceptibility genes, BRCA1 and BRCA2, account together for only around 20% of hereditary breast cancer, elucidating $<5\%$ of the total breast cancer susceptibility. This encouraged extensive efforts to discover additional high penetrance susceptibility genes. With the advent of GWAS, two classes of variants that have an influence on cancer susceptibility have been detected: rare moderate-penetrance variants and common low-penetrance variants. For example, through the direct interrogation of candidate genes encoding proteins involved in the DNA-damage response pathway, in addition to BRCA1 and BRCA2, also ATM, CHECK2 and PALB2 have been associated with an increased breast cancer risk.

The first breast cancer GWAS was published in 2007 by Easton and colleagues. The Authors, using a two-stage GWAS analyzing 227,876 SNPs in 4398 breast cancer cases and 4316 controls, and followed by a third stage in which 30 SNPs were tested for confirmation in 21,860 cases and 22,578 controls, they identified five novel risk loci in *FGFR2* [rs2981582, rs1219648, and rs1078806], *TNRC9* [rs3803662], *MAP3KI* [rs889312], and *LSP1* [rs3817198] genes, with a strong and consistent evidence of association with breast cancer ($P < 10^{-7}$; OR ranging from 1.05 to 1.41). In particular, *FGFR2* and *MAP3K1* are members of the RAS/RAF/MEK/ERK-signaling pathway and could be important tumor markers of breast cancer risk. In particular, it has been showed that FGFR2 rs1219648 and rs2981582 genotypes were significantly associated with breast cancer in estrogen receptor-positive (ER+), progesterone receptor-positive (PR+) and HER2/Neu-negative (HER2−) tumors.

Some of the other susceptibility loci identified in gene-containing regions have not been implicated in cancer previously (e.g., *TOX3*, *STXBP4*, *SLC4A7*, *MRPS30*, *RAD51L1*, *LOC643714*, and *STXBP4*) and are being evaluated for their potential role in carcinogenesis. Nevertheless, the risk associated with each identified SNPs is modest, with ORs ranging from 1.1 to 1.4, and with a contribution to the familial risk of breast cancer of about 8%.

Prostate Cancer

With the increasing incidence of prostate cancer, the identification of common genetic variants that confer risk of developing the disease has become important. In a recent meta-analysis of 87,040 individuals, it have been identified 23 new susceptibility loci for prostate cancer ($P < 5 \times 10^{-8}$), with ORs mostly ranging from 1.2 to 2.0. It has been also demonstrated that this loci in genes like *GOLPH3L* (rs17599629, 1q21), *NEDD9* (rs4713266, 6p25), *HLA-DRB6* (rs115306967, 6p21), *CDKN2B-AS1* (rs17694493, 9p21), *TTC9* (rs8014671, 14q24), *SLC41A1* (rs1775148, 1q32), *TMPRSS2* (s1041449, 21q22), *PEX14* (rs636291, 1p35), *MYO6* (rs9443189, 6q14), among others, combined with the known prostate cancer risk variants, explain 33% of the familial risk for this disease. In particular, variant rs4713266 is located in intron 1 of *NEDD9*, a gene involved in cell adhesion, motility, cell cycle and apoptosis, and is implicated in the progression and metastasis of several cancers. Otherwise, rs9443189 on *MYO6* gene, is described to be a modulator of androgen-dependent gene expression, and it has been found to be overexpressed in prostate cancer driving tumor growth and metastasis.

Moreover, several studies have identified independent risk variants in the 8q24 region (HapC 14 SNPs, rs16901979, DG8S737, rs1447295, rs1016343, rs6983267, rs4242382, rs1006908, rs620861, and rs16902094). Risk SNPs at or near genes (i.e., *MSMB* [rs10993994], *KLK3* and *KLK2* [rs2735839], *NKX3-1* [rs2928679], and *CTBP2* [rs4962416]) with possible roles in prostate carcinogenesis have also been found. Zheng and colleagues also evaluated the impact of carrying multiple risk SNPs on prostate cancer risk, demonstrating that men carrying four out of five possible risk SNPs (rs4430796 [17q12], rs1859962 [17q24.3], rs16901979 [8q24], rs6983267 [8q24], and rs1447295 [8q24]) had a 4.5-fold increased risk of disease. No evidences highlighted that the risk SNPs were associated with disease aggressiveness, earlier age at diagnosis or presence or absence of family history.

Nowadays, the validity and the clinical utility of the use of individual or multiple SNP panels as screening test for prostate cancer have not been demonstrated. Nevertheless, the identification of genetic variants able to predict early-onset or more aggressive disease are in progress.

Lung Cancer

In the field of research investigating the association between SNPs and lung cancer risk, a growing number of studies have been published. In a recent work published by Liu and co-workers, evidences from meta-analyses and GWAS revealed the association between 108 variants and lung cancer. Among these, about 63 were reported to be significantly associated with lung disease, and, in particular, 15 SNPs on or near 12 genes and one miRNA showed strong evidence of association with lung cancer risk, including *TERT* [rs2736098], *CHRNA3* [rs1051730], *AGPHD1* [rs8034191], *CLPTM1L* [rs401681 and rs402710], *BAT3* [rs3117582], *TRNAA* [rs4324798], *ERCC2* [Lys751Gln], miR-146a2 [rs2910164], *CYP1B1* [Arg48Gly], *GSTM1* [null/present], *SOD2* [C47T], *IL-10* [−592C/A and −819C/T], and *TP53* [intron 6]. Among these variants, SNP rs2736098 is localized to telomerase gene *TERT*, a reverse transcriptase component of telomerase, playing an essential role in telomerase enzyme production and maintenance of telomeres. In the literature, it has been reported that TERT gene amplification is commonly described in lung

adenocarcinoma. Among other genes, *CHRNA3* encodes for a member of nicotinic acetylcholine receptor family which may be a key player in nicotine-mediated suppression of apoptosis in lung cancer cells. *CLPTM1L* contributes to the accumulation of DNA damage, which confers susceptibility to tumorigenesis, whereas *BAT3*, a member of the Bcl-2-associated athanogene (BAG) family of proteins, participates in a variety of primary cellular processes, playing also an important role in apoptosis regulation.

In another GWAS for squamous lung cancer, rare variants of BRCA2 (p.Lys3326X [rs11571833]) and CHECK1 (p.Ile157Thr [rs17879961]), in association with the common variation 3q28 (TP6, rs13314271) were also implicated in lung adenocarcinoma.

More recently, a large-scale association analysis has identified new lung cancer susceptibility loci. In this study, 14,803 cases and 12,262 controls of European descent were genotyped and combined with existing data for an aggregated GWAS analysis of lung cancer in 29,266 cases and 56,450 controls. Eighteen new susceptibility loci in several genes (*FUBP1* [rs71658797], *RNASET2* [rs6920364], *CHRNA2* [rs11780471], *BRCA2* [rs11571833], *SEMA6D* [rs66759488], *CHRNA5* [rs55781567], *CYP2A6* [rs56113850], *TP63* [rs13080835], *TERT* [rs7705526], *NRG1* [rs4236709], *CDNK2A* [rs885518], *OBFC1* [rs11591710], *AMICA1* [rs1056562], *SECISBP2L* [rs77468143], *RTEL1* [rs41309931], *MHC* [rs116822326], *RAD52* [rs7953330], *CHEK2* [rs17879961]) were identified, showing the striking heterogeneity in genetic susceptibility across the histological subtypes of lung cancer, with four loci associated with lung cancer overall and six loci associated with lung adenocarcinoma.

The locus 15q21 is predicted to target *SECISBP2L*. The genetic risk allele appears to correlate with decreased expression levels of *SECISBP2L*, already known to be downregulated in lung cancer. rs77468143 was nominally associated with lung function, potentially implicating inflammation of lung as a part of the mechanism at this locus.

Three of the identified variants associated with lung adenocarcinoma are located near gene related to telomerase regulation: rs7902587 and rs41309931 near *OBFC1* and *RTEL1*, respectively, and rs2853677 near *TERT* as previously noted.

The genetic susceptibility alleles described in GWASs explain approximately 12.3% of the familial relative risk. Nevertheless, ongoing studies on target genes will provide new insights into the etiology of lung cancer.

Colorectal Cancer

Among the most common malignancies, colorectal cancer (CRC) has one of the largest proportions of familial cases. It is reported in the literature that approximately 30% of all CRC cases are an inherited form of the disease, and of these, only about 5% of cases are associated with highly penetrant inherited mutations (i.e., common polymorphisms in genes that regulate metabolism or genes that are regulated by environmental or other genetic factors). Inherited CRCs are also likely to be caused by alterations in multiple susceptibility loci that have additive effects. In particular, it has been described that common variants influence CRC risk. In detail, performing different meta-analysis of several GWAS, several CRC risk loci were identified, including 1q41 (rs6691170 and rs6687758), 3q26.2 (rs10936599), 6p21 (rs1321311, *CDKN1A*), 8q23 (rs16892766, *EIF3H*), 8q24 (rs6983267, *LOC727677*, *POU5F1P1*), 11q13.4 (rs3824999, *POLD3*), 12q13.13 (rs11169552 and rs7136702), 18q21.1 (rs4939827, *SMAD7*), 20q13.33 (rs4925386), and Xp22.2 (rs5934683, *SHROOM2*), associated approximately with 0.92 to 1.27-fold increased risk of disease.

These data derived from GWAS on CRC provide important insight into the biological basis of inherited genetic susceptibility to CRC, identifying risk factors able to influence the development of CRC.

Pancreatic Cancer

As CRC, pancreatic cancer is one of the most common cancers, considered as the fourth leading cause of death in the developed world. Both inherited high-penetrance mutations in *BRCA2*, *ATM*, *PALB2*, *BRCA1*, *STK11*, *CDKN2A* and mismatch-repair genes and low-penetrance loci are associated with increased risk.

Conducting GWAS, several loci risk associated with pancreatic cancer have been described: 9q34 (rs505922, *ABO blood group* gene – OR = 1.20), 13q22.1 (rs9543325 and rs9564966, *KLF5* – OR = 1.26 and 1.26, respectively), 1q32.1 (rs3790844, *NR5A2* – OR = 0.77) and 5p15.33 (rs401681, *CLPTM1L-TERT* – OR = 1.19). Recently, to identify new risk loci, Childs and colleagues performed a GWAS on 9925 cases and 11,569 controls, identifying three newly associated regions, including 17q25.1 (*LINC00673*, rs11655237, OR = 1.26), 7p13 (*SUGCT*, rs17688601, OR = 0.88) and 3q29 (*TP63*, rs9854771, OR = 0.89), with high pancreatic cancer risk penetrance.

Two highly correlated variants (rs11655237 and rs7214041) were associated with pancreatic cancer risk. Variant rs7214041 is to *LINC00673* (long intergenic nonprotein coding RNA 673); rs11655237, a noncoding transcript variant, shows significant DNase hypersensitivity in multiple cancer cell lines and binds transcription factors including *P300*, *FOXA1*, *FOXA2*, and the DNA repair protein *RAD21*. A significant association for two variants in high LD (rs9854771 and rs1515496) and located in an intron of TP63, is also described. p63 is a p53 homologue implicated in tumorigenesis and metastasis, playing a role in cell-cycle arrest and apoptosis.

Renal Cell Carcinoma

Similarly, clear cell renal carcinoma (RCC), the most common type of kidney cancer in adults, is responsible for approximately 90%–95% of cases. GWAS approaches in RCC patients have identified several variants conferring risk of the disease. One variant, rs35252396 located at 8q24, has been described to be significantly associated with RCC (OR = 1.27). The conduction of two-stage GWAS of RCC in 3772 affected individuals and 8505 controls, identified two additional risk loci, 2p21 (rs11894252 and rs7579899 mapping to EPAS1) and 11q13.3 (rs7105934, SCARB1), associated with RCC susceptibility, in addition to the more recent seven

new loci at 1p32.3 (rs4381241), 3p22.1 (rs67311347), 3q26.2 (rs10936602), 8p21.3 (rs2241261), 10q24.33-q25.1 (rs11813268), 11q22.3 (rs74911261), and 14q24.2 (rs4903064).

Bladder Cancer

Bladder cancer is also one of the most common cancer in men and its frequent recurrence requires regular screening and intervention. GWAS in bladder cancer identified common variants in four genomic regions on chromosome 3q289 (*TP63*), 4p16.3 (*TMEM129*, *TACC3-FGFR3*), 8q24.219, and 8q24.311 (*PSCA*) all associated with increased risk. Interestingly, the variants on 8q24.21 map to a region centromeric to MYC that has been identified in GWAS of breast, colorectal and prostate cancers, as well as chronic lymphocytic leukemia. Association with bladder cancer risk has been suggested also for variants near the TERT-CLPTM1L locus on chromosome 5p15.33, which has been described to be associated with increased risk of basal cell carcinoma, cutaneous melanoma, lung, brain, and pancreatic cancers, together with three new regions on chromosomes 22q13.1 (rs1014971), 19q12 (rs8102137, *CCNE1*), and 2q37.1 (rs11892031, *UGT1A*).

These findings on common variants associated with bladder cancer risk should provide, together with further discovery, new insights into the mechanisms of carcinogenesis.

Other Malignancies

Other malignancies have been subject of GWAS with the aim to identify genetic variants associated with an increased risk of cancer.

Cutaneous melanoma is a disease of fair-skinned individuals, with an increased risk associated with a family history of the disease. As reported in the literature, the most common high-penetrance melanoma susceptibility gene (*CDKN2A*) maps to 9p21. Germline *CDKN2A* mutations are carried by about 2% of all melanoma cases across populations.

One of the first genome-wide association study of melanoma, conducted of 317K tagging SNPs for 1650 selected cases and 4336 controls, identified three risk loci: 16q24 (*MC1R*, rs258322), 11q14-q21 (*TYR*, rs1393350) and 9p21 adjacent to *MTAP* and flanking *CDKN2A* (rs7023329). In particular, *MC1R* and *TYR* genes are associated with pigmentation and cutaneous sun sensitivity, well-recognized melanoma risk factors.

An international two-stage meta-analysis of 11 cutaneous melanoma GWAS (totaling 15,990 cases and 26,409 controls), has identified some loci not previously associated with melanoma risk ($P < 5 \times 10^{-8}$), including 2p22.2 (*RMDN2/CYP1B1*, rs6750047), 6p22.3 (*CDKAL1*, rs6914598), 7p21.1 (*AGR3*, rs1636744), 9q31.2 (*TMEM38B/RAD23B/TAL2*, rs10739221), 10q24.33 (*OBFC1*, rs2995264), 11q13.3 (*CCND1*, rs498136), and 15q13.1 (*OCA2*, rs4778138).

In addition to this, Barret and colleagues, identified additional new regions with at least one SNPs with $P < 1 \times 10^{-5}$ in *ATM* (rs1801516), in *MX2* (rs45430), a SNP adjacent to *CASP8* (rs13016963), and a fourth locus near *CCND1* (rs1485993).

Ovarian cancer accounts for the most common cause of deaths than all other gynecological cancers combined, with a heritable component explaining less than half of the excess familial risk. Performing GWAS, during the years, identified several common ovarian cancer susceptibility alleles. The prevalent SNPs associated with disease risk ($P < 10^{-8}$) included SNPs at 9p22 (rs3814113, OR = 0.82), variants at 1p36 (nearest gene, *WNT4* [rs56318008]), 4q26 (*SYNPO2* [rs17329882]), 9q34.2 (*ABO* [rs635634]) and 17q11.2 (*ATAD5*) associated with epithelial ovarian cancer (EOC) risk, and at 1p34.3 (*RSPO1* [rs58722170]) and 6p22.1 (*GPX6* [rs116133110]) variants, specifically associated with the serous EOC subtype. In addition, the analysis of *HOXD1* (rs2072590), *MYC* (rs10088218), *TIPARP* (rs2665390), *SKAP1* (rs9303542) and of *BNC2* at 9p22 supports a functional role for these genes in ovarian cancer development.

Gliomas represent about 40% of all primary brain tumors and cause around 13,000 death in the USA each year. These cancers are heterogeneous and typically associated with a poor prognosis irrespective of clinical care, having a median overall survival of only 10–15 months. As described in literature, GWASs identified SNPs in at least eight loci influencing glioma risk: 3q26.2 (near *TERC*), 5p15.33 (near *TERT*), 7p11.2 (near *EGFR*), 8q24.21 (near *CCDC26*), 9p21.3 (near *CDKN2A/CDKN2B*), 11q23.3 (near *PHLDB1*), 17p13.1 (*TP53*), and 20q13.33 (near *RTEL1*).

Moreover, a metaanalysis of four GWASs totaling 4147 cases and 7435 controls identified new glioma susceptibility loci for glioblastoma (GBM) at 12q23.3 (*POLR3B*, rs3851634) and non-GBM at 10q25.2 (*VTI1A*, rs111696067), 11q23.2 (*ZBTB16*, rs648044), 12q21.2 (intergenic, rs12230172), and 15q24.2 (*ETFA*, rs1801591).

In addition, several hematological malignancies including acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL) and Hodgkin's lymphoma, have been addressed within several GWAS to determine the presence of risk loci associated with the disease. Examples included the risk loci 7q12.2 (*IKZF1*, rs4132601), 10q21.2 (*ARID5B*, rs10994982), and 14q11.2 (*CEBPE*, rs2239633) for ALL, the variants 2q13 (*ACOXL/BCL2L11*, rs17483466), 2q37.1 (*SP140*, rs13397985), 2q37.3 (*FARP2*, rs757978), 6p25-p23 (*IRF4*, rs872071), and 19q13.2-q13.3 (*PRKD2/STRN4*, rs11083846) for CLL.

It is well known that the risk of Hodgkin's lymphoma (HL), a common B-cell-derived cancer involving germinal center of lymph nodes, is influenced by HLA genotype variation within the major histocompatibility complex (MHC). Nevertheless, GWASs on this cancer have identified susceptibility loci for HL at 2p16.1 (*REL*, rs1432295), 8q24.21 (*PVT1*, rs2019960), and 10p14 (*GATA3*, rs501764), together with loci mapping to 3p24.1 (*EOMES*, rs3806624), and 6q23.3 (*HBS1L* and *MYB*, rs7745098).

Recently, a metaanalysis performed on three GWASs (1816 cases and 7877 controls) identified a novel variant at 19p13.3 associated with HL (rs1860661; OR = 0.81), located in intron 2 of *TCF3* (also known as *E2A*), a regulator of B- and T-cell lineage commitment known to be involved in HL pathogenesis, suggesting a link between the 19p13.3 locus and HL risk.

In conclusion, despite GWAS provided a direct evidence of polygenic susceptibility, almost all variants identified in several cancers and other diseases have no proven biological or mechanistic relevance to the disease, or medical utility for diagnosis or therapy. Almost all low-penetrance variations discovered to date have weak effects; a meta-analysis approach showed that few loci were replicated in different studies. In this regard, the genetic heterogeneity could be the reason for the limited number of genes that could play an important role in cancer susceptibility exclusively within a limited number of families, and this effect could be lost as soon as they are diluted in the general population. Moreover, the synergistic interactions of low-penetrance genes together with the environmental exposure could characterize an important portion of the heritability of cancer.

For this reason, in order to improve the knowledge regarding the causative relationship between variants and diseases, large-scale association or case-control studies are warranted.

Genome-Wide Association Studies in Pharmacogenetics

Pharmacogenetics is the study of inherited genetic differences in drug metabolic pathways, which can affect the individual response to drug in term of both therapeutic and adverse effects (**Fig. 3**).

Pharmacogenetics is often related to the oncology field, and involves the study of germline mutations, included SNPs, affecting genes coding for liver enzymes and drug carriers responsible for drug metabolism and transport across membranes. Pharmacokinetics and pharmacodynamics are thus involved. Among the most important genes, a relevant role is played by the cytochrome P450 oxidase and genes coding for drug targets like receptor, transporters or enzymes.

Since 2007, GWAS have increasingly been applied in this field. For example, the immunosuppressant thiopurine drugs, used in several cancer treatments, can cause bone marrow suppression associated with inactivating polymorphisms of thiopurine *S*-methyltransferase (TPMT).

In particular, the TPMT c.719A>G SNP has been known to cause a decrease in TPMT activity, increased intracellular thiopurines, and drug toxicities. The majority of low activity phenotypes can be explained by two common variant alleles, TPMT*3A and *3C, both of which are characterized by the nonsynonymous SNP c.719A>G (rs1142345). Similarly, an association has been also described between the uridine diphosphate glucuronosyltransferase 1A1 allele *28 (UGT1A1*28) and neutropenia induced by irinotecan.

One of the first GWA study referred to the analysis of genetic and nongenetic response to the anticoagulant warfarin, was performed on 1053 patients, resulting in an association with two genes: CYP2C9, encoding the main metabolizing enzyme for both drugs, and vitamin K epoxide reductase complex 1 (VKORC1), showing genome wide significance with low *P* values (1×10^{-78}). The role of GWAS in this process is evident in the evaluation of the genetic component of warfarin dosing.

Another example is referred to methotrexate, a chemotherapeutic agent and immune system suppressant used to treat cancer (i.e., breast, leukemia, lung cancer, and lymphoma), and autoimmune diseases (i.e., psoriasis, rheumatoid arthritis, and Crohn's disease). The GWA study, performed on a cohort of 434 children with leukemia, has identified the SLCO1B1 gene affecting methotrexate pharmacokinetics, thus influencing both toxicity of this drug, and possible drug interactions with widely used OATP1B1 substrates, such as statins. In conclusions, ongoing GWAS are demonstrating their potential not only for the identification of genes related to the development and/or progression of a disease, but also in pharmacogenetics and pharmacogenomics. In particular, the complex physiology of drug response highlights the need for analytical methods that address this complexity, using the wealth of information available about drug mechanisms and pathways.

Conclusions

Cancer is an extremely complex phenomenon and, together with the incomplete penetrance of the inherited tumor susceptibility alleles, the interaction with environmental risk factors could substantially alter hereditary susceptibility. The results of currently published GWAS have demonstrated that much of the heritable risk of most common cancers is polygenic, and that the increased risk of cancer associated with the known genetic variants are not medically addressed. With the exception of breast and prostate cancers, the currently identified loci explain only a small proportion of the familial risk of many cancers. Nevertheless, the loci identified through GWAS have greatly expanded the existing knowledge of genes that influence cancer risk. However, determining the functional consequences of data from GWAS could be important in order to gain a deeper understanding of cancer biology and to suggest potential targets for therapeutic and preventive strategies.

Therefore, despite the GWAS limitations, these analysis provide a more complete understanding of the genetic basis of disease, and a wide view of the genetic architecture of complex phenotypes.

Prospective Vision

In contrast to the original hypothesis according to which a common disease may be caused in part by genetic variants (e.g., SNPs) with minor allele frequency (MAF) > 5%, selected diseases are also due to rare variants. GWAS mainly contributed to the identification of common low-penetrance risk variants associated with a low relative-risk to develop a disease. Thus, efforts are currently

being directed towards testing rare variants for association with complex traits. The development of new analytic approaches and the test of rare variants for association with complex traits using imputed variants from the publicly available 1000 Genomes Project resequencing data and from direct resequencing of clinical samples, are now ongoing. In particular, novel analytic approaches are based on the integration of in vitro and in vivo experimental approaches with genomic data.

Therefore, GWAS approaches are powerful tools that have enhanced and will continue to expand our understanding of cancer genetics and will inevitably lead to the identification of novel pathways of carcinogenesis.

Until now, most GWAS evaluated genetic variation depend only on SNPs. At present, the research regarding other forms of genetic variation in the human genome, including genomic structural changes and sequence variation (i.e., CNV), is just beginning to emerge and will provide further insight into the genetic basis of complex diseases. Due to rapid expansion of genomic knowledge, it will be important to build and adequate collaboration between "bench" scientists and "bedside" stakeholders.

See also: Molecular Epidemiology and Cancer Risk.

Further Reading

Daly, A.K., 2010. Genome-wide association studies in pharmacogenomics. Nature Reviews Genetics 11 (4), 241–246.
Han, Y.L., 2016. Data analysis in the post-genome-wide association study era. Chronic Disease and Translational Medicine 2 (4), 231–234.
Houlston, R.S., 2017. Genome-wide association studies of cancer: Current insights and future perspectives. Nature Reviews Cancer 17 (11), 692–704.
Klein, R.J., 2005. Complement factor H polymorphism in age-related macular degeneration. Science 308 (5720), 385–389.
Lee, .J.C., 2017. Beyond disease susceptibility-leveraging genome-wide association studies for new insights into complex disease biology. HLA 90 (6), 329–334.
Mao, J.H., 2011. Cancer evolution and individual susceptibility. Integrative Biology: Quantitative Bioscience from Nano to Macro 3 (4), 316–328.
Motsinger-Reif, A.A., 2013. Genome-wide association studies in pharmacogenomics: Successes and lessons. Pharmacogenetics and Genomics 23 (8), 383–394.
Nickerson, D.A., 2008. The environmental genome project: Reference polymorphisms for drug metabolism genes and genome-wide association studies. Drug Metabolism Reviews 40 (2), 241–261.
Offit, K., 2010. Genome-wide association studies of cancer. Journal of Clinical Oncology 28 (27), 4255–4267.
Raj, T., 2011. Progress and promise of genome-wide association studies for human complex trait genetics. Genetics 187 (2), 367–383.
Stadler, Z.K., 2010. Genome-wide association studies of cancer. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology 28 (27), 4255–4267.
Sud, A., 2017. Genome-wide association studies of cancer: Current insights and future perspectives. Nature Reviews Cancer 17 (11), 692–704.
Witte, J.S., 2010. Genome-wide association studies and beyond. Annual Review of Public Health 31, 9–20.

Relevant Websites

https://ghr.nlm.nih.gov/primer/genomicresearch/gwastudies—National Library of Medicine, understand genetic.
http://www.genome.gov/gwastudies—National Human Genome Research Institute's "Catalog of Published Genome-Wide Association Studies".
http://www.illumina.com/techniques/microarrays/human-genotyping.html; http://www.thermofisher.com/it/en/home/lifescience/microarray-analysis.html—High-troughput technologies for identifying genetic variation.
https://www.ncbi.nlm.nih.gov/variation/news/NCBI_retiring_HapMap/—Hap Map project resources.

Germ Cell Tumors: Pathology and Genetics

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Glossary

Choriocarcinoma Malignant germ cell tumor composed of trophoblastic tissue.

Dermoid cyst Cystic benign tumor of the ovary composed of mature somatic tissues, predominantly from the cranial part of the body.

Developmental state (synonym: pluripotency state) Determines the developmental potential of embryonic stem cells: 2C-state, omnipotent, capable of producing a complete organism; naïve-state (synonym: ground-state), totipotent, capable of producing all lineages of the embryo proper, extraembryonic tissues (yolk sac and trophoblast), and the germ line; primed-state, pluripotent, capable of producing all somatic tissues, but not extraembryonic tissues and the germ line. (Table 1) Plasticity of developmental states allows gradual transitions between the defined states.

Embryonal carcinoma Malignant germ cell tumor composed of transformed embryonic stem cells, so-called embryonal carcinoma cells, the stem cells of nonseminomas.

Genomic imprinting The mechanism whereby the expression of genes may differ depending on parental origin.

Germ cell neoplasia in situ (GCNIS) In situ malignant germ cell tumor confined to seminiferous tubules, composed of transformed gonocytes, located in the spermatogonial niche.

Gonadoblastoma In situ malignant germ cell tumor of dysplastic gonads, composed of transformed gonocytes enveloped by supporting stromal cells, usually granulosa cells.

Hydatidiform mole Benign germ cell tumor composed of trophoblastic tissue.

Nonseminoma Malignant germ cell tumor, resulting from reprogramming of neoplastic primordial germ cell/gonocyte, consisting of embryonal carcinoma, embryoid bodies, teratoma, yolk sac tumor and choriocarcinoma, either pure or in various combinations, and thus representing caricatures of embryonic development. Identical tumors are referred to as nonseminoma in the testis and mediastinum, nondysgerminoma in the ovary, and nongerminoma in the brain.

Parasitic twin Poorly developed, usually monozygotic twin attached to outside of the body, or included within the body.

Pre-GCNIS Lesion of the seminiferous tubule at risk of progressing toward GCNIS.

Seminoma Malignant germ cell tumor composed of transformed primordial germ cells (extragonadal) or gonocytes (testis, ovary, and dysgenetic gonad). Identical tumors are referred to as seminoma in the testis and mediastinum, dysgerminoma in the ovary, and germinoma in the brain.

Spermatocytic tumor Benign germ cell tumor composed of postpubertal spermatogenetic cells before meiosis. Previously referred to as spermatocytic seminoma.

Teratoma Germ cell tumor composed of mature somatic tissues; depending on originating germ cell tumor type (Table 1) teratomas can be benign (type I and IV), malignant (type II), or both (type VI).

Undifferentiated gonadal tissue Combination of granulosa cells and gonocytes in an ambiguous gonad at risk of progressing toward gonadoblastoma.

Yolk sac tumor (synonym primitive endodermal tumor) Malignant germ cell tumor composed of extraembryonic structures (allantois and yolk sac), and intraembryonic endodermal derivatives (primitive gut and liver).

Introduction

Germ cell tumors (GCT) are a heterogeneous family of gonadal and extragonadal tumors derived from germ cells, and their precursors: primordial germ cells (PGC)/gonocytes (Fig. 1). The wide range of clinical manifestations of GCT is due to the different developmental potential and anatomical sites of the originating cells. Characteristics of seven defined types of GCT are summarized in Table 1.

Migration of PGC and Localization of Extragonadal GCT Types I and II

In human embryos PGC can be recognized in the wall of the yolk sac from week 3 to 4 postconception (wpc). They express cKIT, the receptor for KIT ligand (KITLG), which is essential for survival, proliferation, and migration of PGC. In wpc 4–5 PGC leave the gut

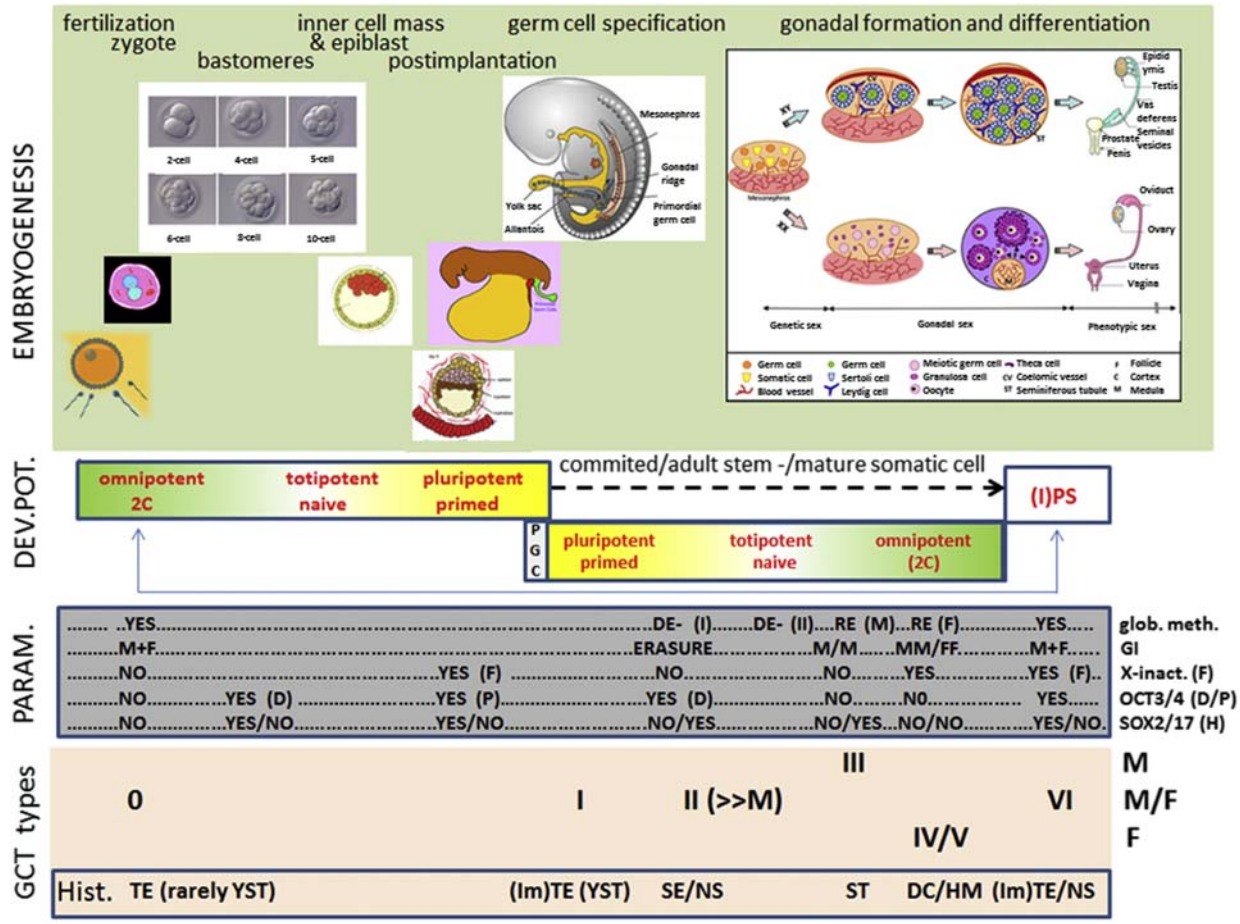

Fig. 1 Unifying model of the pathogenesis of GCT based on the hypothesis that the developmental potential of GCT is determined by the developmental state (2C, naïve, or primed) of the originating cell. Shown in the figure are stages of embryogenesis (upper panel), developmental potential of stem cells in subsequent stages of embryonic development and the germline (second panel), critical features of the involved stem cells (third panel), and corresponding GCT types with gender distribution and their histology, reflecting developmental potential of originating cell (bottom panel, linking **Figure 1** to **Table 1**). Abbreviations in order of appearance: *DEV.·POT.*, developmental potential; *PGC*, primordial germ cell; *iPSC*, induced pluripotent stem cell; *PARAM*, parameters; *M*, male; *F*, female; *DE-(I)*, first wave of demethylation; *DE-(II)*, second wave of demethylation; *RE*, remethylation; *glob. meth.*, Global methylation; *GI*, genomic imprinting; *X-inact*, X-inactivation; *D*, distal enhancer; *P*, proximal enhancer; *H*, human; *GCT*, germ cell tumor; *TE*, teratoma; *YST*, yolk sac tumor; *Im*, immature; *SE*, seminoma; *NS*, nonseminoma; *ST*, spermatocytic tumor; *DC*, dermoid cyst; *HM*, hydatidiform mole. Oosterhuis, J. W. and Looijenga, L. H. J. (2017). Germ cell tumors from a developmental perspective: Cells of origin, pathogenesis and molecular biology; emerging patterns. In F. F. Nogales and R. E. Jimenez (eds.), *Pathology and biology of human germ cell tumors*, Berlin: Springer Nature, pp. 23–129.

epithelium by epithelial mesenchymal transition (EMT) (Fig. 2). In the period wpc 4–6 they are present in the hindgut epithelium, the mesenchyme of the dorsal mesentery, and the developing genital ridge. KITLG activates KIT signaling and facilitates the migration of PGC. After establishment of connections between the enteric and sympathetic nervous systems, PGC follow sympathetic nerve fibers towards the gonads. Numerous PGC are still present in the nervous system by wpc14. PGC failing to exit the nerve branches at the gonadal site may continue along the sympathetic trunk along the midline of the body and may end up in other distant localizations including the retroperitoneum (suprarenal region, adrenal glands), abdomen (stomach), anterior mediastinum, heart, lungs, head and neck, and central nervous system (CNS). These so-called mis-migrated PGC may, unless eliminated by apoptosis (Fig. 3), give rise to GCT in these various extragonadal sites.

Upon arrival in the genital ridges human PGC, from then on called gonocytes, enter a premeiotic stage and upregulate meiotic genes. In the male genital ridge the germ cells enter mitotic arrest as G0/G1 prespermatogonia, due to the low exposure to retinoic acid (RA) within the seminiferous tubules. In the female genital ridge, and extragonadal localizations both in male and female embryos, the germ cells enter meiotic prophase due to exposure to RA.

Migrating PGC undergo progressive germline-specific global demethylation, so-called reprogramming 1. In the gonads reprogramming 2 takes place, including completion of erasure of parental genomic imprinting.

Extragonadal type I GCT occur at all sites where mis-migrated PGC have been demonstrated. In the developing embryo methylated early stage PGC are more robust than late stage PGC, but nonetheless will perish through apoptosis, unless they escape this

Table 1 Characteristics of seven defined types of germ cell tumors (GCT)

Type GCT	*Age (years)*	*Sex*	*Anatomical site*	*Phenotype/ developmental potential*	*Developmental state*	*Precursor cell*	*Genomic imprinting; methylation*	*Karyotype*	*Animal model*
0	Neonates	F/M	Retroperitoneum/ sacrum/skull/hard palate	Included and parasitic twins	2C-state (omnipotent)	Blastomere	Biparentental	Normal diploid	Not available
I	Neonates and children$n < 6$; rarely beyond childhood	F/M	Testis/ovary/sacral region/ retroperitoneum/ anterior mediastinum/neck/ midline brain/other rare sites	(Immature) teratoma (TE)/yolk sac tumor (YST)	Primed-state (pluripotent)	PGC/gonocyte before start of global demethylation	Biparental partially erased	Diploid (TE)/ aneuploid (YST): gain: 1q,12(p13),20q loss: 1p,4,6q	Mouse teratoma
II	After start of puberty. In disorders of sexual developm., Klinefelter's- and Down's syndrome rarely before puberty	>>M	Dysgenetic gonad/ testis/ovary/anterior mediastinum (thymus)/midline brain (pineal gland)	Seminoma/ dysgerminoma/ germinoma reprogrammed to nonseminoma/ nondysgerminoma/ nongerminoma	Naïve-state (totipotent)	PGC/gonocyte undergoing global demethylation	Erased	Aneuploid (+/- triploid) gain: X,7,8,12p,21 loss: Y,1p,11,13,18 In mediastinum and midline brain also (near)diploid and (near)tetraploid with gain of 12p	Not available
III	Older men, usually > 55	M	Testis	Spermatocytic tumor	Spermatogonium to premeiotic spermatocyte	Spermatogonium/ spermatocyte	Partially-completely paternal	Gain: 9	Canine seminoma
IV	After puberty	F	Ovary	Dermoid cyst	Maternally imprinted 2C-state	Oogonia/oocyte (gynogenote)	Partially-completely maternal	(Near) diploid diploid/tetraploid peritriploid gain: X,7,12,15	Mouse gynogenote
V	After puberty	F	Placenata/uterus	Molar pregnancy	Paternally imprinted 2C-state	Empty ovum/ spermatozoa (androgenote)	Completely paternal	Diploid (XX and XY)	Mouse androgenote
VI	Older age, usually >60	F/M	Atypical sites for GCT	Resembling type I or nonseminoma components of type II	Primed state or nonseminoma lineages of naïve-state	Somatic cell induced to pluripotency	Imprinting pattern of originating cell	Depending on precursor cell	Xenografts derived from iPSC

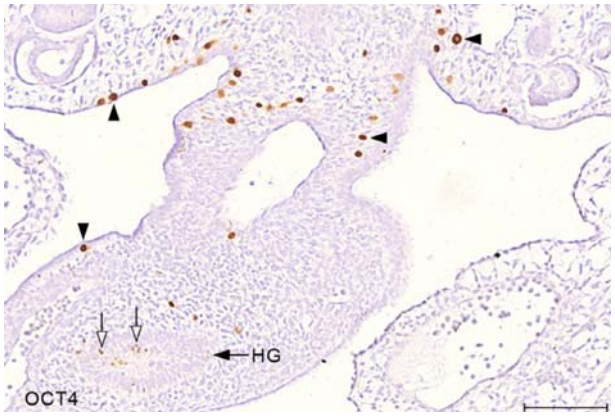

Fig. 2 Transverse section through the hindgut and dorsal mesentery of a human embryo, 4.5 weeks postconception. Remnants of OCT4 reactivity (*open arrows*) are shown in the hindgut epithelium (HG), as well as many migrating, OCT4-positive PGCs (*arrowheads*). Mamsen, L. S., Brochner, C. B., Byskov, A. G., Mollgard, K. (2012). The migration and loss of human primordial germ stem cells from the hind gut epithelium towards the gonadal ridge. *The International Journal of Developmental Biology* **56**, 771–778.

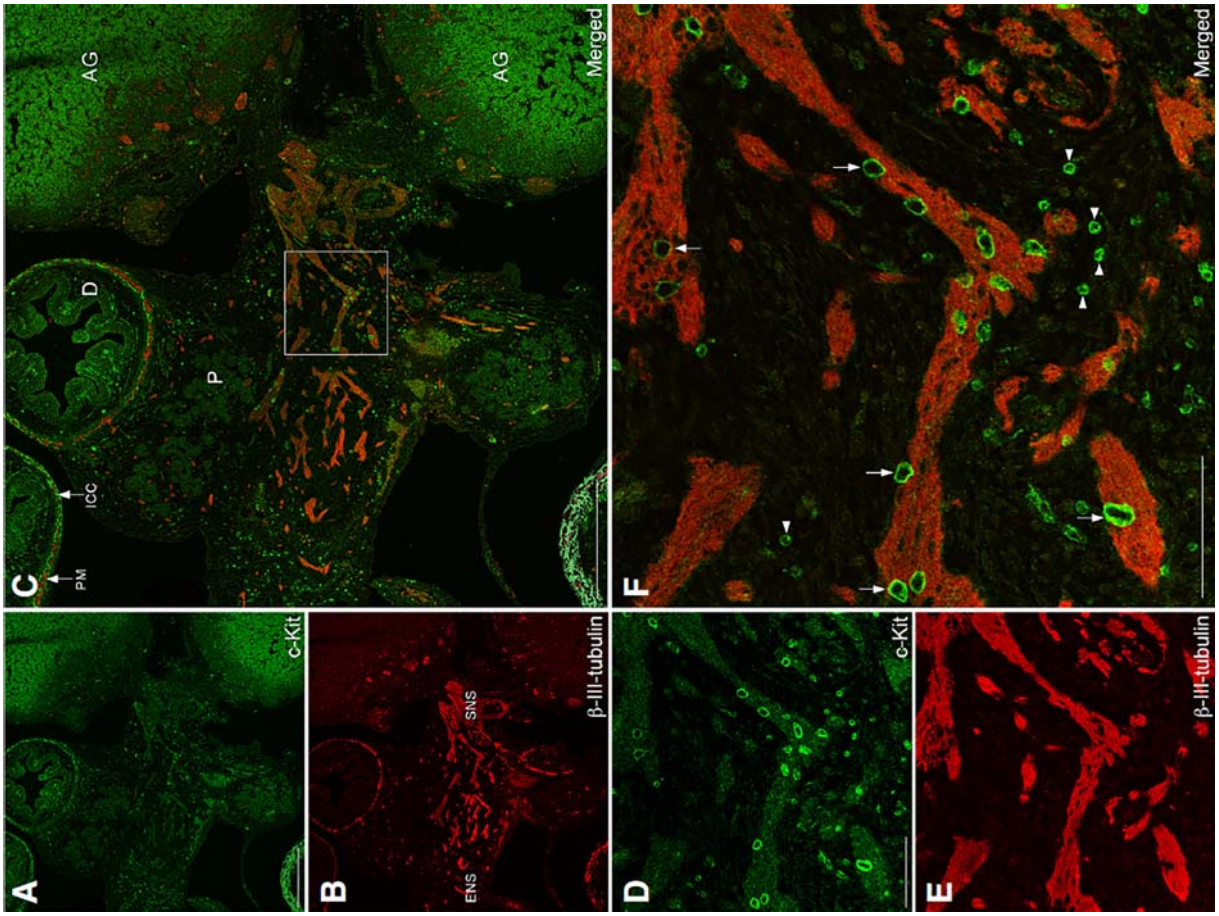

Fig. 3 Horizontal section through abdomen of human embryo, CRL = 30 mm, 7 weeks and 6 days pc, immunofluorescent labeled against c-Kit and b-III-tubulin antibody. Survey depicted (A–C), with commencing connectivity of the enteric (ENS) and sympathetic (SNS) nervous system (B). (C) Some sympathetic nerve fibers are found in the adrenal glands (AG), the pancreas (P), and especially in the dorsal mesentery located in the middle of the section. Positive b-III-tubulin reactivity is seen in nerve fibers of ENS, in general in the plexus myentericus (PM) and similar reactivity is observed in the duodenum (D). Furthermore, c-Kit is also observed in the interstitial cells of Cajal (ICC) of PM. (D) The PGCs in the nerve fibers demonstrate c-Kit reactivity. (E) Higher magnification of (B) demonstrating b-III-tubulin reactivity of SNS. (F) Higher magnification of boxed area in (C). The larger PGCs, with strong membranous c-Kit reactivity are located in close correspondence to the periphery of the individual nerve fibers of the SNS (F, *arrows*). The small, densely labeled c-Kit-positive cells outside of the nerve fibers are mast cells (F, *arrowheads*). Scale bars: (A,B) 500 mm, (C) 200 mm, (D,E) 100 mm, (F) 50 mm. Mamsen, L. S., Brochner, C. B., Byskov, A. G., Mollgard, K. (2012). The migration and loss of human primordial germ stem cells from the hind gut epithelium towards the gonadal ridge. *The International Journal of Developmental Biology* **56**, 771–778.

fate through reprogramming into an embryonic stem cell (ESC) in the primed state, the precursor of type I GCT. Extragonadal type II GCT only occur in the thymus and midline of the brain. This more limited distribution of extragonadal type II GCT compared to type I GCT is probably due to the demethylated, partially erased genome of late stage PGC, which renders them fragile and highly apoptosis-prone. These cells can normally only survive as gonocytes in specific niches in the developing gonads. Presumably, in the thymus and the midline of the brain the conditions of the developing gonads are mimicked to the extent that late stage PGC can occasionally survive there, and give rise to type II GCT.

Pathology and Genetics of GCT Types

Type 0 GCT (Included and Parasitic Twins)

Included and parasitic twins have an estimated incidence of 1/500,000 births. A family history of twinning is a risk factor. Included twins are in 80% localized in the retroperitoneum. External parasitic twins are localized at the same sites where conjoined twins are attached (Fig. 4).

Both are composed of highly organized, mature somatic tissues often with a vertebral axis, limbs and internal organs. Most of the cranial part of the embryo, including the brain, is usually missing. Immature components are rare; progression to yolk sac tumor (YST) is exceptional.

Parasitic twins have the same genetic constitution as the host, consistent with their origin as monozygotic diamniotic twins. Epidemiological data suggest a continuum between twins, conjoined twins, external parasitic twins, acardiacs (external parasitic twins attached via the placenta) internal parasitic twins, and type I teratomas.

The common pathogenesis of these conditions seems proneness of the 2C state, omnipotent blastomeres (Table 1, Fig. 1), to escape control of their developmental potential by the developing embryo. If the resulting twin fails to develop a functional heart it will die. If it succeeds in getting its circulation from the host, it will develop as a parasitic twin or, exceptionally, as a type I teratoma.

Type I GCT (Pediatric GCT; Prepubertal-type GCT)

Epidemiology and risk factors

The incidence of type I GCT is estimated at 1–1.5 per 100,000; more than half are benign teratomas. A family history of twinning is a risk factor, and possibly Down's syndrome.

The age distribution of GCT shows a small neonatal peak (Fig. 5), before the age of six. This represents type I GCT of the sacrococcygeal area/pelvis, retroperitoneum, anterior mediastinum, head and neck (not shown), brain, and testis. Type I GCT of the ovary occur from birth through adulthood, partially overlapping with the age range of type II and IV GCT. Rarely type I GCT occur in older individuals at other sites than the ovary. Type I GCT occur more often in girls than in boys. Multiplicity of type I GCT is rare (Table 1).

Anatomical localization

Type I GCT occur in the testis, ovary, and, outside the gonads, along the midline of the body at sites where mis-migrated PGC have been demonstrated. They occur also in the thymus where PGC have not yet been identified.

Pathology

Extragonadal type I GCT result from accidental reprogramming of mis-migrated, early stage, still methylated PGC that have escaped immediate apoptosis. Reprogramming, due to failure of cell intrinsic factors that check developmental potential (SOX 17, BLIMP1,

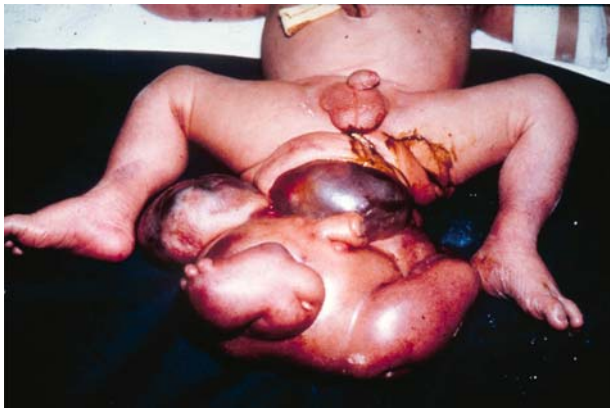

Fig. 4 Neonate with parasitic twin (Type 0 GCT) attached to sacrum; clearly recognizable foot. Oosterhuis, J. W., Stoop, H., Honecker, F., Looijenga, L. H. (2007). Why human extragonadal germ cell tumours occur in the midline of the body: Old concepts, new perspectives. *International Journal of Andrology* **30**, 256–263.

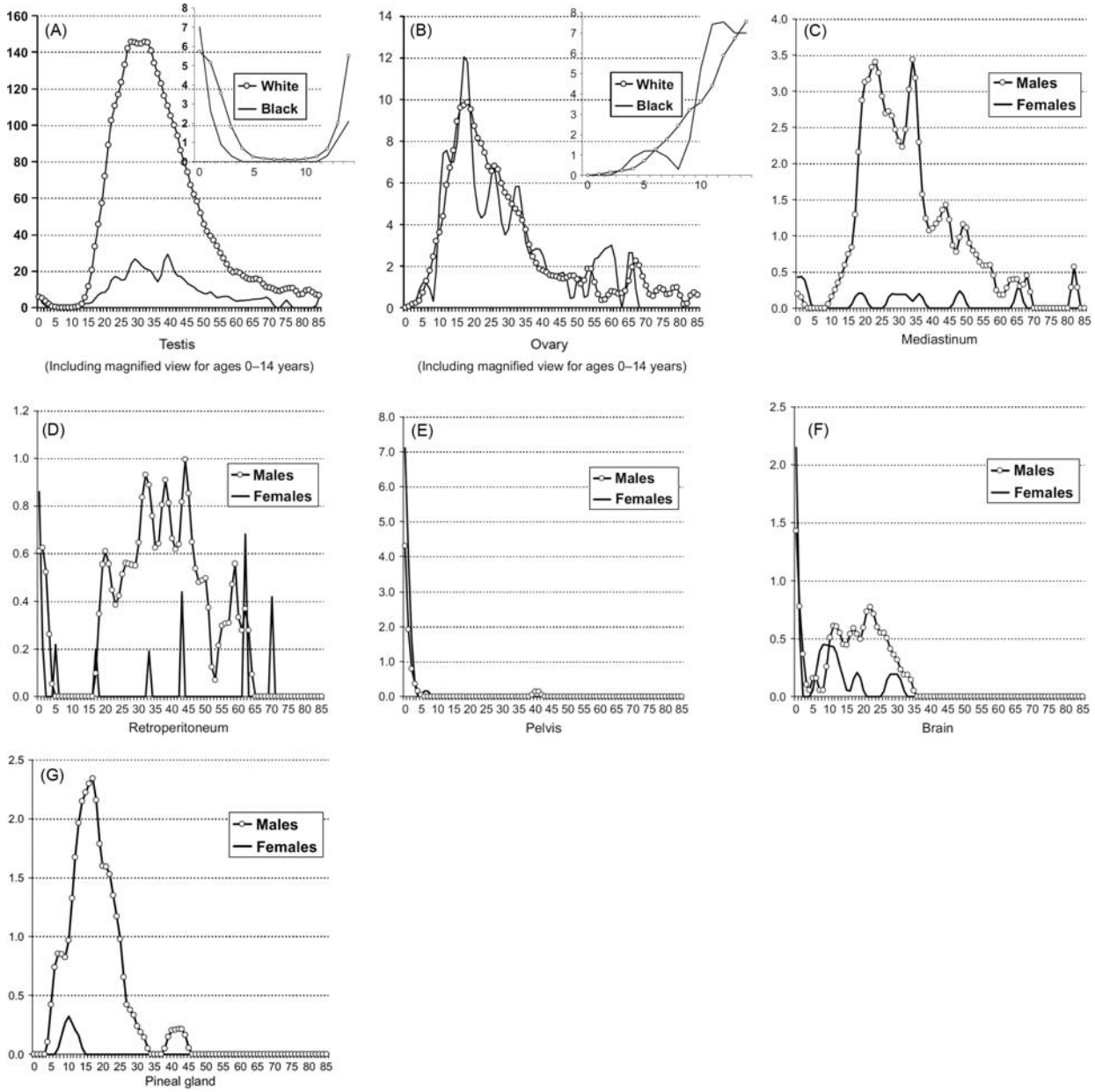

Fig. 5 Age-specific incidence rates of gonadal germ cell tumors in the United States by race, US SEER-9, 1973–2007 (Cases per 1 million) for testis (A); ovary (B); mediastinum (C); retroperitoneum (D); pelvis (E); brain (F); pineal gland (G). Note the early peak before age 6 in testis, mediastinum, retroperitoneum, pelvis, and brain representing type I GCT. This peak is missing in the ovary due the broad age range of ovarian type I GCT. In the pineal gland type I GCT do not occur, only type II GCT. The large majority of extragonadal type II GCT occur in males. Stang, A., Trabert, B., Wentzensen, N., Cook, M. B., Rusner, C., Oosterhuis, J. W., McGlynn, K. A. (2012). Gonadal and extragonadal germ cell tumours in the United States, 1973–2007. *International Journal of Andrology* **35**, 616–625.

and OCT4), results in apoptosis resistant ESC in the primed state. In the testis and ovary preerased gonocytes are reprogrammed to ESC in the primed state due to failure of control of developmental potential by germ cell-intrinsic-and niche factors, such as GDNF. This developmental origin of type I GCT explains the rarity of driver mutations in these tumors.

Most type I GCT consist of immature and/or mature teratoma, in accordance with the developmental potential of ESC in the primed state. OCT4-positive stem cells, which are negative for other pluripotency markers and CD30, are occasionally found in the immature teratoma component. Small foci of YST, often only detectable with the use of immunohistochemical markers such as AFP and glypican 3, may also be present, associated with immature teratoma. The YST component will eventually outgrow and obscure the teratoma component, resulting in pure YST. Histologically, YST can be composed of intraembryonic (primitive gut and liver) and extraembryonic structures (allantois and yolk sac). Trophoblastic extraembryonic differentiation rarely occurs (Fig. 6).

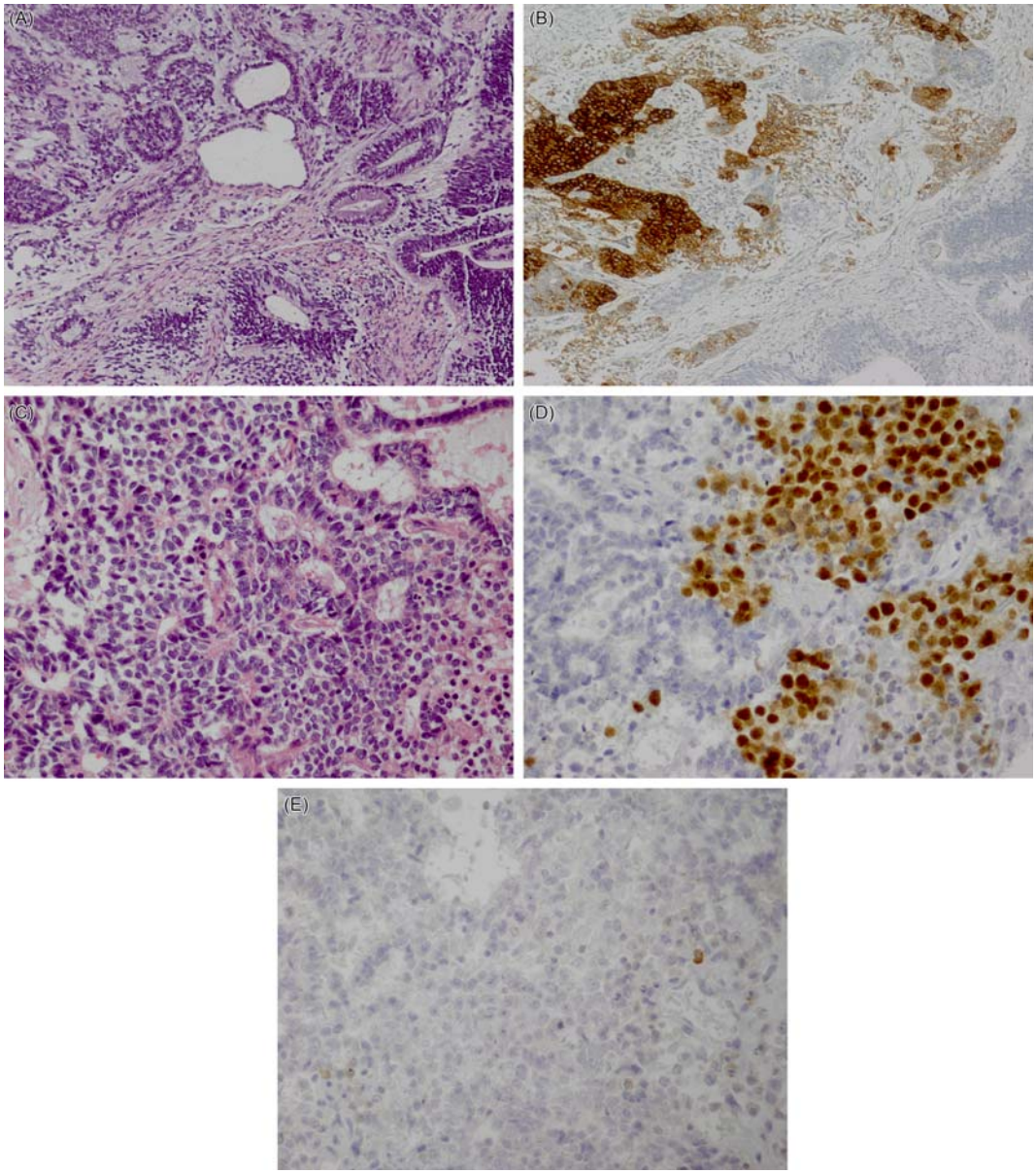

Fig. 6 Histology of testicular type I GCT: immature teratoma (A, H and E, ×200); appears to contain foci of YST on staining for glypican 3 (B, glypican 3 *brown*, ×200); area of pluripotent stem cells (C, H, and E, ×200); with nuclei staining positive for OCT4 (D, OCT4 *brown*, ×200); but, apart from one lymphocyte, negative for CD30 (E, no membranous brown staining, CD30 *brown*, ×200).

Apart from high-grade immature teratoma of the ovary, type I immature and mature teratoma is benign. A YST-component, overall recognized in 5%–10% of the cases at birth, and clinically detectable by increased levels of serum AFP, is the only predictor of malignant behavior at any site. When not, or incompletely removed, type I teratoma has a high risk of progressing to YST.

(Cyto)genetics and epigenetics

A genome wide association study (GWAS) suggests that a single nucleotide polymorphism (SNP) variant in *BAK1*, a gene involved in suppression of apoptosis, might be associated with type I GCT.

Type I immature and mature teratoma is diploid, lacking chromosomal rearrangements. YST of type I, pure or combined with teratoma, is usually near-diploid and may have gains of 1q, 3, 3p, 8q24, 12p13, 20q, 22, and X, and losses of 1p (1p36), 4, 4q, 6q (6q24-qter), 16q, and 20p (Fig. 7). Overrepresentation of the whole of 12p or the region 12p11.2–p12.1, characteristic for type II GCT, is not a feature of type I GCT. Possibly involved genes in the affected chromosomes are *MYC* (8q24) and the pluripotency genes *STELLAR*, *NANOG*, and *GDF3* (12p13). Some of the changes, such as gain of 1q and loss of 1p and 6q, are shared by type II YST and may be related to the phenomenon of progression toward YST rather than being specific for type I GCT.

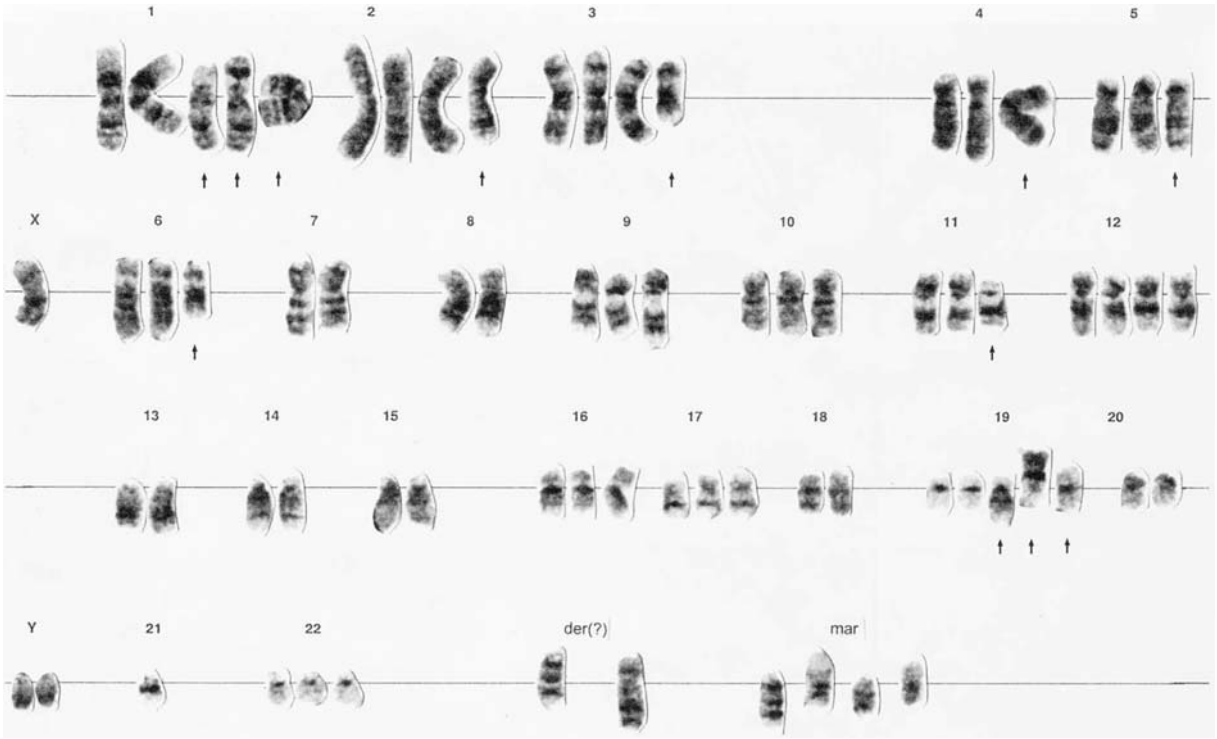

Fig. 7 Karyotype of testicular type I YST, note aneuploidy with characteristic gain of 1q and loss of 6q, and absence of overrepresentation of 12p.

The Wnt/beta-catenin and TGFbeta/BMP signaling pathways are strongly expressed in type I and II YST. Methylation of APC and LOH at 5q21-22 suggests that APC might be involved in the activation of the Wnt pathway.

Type I GCT by anatomical site

- Sacral region
  The sacrococcygeal region is the most common site of type I GCT. The male-to-female ratio is 1:3.5. With a frequency of 1/35,000 live births they constitute about a third of all type I GCT. It is the most frequent neonatal tumor, rarely diagnosed beyond the age of 2 years; exceptionally they remain undetected until adult age, and are diagnosed as a type I GCT beyond infancy. The continuum between type 0 and type I GCT makes the distinction sometimes difficult and arbitrary.
- Other extragonadal sites
  Other localizations of type I GCT are: retroperitoneum (10%); stomach (2%–3%); mediastinum/thymus (2%–3%); heart (5%); head and neck (15%); brain (10%). Females are more often affected than males.
- Testis and ovary
  Ten percent of type I GCT occur in the testis (Fig. 8). Type I GCT of the testis are not associated with germ cell neoplasia in situ (GCNIS) and testicular dysgenesis syndrome (TDS), and do not share the risk factors of testicular type II GCT nor their increasing incidence.
  About 20% of all type I GCT are localized in the ovary, the second most frequent site, with a broad age range from neonates to adults.

Type I GCT beyond infancy

At sites other than the ovary, type I GCT are exceedingly rare beyond the age of 6. However, recently so-called prepubertal type-teratomas have been described in the testis after puberty. It is clinically important to distinguish these type I teratomas from type II teratoma, which is malignant, notwithstanding its deceptively bland histology.

Remarkably, these prepubertal type teratomas of the postpubertal testis may grossly be partly solid, partly cystic, and even present as dermoid cysts, sometimes containing hair, like type IV GCT, the dermoid cysts of the postpubertal ovary. Similar partly cystic teratomas may also occur beyond infancy at extragonadal sites, like the mediastinum and brain. Particularly in the mediastinum, mature teratomas may have the gross and microscopic appearance of a dermoid cyst, containing hair and even tooth structures (Fig. 9).

Thus, type I teratomas beyond infancy may show phenotypes intermediate between typical type I and type IV GCT. In the ovary, typical type I teratomas may occur side by side with type IV teratomas, both uni- and bilaterally. The explanation may be that PGC at

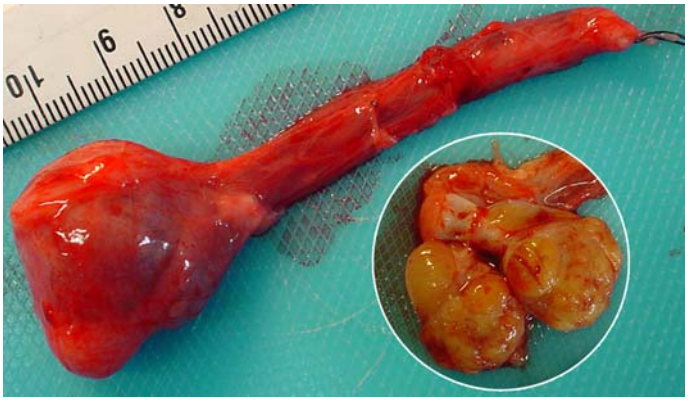

Fig. 8 Gross image of surgically removed testis of infant. On cross section friable yellowish tissue of type I YST; left most nodule is remnant of testis.

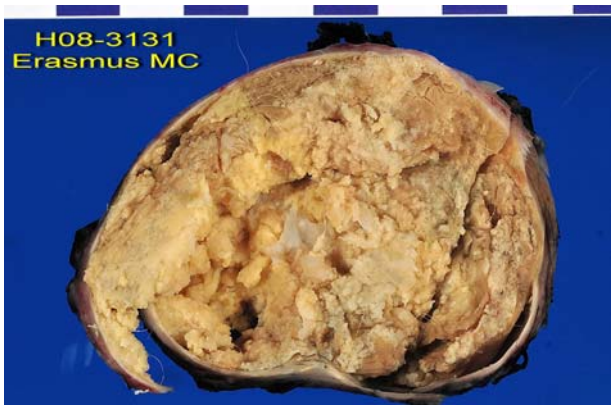

Fig. 9 Surgical specimen of mediastinal type I GCT beyond infancy in a 48 year old women showing a cystic tumor containing sebaceous material and hairs, resembling a type IV GCT (dermoid cyst) of the ovary. Probably an intermediate phenotype between a type I and a type IV GCT. Oosterhuis, J. W. and Looijenga, L. H. J. (2017). Germ cell tumors from a developmental perspective: Cells of origin, pathogenesis and molecular biology; Emerging patterns. In: Nogales, F. F. and Jimenez, R. E. (eds.), *Pathology and Biology of Human Germ Cell Tumors*, Berlin: Springer Nature, pp. 23–129.

all sites, except in the seminiferous tubules, both in females and males enter meiotic prophase like in the ovary. These cells resemble primary oocytes (the precursors of type IV GCT), perhaps with a genotype in between that of gonocyte/oogonium and primary oocyte, with partial erasure of GI and some degree of maternal imprinting. Such cells may give rise to teratomas beyond infancy with a phenotype intermediate between type I and type IV GCT.

Type II GCT (Seminoma and Nonseminoma)

Epidemiology

Worldwide the average incidence is 1.5/100,000 with a 20-fold difference between areas with the lowest and highest incidence. Over 90% of type II GCT occur in the testis, the most frequent cancer of males aged 25–45 in Western white Caucasian populations. The remainder develop in dysgenetic gonads/ovary (about 4%) and in the extragonadal sites: anterior mediastinum/thymus and brain midline/pineal gland (about 3%).

Type II GCT occur in patients in whom puberty has started or is completed, except for rare cases associated with disorders of sex development (DSD), Down's syndrome, and Klinefelter's syndrome. The peak age of type II GCT is about 30 for of the testis, 25 for ovary and mediastinum, and 15 for the brain.

At each of the extragonadal sites, males greatly outnumber females. Apparently, type II GCT is very much a disease of adolescent and adult males, probably related to the presence of the TSPY gene on the Y chromosome (Table 1).

Testicular and ovarian type II GCT are bilateral, respectively, in 3%–5% and over 6%. Rarely, gonadal tumors may be combined with extragonadal type II GCT in the same individual.

Type II GCT of the testis have a strong familial component: over 5% of patients with a testicular type II GCT have a relative with a similar tumor; an estimated 25% of testicular cases is due to familial susceptibility. Although much rarer, familial clustering is also documented for ovarian tumors, and testicular- may cluster with ovarian type II GCT. The only difference between sporadic and

familial cases is a younger age of clinical manifestation: 2–3 years for testicular tumors and about 7 years for ovarian cases. Gonadal type II GCT may cluster in families with I GCT, and very rarely with type III GCT (spermatocytic tumor).

Anatomical localization

Type II GCT occur at sites that provide niches for late stage, demethylated PGC/gonocytes: dysgenetic gonads, testis and ovary. Outside the gonads they occur only in the thymus and the midline of the brain, which offer surrogate niches for late stage PGC. So-called retroperitoneal extragonadal type II GCT are not primary tumors, but metastases from unrecognized primary testicular type II GCT.

Pathology

Type II GCT are derived from late stage, demethylated PGC/gonocytes. They can be transformed into seminoma cells in two different ways. Firstly, and most frequently, the "developmental pathway" involving TSPY in the GBY region on Y in concert with OCT4. Secondly, the much rarer "somatic mutation pathway," usually in the absence of TSPY.

Identical tumors are traditionally named seminoma and nonseminoma in the testis and thymus; dysgerminoma and nondysgerminoma in the ovary and dysgenetic gonad; germinoma and nongerminoma in the midline of the brain (Table 1). From here on we will only use the terms seminoma and nonseminoma.

At all anatomical sites, over half of all primary type II GCT are pure seminomas. The younger the patient population, the higher the proportion of seminoma (in dysgenetic gonads and the brain about 80%, in the ovary 60%, in the mediastinum 55%, and in the testis about 50%), suggesting that reprogramming to nonseminoma is a chance event accumulating over time. Reprogramming occurs when due to chromosomal imbalances NODAL- prevails over BMP-signaling (with downregulation of BLIMP1, SOX17, OCT4), whereby niche factors (such as GDNF in the testis) fail to check the potential omnipotency seminoma cells. In invasive seminoma stromal NOGGIN may start reprogamming by inhibiting BMP in the tumor cells.

Seminomas are by definition pure tumors of neoplastic late stage PGC/gonocytes. The only other cells are scattered trophoblastic cells occurring in less than 10% of cases. Reactive host lymphocytes are a consistent histological feature (Fig. 10).

Nonseminomas contain combinations of embryonal carcinoma (EC), YST, choriocarcinoma, immature, and mature teratoma with or without a seminoma component. Early germ cell differentiation is occasionally encountered. The proportions vary by primary site (Fig. 11).

The more aggressive biology of nonseminoma explains its earlier clinical manifestation than seminoma, at all sites. Somatic tissues in nonseminomas may progress to form somatic-type malignancies that closely resemble their somatic counterparts (Fig. 12).

Seminomas and nonseminomas may metastasize to regional lymph nodes and from then on to lungs, liver, brain, and bone, so-called visceral metastasis. Choriocarcinoma has a propensity for blood–borne metastases, sometimes the first clinical manifestation of the tumor. In up to 44% of cases, seminoma cells at metastatic sites are reprogrammed to nonseminoma. In nonseminoma, EC cells are the main metastatic cell type. This is microscopically apparent as tumor emboli are virtually always composed of EC cells only (Fig. 13). At the site where these tumor stem cells eventually lodge, they may resume differentiation, mimicking the histology of the primary tumor. The level of differentiation at the metastatic site is in general less than in the primary tumor.

(Cyto)genetics and epigenetics

Testicular type II GCT are virtually always peritriploid (GCNIS and seminoma hypertriploid, and nonseminoma hypotriploid), whereas in the ovary, mediastinum, and brain, type II GCT may be (near)diploid or (near)tetraploid.

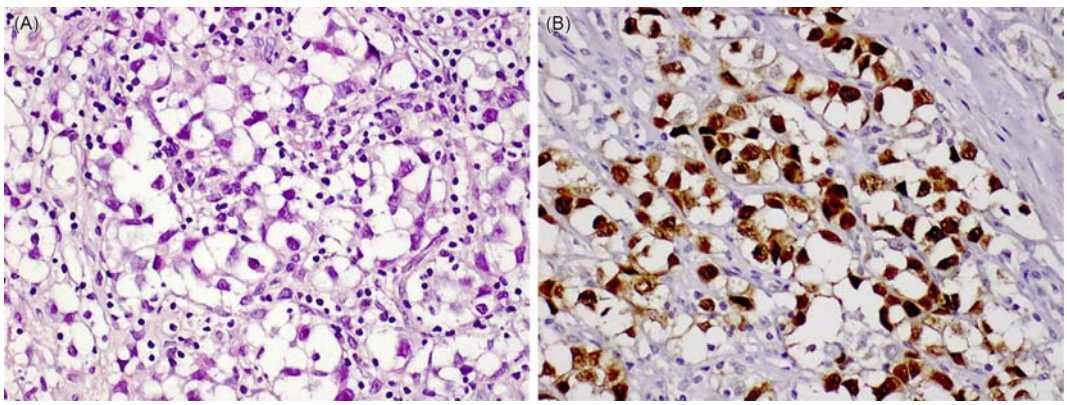

Fig. 10 Histology of seminoma, a tumor composed of transformed gonocytes, large pale cells with angulated nuclei, eliciting a host response, apparent from the many lymphocytes (dark small nuclei) in between the tumor cells (A: H and E, ×200). The seminoma cells show a strictly nuclear staining for OCT4 (B, OCT4 *brown*, ×200).

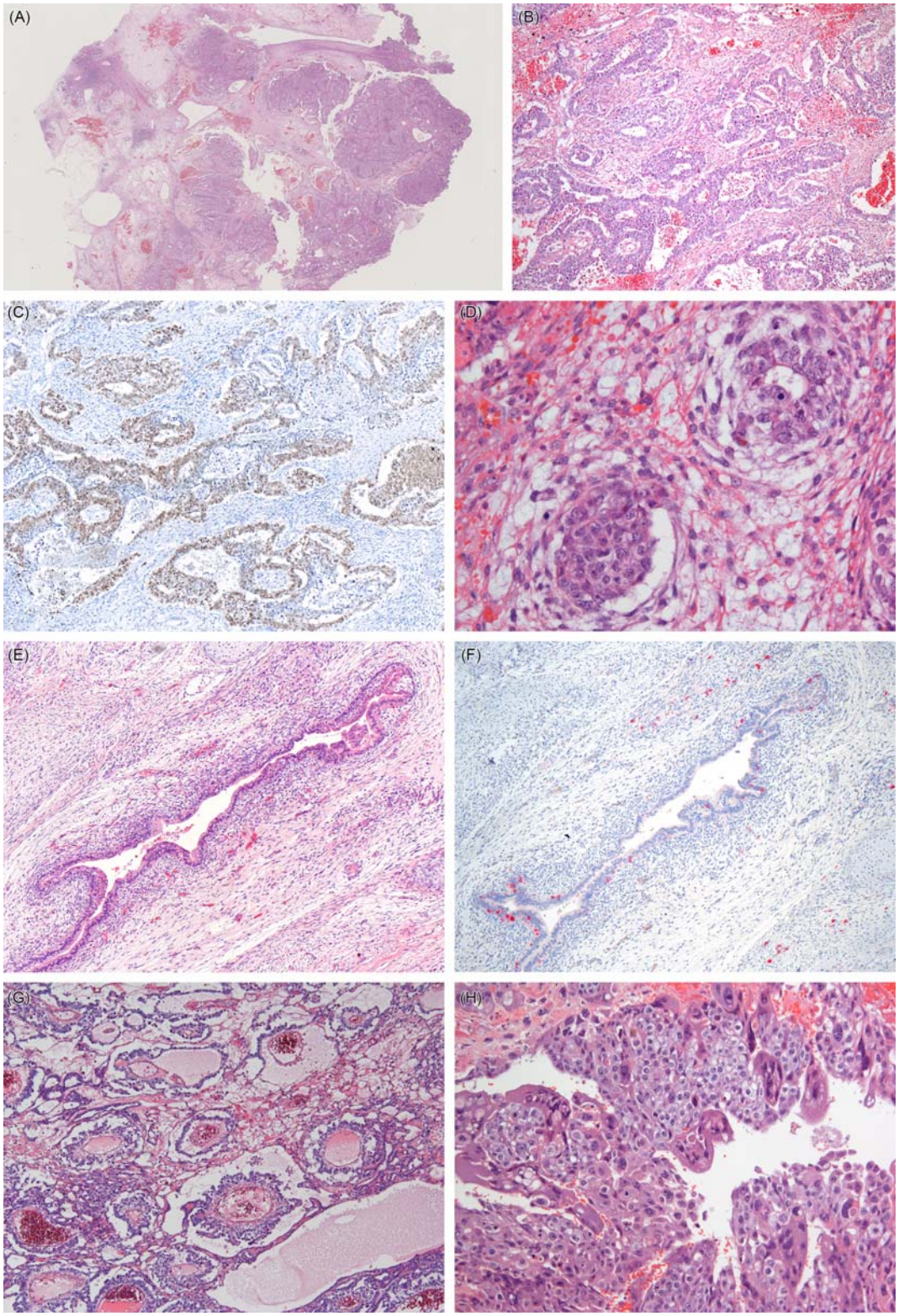

Fig. 11 Histology of nonseminoma with a variegated appearance on low power; the dark areas represent EC, the lighter areas mainly consist of YST, the *red* areas are bleedings caused by trophoblastic giant cells (A: H and E, ×12.5); EC cells arranged in strands and tubular structures (B: H and E, ×50); these totipotent stem cells express OCT4 in the nucleus and the cytoplasm (C: OCT4 *brown*, ×50); EC cells may form embryoid bodies, representing early embryos (D: H and E, ×200); gut-like structure in mature teratoma (E: H and E, ×50); upon staining for TSPY, an early marker of germ cell differentiation, it appears that there are germ cell present in the gut epithelium and the surrounding stromal tissue (F: TSPY *red*, ×50); YST with characteristic Schiller–Duval bodies, concentric structures centered around blood vessels, representing yolk sac elements (G: H and E, ×40); choriocarcinoma, in fact, malignant placenta-like tissue with large multinucleated syncytiotrophoblastic giant cells alternating with mononuclear cytotrophobastic cells (H: H and E, ×100).

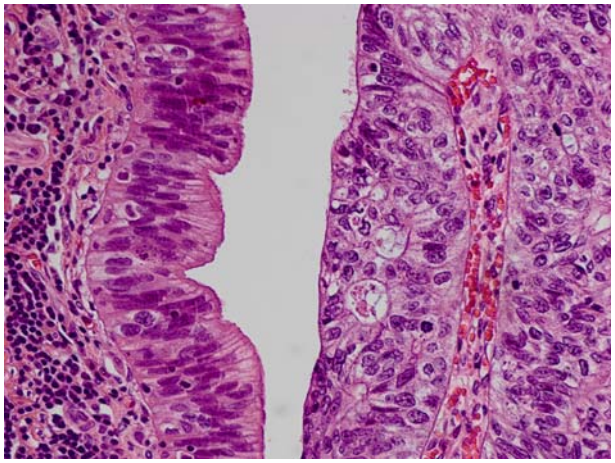

Fig. 12 Teratoma showing progression to somatic-type malignancy; to the left glandular tissue lacking features of malignancy, and to the right glandular tissue with malignant features, to be classified as adenocarcinoma (H and E, ×200). Oosterhuis, J. W., Peeters, S. H., Smit, V. T., Stoop, H., Looijenga, L. H., Elzevier, H. W., Osanto, S. (2013). Patient with two secondary somatic-type malignancies in a late recurrence of a testicular non-seminoma: Illustration of potential and flaw of the cancer stem cell therapy concept. *The International Journal of Developmental Biology* **57**, 153–157.

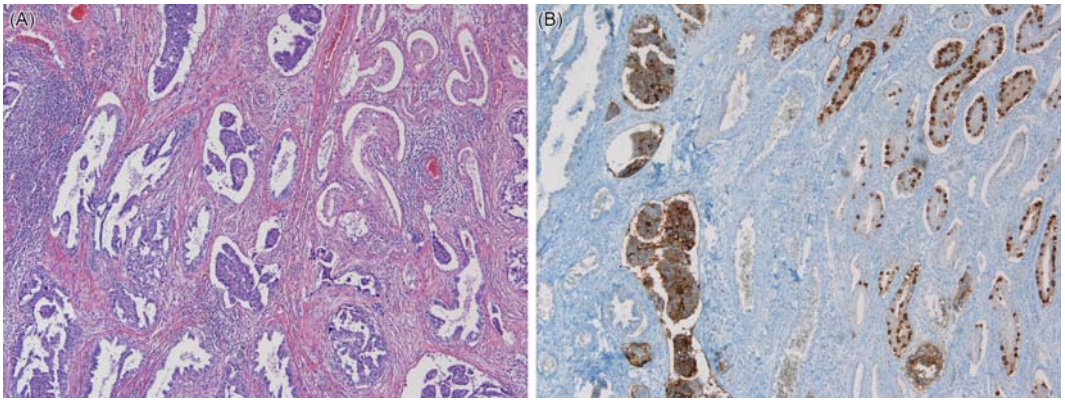

Fig. 13 EC cells, the stem cells of nonseminoma, are the cells invading lymphatics and blood vessels; shown here are numerous tumor emboli in vessels (A: H and E, ×40); similar area stained for D2–40 which demonstrates that the vessels are lymphatics, and the tumor cells within the vessels are EC cells; the right half of the panel shows GCNIS cells with membranous staining for D2–40 within seminiferous tubules (B: D2–40 *brown*, ×40).

At all anatomical sites type II GCT are characterized by gain of (parts of) the short arm of chromosome 12, most often in the form of an isochromosome of 12p (i(12p)) (Fig. 14). In the testis, it occurs in almost 100% of cases, in the ovary/dysgenetic gonad in about 75%, in the mediastinum in close to 90%, and in the midline of the brain in 60%. Especially in invasive components the more proximal parts of 12p, in particular 12p11.2-p12.1, may be amplified. The proportion of tumors with 12p gain increases with the age of clinical presentation. The very high rate of 12p gain and the peritriploidy in testicular tumors is probably due to the long period of intratubular karyotype evolution.

Isochromosome 12p is of uniparental origin, arising from an erroneous centromeric division during mitotic anaphase preceded by tetraploidization. Among the genes involved in the 12p aberrations are *NANOG*, *STELLAR*, *GDF3*, and *EDR1* for maintaining pluripotency; *cyclin D2* and *KRAS* providing proliferative advantage; genes involved in glucose or glycolytic metabolism, including *GLUT3*, *GAPDH*, and *TPI1* for energy metabolism in a low-oxygen environment; and genes involved in suppression of apoptosis such as *EKI1*, *SOX5*, and *DAD-R*. Expression of these genes maintains the PGC/gonocyte-phenotype of the tumor cells and allows them to survive and proliferate in the proper niches.

In addition to 12p gain the tumors have numeric changes involving other chromosomes: gain of chromosomes X, 7, 8, 12, and 21 and loss of chromosomes Y, 1p, 11, 13, and 18. The pattern is explained by early tetraploidization of the tumor cells, followed by nonrandom losses and gains of (parts of) chromosomes. The large stretches involved, often entire chromosome arms, suggest that aberrant meiotic division plays a role in the evolution of these chromosomal aberrations. The resulting polyploid genome, combined with hypomethylation, renders Type II GCT chromosomally instable. Chromosomal instability likely drives tumor progression of type II GCT.

Mutations and amplifications of oncogenes are rare in type II GCT, which is at 0.5 mutations per Mb lower than in any other solid cancer of adults. *KIT* is most frequently mutated and mainly involved in seminoma: of the testis in about 30%; of the ovary in

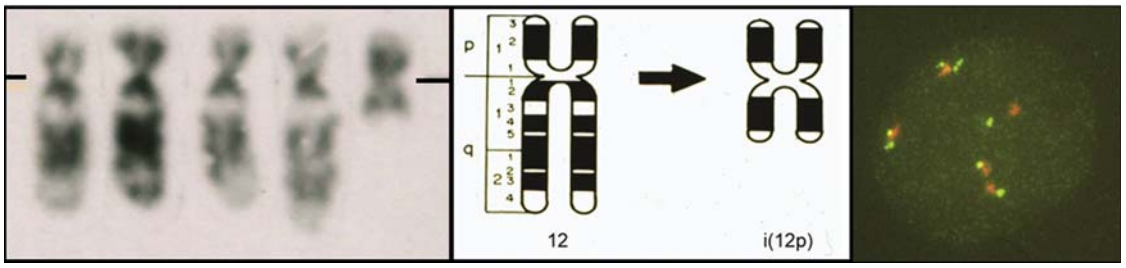

Fig. 14 Left panel, partial karyotype showing four copies of chromosome 12 and one isochromosome 12p; middle panel, cartoon of chromosome 12 and isochromosome 12p; right panel, in situ hybridization of type II GCT cell with three copies of chromosome 12, and two isochromosomes 12p (*red dots*, centromeres of chromosome 12; *green dots*, marker on 12p). Ulbright, T. M., Amin, M. B., Balzer, B., Berney, D. M., Epstein, J. I., Guo, C., et al. (2016). Germ cell tumours. In: Moch, H., Humphrey, P. A., Ulbright, T. M., Reuter, V. E. (eds), *WHO classification of tumors of the urinary system and male genital organs*, 4th edn. Lyon: IARC Press, p. 189–226.

up to 50%; of the mediastinum in 40%; and of the brain in over 50%. In nonseminomas, KIT mutations are rare, less than 1.5%; a similar low figure has been reported in gonadoblastoma and derived seminomas. *KIT* mutations are activating and occur predominantly in the activation loop (exon 17, usually in codons 816, 820, 822, 823, and 825) of the second TK domain.

In seminomas mutations of *KIT* appear to be only one of the mechanisms of activation of KIT signaling and its downstream pathways: KRAS/RAF/MEK/ERK and AKT/mTOR. In these tumors, KIT signaling is always activated either by upregulation of expression or genetically by mutation or amplification (**Figs. 15–17**).

The fact that *KIT* mutations are predominantly found in seminomas and only rarely in nonseminomas suggests that they are in general involved in the progression of seminomas, rather than in initiation of type II GCT. Activating *KRAS* mutations occur in some type II GCT, more or less at the same rate in seminomas and nonseminomas. *KIT* and *KRAS* mutations are mutually exclusive in type II GCT.

Due to early tetraploidization, loss of heterozygosity is an unlikely event in type II GCT. In addition, inactivation of tumor suppressor genes by promoter hyper-methylation is rare. Therefore, tumor suppressor genes probably play a minor role in initiation and progression of type II GCT. In somatic-type malignancies the same mutations may be found as in their somatic counterparts, such as 2q37 rearrangements in rhabdomyosarcoma, p53 mutations in sarcomas, and t(11;22) translocations in PNET.

Type II GCT including GCNIS and gonadoblastoma are globally demethylated, with permissive histone modifications and an open chromatin structure; parental genomic imprinting is erased. In nonseminomas only Alu repeats and some CpG islands are methylated. Mature teratoma components are methylated. The developmentally important miRNAs, miR-371-373 as well as miR-302 and miR-367, which have a crucial role in development of embryonic stem cells, are highly expressed in type II GCT.

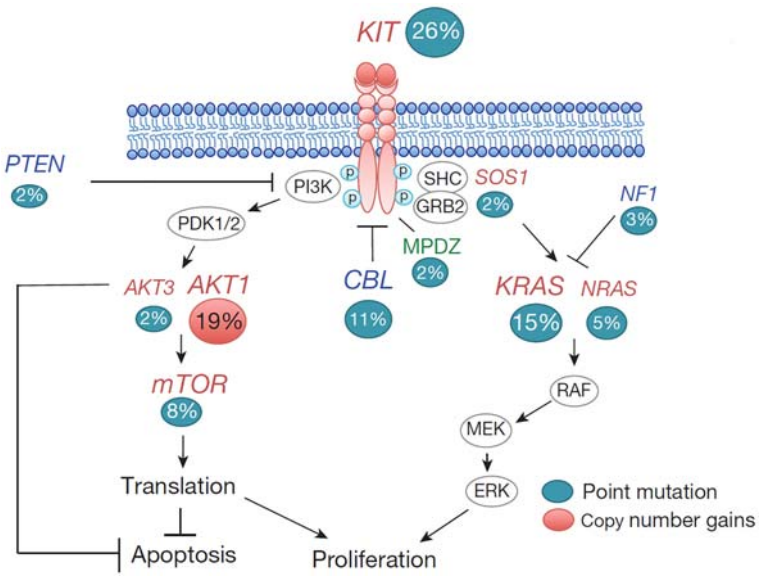

Fig. 15 KIT/RAS and AKT/mTOR pathway interactions showing frequencies of somatic alterations in key genes. Alteration frequencies are expressed as a percentage of all intracranial GCT patients. *Red text*, protein positively regulates signaling; *blue text*, protein negatively regulates signaling; green text, physically interacting protein. Wang, L., Yamaguchi, S., Burstein, M. D., Terashima, K., Chang, K., Ng, H. K. et al. (2014). Novel somatic and germline mutations in intracranial germ cell tumors. *Nature* **511**, 241–245.

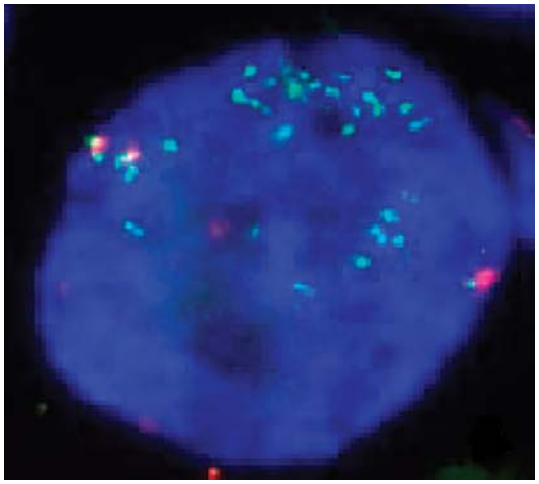

Fig. 16 Nucleus of a seminoma cell case showing multiple copies of KIT (*green*) relative to the centromere of chromosome 4 (*red*). McIntyre, A., Summersgill, B., Grygalewicz, B., Gillis, A. J., Stoop, J., van Gurp, R. J. et al. (2005). Amplification and overexpression of the KIT gene is associated with progression in the seminoma subtype of testicular germ cell tumors of adolescents and adults. *Cancer Research* **65**, 8085–8089.

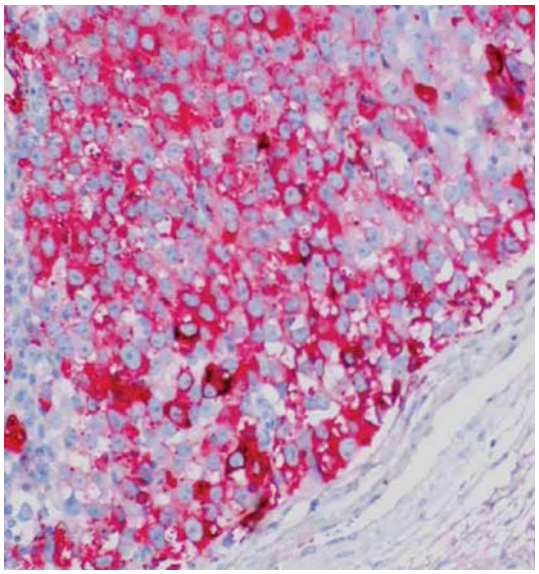

Fig. 17 Seminoma with strong expression of KIT (KIT *red*, ×100).

Treatment sensitivity and resistance

Like their normal counterparts, PGC and ESC, seminoma cells and EC cells have an open chromatin structure, and a hypersensitive DNA damage response. The latter characterized by inducibility of wild-type p53 jointly with the absence of p21-induced cell cycle arrest, whereby cells with damaged DNA are not repaired but eliminated due to a low threshold for apoptosis. This mechanism is a physiologic protection against propagation of repair errors via germ cells into the next generation or via ES cells into the developing embryo. The open chromatin structure in combination with a hypersensitive DNA damage response explains the high sensitivity of type II GCT for DNA damaging agents. As a consequence, Type II GCT are the solid tumors in adults with the highest sensitivity to DNA-damaging agents with cure rates of over 80% in disseminated disease. Nonseminoma lacks the exquisite sensitivity to radiotherapy of seminoma.

The chemotherapy sensitive germ cell/embryonic phenotype gets lost upon somatic differentiation into teratoma, as adult tissue stem cells are able to survive DNA damage by prolonged G1 and G2 arrest and proficient repair. This explains why characteristically the teratoma component survives chemotherapy of a Type II GCT as so-called residual mature teratoma. Primary and acquired resistance is relatively rare in type II GCT because of the low mutation rate in these tumors. In general, loss of germ cell- and embryonic phenotype and acquisition of somatic mutations are factors inducing treatment resistance. A final important mechanism causing resistance of type II GCT is further progression of teratoma- and YST-elements due to accumulation of mutations commonly found in adult cancers.

Type II GCT by anatomical site

Type II GCT of dysgenetic gonads in patients with disorders of sex development (DSD)

Gonadoblastoma is a rare lesion that develops in the dysgenetic gonad of patients with of DSD. At high risk are 46,XY patients with mutations in *WT1* (including Denys–Drash, Fraser, and WAGR syndromes), *SRY*, *SOX9*, *DHH*, *ARX*, *RR5A1*, or *TSPYL1*. These germ line mutations in the presence of Y-chromosomal sequences result in a dysgenetic testis.

- Pathology: Gonadoblastoma is most often caused via the developmental pathway. Mutations in genes involved in male gonadal development in the presence of GBY/TSPY result in a hypovirilized gonad allowing sustained coexpression of OCT4 and TSPY in gonocytes in undifferentiated gonadal tissue (UGT) (Fig. 18), later accompanied by overexpression of KITLG. Initiating somatic mutations, particularly in *KIT* are rare in the presence of GBY/TSPY; probably more frequent in its absence. The usual precursor of type II GCT in dysgenetic gonads is gonadoblastoma (Fig. 19), and much less frequently GCNIS; rarely gonadoblastoma and GCNIS are simultaneously present (Fig. 20). Gonadoblastoma may develop in streak gonads in the abdomen, and in dysgenetic testes in abdominal, inguinal and scrotal position. In 40% of cases gonadoblastoma is bilateral.

Microscopically, gonadoblastoma consists of solid sheets composed of two cell types: nonneoplastic immature granulosa cells, the niche cells, and germ cells, in various arrangements associated with small round depositions of basement membrane-like matrix, called Call-Exner bodies, or psammoma bodies when calcified (Fig. 19). Some of the germ cells resemble oogonia, others, the actual gonadoblastoma cells, have the same atypical morphology of transformed gonocytes as the cells of GCNIS and seminoma, with

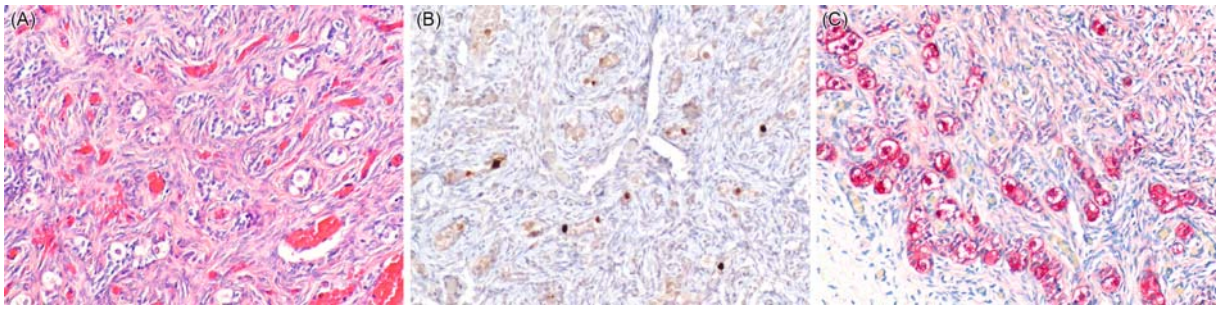

Fig. 18 Undifferentiated gonadal tissue (UGT) composed of strands of small stromal cells resembling granulosa cells, enveloping large pale cells, the germ cells (A: H and E, ×200); some of them, the gonocytes, express OCT4 (B: OCT4 *brown*, ×200); all germ cells express TSPY (C: TSPY *red*, ×200).

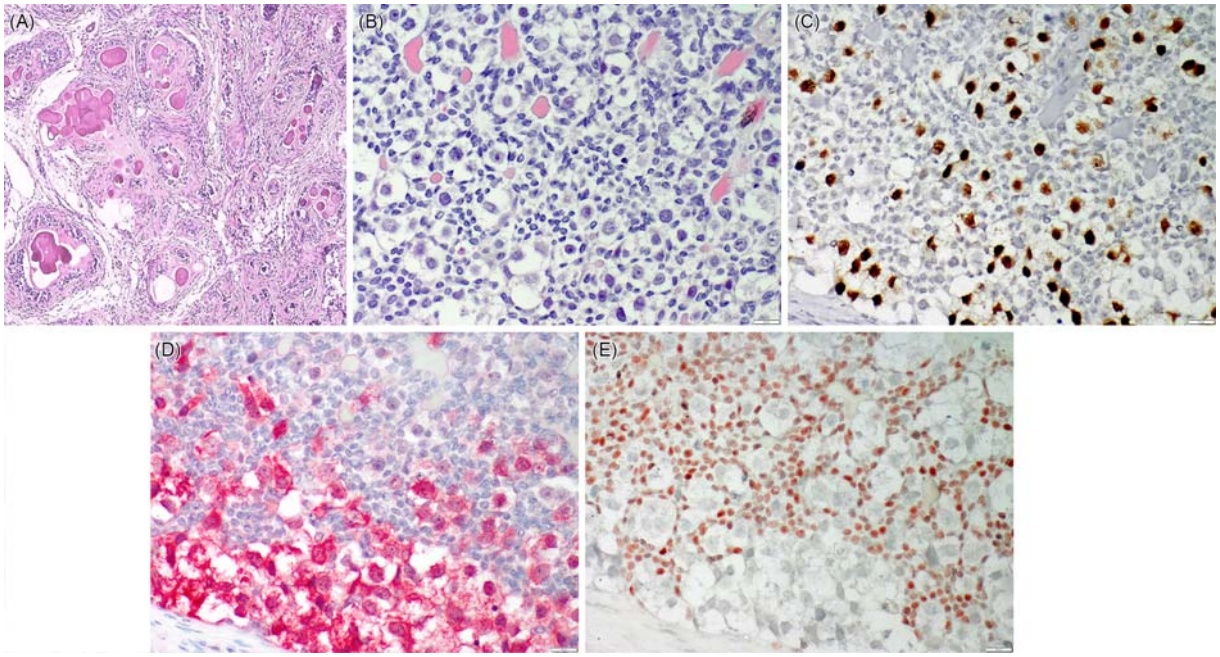

Fig. 19 Gonadoblastoma: areas of immature granulosa cells containing coarse, mulberry-like psammoma bodies and lager pale cells (A: H and E, ×50); Gonadoblastoma: oogonia and gonadoblastoma cells embedded in area of immature granulosa cells, as well as *pink-colored* Call-Exner bodies (B: H and E, ×200); gonadoblastoma cells express OCT4, oogonia do not (C: OCT4 *brown*, ×200); oogonia and gonadoblastoma cells express TSPY (D: TSPY *red*, ×200); the granulosa cells express FOXL2 (E: FOXL2 *reddish brown*, ×200).

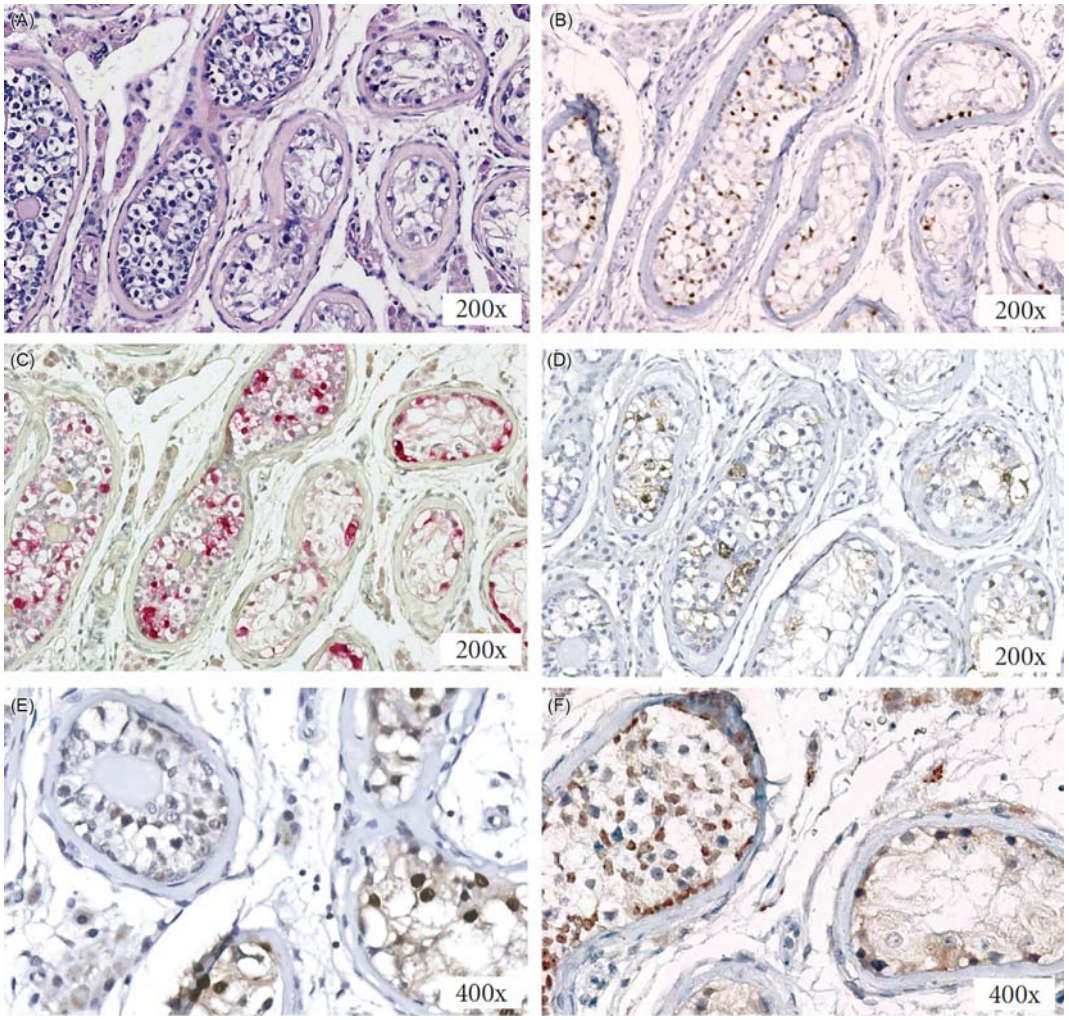

Fig. 20 Gonadoblastoma (left side of each photograph) and GCNIS (right side of each photograph) both within abnormal testicular tubules (A: H and E, ×200); both gonadoblastoma and GCNIS cells have nuclear expression of OCT4 (B: OCT4 *brown*, ×200); both express TSPY (C: TSPY *red*, ×200); the supportive cells express KITLG in gonadoblastoma and GCNIS (D: KITLG *brown*, ×200); Sertoli cells supporting GCNIS express SOX9 (E: SOX9 *brown*, ×400), whereas the granulosa cells supporting gonadoblastoma express FOXL2 (F: FOXL2 *brown*, ×400). Hersmus, R., Stoop, H., White, S. J., Drop, S. L., Oosterhuis, J. W., Incrocci, L., Wolffenbuttel, K. P., Looijenga, L. H. (2012). Delayed recognition of disorders of sex development (DSD): A missed opportunity for early diagnosis of malignant germ cell tumors. *International Journal of Endocrinology* **2012**, 671209.

enlarged angulated nuclei with large nucleoli and abundant clear cytoplasm. Immunohistochemically, the morphologically normal germ cells express early differentiation markers of oogonia, such as TSPY and VASA, and are negative for the pluripotency marker OCT4. The gonadoblastoma cells coexpress markers of gonocytes (e.g., OCT4, PLAP, AP-2gamma, and KIT), and of oogonia (TSPY and VASA). The immature granulosa cells express FOXL2, a key regulatory protein for ovarian development of the undifferentiated gonad (Fig. 19). In dysgenetic testes the tubules are lined by immature Sertoli cells expressing SOX9, the key regulator for testicular development (Fig. 20). Occasionally, tubules in transition zones between testicular and ovarian development may be lined by FOXL2 positive primitive granulosa cells. KITLG is also expressed in gonadoblastoma, probably both in the granulosa and gonadoblastoma cells (Fig. 20).

Gonadoblastoma cells will eventually outgrow the nonneoplastic oogonia, and by way of further progression become an invasive type II GCT: seminoma (80%), and upon reprogramming, nonseminoma (20%). Most mixed nonseminomas contain a seminoma component.

Type II GCT of the testis

- Epidemiology and risk factors
  Africa and parts of Asia have the lowest incidence figures of less than 0.5 for testicular type II GCT. The incidence is a factor 10–20 higher among most white Caucasian populations in Western societies. Worldwide, the incidence has increased in the last decades. In most Western and Northern European countries it has doubled. In the United States the incidence among Caucasians is 6.6 versus 1.2 in blacks. In both groups, the rates have increased in the past 30 years.

The large incidence differences among ethnic groups within the same society demonstrate the importance of genetic factors. However, the worldwide increasing incidence of testicular type II GCT, and the changing incidence among immigrant populations toward the country of destination, point to an important role of environmental factors, associated with Western lifestyle, favoring hypovirilization of the developing male embryo.

The strongest risk factors for testicular type II GCT are cryptorchidism (OR 4.3), previous inguinal hernia (OR 1.63), hypospadias, and impaired spermatogenesis. These conditions are considered part of the so-called testicular dysgenesis syndrome (TDS), a relatively mild disturbance of sex differentiation, due to hypovirilizing factors in utero. Other established risk factors are: previous testicular cancer, and a family history of testicular type II GCT.

- Genetic risk factors

  Familial risk is among the highest in cancers. The small size of affected families, usually a father and a son or two brothers, and the high risk in monozygotic compared to dizygotic twins are consistent with multiple autosomal recessive low-penetrance susceptibility genes. The first identified risk locus was the gr/gr deletion in azoospermia factor c region of Y, and recently a deleterious probably causative germline mutation in *PDE11A* was discovered in familial and sporadic cases. Both mutations explain only a few percent of the familial cases.

  GWAS have discovered variants in over 30 tumor-biologically plausible genes, which increase susceptibility for testicular type II GCT. The ones with the highest OR are *KITLG*, *BAK1*, *SPRY4* and *PDE11A*, which are involved in KIT/KITLG signaling, and thus in survival and proliferation of PGC/gonocytes. *DMRT1* is a niche factor involved in sex determination and regulation of meiotic division. These variants explain still only 20% of the excess familial risk compared to testicular type II GCT families. Obviously more low penetrance genes are involved, like variants in *TGFBR3* and *BMP7* and *AR*, which are associated with TDS. The different distribution of variants, such as in *KITLG* and *AR*, in Caucasian and black populations may partly explain the 20-fold ethnic difference in the incidence of testicular type II GCT.

  Homozygosity jointly for variants that target the PGC/gonocyte and those targeting niche cells substantially increase risk. For example, men with testicular type II GCT have a 14 times higher chance than controls to be homozygous for the two risk alleles, *KITLG* and *DMRT1*.

- Pathology

  The developmental pathway, due to hypovirilization of the niche of the gonocytes in the developing testis is the most important mechanism of initiation of type II GCT. The disturbed niche interferes with downregulation of OCT4 in gonocytes relocated from the center of the tubules to the prespermatogonial niche, thereby creating a window for coexpression of OCT4 and TSPY. In due time accompanied by overexpression of KITLG, which enhances proliferation of the maturation-disturbed prespermatogonia, and thereby promotes neoplastic transformation.

  The earliest changes, preceding overt GCNIS, are maturation delay, and pre-GCNIS, (**Figs. 21 and 22**) which have been studied in cryptorchid testes and androgen insensitivity syndromes.

  GCNIS is the common precursor of seminoma and nonseminoma of the testis (**Fig. 23**); it is bilateral in 3%–5% of the patients. This high rate of bilaterality is probably because the germ cell niche is disturbed in both testes as a consequence of TDS. Noteworthy, in DSD, where the disturbance of the niche is more severe, bilaterality of gonadoblastoma may occur in up to 40%. Supposedly GCNIS when left untreated, will always progress to an invasive type II GCT. By default GCNIS develops into intratubular seminoma (**Fig. 24**), whereby the lumen of the tubule is packed with GCNIS cells, often accompanied by lymphocytes, like in seminoma. Remarkably, necrosis is rare in intratubular seminoma. Apparently the tumor cells, like gonocytes, are well adapted to the low oxygen environment within the tubules. In general, intratubular seminoma becomes invasive by overextension and destruction of the seminiferous tubules.

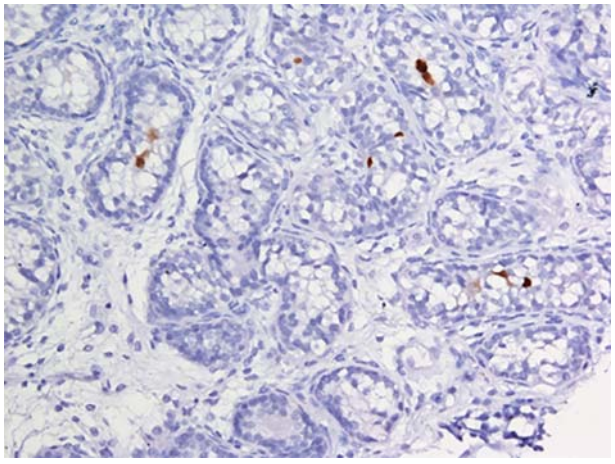

Fig. 21 Maturation delay: gonocytes located centrally in the tubules still express OCT4, although the infant is older than 6 months (OCT4 *brown*, ×200).

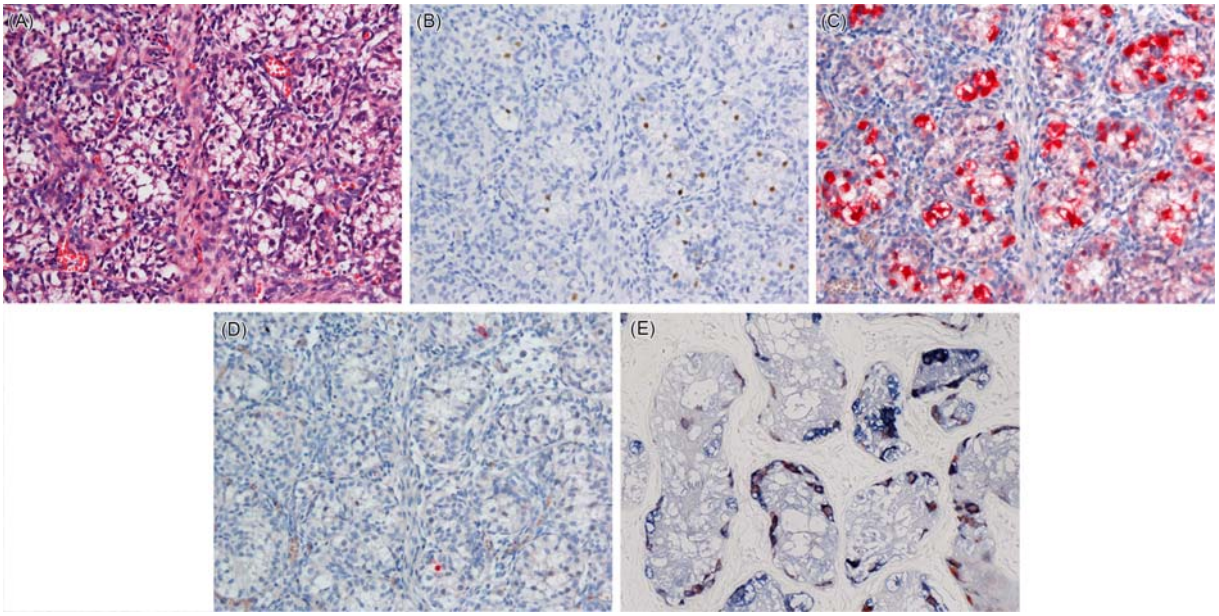

Fig. 22 Pre-GCNIS is characterized by coexpression of OCT4 and TSPY by germ cells located in the prespermatogonial niches and focal expression of KITLG. Immature tubules of a young boy contain gonocytes (centrally in the tubules) and prespermatogonia (peripherally in the tubules) (A: H and E, ×200); OCT4 is still expressed in the germ cells, not only centrally, but also in the periphery of tubules by germ cells in the prespermatogonial niches (B: OCT4 *brown*, ×200); all germ cells express TSPY (C: TSPY *red*, ×200); focal expression of KITLG (D: KITLG *red*, ×200). Double staining for OCT4 (*brown*) and TSPY (*blue*) demonstrates germ cells in the spermatogonial niches with coexpression of OCT4 and TSPY, consistent with pre-GCNIS (E, ×200).

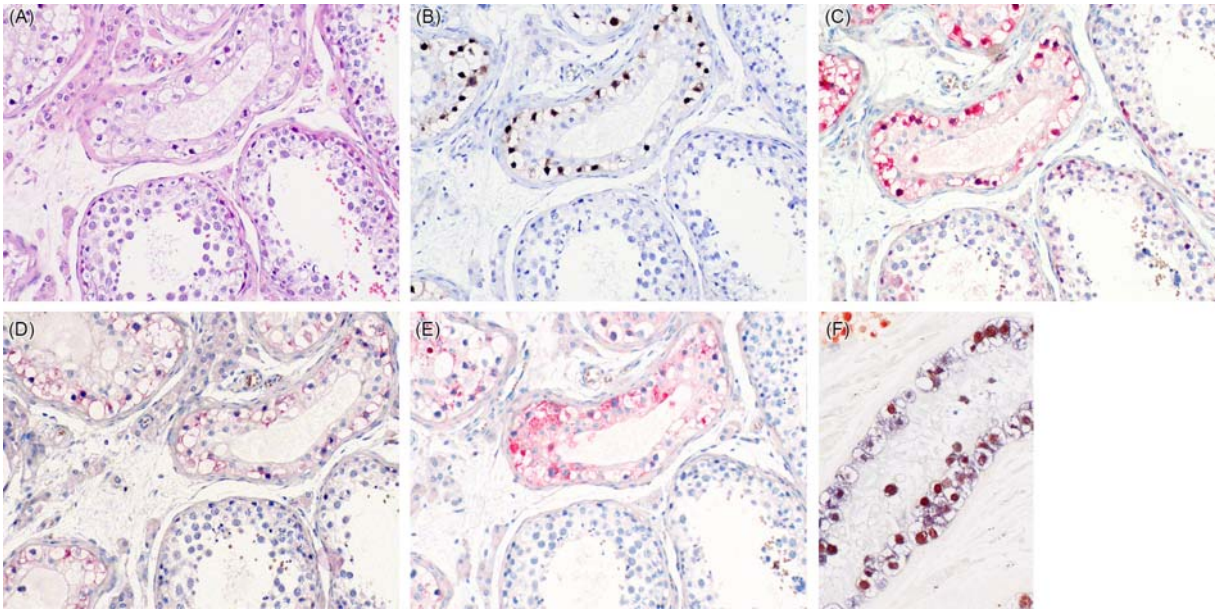

Fig. 23 In the upper left corner of the photograph are tubules with GCNIS, in the lower right corner tubules with spermatogenesis. The tubules with GCNIS have a narrower than normal caliber and a thickened wall; GCNIS cells have dark, enlarged, angular nuclei (A: H and E, ×200); GCNIS cells have nuclear expression of OCT4 (B: OCT4 *brown*, ×200), and membranous and cytoplasmatic expression of TSPY (C: TSPY *red*, ×200), as well as membranous expression of KIT (D: KIT *red*, ×200); the Sertoli cells diffusely express KITLG in a granular fashion (E: KITLG *red*, ×200); GCNIS cells consistently coexpress OCT4 (*reddish brown*, nuclear) and TSPY (bluish, membranous, and cytoplasmatic) (F, ×400).

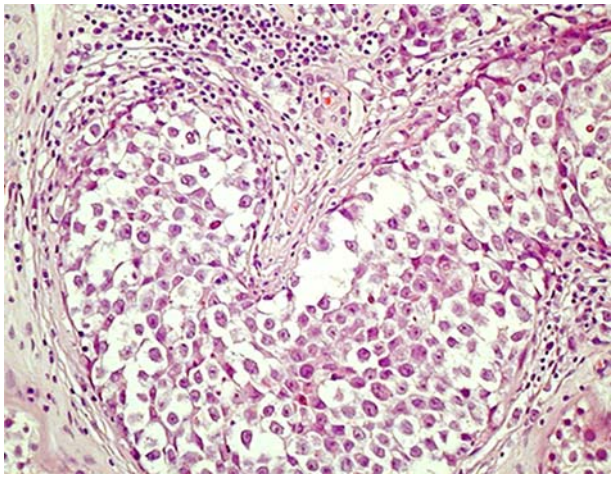

Fig. 24 Intratubular seminoma: seminiferous tubule packed with seminoma cells, extending the tubule (H and E, ×200).

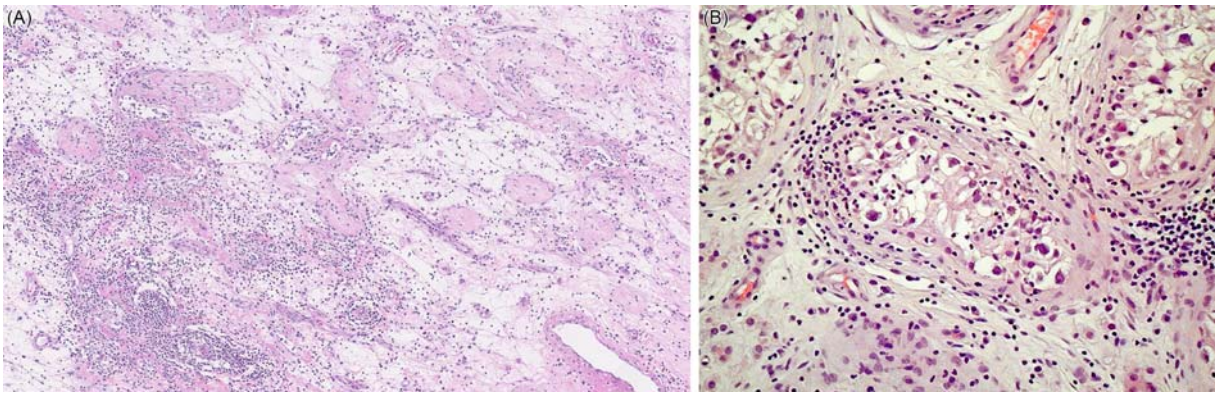

Fig. 25 The immune reaction of the host elicited by seminoma, apparent from the inflammatory cells, is capable of destroying invasive seminoma cells, and GCNIS cells within the seminiferous tubules, leaving fibrotic tubules and interstitial tissue (A: H and E, ×100); tubule with GCNIS, infiltrated by lymphocytes (B: H and E, ×200).

Upon invasion, seminoma invariably elicits an inflammatory host response, usually composed of lymphocytes, macrophages, plasma cells, and often a granulomatous reaction (Fig. 25), which may destroy tumor cells, leaving scar tissue. It is probably clinically relevant as in patients with AIDS the median age of seminoma is 25, 10 years younger than in the general population. Deviation from the default development of seminoma occurs when a seminomatous cell, either a GCNIS cell or an intratubular-, invasive- or metastatic seminoma cell, is reprogrammed to an EC cell, the totipotent stem cell of nonseminoma. One in three nonseminomas has a seminoma component, suggesting that reprogramming has occurred in an invasive seminoma (Fig. 26). Probably the remaining nonseminomas are due to reprogramming of a GCNIS- or an intratubular seminoma cell.

Virtually without exception intratubular nonseminoma has the morphology of EC. It is always partly necrotic (Fig. 27), apparently not adapted to the low oxygen intratubular environment. This relative anoxia may induce hypoxia factors, like MET, which triggers intratubular EC to invasion. Interestingly, upon invasion the EC cells often start to differentiate (Fig. 28). The intratubular environment seems to suppress differentiation of EC, while stromal factors stimulate differentiation. Candidate stromal factors are TGF-β, FGF, and BMP, which may derepress differentiation-promoting genes by removing polycomb repressive complexes recruited to the promotor sites of these genes by the pluripotency proteins OCT4, SOX2, and NANOG.

Type II GCT of the ovary

The incidence of ovarian type II GCT is about 20-fold lower than those of the testis. Ninety-five percent of ovarian type II GCT become manifest after puberty in women with a normal 46,XX karyotype, a couple of years earlier than in males, in accordance with the earlier onset of puberty in females. The remaining 5% are diagnosed before puberty in individuals with manifest or silent DSD. Overall, 6% are bilateral.

An established risk for (bilateral) ovarian type II GCT are various forms of DSD in phenotypic females who carry the GBY/*TSPY* region in their genome. As most studies of risk factors do not distinguish between type I and II GCT, the results have to be

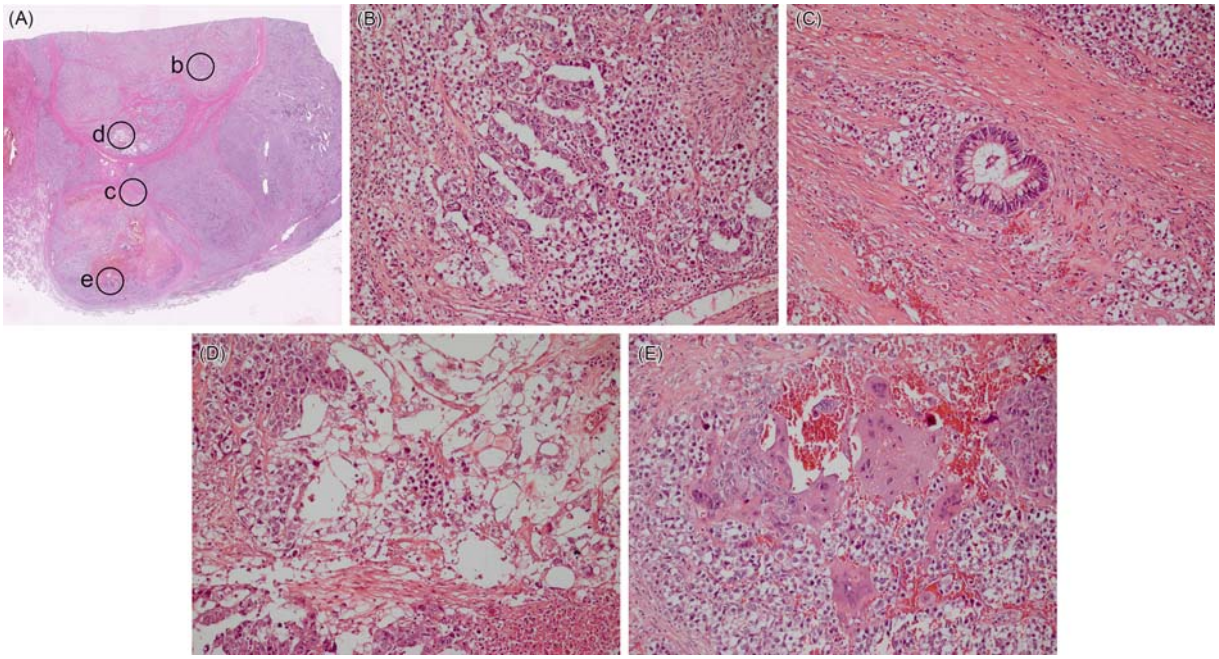

Fig. 26 Seminoma with reprogramming to EC, teratoma, YST, and choriocarcinoma (A: H and E, ×12.5). Embedded in seminoma are EC (B: H and E, ×200); teratomatous tubule lined with goblet cells (C: H and E, ×200); YST (D: H and E, ×200); choriocarcinoma (E: H and E, ×200).

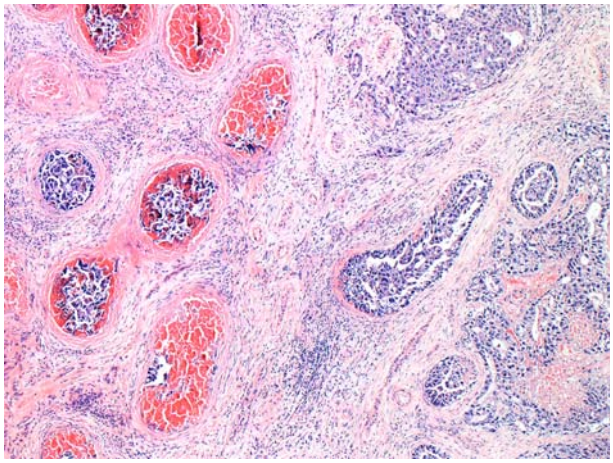

Fig. 27 Intratubular nonseminoma consists of EC only that is largely necrotic (pink staining material) (H and E, ×50).

interpreted with caution. Reported risks with the highest OR, such as exogenous hormones during pregnancy, maternal obesity and early regular menstruation after menarche, are related with hormonal regulation of reproduction.

An estimated 20% of ovarian type II GCT develop via the developmental pathway in women carrying GBY/*TSPY*: 6% with overt, and the remaining with clinically silent DSD. Ovarian seminomas in phenotypically and genotypically normal women have *KIT* mutations in about 50%. Only a minority of these being initiating, it leaves the pathogenesis of about three-quarters of ovarian type II GCT unexplained.

It is possible that in the ovary, the majority of type II GCT develop in the context of mild dysgenesis, comparable to TDS of the male, mainly caused by imbalances of factors regulating gonadal development and maintenance, such as FOXL2 (Fig. 29). Down-regulation of these factors during gonadal development, even only transiently, in the stage before oocytes become arrested in the prophase of meiosis I, would create a hypovirilized testis-like environment favoring disturbance of maturation oogonia/gonocytes and a window for the development of a type II GCT. However, due to the absence of GBY/TSPY, at a much lower rate than in males, or in DSD patients carrying GBY in their genome, who have an up to 70-fold risk of developing a type II GCT as compared to normal females.

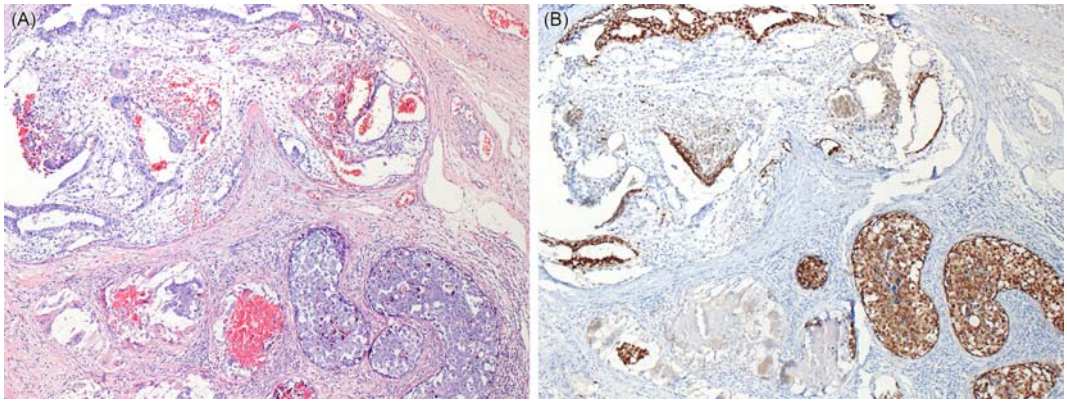

Fig. 28 Intratubular nonseminoma (right lower corner of the photograph) consisting of EC only, that starts to differentiate upon invasion (A: H and E, ×50; b, OCT4 *brown*, ×50). Oosterhuis, J. W. and Looijenga, L. H. J. (2017). Germ cell tumors from a developmental perspective: Cells of origin, pathogenesis and molecular biology; Emerging patterns. In Nogales, F.F. and Jimenez, R.E.(eds.) *Pathology and Biology of Human Germ Cell Tumors*, Berlin: Springer Nature, pp. 23–129.

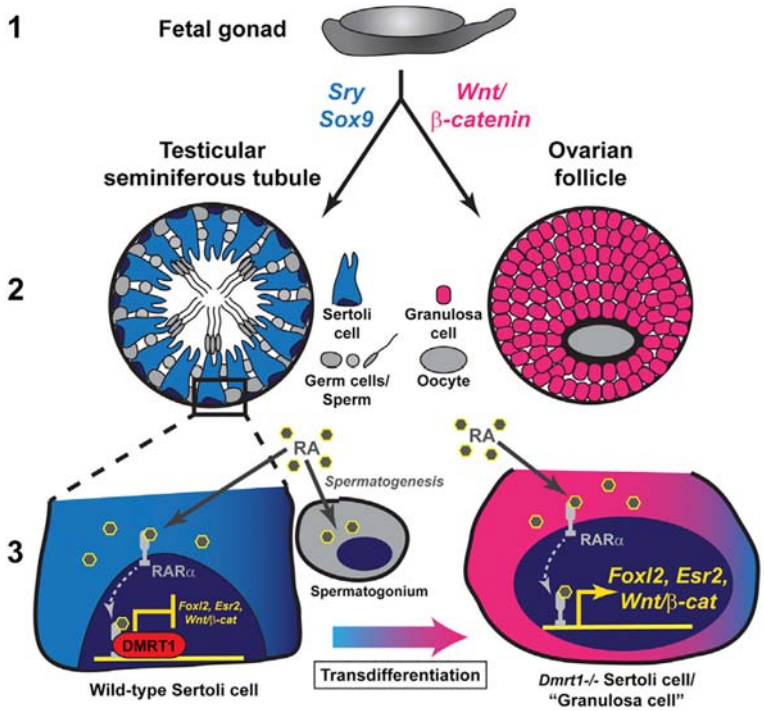

Fig. 29 Dmrt1 silences RA-dependent feminization genes to ensure postnatal sex maintenance during fetal sex determination (1), the bipotential gonad makes a choice between male (*blue*) and female (*pink*), largely guided by the presence or absence of Sry. The sexual differentiation machinery downstream of sex determination transforms the undifferentiated gonad into a mature testis or ovary (2), manifested in the formation of Sertoli-cell-containing seminiferous tubules in the male and granulosa-cell-containing ovarian follicles in the female. Postnatal sex maintenance within Sertoli cells (3) is achieved via the silencing of RA signaling-dependent feminization genes (such as Foxl2) by the transcriptional regulator Dmrt1. RA is thereby allowed to act in adjacent spermatogonia to promote spermatogenesis within the seminiferous tubule. In Dmrt1 mutant Sertoli cells, however, RA acting through RARa activates feminizing genes and reprograms the Sertoli cell into a granulosa-like cell through the process of trans-differentiation. DeFalco, T. (2014). DMRT1 keeps masculinity intact. *Developmental Cell* **29**, 503–504.

Type II GCT of the mediastinum

Mediastinal type II GCT constitute 50%–70% of extragonadal type II GCT. Both in whites and blacks over 95% occur in males, thus being male is the most important a risk factor. Mean age for seminomas is about 30 and for nonseminomas 25 years. Over 20% occur in individuals with Klinefelter's syndrome, who have a 67-fold risk compared to males without this condition. They present at median age of 17 (range 4–31), substantially younger than in non-Klinefelter patients. Klinefelter cases younger than 12 virtually always have precocious puberty, due to β-HCG produced by the tumor. In Klinefelter patients, increased levels of gonadotropins in

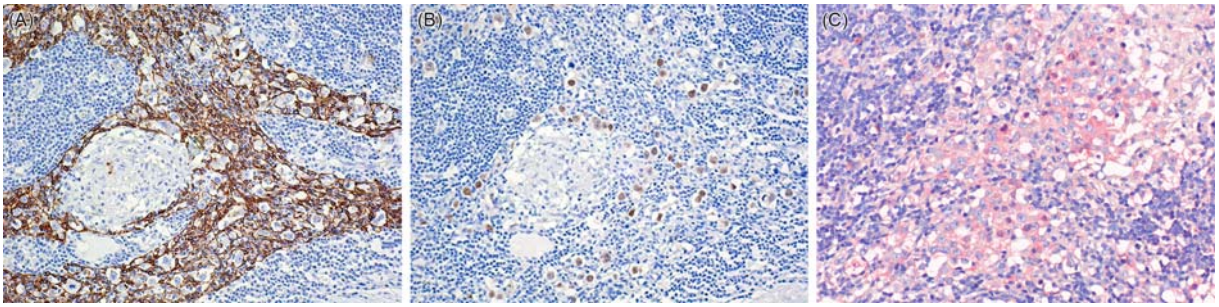

Fig. 30 Gonadoblastoma-like lesion in the thymus with seminoma cells enclosed by thymic epithelium (A: cytokeratin *brown*, ×200); the seminoma cells are OCT4 positive (B: OCT4 *brown*, ×200); thymic epithelium expresses KITLG (C: KITLG *red*, ×200). Oosterhuis, J. W., Stoop, H., Honecker, F., Looijenga, L. H. (2007). Why human extragonadal germ cell tumors occurin the midline of the body: Old concepts, new perspectives. *International Journal of Andrology* **30**, 256–263.

response to testicular atrophy may enhance malignant transformation of PGC in the thymus, explaining their 67-fold risk of these tumors compared to other males.

Mediastinal type II GCT originate from demethylated mis-migrated PGC that survived in the surrogate niche offered by thymic epithelium, shown to express KITLG, (Fig. 30), the critical factor for survival and proliferation of PGC. Primary mediastinal type II GCT are only localized in the anterior or antero-superior mediastinum within, or in association with the thymus. Apart from a somewhat higher proportion of pure YST and pure choriocarcinoma, the distribution of seminoma, and nonseminoma subtypes is similar as in the testicular type II GCT. In Klinefelter's syndrome the tumors are virtually always nonseminomas with 15% pure choriocarcinoma, and trophoblastic differentiation in 85%.

Of mediastinal nonseminomas 10%–20% develop a solid somatic-type malignancy. The distribution of the various histological types is largely similar to that in the testis, but angiosarcomas are more frequent. Hematologic malignancies, developing in 2%–6%, are uniquely associated with mediastinal YST. The most frequent types are megakaryoblastic leukemia, malignant and benign histiocytosis, and myelomonoblastic leukemia.

The (cyto)genetic aberrations are largely the same as in testicular type II GCT. Excess copies of chromosome X are partly due to Klinefelter cases.

Type II GCT of the central nervous system

- Epidemiology and risk factors
  The combined incidence of type I and II GCT of the brain is 0.143 for males and 0.046 for females in Japan and 0.118 for males and 0.030 for females in the United States. Probably more than 90% are type II.
  The overall male-to-female ratio of CNS GCT is about 4:1. The incidence of nonmalignant GCT is similar in males and females, 0.029 and 0.020, respectively. The male-to-female ratio for malignant GCT is 16:1 in the pineal region and 2.1:1 in the rest of the CNS. Being male is the most important a risk factor for Type II GCT of the CNS. More than half of all malignant GCT are located in the pineal region, virtually all of them type II.
  Type II GCT of the CNS can be familial and may cluster with type II GCT of the gonads and the mediastinum, sometimes in Klinefelter's and Down's syndrome. In Klinefelter's syndrome Type II GCT are the most frequent malignant CNS tumors, suggesting that it is a risk factor for these tumors.
- Anatomical localization
  Over 80% of type II GCT of the brain are located in the pineal gland, suprasellar region (neurohypophysial axis; occasionally within the neurohypophysis), hypothalamus, and the wall of the third ventricle. In these midline structures, the large majority are in male patients, malignant, and seminomas. Seminomas occur also in the basal ganglia, cerebral hemispheres, and in the posterior fossa. However, in these and other nonmidline anatomical sites, the proportion of females, nonseminomas and benign GCT (the latter probably of type I) is higher. In fact, type I GCT of the brain have an anatomical distribution resembling that of the type II nonseminomas. The anatomical distribution of type II GCT in Klinefelter's syndrome is similar to that in the general population. In Down's syndrome, where there is a high percentage of nonseminomas, the tumors lie most often outside the typical midline sites.
- Pathology
  The midline of the brain, in particular the pineal gland and the supra-sellar region, offers a niche where some mis-migrated, totipotent PGC can survive long enough to undergo transformation to a seminomatous precursor cell. Secretion of gonadotropins in the diencephalic centers at the inception of puberty may stimulate neoplastic transformation, and explain the young age of tumor manifestation.
  Eighty percent of type II GCT of the CNS are seminomas; most nonseminomas are mixed, followed by pure mature and immature teratoma, EC, YST and choriocarcinoma. Of the mixed tumors 75% have a seminoma component. Somatic-type malignancies rarely develop.

The strong male predominance suggests initiation via the developmental pathway with a crucial role for coexpression of OCT4 and TSPY. Probably only the minority of the tumors is initiated via somatic mutation of one of the genes involved in KIT signaling and downstream pathways (Fig. 15).

Eighty percent of the tumors developing in the midline is seminoma. Away from the midline type II GCT are rarer and more often nonseminomas. In these nonmidline sites, the conditions are supposedly less suitable for neoplastic PGC, favoring precursors that have undergone reprogramming to an ESC-like precursor, with a developmental potential in between the toti- and the pluripotent state. Indeed, the spectrum of histologies of type II nonseminomas of the brain (and to a lesser degree of the mediastinum) resembles that of type I GCT: relatively high proportions of pure (immature) teratoma, pure YST, and pure choriocarcinoma and rarely pure EC. Yet these tumors occur most often beyond the age of six and have the (cyto)genetic characteristics of type II GCT. In fact, in the brain, particularly away from the midline, there seems to be a gray area with a gradual transition between GCT of type I and II, featuring tumors that are type II by genotype and age but resembling type I by phenotype.

Klinefelter patients have the same distribution of type II GCT as non-Klinefelter patients, completely different from mediastinal type II GCT in Klinefelter's syndrome, where seminomas are virtually lacking.

In Down's syndrome, type II GCT of the brain are less frequently seminomas than in the general population (55%). The anatomical localization of the tumors is atypical, with only one in the pineal gland. Of five nonseminomas, four were YST and one teratoma.

- (Cyto)genetics

  Activating mutations in *KIT* (47%), *KRAS* (18%), and *NRAS* (6%) and inactivating mutations in *CBL* (6%), a negative regulator of KIT expression, are all mutually exclusive and occur most often in seminomas and mixed GCT that lack gain of 12p. The complementary character of these genetic events and their preferred occurrence in seminomas and mixed GCT indicate that they are probably not initiating but engaged in the progression of seminoma. The AKT/mTOR signaling pathway is activated in about 20% of cases, mostly by focal amplification of 14q32.33, containing the AKT1 locus, often in tumors lacking gain of 12p (Fig. 15).

Type III GCT (Spermatocytic Tumor)

Epidemiology

The incidence of spermatocytic tumor is 0.4 per million, without a significant increase over the past 20 years. The median age of clinical presentation is 55. In about 9% the tumor is bilateral.

Anatomical localization

Spermatocytic tumors occur only in the postpubertal testis, virtually always in scrotal position, and sporadically in a maldescended testis (Table 1).

Pathology

Spermatocytic tumor is a benign neoplasm, of which the cells resemble postpubertal germ cells with early, premeiotic differentiation. The nuclei appear in three size classes corresponding to A-dark spermatogonia (reserve spermatogonial stem cells) with the smallest nuclei with dense chromatin; A-pale spermatogonia (self-renewing stem cells) with intermediate and large paler nuclei with finely granular filamentous chromatin; B spermatogonia, and leptotene spermatocytes (Fig. 31). The tumor cells express

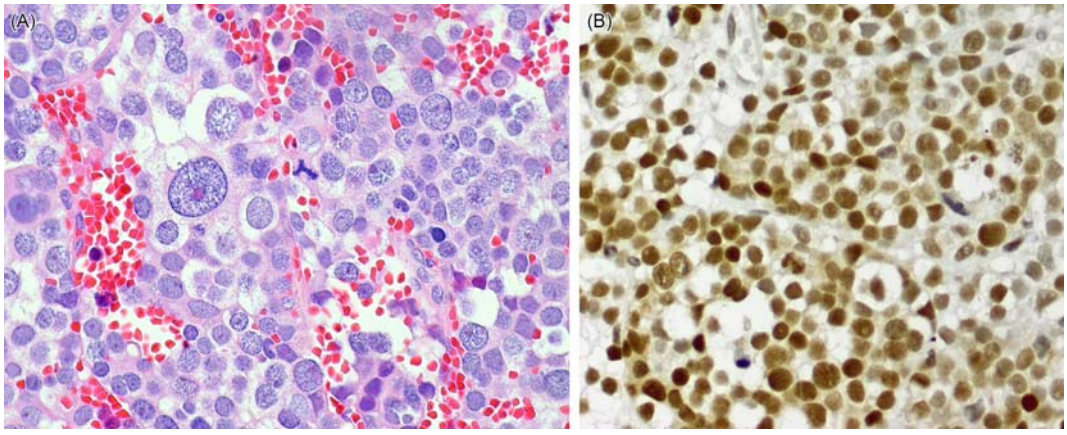

Fig. 31 Spermatocytic tumor with small dark nuclei, intermediate nuclei, and large nuclei with finely granular filamentous chromatin (A: H and E, ×400); the nuclei express DMRT1 (B: DMRT1 *brown*, ×400).

markers of these cell types, such as DMRT1, which is a diagnostically useful immunohistochemical marker (Fig. 31). Markers of gonocytes, such as OCT4 and PLAP have been switched off, and markers of meiosis are not yet expressed. Spermatocytic tumor lacks the characteristic host response of seminoma. Adjacent seminiferous tubules may contain intratubular spermatocytic tumor. The occasional finding of an exclusively intratubular spermatocytic tumor without an invasive component proves that the tumor has its origin within the seminiferous tubules (Fig. 32).

Exceptionally, the tumor is associated with a sarcomatous component, usually undifferentiated sarcoma, and rarely rhabdomyo- or chondrosarcoma. These are highly malignant and readily metastasize to regional lymph nodes or, blood–borne, to visceral organs.

Disturbance of the niche- and spermatogonial factors involved in the regulation of spermatogenesis are the factors driving tumorigenesis (Figs. 33 and 34). Like in the other types of GCT, in spermatocytic tumors, initiation is primarily due to

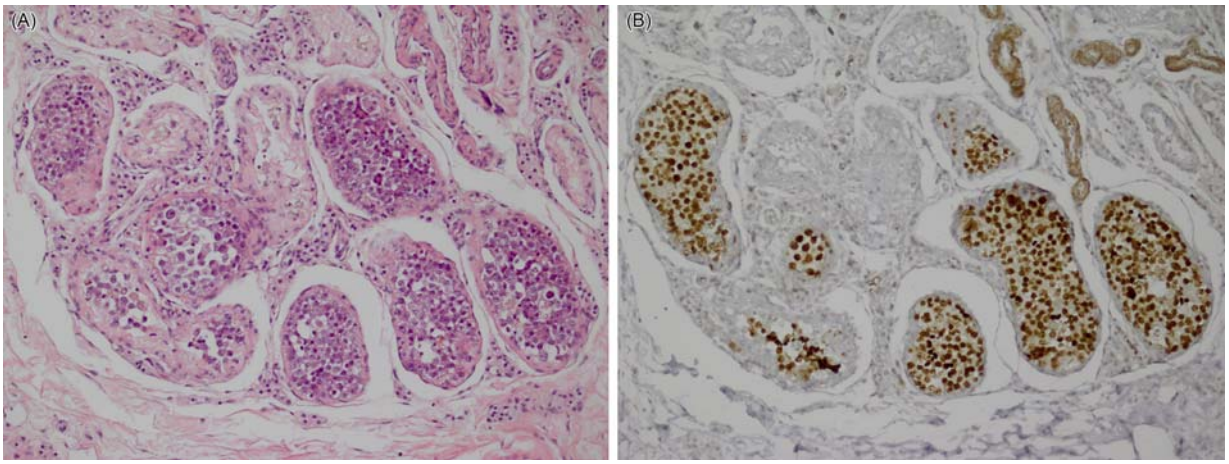

Fig. 32 Intratubular spermatocytic tumor (A: H and E, ×200); expressing DMRT1 (B: DMRT1 *brown*, ×200). Oosterhuis, J. W. and Looijenga, L. H. J. (2017). Germ cell tumors from a developmental perspective: Cells of origin, pathogenesis and molecular biology; Emerging patterns. In Nogales, F.F. and Jimenez, R.E.(eds.), *Pathology and biology of human germ cell tumors*, Berlin: Springer Nature, pp. 23–129.

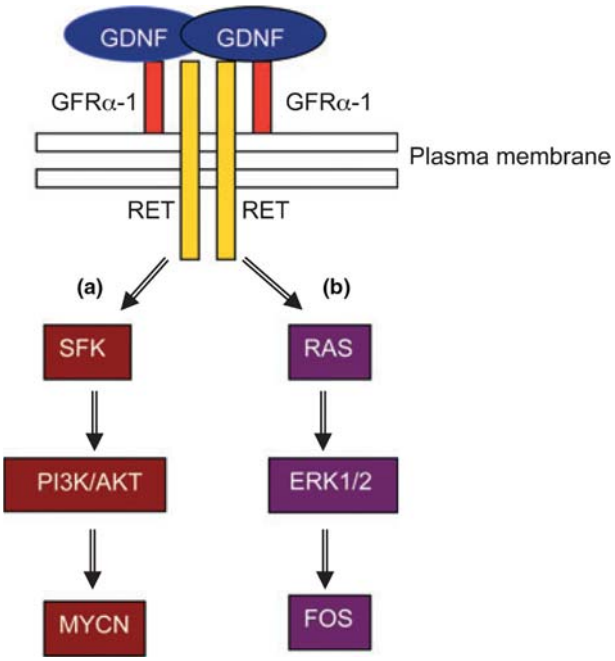

Fig. 33 Signaling pathways triggered by GDNF in spermatogonial stem cells. GDNF dimerizes and binds to the GFRA1/RET receptor complex. (A) Binding of GDNF activates RET, which triggers SRC-kinase phosphorylation and the downstream activation of PI3K/AKT. Ultimately the transcription factor MYCN is upregulated. (B) Binding of GDNF also can activate the RAS-mediated signaling pathway, which triggers ERK1/2 phosphorylation and upregulation of the transcription factor FOS. Waheeb, R., and Hofmann, M.-C. (2011). Human spermatogonial stem cells: A possible origin for spermatocytic seminoma. *International Journal of Andrology* **34**, e296–e305.

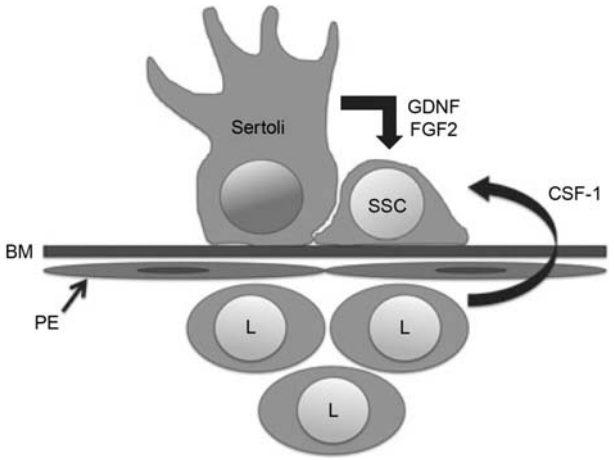

Fig. 34 A simplified scheme of the spermatogonial stem cell niche showing the main extrinsic factors driving spermatogonial stem cell (SSC) maintenance and self-renewal. Sertoli cells and spermatogonial stem cells (SSCs) are both attached to the basement membrane (BM). Sertoli cells provide for structural support and produce glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF) which are crucial for SSC self-renewal in vitro and in vivo. Leydig cells (L) and peritubular cells (PE) produce colony-stimulating factor-1 (CSF-1), also essential for self-renewal. Waheeb, R. and Hofmann, M-C. (2011). Human spermatogonial stem cells: A possible origin for spermatocytic seminoma. *International Journal of Andrology* **34**, e296–e305.

a developmental deregulation. Somatic mutations, and whole chromosome-aneuploidy are probably mainly progression related. The tumor promoting niche factors explain the high percentage of bilaterality of spermatocytic tumor.

(Cyto)genetics and epigenetics

Most spermatocytic tumors are (near)diploid, the second largest group is (near)tetraploid, and a few are peritriploid. The only consistent cytogenetic aberration is extra copies of chromosome 9 (Fig. 35). The candidate gene on this chromosome involved in tumorigenesis is *DMRT1* (Fig. 36). A small number of tumors, usually in the oldest half of the patients, have mutually exclusive, paternal age-related mutations in *FGFR3*, *HRAS* or *NRAS*. In 80% of spermatocytic tumors p53 is expressed.

Spermatocytic tumors have heterogeneous pattern of DNA methylation and histone modification, different from the regular patterns in normal spermatogenesis, probably because the tumor cells are deprived of regulatory niche factors.

Type IV GCT (Dermoid Cyst of the Ovary)

Epidemiology and risk factors

The dermoid cyst of the ovary is the most frequent GCT, with an estimated incidence between 10 and 15, occurring at the reproductive age (median age 30, range 10–90) (Fig. 37). Over 10% of the patients have bilateral tumors.

Anatomical localization

Dermoid cysts occur exclusively in the ovaries. On rare occasions, they become detached from the ovary and reimplanted in either omentum, fallopian tube, or Douglas' pouch. Tumors in the testis and extragonadal sites resembling dermoid cysts are type I GCT beyond infancy, with a developmental potential in between that of type I and type IV GCT.

Pathology

Dermoid cysts are derived from parthenogenetically activated primary oocytes (Fig. 38) that have escaped the normal, postpubertal regulation of meiotic arrest, and started uncontrolled meiosis, which explains why they occur at reproductive age. Genetic variants in the factors regulating meiotic arrest may be involved in proneness for bilaterality and familial occurrence of type IV GCT. Thus, it seems probable that, as in other GCT, the origin of type IV GCT is mainly determined by developmental factors, with a minor role for genetic events. Half of the dermoid cysts have the molecular characteristics of primary oocytes that have been parthenogenetically activated after meiosis I (Type-I), a quarter after meiosis II (Type-II), and the remaining quarter after endoreduplication (Type-III) (Fig. 39).

Dermoid cysts are completely mature teratomas, which present as a thin-walled cyst lined with epidermis with attached appendages and filled with sebaceous material and hairs. Usually one solid nodule (Rokitansky's protuberance) protrudes from the wall into the lumen of the cyst. It is mainly composed of cranial tissues: skin covered with hair (scalp), bone (skull), choroid plexus and glial tissue (intracranial tissues), retinal epithelium (eyes), teeth (jaw), and thyroid (neck). In fact, a dermoid cyst represents the rostral part of an attempted embryo turned inside out (Fig. 40). In addition, virtually any adult tissue can be present. Exceptional tumors are predominantly solid with highly organized structures resembling a fetus, however, lacking extraembryonic tissues, as do

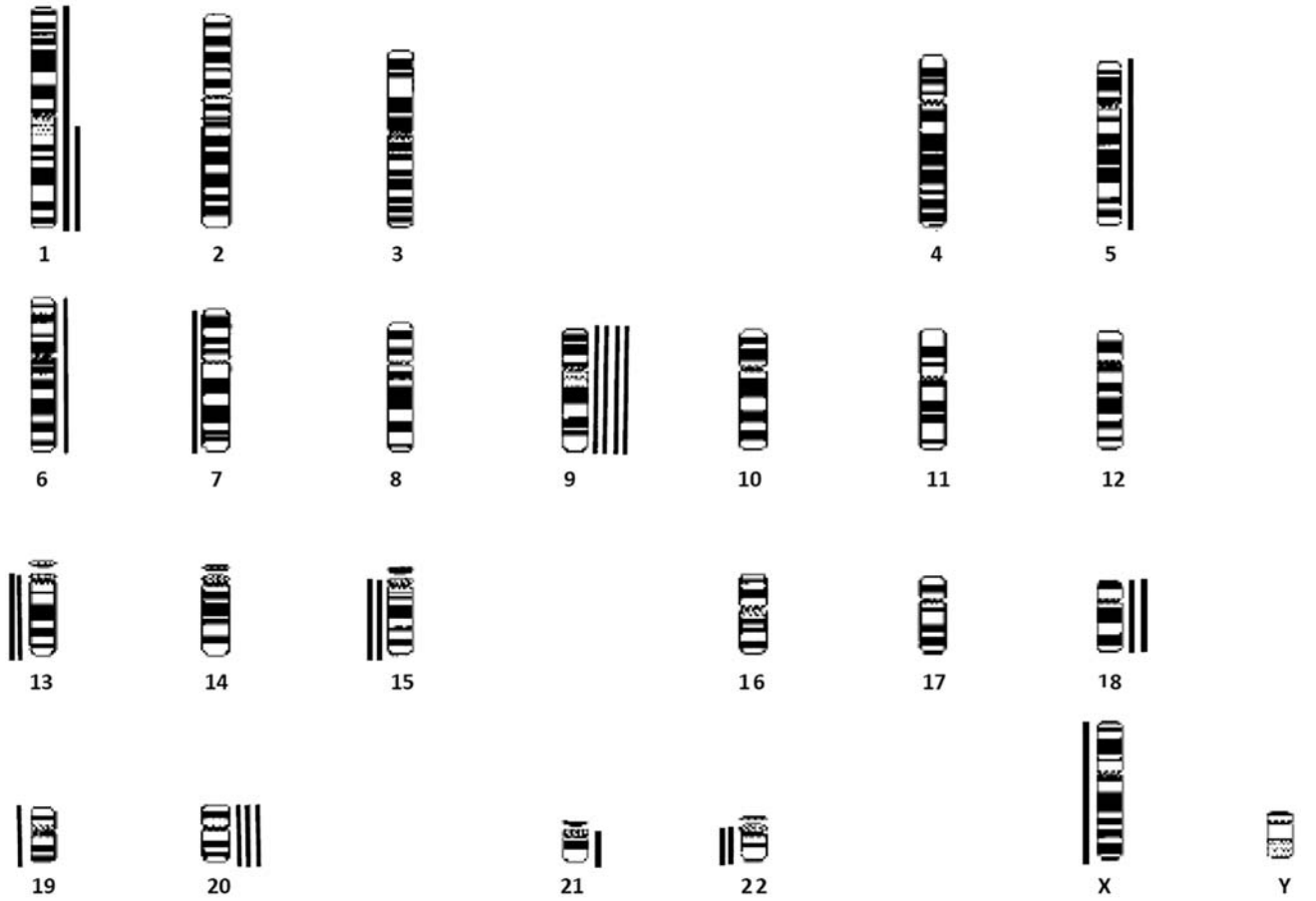

Fig. 35 Combined CGH data of four spermatocytic tumors; underrepresentation of chromosomes is indicated lines on the left of the chromosome-ideograms, overrepresentation on the right. Chromosome 9 is overrepresented in each of the four cases. Rosenberg, C., Mostert, M. C., Schut, T. B., van de Pol, M., van Echten, J., de Jong, B., Raap, A. K., Tanke, H., Oosterhuis, J. W., Looijenga, L. H. (1998). Chromosomal constitution of human spermatocytic seminomas: Comparative genomic hybridization supported by conventional and interphase cytogenetics. *Genes Chromosomes Cancer* **23**, 286–291.

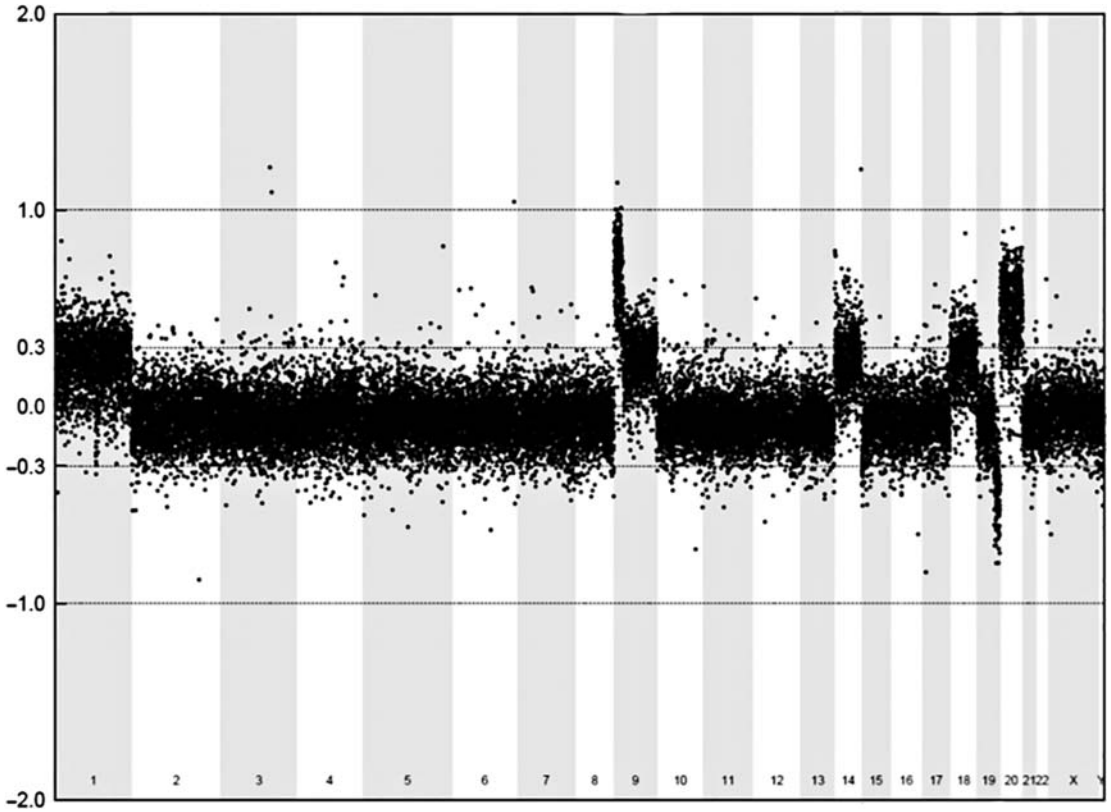

Fig. 36 Detailed representation of chromosome 9 pattern showing a subchromosomal amplification of the 9p21.3-pter region containing *DMRT1*. Looijenga, L. H., Hersmus, R., Gillis, A. J., Pfundt, R., Stoop, H. J., van Gurp, R. J., Veltman, J., Beverloo, H. B., van Drunen, E., van Kessel, A. G., Pera, R. R., Schneider, D. T., Summersgill, B., Shipley, J., McIntyre, A., van der Spek, P., Schoenmakers, E., Oosterhuis, J. W. (2006). Genomic and expression profiling of human spermatocytic seminomas: primary spermatocyte as tumorigenic precursor and DMRT1 as candidate chromosome 9 gene. *Cancer Research* **66**, 290–302.

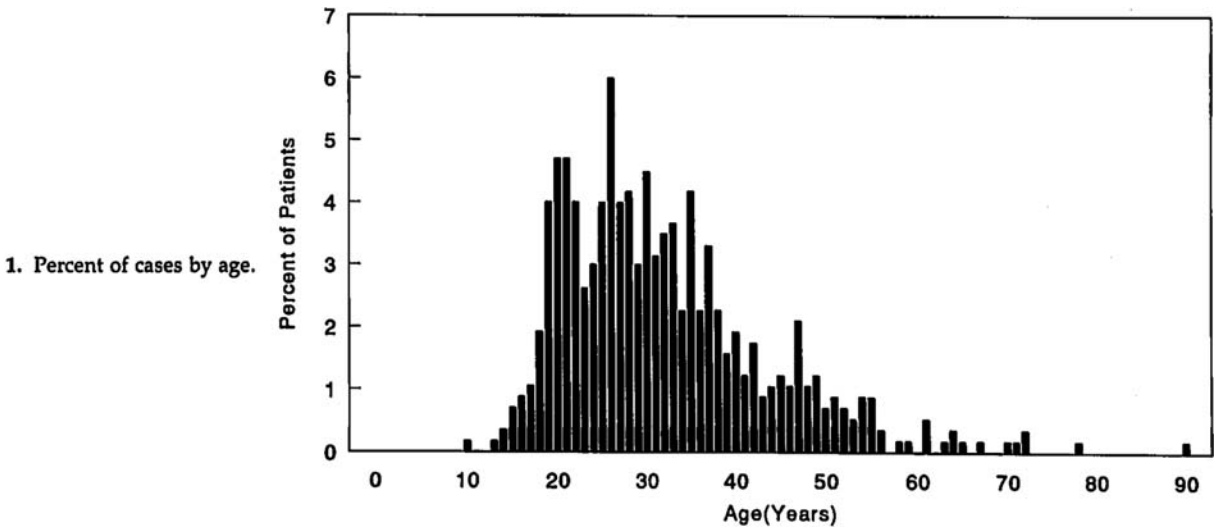

Fig. 37 Age distribution of patients with type IV GCT (dermoid cysts) showing that these GCT occur in postpubertal women. Comerci, J. T., Licciardi, F., Bergh, P. A., Gregori, C., Breen, J. L. (1994). Mature cystic teratoma: A clinicopathologic evaluation of 517 cases and review of the literature. *Obstetrics & Gynecology* **84**, 22–28.

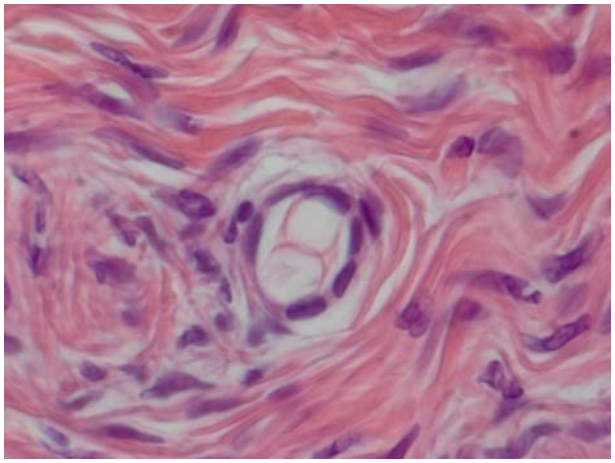

Fig. 38 Two cell-stage embryo due to parthenogenetic activation of an ooctye in a dysgenetic ovary (H and E, ×640).

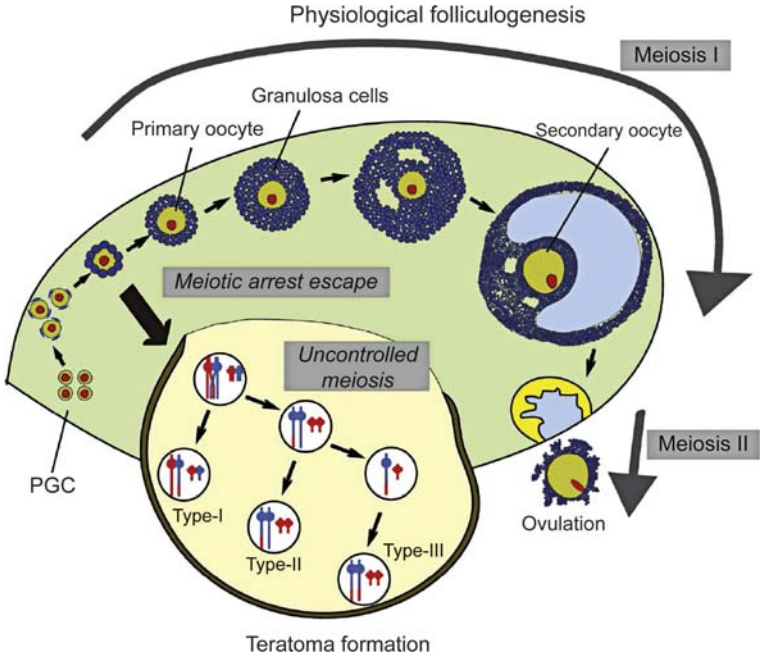

Fig. 39 The postulated mechanism of human ovarian teratoma formation. The source cells are proposed to be primary oocytes that escaped from meiotic arrest. Subsequent uncontrolled meiotic division could produce ovarian teratomas. PGC, primordial germ cell. Kaku, H., Usui, H., Qu, J., Shozu, M. (2016). Mature cystic teratomas arise from meiotic oocytes, but not from pre-meiotic oogonia. *Genes Chromosomes Cancer* **55**, 355–364.

dermoid cysts. Benign tumors, such as struma ovarii and carcinoids, may arise from organ anlages within a dermoid cyst. Somatic-type malignancies develop reportedly in 0.2%–3% of dermoid cysts, of which about 90% are squamous cell carcinomas. Dermoid cysts may sporadically contain immature foci, even more exceptionally combined with YST.

(Cyto)genetics and epigenetics

Virtually all dermoid cysts are diploid, a few percent have chromosomal abnormalities including trisomy for chromosomes 7, 8, 12, 15, and X. Trisomy for chromosomes 8, 12, and X may be shared by immature teratomas of the ovary. Somatic-type malignancies have the same genetic changes as their somatic counterparts. For example, malignant struma ovarii has the same *BRAF* point mutations as papillary carcinomas of the thyroid.

The genomic profile of dermoid cysts reflects the stage of meiosis of the oocytes from which they have originated. Most significantly, the chromosomes of dermoid cysts have an exclusively maternal imprinting (Fig. 41), inhibiting the development of trophoblast, like in the mouse gynogenote (Fig. 42). (Table 1).Thus, the developmental potential of type IV GCT corresponds to the 2C state, whereby, due to the exclusively maternal imprinting, only somatic tissues are formed.

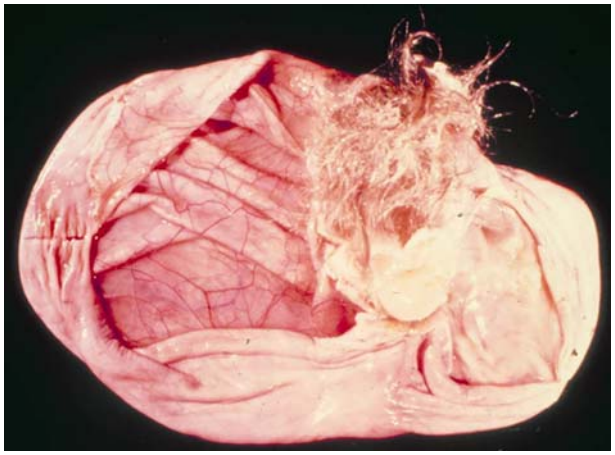

Fig. 40 Gross image of type IV GCT (dermoid cyst), fully developed hair on a scalp-like covering of Rokitansky's protuberance (dermoid hill).

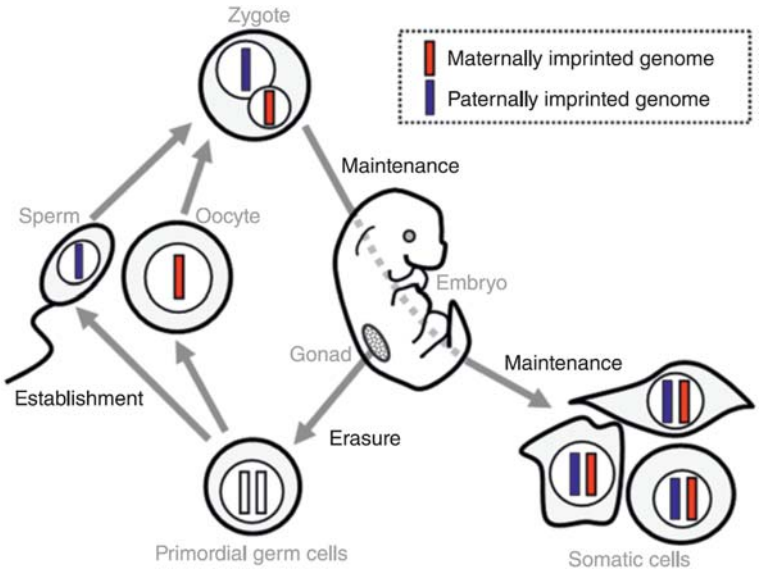

Fig. 41 Cycle of genomic imprinting (GI). Upon fertilization, the zygote acquires a haploid set of paternally imprinted chromosomes from the father and a haploid set of maternally imprinted chromosomes from the mother; the cells of the embryo therefore have a biparental GI pattern. In the germline, GI is erased; during spermatogenesis and oogenesis, respectively, paternal and maternal imprinting is reestablished. Hirasawa, R. and Feil, R. (2010). Genomic imprinting and human disease. *Essays in Biochemistry* **48**, 187–200.

Type V GCT (Molar Pregnancies)

Epidemiology and risk factors

The incidence of gestational trophoblastic disease, mostly complete hydatidiform moles, is 1 in 120 pregnancies in some parts of Asia and South America, a factor 10 higher than in Western societies. Risk factors are pregnancy at young or old age, prior gestational trophoblastic disease, Asian ethnicity, and possibly dietary deficiencies and low socioeconomic status.

Genetic risk factors are maternal mutations of *NALP7/NLRP7* on 19q13.4, causing abnormal imprinting with overexpression of the paternal genome, which results in recurrent familial biparental complete hydatidiform moles and reproductive wastage.

Anatomical localization

Molar pregnancies occur virtually always in the uterus, occasionally in a fallopian tube as an ectopic pregnancy.

Pathology

Complete hydatidiform moles are caused by androgenesis with two, rarely four, haploid sets of paternal chromosomes, arising from fertilization of an anuclear empty ovum by one 23,X sperm that replicates its chromosomes. Those with a 46,XY karyotype result from fertilization of an empty ovum by two sperm. Both mechanisms create a zygote with an exclusively paternal imprint, which

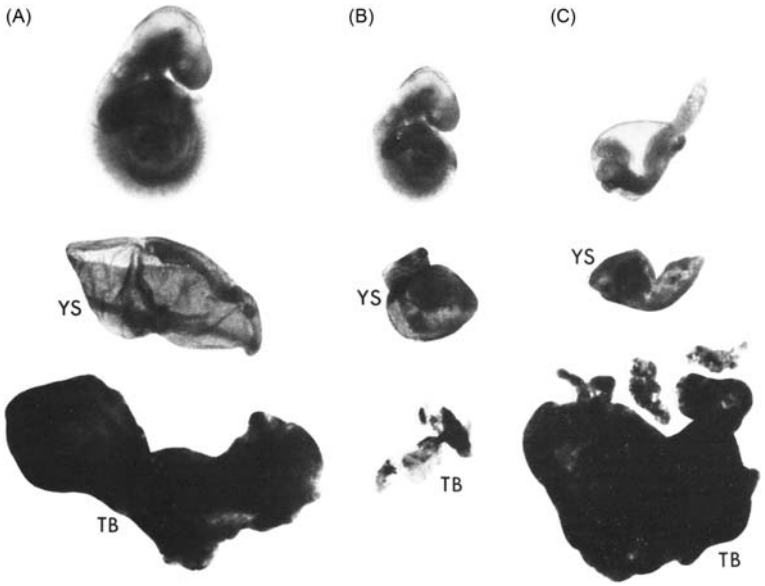

Fig. 42 Compare development of control embryo (A) with that obtained from eggs with two maternal genomes, gynogenotes (B), in which a small but well-advanced 25-somite embryo was the maximum development but with poor extraembryonic tissues. The eggs with two paternal nuclei, androgenotes (C), developed maximally to about the 6- to 8-somite stage but with extensive trophoblast development. YS yolk sac, TB trophoblast. Surani, M. A., Barton, S. C., Norris, M. L. (1986). Nuclear transplantation in the mouse: Heritable differences between parental genomes after activation of the embryonic genome. *Cell* **45**, 127–136.

gives rise to placental tissue only and no somatic tissues of the embryo proper, similar to experimentally produced mouse androgenotes (Fig. 42) (Table 1) The precursor cell of the hydatidiform mole has the 2C-state developmental potential, except for the ability to form somatic tissues of the embryo proper.

Consistent with this pathogenesis complete hydatidiform moles, type V GCT, are composed of hypertrophic, dysplastic placental tissue only, lacking somatic tissues of the embryo proper (Fig. 43). Thus, these growths are the mirror image of dermoid cysts, which are composed of somatic tissues only, and lack trophoblastic tissue. They progress to choriocarcinoma in 2%–3% of cases, possibly driven by hypomethylation-associated genomic instability.

Genetics and epigenetics

Complete hydatidiform moles are generally diploid with a 46,XX (90%) or 46,XY (10%) karyotype; rare cases are tetraploid.

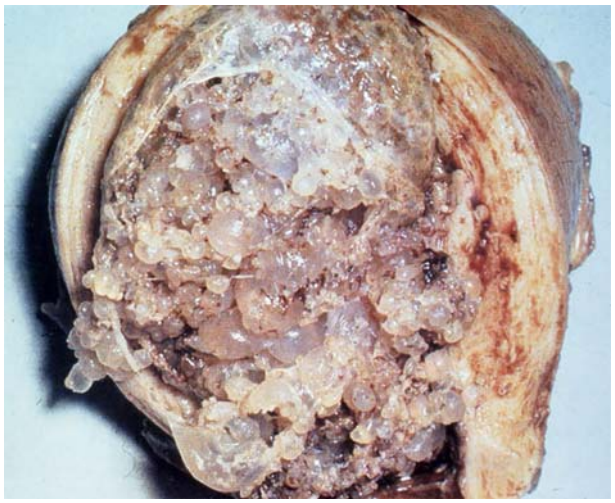

Fig. 43 Gross image of hydatidiform mole, resembling a bunch of grapes, shown in an opened uterus. The individual grapes consist of hypertrophic, edematous placental villi.

Type VI GCT

Definition

Type VI GCT are defined as neoplasms resembling GCT as to their developmental potential. However, they are not derived from germ cells, but from somatic cells induced to pluripotency (Table 1).

Epidemiology and risk factors

As type VI GCT are a new concept, epidemiological data are scarce. With a median age of over 50, the patients are old for a germ cell tumor. Most type VI GCT develop in advanced cancers, in particular common epithelial cancer of the ovary; few arise de novo.

Anatomical localization

The anatomical distribution follows the various types of cancer in which of GCT components may develop, in particular of the ovary and stomach. Sinonasal teratocarcinosarcomas are virtually always located in the nasal cavity and/or ethmoid sinus. The few published de novo type VI GCT with balanced translocations were localized at atypical sites for GCT, certainly in view of the old age of the patients: retroperitoneum, posterior mediastinum, and inside the sacrum.

Pathology

Type VI GCT developing in somatic cancers may contain all elements of GCT apart from seminoma, either pure or in various combinations. Probably the most frequent types are YST and immature teratoma. Thus their developmental potential resembles that of type I GCT and the nonseminoma variant of type II GCT, like genetically engineered human induced pluripotent stem cells (iPSC) (Fig. 44).

(Cyto)genetics

Little is known on the genetics of GCT originated in somatic cancers. Overrepresentation of 12p has not been demonstrated. In a subpopulation of tumor cells of a nasal teratocarcinosarcoma an extra copy of 12p13 (a feature of type I GCT) was demonstrated by ISH.

The published de novo type VI GCT showed complex balanced translocations, with 6p21 being a common breakpoint in each of them; chromosome 12 was not involved. (Fig. 45) A nasal immature teratoma was diploid with a balanced translocation t(1;11)(q12;p15).

In type VI GCT developing in somatic cancers, various genetic and epigenetic changes may de-repress pluripotency genes. *MYC* might play a crucial role, since it is a central player in oncogenesis and pluripotency. Indeed, more aggressive cancers may express both the core pluripotency genes (OCT4, NANOG, SOX2, and KLF4) and MYC-centered networks.

The few cytogenetically characterized de novo type VI GCT suggest that breakpoints in certain chromosomal regions might activate the pluripotency program. The breakpoint in 6p21–22 in these tumors could involve OCT4. The overrepresentation of 12p13 in a sinonasal teratocarcinosarcoma may have led to overexpression of the pluripotency cluster NANOG, STELLAR, and GDF3.

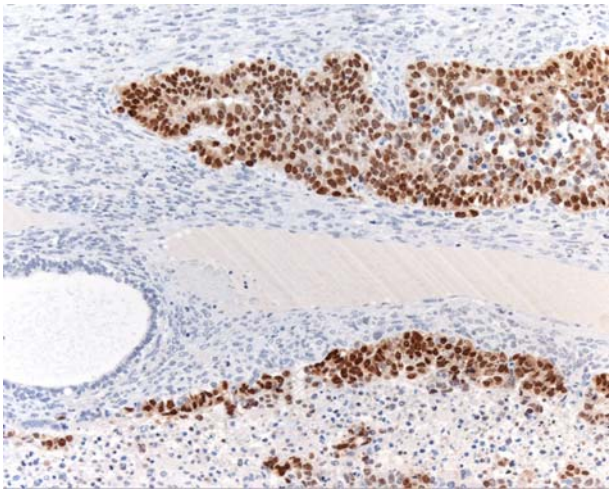

Fig. 44 Nonseminoma component of a type VI GCT developed in a clear cell carcinoma of the ovary with EC cells expressing OCT4. Nogales, F. F., Prat, J., Schuldt, M., Cruz-Viruel, N., Kaur, B., D'Angelo, E., Matias-Guiu, X., Vidal, A., McCluggage, W. G., Oosterhuis, J. W. (2018). Germ cell tumour growth patterns originating from clear cell carcinomas of the ovary and endometrium: A comparative immunohistochemical study favouring their origin from somatic stem cells. *Histopathology* **72**, 634–647.

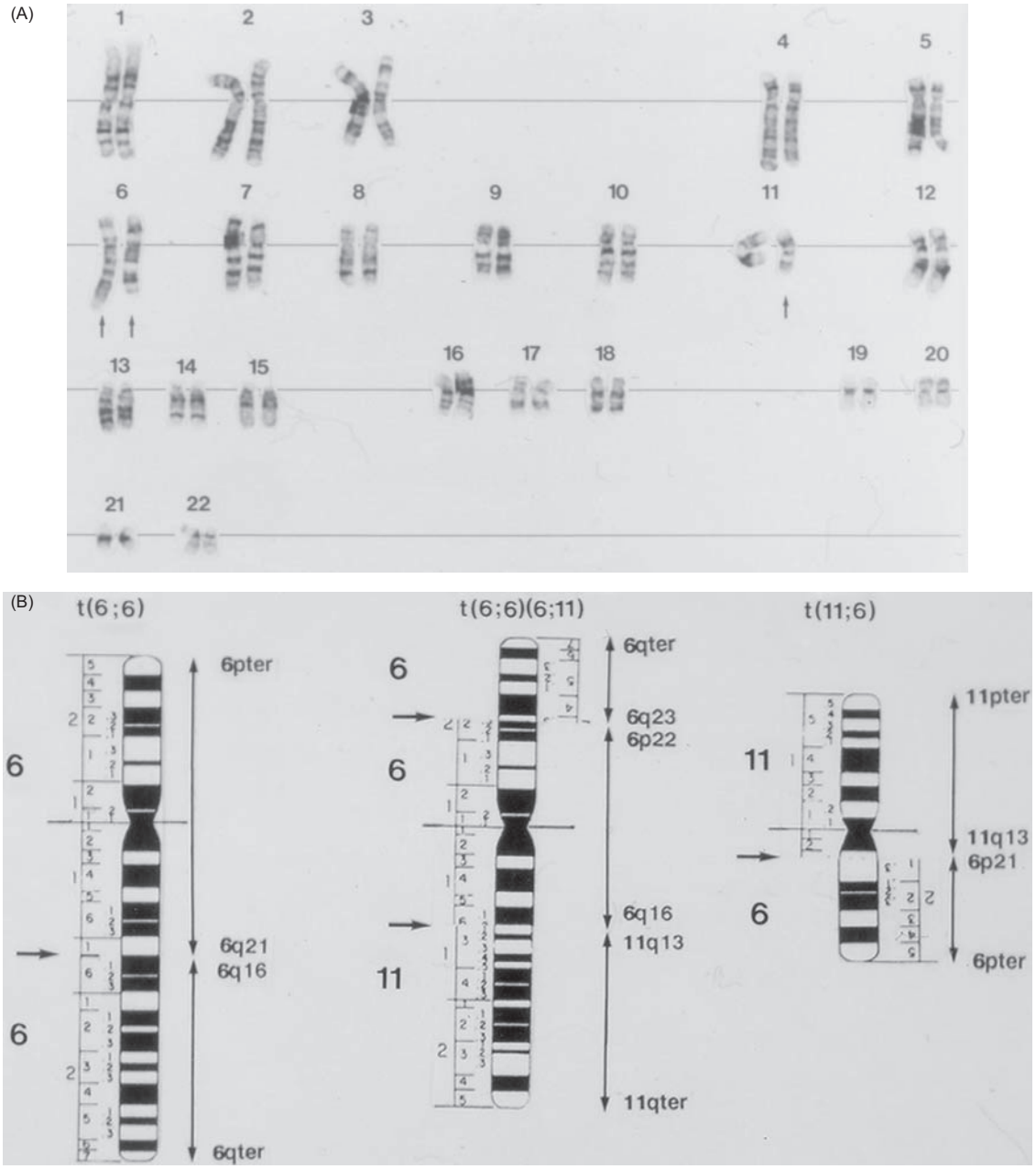

Fig. 45 Karyotype (A), and cartoon of the translocations (B) of a de novo type VI GCT arisen in posterior mediastinum, showing breakpoints in 6p21, 6p22, 6q23, and 11q13, with the chromosomal regions, 6p22::6q23 and 6p21::11q13, involved in fusions. Van Echten, J., de GJong, B., Sinke, R. J., Weghuis, D. O., Sleijfer, D. T., Oosterhuis, J. W. (1995). Definition of a new entity of malignant extragonadal germ cell tumors. *Genes Chromosomes Cancer* **12**, 8–15.

GCT With Intermediate Phenotypes

Consistent with the plasticity of the developmental states of embryonic stem cells, there are intermediate phenotypes between the seven defined types of GCT.

There is continuum between multiple pregnancies, conjoined twins, parasitic twins, and type I GCT, with intermediate types between type 0 and type I GCT, which could arbitrarily be classified in either type.

Type I GCT and type II GCT have gradual transitions, in particular among prepubertal GCT of the mediastinum in Klinefelter's and among GCT of the brain in patients with Down's syndrome. Such tumors are genotypically type II but phenotypically resemble type I, suggesting that the genomic changes typical for type II, particularly gain of 12p, have occurred in a PGC that is still too heavily methylated to allow the full spectrum of the totipotent developmental potential of a type II GCT.

The type I GCT beyond infancy, composed of (immature) teratoma combined with dermoid cysts likely represent a transitional phenotype between type I and type IV GCT. It may be hypothesized that such tumors are derived from a precursor cell somewhere in between an oogonium and a type I oocyte, in which maternal GI is not yet completed.

There is at least one published case of a spermatocytic tumor that is intermediate between a type II and a type III GCT, in terms of morphology, chromosomal composition, and behavior: a seminomatous morphology, lacking lymphocytes, gain of 12p and chromosome 9, and metastasis.

Type VI tumors have developmental characteristics with features of type I GCT and type II nonseminomas, and intermediate phenotypes.

Conclusions

GCT are indeed one family of gonadal and extragonadal tumors derived from germ cells and their precursors. Their developmental potential/pathology is related to a developmental state rather than a specific cell of origin, as a particular originating cell may assume different developmental states, and different cell types may have the same developmental potential, in agreement with developmental state plasticity. A new concept is the type VI GCT, derived from somatic cells, most often cancer cells, induced to pluripotency.

Apart from type VI, most GCT are caused via the so-called developmental pathway: disturbance of niche-, and cell intrinsic factors that control the developmental potential of germ cells and their precursors. The somatic mutation pathway is much rarer; most mutations occur during tumor progression.

This developmental pathogenesis explains the strong familial component of most GCT, including clustering of different types of GCT, their frequent bilaterality, and their intact p53 response, which renders malignant GCT highly sensitive to DNA-damaging agents.

Having *TSPY*, located in the GBY region of the Y chromosome, is the most important risk factor for a type II GCT, regardless of anatomical site and phenotypic gender. Coexpression of OCT4 and TSPY in maturation delayed PGC/gonocytes emerges as a plausible factor for initiation of type II GCT via the developmental pathway.

Clinically it is important to differentiate between type I and IV teratomas, which are benign, and type II teratoma, which may look identical microscopically but behaves malignant.

Prospective Vision

For the advancement of the field of GCT it is important to reconsider the traditional site-oriented approach, which has led to the present confusing nomenclature, and blurred entities. GCT should rather be considered as one disease manifesting in different organs, like for example soft tissue tumors and lymphomas. This change will stimulate the development of a unifying, biologically plausible, and clinically relevant classification of existing and emerging entities, including tumors in the wake of clinical application of iPSC. Finally, it will provide a reliable basis for genotype/phenotype correlations carried out in joint efforts of basic scientists and clinicians.

Further Reading

Ben-David, U., Benvenisty, N., 2011. The tumorigenicity of human embryonic and induced pluripotent stem cells. Nature Reviews Cancer 11, 268–277.

Cools, M., Drop, S.L., Wolffenbuttel, K.P., Oosterhuis, J.W., Looijenga, L.H., 2006. Germ cell tumors in the intersex gonad: Old paths, new directions, moving frontiers. Endocrine Reviews 27, 468–484.

De Backer, A., 2006. Surgical treatment and outcome of extracranial germ cell tumors in childhood [Doctoral Thesis]. Erasmus University Medical Center, Rotterdam, ISBN 90-8559-166-X.

Giannoulatou, E., Maher, G.J., Ding, Z., et al., 2017. Whole-genome sequencing of spermatocytic tumors provides insights into the mutational processes operating in the male germline. PLoS One 12 (5), e0178169s. https://doi.org/10.1371/journal.pone.0178169.

Greene, M.H., Mai, P.L., Loud, J.T., et al., 2015. Familial testicular germ cell tumors (FTGCT)—Overview of a multidisciplinary etiologic study. Andrology 3, 47–58.

Hackett, J.A., Surani, M.A., 2014. Regulatory principles of pluripotency: From the ground state up. Cell Stem Cell 15, 416–430.

Kaku, H., Usui, H., Qu, J., Shozu, M., 2016. Mature cystic teratomas arise from meiotic oocytes, but not from pre-meiotic oogonia. Genes, Chromosomes & Cancer 55, 355–364.

Killian, J.K., Dorssers, L.C.J., Trabert, B., et al., 2016. Imprints and DPPA3 are bypassed during pluripotency and differentiation-coupled methylation reprogramming in testicular germ cell tumors. Genome Research 26, 1490–1504.

Lindeman, R.E., Gearhart, M.D., Minkina, A., et al., 2015. Sexual cell-fate reprogramming in the ovary by DMRT1. Current Biology 25, 764–771.

Mamsen, L.S., Brochner, C.B., Byskov, A.G., Mollgard, K., 2012. The migration and loss of human primordial germ stem cells from the hind gut epithelium towards the gonadal ridge. The International Journal of Developmental Biology 56, 771–778.

Oosterhuis, J.W., Looijenga, L.H.J., 2005. Testicular germ-cell tumors in a broader perspective. Nature Reviews Cancer 5, 210–222.

Oosterhuis, J.W., Looijenga, L.H.J., 2017. Germ cell tumors from a developmental perspective: Cells of origin, pathogenesis and molecular biology; emerging patterns. In: Nogales, F.F., Jimenez, R.E. (Eds.), Pathology and biology of human germ cell tumors. Springer Nature, Berlin, pp. 23–129.

Skakkebaek, N.E., Rajpert-De Meyts, E., Main, K.M., 2001. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Human Reproduction 16, 972–978.

Spencer, R., 2001. Parasitic conjoined twins: External, internal (fetuses in fetu and teratomas), and detached (acardiacs). Clinical Anatomy 14, 428–444.

Surani, M.A., Barton, S.C., Norris, M.L., 1986. Nuclear transplantation in the mouse: Heritable differences between parental genomes after activation of the embryonic genome. Cell 45, 127–136.

Glioblastoma: Biology, Diagnosis, and Treatment

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Glossary

Akt/PI3K signaling pathway A signal transduction pathway involving the phosphatidylinositol 3-kinase (P13K) and protein kinase B (Akt) proteins that promotes survival and growth in response to extracellular signals.
Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) A protein receptor that functions as an immune checkpoint and can downregulate immune response. It is over-expressed in tumor cells, thus helping them evade the body's immune system.
Epidermal growth factor receptor (EGFR) A transmembrane protein that is a receptor for members of the epidermal growth factor family of extracellular protein ligands. Mutations that lead to its over-expression are associated with various cancers including glioblastoma.
Glial fibrillary acidic protein (GFAP) A protein expressed by numerous cell types of the central nervous system including astrocytes, and involved in cell communication and the blood brain barrier. It is heavily expressed in glioblastoma.
Isocitrate dehydrogenase (IDH) An enzyme that catalyzes the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate and CO_2. Mutations in the isocitrate dehydrogenase gene IDH1 have been found in several brain tumors including glioblastoma and hold prognostic implications.
Mammalian target of rapamycin (mTOR) kinase A protein kinase that is involved in regulating cell growth, cell proliferation, cell motility and transcription, amidst other functions, and is implicated in the development of glioma.
Mitogen-activated protein (MAP) kinase A protein kinase involved in regulation of cell functions including proliferation, differentiation, mitosis, cell survival and apoptosis, and is implicated in the development of glioma.
O-6-methylguanine-DNA methyltransferase (MGMT) An enzyme encoded by the MGMT gene and is crucial for genome stability through its mechanism of repairing mutagenic DNA lesions and preventing mismatch and errors during DNA replication and transcription.
Phosphatase and tensin homolog (PTEN) gene The PTEN protein encoded by this gene acts as a tumor suppressor by negatively regulating the Akt/PKB signaling pathway.
Programmed cell death ligand 1 (PD-L1) A protein that suppresses the immune system by reducing proliferation and inducing apoptosis of T cells. Cancer cells upregulate expression of this protein and this may help them evade the body's immune system.
Telomerase reverse transcriptase (TERT) Subunit of the enzyme telomerase which comprises an important unit of the telomerase complex.
Tumor treating fields (TTF) Electromagnetic field therapy that applies low-intensity electrical fields to target areas containing proliferating tumor cells, impairing mitosis in these cells and thus selectively causing tumor cell death.
Vascular endothelial growth factor (VEGF) An important signaling protein that stimulates the formation of blood vessels.
World Health Organization (WHO) classification Classification of tumors of the central nervous system

History

In the mid-1800s German pathologist Rudolf Virchow coined the term "glioma" and became the first to attempt to classify this group of neoplasms. Glioblastoma, the most aggressive of these gliomas, was introduced by its name in 1926 by Percival Bailey and Harvey Cushing in their book "*A classification of the tumors of the glioma group on a histogenetic basis with a correlated study of prognosis*." The term was inspired by the fact that the tumor appeared to originate from primitive precursors—"glial cells"—and had a highly variable "multiform" appearance due to necrosis, hemorrhage, cysts, and blood vessels. Cushing and Bailey attempted to organize the gliomas into 10 groups, correlating with patient survival. Over the next few decades, various different classification systems were used until 1993, when the World Health Organization (WHO) introduced its grading system. Morphologic features continue to form the basis for classifying gliomas. Four distinct grades of glioma exist, with grades I representing circumscribed, generally slow-growing tumors and grades II, III, and IV representing diffusely infiltrating tumors with variable rates of growth. This classification was recently revised in 2016 and is discussed in greater detail below.

Epidemiology

Primary brain tumors have an incidence rate of 7 per 100,000 with a prevalence of almost 222 per 100,000 individuals. The incidence of primary brain tumors appears to be increasing over the last 30–40 years, especially in the elderly. Almost 35,000 new

diagnoses will be this year, and in 2017 alone, the condition will lead to almost 17,000 deaths. Of the primary brain tumors, 33% are malignant in nature. Glioblastoma is the most common form of malignant glioma, accounting for up to 70% of the cases. It is also the most aggressive and has the highest histological grade of the gliomas (WHO Grade IV). The typical age at presentation is between 45 and 55 years of age. Malignant gliomas including glioblastoma are almost twice as likely to occur in men than women, and twice as common in whites as in blacks.

Classification

The 2016 WHO classification of brain tumors categorizes glioblastoma by isocitrate dehydrogenase (IDH) status into IDH-wild type, IDH-mutant, and not-otherwise-specified subtypes. The IDH-wild type glioblastoma generally presents de novo (primary glioblastoma) in older (>50 years of age) patients, and 90% of glioblastomas fall into this subtype. These tumors exhibit epidermal growth factor receptor (*EGFR*) amplification and mutations, loss of heterozygosity of chromosome 10q, deletion of the phosphatase and tensin homolog (*PTEN)* on chromosome 10, and p16 deletion. The IDH-mutant glioblastoma typically present in younger patients with or without a previous diagnosis of a lower grade glioma that transforms to an aggressive, grade IV secondary glioblastoma. These have mutations in the p53 tumor suppressor gene, overexpression of the platelet derived growth factor receptor (PDGFR), abnormalities in the p16 and retinoblastoma pathways, and loss of heterozygosity of chromosome 10q. Primary and secondary glioblastomas differ at the molecular level, with differences in DNA copy number and their transcriptional patterns, but on a morphological level appear essentially identical. The glioblastoma NOS (not otherwise specified) is reserved for cases where the IDH status cannot be clarified further (**Table 1**).

A new variant to the 2016 classification is the epithelioid glioblastoma, which falls under the IDH-wildtype category. These tumors typically occur in children and young adults and are located in the cerebrum or diencephalon. They are characterized by large epithelioid cells with abundant eosinophilic cytoplasm and frequently have BRAFV600E mutations.

Etiology

The only environmental risk factor that has consistently demonstrated brain tumor risk has been exposure to therapeutic doses of ionizing radiation. Several studies have raised concern about cell phone usage and glioma, but to date the evidence overall has been inconsistent. These studies may be influenced by recall and selection bias. Diseases such as diabetes mellitus, hyperlipidemia, obesity, etc., have been evaluated and are not associated with any increased risk of developing glioblastoma. Epidemiological evaluations have noted that women have a lower glioma risk, and this protective effect is more obvious premenopause. However, the protective role of female hormones remains unclear and has still to be established conclusively. Head injury, diet, smoking, alcohol consumption, exposure to electromagnetic fields, prenatal exposure to drugs or medications, are some of the many factors that have been studied and the evidence has been minimal or inconclusive at best. Genetic factors have also been evaluated. While specific genetic polymorphisms are associated with specific types of gliomas, and may impact DNA repair and cell cycle regulation, patterns of occurrence are not always consistent with hereditary disease. Certain hereditary syndromes such as neurofibromatosis, Turcot and Li–Fraumeni syndromes are associated with increased brain tumor risk, but these account for a very small proportion of incident cases.

There is evidence that immunological factors that are related to allergic conditions and infections may have an impact on glioblastoma risk. Many studies evaluating risk of glioma in patients with allergic conditions (such as asthma or hay fever) or auto-immune diseases have found that risk of developing a glioma or glioblastoma is actually decreased in these conditions. Prior infection with certain viruses (herpes virus or varicella zoster virus, for instance) may also be associated inversely with adult glioma risk.

Table 1 Classification of glioblastoma under the 2016 WHO Guidelines

	Glioblastoma	
	IDH Wildtype	*IDH Mutant*
Type	Spontaneous, de novo	Transformation of lower grade glioma
Age distribution	Generally >50 years of age	Generally younger adults
Molecular characteristics	EGFR amplification	p53 Tumor suppressor
	Loss of heterozygosity of chromosome 10q	Gene mutations
	Deletion of PTEN	Overexpression of PDGFR
Prognosis	Worse	Better

Pathological Features

As already mentioned, glioblastoma is classified as WHO Grade IV, and thus has the highest grade of the malignant gliomas. Anaplastic astrocytomas, which are WHO Grade III, demonstrate high cell density, nuclear atypia, and mitotic activity on pathology. Glioblastoma is distinguished from the grade III pathology by the addition of abundant areas of endothelial proliferation or necrosis. Pseudopalisading necrosis may or may not be seen. IDH evaluation is routinely performed on all suspected glioblastomas, since the new classification system relies on knowing the wildtype or mutant status of the IDH gene. The tissue may also be evaluated for presence or absence of O-6-alkylguanine DNA alkyltransferase (*MGMT*) promoter methylation. Methylation of the MGMT promoter prevents gene transcription, reducing tumor cell DNA damage repair. Thus, methylation of the *MGMT* promoter makes the tumor cell more susceptible to chemotherapeutic agents that cause DNA damage, and subsequently, cellular death.

Cell of Origin and Signaling Pathways

Origins of glioblastomas and malignant gliomas have been explored since the first time the tumor was described by Dr. Harvey Cushing. Neural stem cells are proposed to be the primary origin of these tumor cells, primarily due to similarities in protein and cell surface marker expression. Neural stem cells have been known to express nestin, glial fibrillary acidic protein, and also a CD-133 cell surface marker that may lend properties of self-renewal to tumor cells.

Epidermal growth factor receptor (*EGFR*) and platelet derived growth factor receptor (*PDGFR*) are oncogenes that are often overexpressed in 40%–50% of primary glioblastomas. These likely create an autocrine loop that stimulates tumor proliferation. Moreover, several signal transduction mediators may impact the phosphatidylinositol-4,5-bisphosphate 3-kinase (*PI3-K*) pathways in these cells—these kinases are involved in cellular growth, proliferation, differentiation, and survival. These include the Ras-mitogen activated protein (*MAP*) kinase pathway and the mammalian target of rapamycin (*mTOR*) pathway. Mutation in or deletions of phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene, may result in increased activity of the *P13-K* pathway also. Molecular therapeutics are currently targeting these pathways in an attempt to inhibit cell growth and encourage apoptosis of tumor cells.

Clinical Features and Diagnostic Approach

Primary brain tumor patients can present with either focal or generalized signs and symptoms. Headaches, nausea, vomiting, fatigue, confusion or altered/depressed mental status are more generalized and fairly nonspecific symptoms. Focal seizures, weakness and/or sensory loss of a particular side or limb, language and visuospatial difficulties are focal symptoms that may be more alarming and prompt earlier care.

Headache has been known to be the most common initial presenting symptom, as evaluated in many studies of patients with high grade primary brain tumor. A new diagnosis of headache or a distinct change in headache pattern in a patient older than 50 should be considered a red flag. Chronic, persistent headache with protracted nausea, vomiting, especially with positional worsening should also be evaluated carefully. This is also true for headaches that present in the morning, or wake the patient up from sleep, or are provoked by Valsalva maneuvers, coughing or straining.

When concerned, evaluation can generally be initiated with a contrast enhanced computed tomography (CT) scan of the head. At times it may be reasonable to go directly towards a gadolinium enhanced magnetic resonance imaging (MRI) scan if index of suspicion is high enough. An MRI can also provide many clues toward the nature of the tumor, based on location, presence and degree of enhancement, diffusion restriction, and perfusion, but definitive diagnosis is still made by pathological examination of tissue after excision or biopsy. Imaging in glioblastomas often reveals irregularly shaped borders, and a ring-shaped zone of contrast enhancement around a dark central area of necrosis. Significant peritumoral edema may be seen on fluid attenuation inversion recovery (FLAIR) sequences. Necrotic tumors will also have a high apparent diffusion coefficient (ADC). ADC may be also helpful in estimating cellularity of a tumor (**Fig. 1**).

Tumor perfusion, as measured by relative cerebral blood volume (rCBV), tends to be elevated in glioblastoma. Proton magnetic resonance spectroscopy or MRS can detect levels of metabolites. Areas with malignant gliomas show an increase in the choline peak and a decrease in the *N*-acetyl aspartate or NAA peak when using the MRS. Positron emission tomography (PET) is being increasingly investigated, but thus far the data are insufficient to support its use in routine evaluation of central nervous system tumors (**Fig. 2**).

Prognosis

Overall median survival is still less than 15 months in unselected patients and further reduced in the elderly population. Two-year survival rate in patients 65–74 years of age is only 8.9%, and only 3.2% for those greater than 75 years of age. Over the years, several factors have been demonstrated to be related to prognosis in glioblastoma patients. In addition to age, these include the Karnofsky Performance Status score, and the extent of resection.

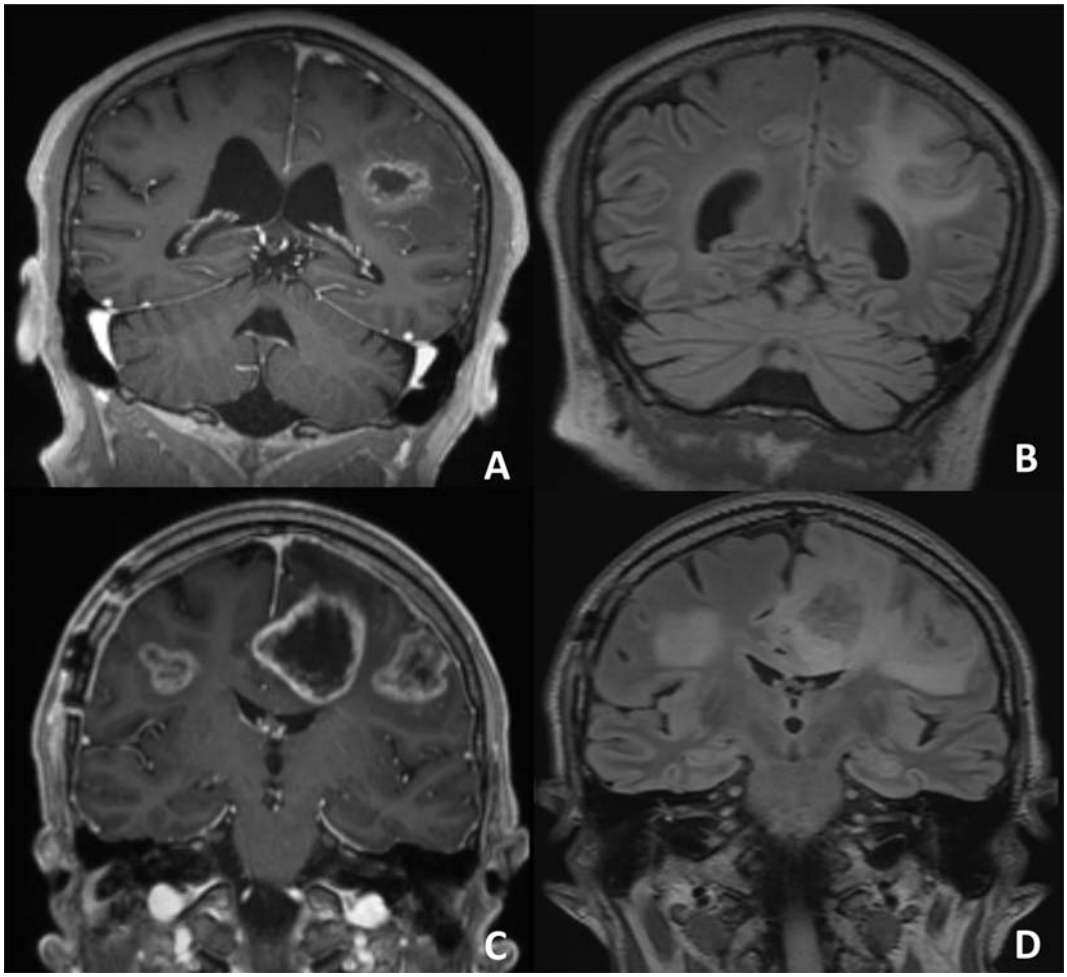

Fig. 1 Panel A and B Postcontrast T1-weighted and FLAIR MRI images showing left frontal lobe IDH wildtype glioblastoma; Panel C and D Postcontrast T1-weighted and FLAIR MRI images of a different patient with multifocal glioblastoma.

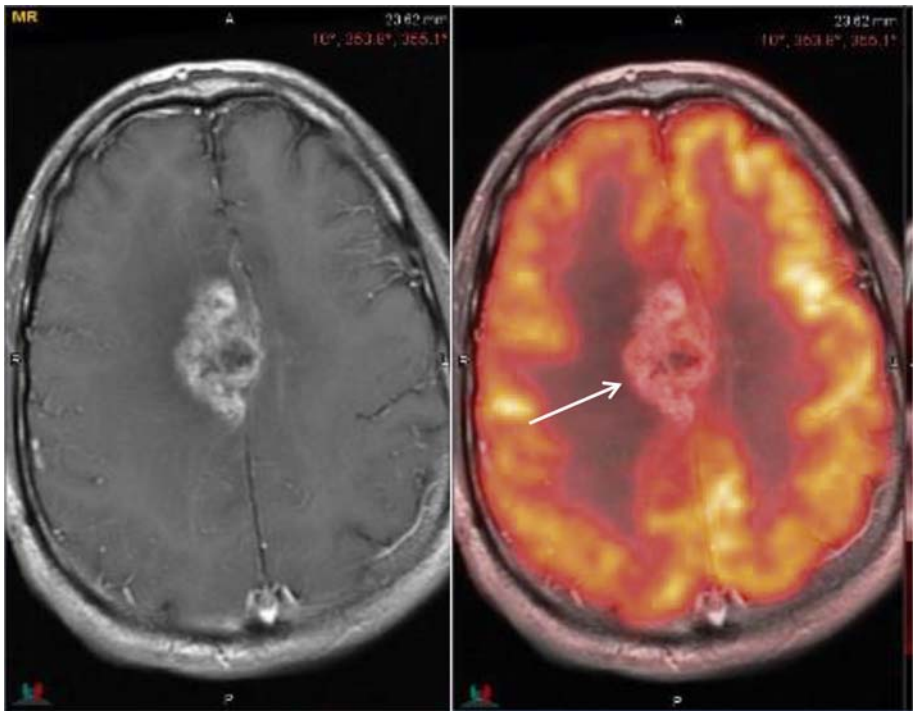

Fig. 2 PET/MRI of recurrent high grade glioma. *Arrow* points toward areas of increased uptake of radioactive label suggesting active disease.

Tumor IDH *mutation and MGMT* promoter methylation are extremely strong prognostic factors. Median survival of patients with IDH-mutant glioblastoma (which is a much smaller percentage) can be as high as 66.8 months in some cases. Similarly, patients with methylation of the *MGMT* promoter had a median survival of 21.7 months on standard of care versus 15.3 months for those with unmethylated *MGMT* promoter. A combination of MGMT promoter methylation and IDH1 mutation may have the best prognosis, thus, with an overall survival of up to 70 months in some series. It has also been noted that glioma patients with telomerase reverse transcriptase (TERT) mutations have poorer overall survival (**Figs. 3 and 4**).

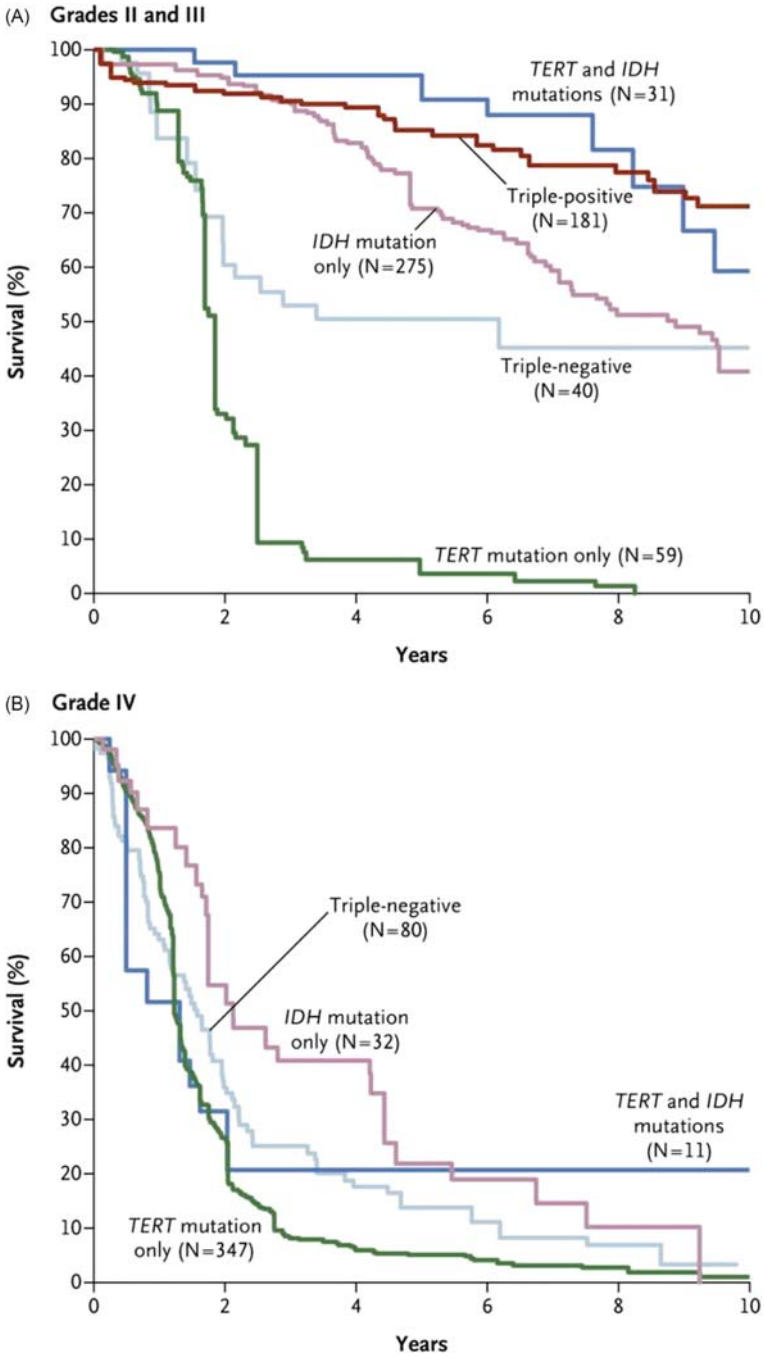

Fig. 3 Survival in glioblastoma by molecular subtype. Glioma patients with TERT mutations have poorer survival, while IDH mutations confer a survival benefit. Mutations of both TERT and IDH appears to be helpful for survival. From Eckel-Passow, J. E., Lachance, D. H., Molinaro, A. M., Walsh, K. M., Decker, P. A., Sicotte, H., et al. (2015). Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. *The New England Journal of Medicine* **372**(26), 2499–2508.

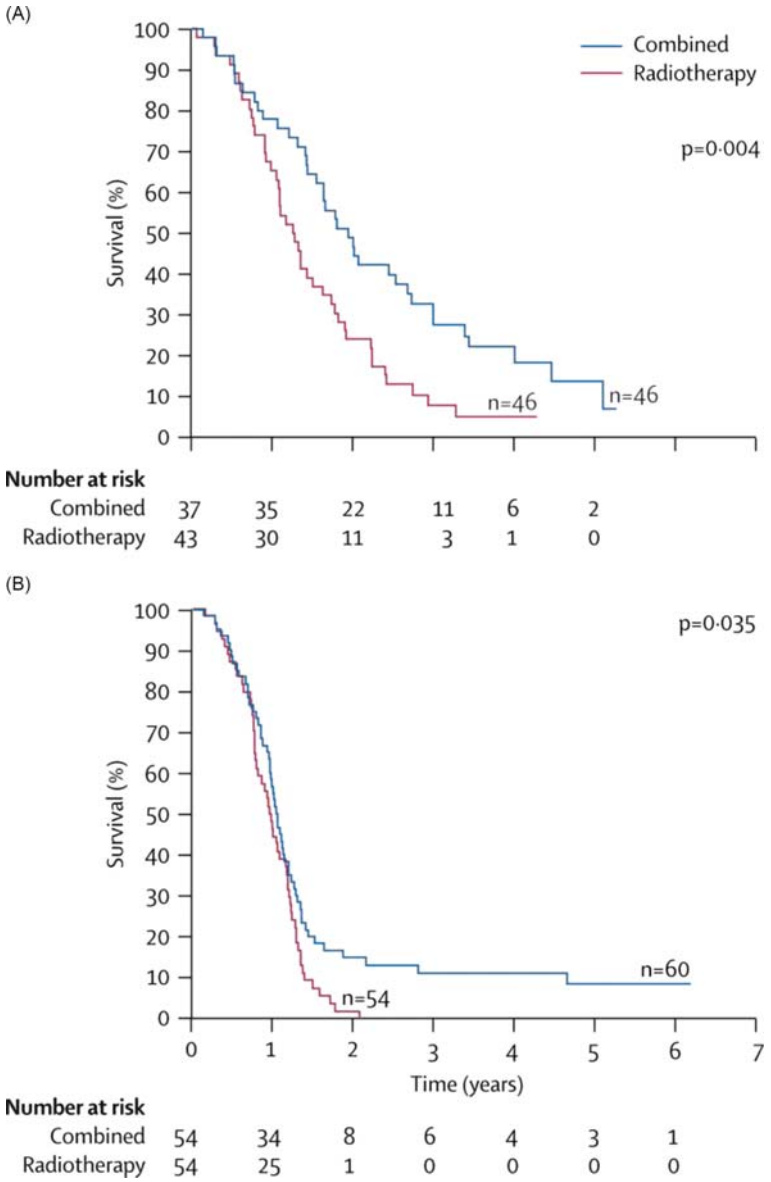

Fig. 4 Survival in glioblastoma by MGMT methylation status. Survival is longer with the combination of radiation therapy plus temozolomide in patients with either methylated or unmethylated tumors, but the treatment effect is found to be greater in patients with methylated tumors. From Stupp, R., Hegi, M. E., Mason, W. P., van den Bent, M. J., Taphoorn, M. J., Janzer, R. C., et al. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncology* **10** (5), 459–466.

Treatment

There is currently no cure for glioblastoma. Whenever possible, patients should be offered a clinical trial as a part of their therapy plan. Maximal safe resection has been recommended as the standard of care for high grade gliomas such as glioblastoma. Various retrospective studies and meta-analyses have established that obtaining a gross total resection (GTR) when possible can improve outcomes including overall survival when compared to a subtotal resection or biopsy alone. Tumor location often dictates safety and feasibility of the resection, since morbidity of the surgery has to be balanced with the benefit from resection.

Radiation is the second pillar of therapy in glioblastoma. It should be initiated as soon as it is safe after surgical intervention, and is generally started between 3 and 6 weeks from surgery, barring any complications or infection. For patients who are younger than 65 with Karnofsky performance status greater or equal to 60, optimal dose fractionation for external beam radiation therapy after resection or biopsy is 60 Gy in 2-Gy fractions, delivered over 6 weeks. This schedule has been repeatedly demonstrated to have the maximal benefit. Elderly patients greater than 65 with a Karnofsky greater or equal to 50 are also advised external beam radiation therapy, but recent studies have demonstrated that hypofractionated radiotherapy has similar survival, lesser side effects, including

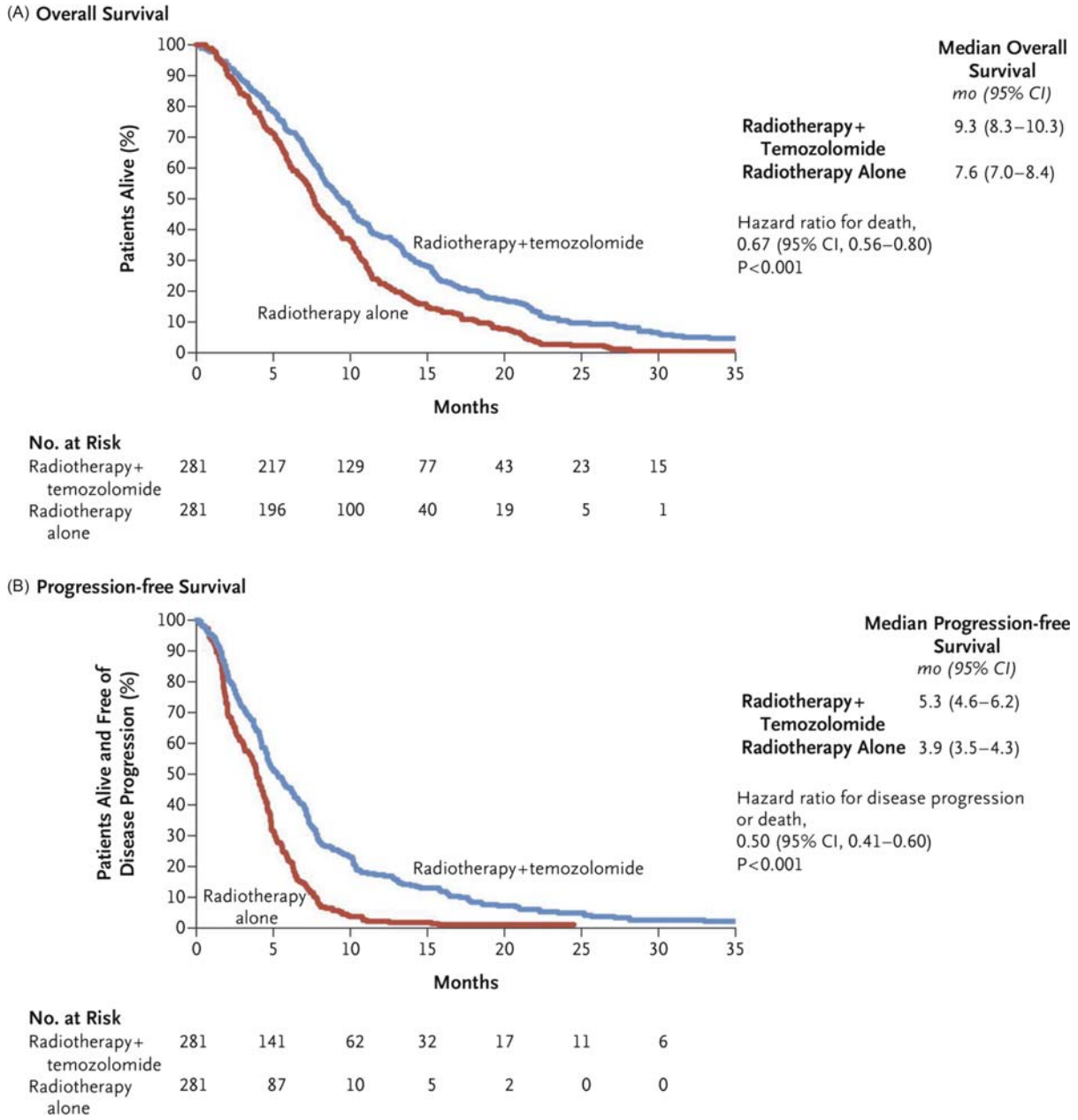

Fig. 5 Overall and progression-free survival in elderly patients treated with hypofractionated radiation in combination with chemotherapy. From Perry, J. R., Laperriere, N., O'Callaghan, C. J., Brandes, A. A., Menten, J., Phillips, C. et al. (2017). Short-course radiation plus temozolomide in elderly patients with glioblastoma. *The New England Journal of Medicine* **376**(11), 1027–1037.

lower steroid requirement during treatment. This is a dosing of 40 Gy in 15 fractions over 3 weeks instead of the schedule described above. Patients with poor Karnofsky performance status may receive hypofractionated radiotherapy alone, chemotherapy alone, or best supportive care. Partial brain radiation therapy is recommended, since no benefit has been seen with whole-brain radiation therapy. Radiation techniques involve using image guided radiotherapy and intensity modulated radiotherapy to deliver a high dose of radiation to the target area, while delivering a lower dose to the margin, thus sparing healthy surrounding tissue. While the evidence is weak, reirradiation to focal areas of recurrence with stereotactic radiosurgery or hypofractionated radiotherapy has been tried and may improve outcomes (Fig. 5).

Chemotherapy is the third and integrally important component of treatment. Prior to 2005, standard therapy consisted of maximal safe resection followed by radiotherapy and adjuvant carmustine, a nitrosourea. Significant survival benefit was not established with this regimen. In 2005, reported results using the "Stupp protocol" changed the management of glioblastoma patients. Patients receiving temozolomide, an oral methylating agent, with concurrent radiotherapy, followed by six monthly cycles of adjuvant temozolomide experienced prolongation of median survival by 2.5 months. Two year survival with this regimen

improved by 16%. In addition, temozolomide is taken orally, and is generally well tolerated. The more common side effects included fatigue, headache, nausea, gastrointestinal discomfort. Grade 3 or 4 cytopenias are uncommon. Following the results of this trial, standard of care for newly diagnosed glioblastoma transitioned to maximal surgical resection followed by radiotherapy, generally at a dose of 60 Gray given in 30 fractions over 6 weeks, with concomitant temozolomide at 75 mg/m^2 per day for the 42 days of radiotherapy. Four weeks after radiation, adjuvant temozolomide is given at 150–200 mg/m^2 on days 1–5 of each 28 day cycle, for 6 cycles. Treatment effect is greatest in patients with MGMT promoter-methylated tumors.

Carmustine wafers are another Food and Drug Administration (FDA) approved chemotherapeutic approach that may be undertaken with newly diagnosed glioblastoma at time of resection. Small polymers containing carmustine are implanted into the tumor bed, with the idea that slow release of the drug over the course of several weeks may kill residual tumor cells. Median survival has been seen to improve by approximately 2 months with integrated use of these polymers in newly diagnosed glioblastoma. These wafers do have significant limitations, and cannot be offered to every patient. Adverse effects in most studies include brain edema, seizures, wound-healing problems, infection, thrombosis, and intracranial hypertension.

The NovoTTF-100A system known as Optune was recently approved by the FDA for patients with recurrent and newly diagnosed glioblastoma. This is a portable, noninvasive device that generates low intensity, intermittent frequency alternating electric fields and delivers them via transducer arrays that are placed directly on the patient's scalp. The electrical fields, also called tumor-treating fields, have antimitotic effects by interfering with mitotic spindle cell formation and chromosomal segregation during tumor cell division. Usage compliance rates of >18 h a day are required for the best outcomes. Treatment begins in conjunction with temozolomide following completion of radiation therapy. When combined with temozolomide, median survival has been shown to improve to 20.5 months from 15.6 months with chemotherapy alone. Recent data from Stupp and colleagues indicate a 2 year survival rate of 43% in the TTF-treated group versus 31% in the group treated with standard of care alone. At 5 years, the difference remained—13% in the treatment group and 5% with standard of care alone.

Unfortunately glioblastoma almost always recurs. When it does recur, treatment options vary. Reoperation may be feasible and is often offered depending on the functional status of the patient and tumor (recurrence) location, size, and safety of the resection. There are no definitive data yet that the second surgery provides survival benefit, but it may be helpful to confirm the lesion is indeed recurrence and not radiation necrosis or inflammation. Radiotherapy for recurrent glioblastoma is controversial at this time and though frequently used, has not been established as having clear survival benefit. This is being evaluated in clinical trials. Stereotactic radiosurgery may be an option that can benefit these patients that have a small relatively well-circumscribed pattern of recurrence.

Carmustine wafers have been used in patients with recurrent glioblastoma with modest increase in time to progression. Several other chemotherapy agents have been assessed, including lomustine, procarbazine with lomustine, and vincristine (PCV), carboplatin, irinotecan, etoposide, and others. However, benefits of these agents are modest at best, with no evidence that any of the agents improves survival. Rechallenge with temozolomide has been studied, with inconclusive results.

Bevacizumab, a vascular endothelial growth factor (VEGF) inhibitor was approved for use in recurrent glioblastoma in 2009. It can be used as a single agent or in combination with the drugs mentioned above. Clinical trials thus far have demonstrated that bevacizumab may improve progression free survival, but does not improve overall survival. It does prove to be very useful in treating peritumoral edema and radiation necrosis in the appropriate patient, thus improving quality of life.

The NovoTTF-100A device has also been evaluated in patients with recurrent glioblastoma and compared with physician's choice of chemotherapy. There was no difference in outcomes between the two treatments.

General Medical Management

Apart from treating the tumor, patients with glioblastoma also require management of morbidity associated with their condition and/or its treatment. Seizures, peritumoral edema, venous thromboembolism, fatigue, headaches, and cognitive dysfunction are some of the most common problems encountered in care of the glioblastoma patient. Patients may also develop significant functional disability over the course of their disease.

Prevalence of seizures can be as high as 50% in glioblastoma patients. Patients with temporal lobe tumors and those with hemorrhage in the bed of the tumor may be at a higher risk of seizures. No statistically significant benefit has been seen thus far for the prophylactic use of antiepileptics in patients with tumors, even in those treated with a craniotomy for resection. Antiepileptics should only be started when patient has a clearly witnessed seizure or provides history that is concerning enough for a seizure. A single event is usually enough to start an antiepileptic in this high risk population, and an EEG is not always required if the clinical history is convincing.

The choice of antiepileptics is based on many considerations, including ease of dosing and tolerance, the side effects and the drug interactions. Generally, drugs that induce the hepatic cytochrome *p*-450 enzymes (such as phenytoin or carbamazepine) are avoided since they can increase the metabolism of many chemotherapeutic agents. Phenytoin should also be avoided in patients once patients have received cranial radiation, given higher risk of rash in this population. Levetiracetam, lamotrigine, pregabalin, and lacosamide are generally more preferred in this population. They appear to be better tolerated and have fewer drug interactions. Levetiracetam is perhaps the most frequently prescribed antiepileptic for this patient population. Side effects in a small percentage of people include irritability, aggression, hostility, suicidality, and depression. Lacosamide also has few drug interactions, and has fewer of these psychiatric side effects, but can have cardiac side effects and is quite expensive. Valproic acid in recent years has been

investigated for antiglioma properties in addition to anticonvulsant properties, as it may function as a radiosensitizer in glioblastoma patients. However, it does have side effects of teratogenicity, hyperammonemia, alopecia and weight gain, and thus has to be used in the appropriate patient.

Patients often complain of fatigue more than any other symptom, and can impact their overall sense of well-being. The disease as well as radiation and chemotherapy all contribute to this symptom. Management should include addressing reversible factors such as medications (anticonvulsants, opioids, antiemetics), diet and vitamin intake, sleep quality, anemia, and nutritional deficiencies. Aerobic exercise and steroids may improve fatigue. Psychostimulants such as modafinil and methylphenidate have been studied with mixed results, but may be appropriate for some patients.

Headache is experienced by up to 50% of patients with brain tumors and can get worse toward the end of life. Management of headache in glioblastoma depends on the stage of disease and overall functionality. Steroids may alleviate symptoms when edema is contributing but cannot be used indefinitely due to side effects. Antiinflammatory agents may be helpful when used in a limited manner to avoid overuse and kidney or gastrointestinal injury. Prophylactic agents such as gabapentin and topiramate may be beneficial but have side effects that may outweigh the benefits in some patients. At the end of life, opioids may be preferred, weighing the risk of sedation with pain control.

Peritumoral edema can be quite disabling, resulting in focal weakness and even coma. Steroids can treat and control edema in the glioblastoma patient, rapidly alleviating the symptoms. Steroids also work in alleviating nausea, treating headaches, and improving appetite. However, steroids also have significant side effects such as hyperglycemia, weight gain, myopathy, psychosis, delirium, and irritability. In an acute setting, the glioblastoma patient may be disabled by edema from the tumor. The goal should be to start with the lowest possible dose and taper down as quickly and safely as possible. Calcium/vitamin D supplementation and a proton pump inhibitor or histamine receptor blocker should be prescribed with the steroids for bone health and gastrointestinal prophylaxis, respectively. Bevacizumab may be another agent that can decrease peritumoral edema in these cases and reduce the need for steroids. Wider use for this indication, however, is limited due to high cost.

Glioblastoma patients are at an increased risk of venous thromboembolism. Anticoagulation therapy is recommended and safe for venous thrombo-embolism unless there is obvious systemic or intracerebral hemorrhage. Intratumoral hemorrhage rates in anticoagulated GBM patients are relatively low. Low molecular weight heparin can be safe and more effective than warfarin in this population, and is thus frequently used. Oral thrombin inhibitors have not yet been studied in this specific patient population; in fact, data on their safety and efficacy in cancer patients receiving chemotherapy at large is lacking.

Neurocognitive problems may be a presenting symptom of brain tumors or present later in the course of the disease. Impairment in executive functioning, memory, and attention are the areas where deficits are most commonly noted. Memantine has been trialed in a small group of patients—when initiated within 3 days of radiotherapy for 24 weeks, cognitive function over time may be better preserved. No other preventive interventions have been clearly established thus far. Several small studies have noted that methylphenidate at 10 mg twice a day can improve cognition and mood as well as functional status in high grade glioma patients, and can be taken with minimal adverse effects. Modafinil has also been seen to have a beneficial effect in speed of processing and executive function requiring attention in some studies. Fatigue and mood may also improve with this drug.

Prospective Vision

The neuro-oncology field, similar to the cancer therapy field at large, is in the midst of a variety of exciting new research that hopes to create new targeted therapy for primary brain tumors.

One of these areas is immunotherapy—agents developed for advanced systemic tumors have demonstrated significant clinical benefit but this has not yet been true for glioblastoma despite decades of research in the field. The T cell response is key in this process—antigens released from tumor cells at their death are presented to T cells by antigen presenting cells, which in turn prime the T cells against these antigens. Checkpoint pathways between the antigen presenting cells, tumor cells, and T cells, provide signals that activate or inhibit T cells and thus regulate this immune response. The brain, despite being highly protected by the blood brain barrier, does communicate with the immune system since we now know that activated cells are able to traffic through the barrier and the CNS lymphatic system also facilitates immune surveillance. The rationale is that antibodies to checkpoint inhibitors—specifically to cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed cell death-ligand (PD-L1)—could result in increased susceptibility of glioblastoma to an immune response. These are being investigated in clinical trials but at this point, a single phase III trial using one of these agents showed no benefit in the setting of recurrent glioblastoma, but numerous other clinical trials are in early stages and hold promise. Studies are also investigating the combination of these immunotherapies with established therapies, and with radiotherapy. One limitation is that we are still working on understanding how these specific pathways work in the brain and how glioblastoma specifically evades the immune system. It is important to ensure that these treatments will be safe in the CNS, where an intense immune response could have the potential of inflammatory damage to the brain. Assessing response in glioblastoma, and differentiating inflammation from progression, will also be key challenges.

Various vaccine-based therapies are also under investigation. One approach utilizes a mixture of autologous tumor cells with allogeneic glioblastoma cell populations that have been inactivated with irradiation. Vaccines with antigen-presenting dendritic cells primed ex vivo with glioblastoma antigens and peptide vaccines to enhance the immune response are in clinical trials. For

example, rindopepimut, an EGFRvIII peptide combined with an adjuvant, failed to demonstrate survival benefit in patients with newly diagnosed glioblastoma, but is currently being studied in combination with bevacizumab in patients with recurrent disease.

In recent years T cell therapy has gained a lot of attention in the field of oncology in general, and neuro-oncology is no different. Tumor infiltrating lymphocytes can be harvested, expanded in vitro and re-infused into the patient with the hope that a cytokine immune response will lead to cell death of the tumor. Engineered T cells—high avidity tumor specific transgenic T cells or TCR, and chimeric antigen receptor or CAR T cells—are being investigated specifically in glioblastoma.

Viruses that can infect tumor cells and lead to host cell death are an attractive concept. Various viruses are being investigated in phase I and II trials at this point, including herpes simplex virus-1, adenovirus, poliovirus, zika virus, measles virus, and parvovirus. However, many challenges must be overcome at this point. One is safety—only malignant tumor cells must be infected and healthy brain tissue surrounding the tumor margins must be spared. In addition, the likely immune response in the brain to the virus must protect the brain without irreversible inflammatory damage, and also not diminish the impact of the viral agent itself. This area continues to carry much excitement and hope for the future, as researchers continue to troubleshoot these aspects.

Advances in surgical and imaging techniques also hold promise for the future. While it has been established that maximal safe resection improves prognosis in glioblastoma, achieving that gross total resection has been a challenge on the operating table given limitations in visualizing tumor margins. To this end, advances in imaging have allowed for improved volumetric analyses and surgical planning. Fluorescence guided surgery ("tumor paint") has been noted to help improve extent of resection and in turn overall survival in advanced glioma. Intraoperative MRI guidance has also been used in several centers to achieve maximal resection with subsequent improvement in progression free survival. Other technologies increasingly available to the surgeon include cortical and subcortical stimulation mapping and intraoperative ultrasonography, techniques that can help preserve normal and essential brain tissue while helping resection of tumor.

The field of radiation therapy has had many exciting advances in recent years and we have learned more about what is both efficacious and safe for our patients. Proton beam radiotherapy has received significant attention in recent years for several malignancies, including glioblastoma. The hope is that proton therapy will offer the same or improved benefit with less toxicity since the radiation exposure to normal tissue is less than with conventional photon beam therapy. Trials investigating this in glioblastoma are on going. The future also holds promise with tractography and functional mapping to better diagnose and delineate anatomy. Diffusion tensor imaging tractography may be able to help differentiate between gross tumor and infiltrative margins, allowing for safer treatment. [^{18}F]-fluoromisonidazole has been used to identify hypoxic areas in tissue and is being explored as a means to identify specific different regions within the tumor and tailor treatment doses accordingly. Improved imaging techniques will continue to enable the radiation oncologist to provide the best treatment for the tumor bed, thus impacting overall and progression free survival.

See also: Glioblastoma: Pathology and Genetics.

Further Reading

Bernstein, M., Berger, M.S., 2014. Neuro-oncology: The essentials, 3rd edn. Thieme, Stuttgart.

Brown, C.E., Alizadeh, D., Starr, R., Weng, L., Wagner, J.R., Naranjo, A., et al., 2016. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. New England Journal of Medicine 375 (26), 2561–2569.

D'Amico, R.S., Englander, Z.K., Canoll, P., Bruce, J.N., 2017. Extent of resection in glioma—A review of the cutting edge. World Neurosurgery 103, 538–549.

Diaz, R.J., Ali, S., Qadir, M.G., De La Fuente, M.I., Ivan, M.E., Komotar, R.J., 2017. The role of bevacizumab in the treatment of glioblastoma. Journal of Neuro-Oncology 133, 455–467.

Eckel-Passow, J.E., Lachance, D.H., Molinaro, A.M., Walsh, K.M., Decker, P.A., Sicotte, H., et al., 2015. Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. New England Journal of Medicine 372, 2499–2508.

Farrell, C.J., Plotkin, S.R., 2007. Genetic causes of brain tumors: Neurofibromatosis, tuberous sclerosis, von Hippel-Lindau, and other syndromes. Neurologic Clinics 25, 925–946.

Fisher, J.L., Schwartzbaum, J.A., Wrensch, M., Wiemels, J.L., 2007. Epidemiology of brain tumors. Neurologic Clinics 25, 867–890.

Gardeck, A.M., Sheehan, J., Low, W.C., 2017. Immune and viral therapies for malignant primary brain tumors. Expert Opinion on Biological Therapy 17, 457–474.

Halani, S.H., Babu, R., Adamson, D.C., 2017. Management of glioblastoma multiforme in the elderly: A review of the literature. World Neurosurgery 105, 53–62.

Hegi, M.E., Diserens, A.C., Gorlia, T., Hamou, M.F., de Tribolet, N., Weller, M., et al., 2005. MGMT gene silencing and benefit from temozolomide in glioblastoma. New England Journal of Medicine 352, 997–1003.

Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Ellison, D.W., Figarella-Branger, D., Perry, A., Reifenberger, G., Deimling, A., 2016. WHO classification of tumors of the central nervous system, 4th edn. World Health Organization, Geneva.

Mrugala, M.M., 2013. Advances and challenges in the treatment of glioblastoma: A clinician's perspective. Discovery Medicine 15, 221–230.

Ostrom, Q.T., Gittleman, H., Xu, J., Kromer, C., Wolinsky, Y., Kruchko, C., et al., 2016. CBTRUS statistical report: Primary brain and other central nervous system tumors diagnosed in the United States in 2009–2013. Neuro-Oncology 18, v1–v75.

Preusser, M., Lim, M., Hafler, D.A., Reardon, D.A., Sampson, J.H., 2015. Prospects of immune checkpoint modulators in the treatment of glioblastoma. Nature Reviews Neurology 11, 504–514.

Schiff, D., Lee, E.Q., Nayak, L., Norden, A.D., Reardon, D.A., Wen, P.Y., 2015. Medical management of brain tumors and the sequelae of treatment. Neuro-Oncology 17, 488–504.

Stupp, R., Mason, W.P., van den Bent, M.J., Weller, M., Fisher, B., Taphoorn, M.J., et al., 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. New England Journal of Medicine 352, 987–996.

Stupp, R., Taillibert, S., Kanner, A.A., Kesari, S., Steinberg, D.M., Toms, S.A., et al., 2015. Maintenance therapy with tumor-treating fields plus Temozolomide vs Temozolomide alone for glioblastoma: A randomized clinical trial. Journal of the American Medical Association 314, 2535–2543.

Walbert, T., Chasteen, K., 2015. Palliative and supportive care for glioma patients. Cancer Treatment and Research 163, 171–184.

Relevant Websites

http://www.abta.org—American Brain Tumor Association.
http://www.braintumor.org—National Brain Tumor Foundation.
http://www.cbtrus.org/—Central Brain Tumor Registry of the US (CBTRUS).
http://www.nccn.org—National Comprehensive Cancer Network Central Nervous System Guidelines.
http://www.sciencedirect.com/science/referenceworks/9780080450469—Encyclopedia of Neuroscience.
https://seer.cancer.gov/—Surveillance, Epidemiology, and End Results Program.
http://www.uptodate.com—UpToDate.

Glioblastoma: Pathology and Genetics

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Introduction

Gliomas are a very diverse group of glial neoplasms and account for the great majority of tumors originating in the parenchyma of the central nervous system (CNS). Two main categories of gliomas are recognized: diffuse gliomas, by far the most frequent gliomas in adult patients and characterized by extensive infiltrative growth into the surrounding CNS parenchyma, and gliomas with a much more circumscribed growth pattern ("nondiffuse gliomas"). Traditionally, diffuse gliomas are further classified based on their microscopic similarities with (precursors of) glial cells and then designated as astrocytoma, oligodendroglioma, or mixed glioma/oligoastrocytoma. After assessment of the glioma type, a malignancy grade is assigned to these tumors based on especially the following histologic features: marked mitotic activity, florid microvascular proliferation, and necrosis. Diffuse astrocytic tumors showing marked mitotic activity, as well as necrosis and/or florid microvascular proliferation are diagnosed as glioblastoma. Glioblastomas account for about 15% of all primary CNS tumors and for over 50% of all gliomas. Even though extracranial metastases of gliomas are very rare, most glioblastoma patients die within 1–2 years after diagnosis, despite today's standard of care (surgery, radiotherapy, and chemotherapy). Many patients with a lower grade (WHO grade II or III) diffuse astrocytoma survive (much) longer, but virtually all these patients will eventually experience progression to glioblastoma resulting in fatality.

For a long time, microscopic evaluation has provided the gold standard for the diagnosis of gliomas, assessment of prognosis and formed the basis for therapeutic management. Multiple studies, however, showed that a purely histopathologic classification suffers from considerable inter- and intraobserver variability. Especially in the course of the last decade it became clear that molecular characteristics provide a more robust and objective basis for subtyping of diffuse gliomas. Indeed, in the revised 4th edition of the WHO classification of CNS tumors (published in 2016), classification of diffuse gliomas has fundamentally changed as for the first time presence/absence of particular molecular aberrations is now part of the definition of these tumors. After providing some essentials of the traditional, histopathology-based classification of glioblastomas, this review discusses novel insights in molecular characteristics of (different subgroups of) these neoplasms and how this information is now used for a combined histomolecular diagnosis. Furthermore, information is provided on how based on tools like expression profiling and methylation profiling additional subgroups can be recognized, while in the last part some future perspectives are given with regard to where the field is or may be heading.

From Histopathologic to Histomolecular Classification

For over a century, microscopic analysis of tumors of the CNS has been performed on histochemically, especially hematoxylin-and-eosin (H&E) stained sections, later on supplemented by electron microscopy and immunohistochemistry. Diffuse gliomas represent the vast majority of glial neoplasms in adult patients and are microscopically characterized by diffuse infiltrative growth within the CNS parenchyma, with tumor cells extensively invading individually or in small groups in the neuropil along myelinated fiber tracts and along blood vessels. This invasive growth may well extent over several centimeters outside the abnormal area as seen on magnetic resonance imaging (MRI) scans. Also, not infrequently the tumor cells of diffuse gliomas cross the corpus callosum, resulting in a "butterfly glioma" pattern on MRI. Especially in the less cellular areas of diffuse gliomas the matrix may consist of relatively intact preexistent gray and white matter with aggregation of neoplastic cells around neurons (perineuronal satelitosis), blood vessels, and under the pial membrane. Such "secondary structures of Scherer" are nearly pathognomonic of diffuse glioma. Occasionally, a diffuse glioma may present radiologically as multiple lesions (multifocal or multicentric glioma) resembling brain metastases or abscesses while it in fact concerns multiple foci of high cellularity, microvascular proliferation and/or necrosis in a widespread diffuse glioma.

Based on their histopathologic phenotype, diffuse gliomas are traditionally typed as astrocytic, oligodendroglial, or mixed oligodendroglial-astrocytic, and graded as WHO grade II (low grade), III (anaplastic), or IV (glioblastoma). Diffuse gliomas without mitotic activity, microvascular proliferation, and necrosis are graded as low-grade (WHO grade II). With increased mitotic activity, the diagnosis of anaplastic astrocytoma or anaplastic oligodendroglioma (WHO grade III) is rendered, while in astrocytic tumors the presence of necrosis and/or microvascular proliferation leads to a diagnosis of glioblastoma (WHO grade IV). In contrast, pure oligodendroglial tumors with necrosis and florid microvascular proliferation are still graded as WHO grade III. Necrosis in glioblastomas often consists of irregular, serpiginous foci surrounded by densely packed, somewhat radially oriented tumor cells ("palisading necrosis").

Glioblastoma is by far the most frequent and most malignant diffuse glioma and was previously called "glioblastoma multiforme" because of the often striking intratumoral cytologic and histologic heterogeneity. Furthermore, several histologic subtypes of glioblastoma are listed in the WHO 2016 classification, including the small cell variant of glioblastoma (predominantly showing small, relatively monomorphous tumor cells with little cytoplasm which may resemble anaplastic oligodendroglioma),

gliosarcoma (with a sarcoma-like, reticulin-rich component), giant cell glioblastoma (characterized by the presence of many large, multinucleated tumor cells), epitheloid glioblastoma (often apparently more circumscribed tumors showing prominent epitheloid or even rhabdoid morphology and thereby showing resemblance with metastastic carcinoma or melanoma), glioblastomas with primitive neuronal component (with nodular foci of primitive neuronal cells resembling embryonal tumor), and granular cell glioblastoma (with histiocyte-like cytoplasmic granularity and vacuolation). (Fig. 1).

Until the most recent World Health Organization (WHO) classification of CNS tumors published in 2016, the histologic diagnosis was the gold standard for classification of gliomas. In daily clinical practice, however, unequivocal typing and grading of gliomas can be difficult. For instance, because diffuse gliomas often show marked phenotypic heterogeneity with spatial differences in cellular phenotype, degree of anaplasia and mitotic activity there is a danger of underestimation of malignancy grade because of sampling error. Furthermore, the minimum diagnostic criteria of, for example, florid microvascular proliferation are not clear. Even identification of necrosis may be troublesome in biopsy samples that are small or poorly preserved, and in recurrent gliomas discrimination of native tumoral from therapy-induced necrosis may be difficult.

Meanwhile, since 2008 multiple studies showed that based on the presence or absence of mutations in the *isocitrate dehydrogenase 1* (*IDH1*) or *IDH2* gene and of complete, combined loss of the short arm of chromosome 1 and of the long arm of chromosome 19 (complete 1p/19q-codeletion) three major, clinically relevant subgroups of diffuse glioma can be defined. Indeed, the International Society for Neuropathology—Haarlem Consensus Guidelines and the subsequently published, revised 4th edition of the WHO classification of CNS tumors published in 2016 embraced this notion of an integrated histomolecular classification of diffuse gliomas, and the WHO classification nowadays recognizes the following three major molecular diffuse glioma subgroups:

- *IDH-wildtype*: most of these histologically represent astrocytic tumors, a large percentage belonging to the highest malignancy grade, that is, glioblastomas;
- *IDH-mutant and 1p/19q-non-codeleted*: these tumors also generally have an astrocytic phenotype, but a much larger percentage is at first diagnosis histologically lower grade/WHO grade II or III;
- *IDH-mutant and 1p/19-codeleted*: most of these are characterized by a prominent oligodendroglial phenotype of the tumor cells.

In this new classification, the presence of complete 1p/19q-codeletion in an IDH-mutant diffuse glioma is considered pathognomonic for oligodendroglioma, while IDH-mutant oligodendroglioma-appearing tumors lacking this event are reclassified as astrocytoma, IDH-mutant. Following this strategy, the diagnosis of oligoastrocytoma can be expected to largely disappear, except when additional molecular tests cannot be performed or do not provide unequivocal results; in that situation, not-otherwise-specified (NOS) should be added to the diagnosis to indicate that ideally such samples require further workup. Similarly, molecular studies have now also revealed that most cases previously diagnosed as glioblastoma with oligodendroglioma component (GBM-O) fit in one of the three categories just mentioned (i.e., can be diagnosed as glioblastoma IDH-wildtype, glioblastoma IDH-mutant, or as anaplastic oligodendroglioma IDH-mutant and 1p/19q-codeleted) and GBM-O has therefore been removed from the WHO 2016 classification as a diagnosis.

In the following paragraphs three molecularly defined groups of glioblastoma will be discussed in more detail: IDH-wildtype glioblastoma, IDH-mutant glioblastoma, and diffuse midline glioma H3 K27M-mutant. This last category was added in the

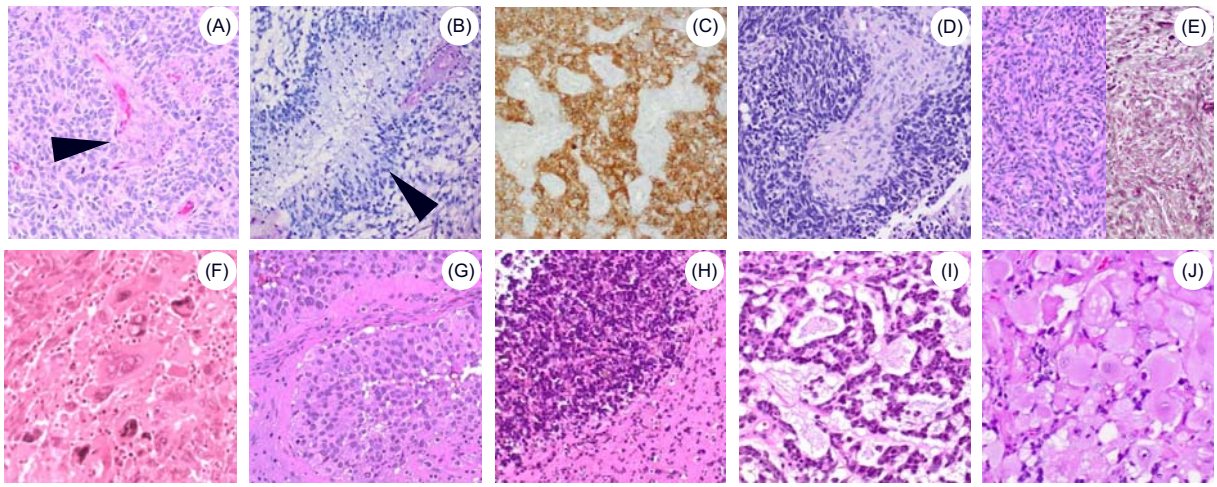

Fig. 1 Examples of histopathology of glioblastoma, including some histologic variants (=commonly accepted subtypes associated with particular clinical features) and patterns (=histological appearances without any currently identified clinical significance). (A) Florid microvascular proliferation (arrowhead); (B) palisading necrosis (arrowhead); (C) immunohistochemical demonstration of IDH1 R132H mutant protein in glioblastoma, IDH-mutant (molecularly defined entity); (D) small cell glioblastoma (pattern); (E) gliosarcoma (variant); (F) giant cell glioblastoma (variant); (G) epitheloid glioblastoma (variant); (H) glioblastoma with primitive neuronal component (pattern); (I) adenoid glioblastoma (pattern); (J) granular cell glioblastoma (pattern). Images A, B and D–J are from hematoxylin-and-eosin stained sections, apart from the right half of image E where a reticulin stain highlights the widespread presence of reticulin fibers in between the sarcomatoid tumor cells.

WHO 2016 classification as a separate entity. H3 K27M-mutant diffuse midline glioma generally occurs in children and should be located in the "midline" of the CNS (defined as brainstem, thalamus, cerebellum and/or spinal cord). Diffuse intrinsic pontine glioma (DIPG) is a frequent representative in this category.

Glioblastoma, IDH-Wildtype

The current WHO classification thus separates glioblastomas according to their IDH and H3 K27M status. Approximately 90%–95% of adult patients with glioblastoma have an IDH-wildtype (and H3 K27-wildtype) tumor. In most of these patients the tumor presents as a "primary glioblastoma," that is, a glioblastoma without clinical, radiological and/or pathological evidence of a lower grade precursor lesion. Data from large epidemiological cohorts are currently not yet available for IDH-wildtype versus IDH-mutant glioblastomas. The most recent CBTRUS report (published in 2017) on the occurrence of CNS tumors in the USA in the period 2010–14 still presents glioblastomas as one group. In the majority of patients IDH-wildtype glioblastoma is diagnosed in their 6th or 7th decade of life. Of note, many glioblastomas in childhood are also IDH-wildtype but the molecular underpinnings of these tumors is generally different from that in adult patients (see also paragraph on diffuse midline glioma, H3 K27M-mutant).

Large consortia such as the International Cancer Genome Consortium (ICGC) (https://icgc.org), The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov), and the Chinese Cancer Genome Consortium (CCGC) (http://cancerdb.genomics.org.cn) have provided a wealth of new information on molecular alterations in different subgroups of glioblastoma. Different molecular classification schemes have been proposed over the last decade, some of these using a restricted set of molecular markers (such as IDH, H3 K27 and 1p/19q status), others using information on additional markers and/or on data obtained by global gene expression or methylation profiling analysis. Salient molecular aberrations in IDH-wildtype glioblastomas are: loss of chromosome 10 combined with gain of chromosome 7 (+7/−10), amplification of the epidermal growth factor receptor (*EGFR*) gene, mutation of the promoter of the telomerase reverse transcriptase (*TERT*) gene. These molecular markers will now be discussed in more detail. Additionally, in this paragraph some information will be provided on the methylation of the promoter of *MGMT* gene (encoding O^6-methylguanine-DNA methyltransferase). Of note, *MGMT* promoter methylation status is an epigenetic marker with predictive value for response to alkylating chemotherapy in patients with glioblastoma rather than a marker of use for assigning a particular WHO diagnosis to a glioma.

The vast majority of IDH-wildtype glioblastomas show (often whole chromosome) gain of one copy of chromosome 7 and loss of one copy of chromosome 10 (+7/−10). (**Fig. 2**) This aberration is considered as a very early event in the oncogenesis of glioblastomas. Several oncogenes that have been implicated in gliomagenesis are located on chromosome 7, for example, Cyclin Dependent Kinase 6 (*CDK6*), MET Proto-Oncogene (*MET*) and *EGFR*, while several tumor suppressor genes are located on chromosome 10, including Tet family member Tet Methylcytosine Dioxygenase 1 (*TET1*) and Phosphatase and Tensin Homolog (*PTEN*). It is still unclear, however, how exactly +7/−10 plays a role in gliomagenesis. With a reported mutation frequency of

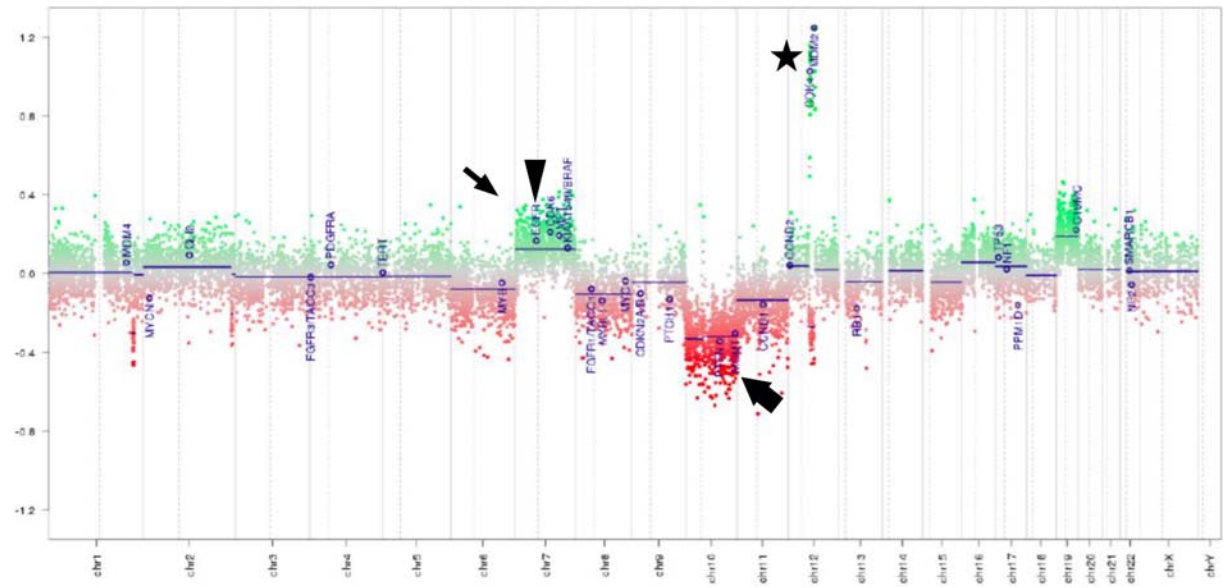

Fig. 2 Chromosomal copy number variation (CNV) plot of an IDH-wildtype glioblastoma based on Illumina EPIC/850k methylation profiling analysis. This glioblastoma shows +7/−10 in the form of whole chromosome 7 gain (thin arrow) combined with whole chromosome 10 loss (thick arrow). Additionally, amplification of *CDK4* and *MDM2* is present (asterisk), but high copy *EGFR* amplification is lacking (arrowhead). Of note, demonstration of +7/−10 and/or EGFR amplification in an IDH-wildtype diffuse astrocytic tumor of histologically WHO grade II or III is a very strong indication that the tumor will clinically behave as glioblastoma (WHO grade IV).

54%–84%, *TERT* promoter mutation is the most frequent mutation in IDH-wildtype glioblastomas. This gene encodes the telomerase, which induces the extension of the telomeres. Interestingly, the *TERT*-related mutations are thus not located in the coding area of the gene, but in the promoter, with approximately 80% of the C228T type, and 20% of the C250T type. The consequence of these mutations is an increased expression of telomerase by a factor of five. Glioblastoma patients with a *TERT* promoter mutation are older and tend to have a worse prognosis. Of note, IDH-mutant and 1p/19q-codeleted oligodendrogliomas also show a very high frequency of *TERT* promoter mutations but in those tumors this marker does not have a negative prognostic significance. In contrast, diffuse, IDH-mutant astrocytomas, including IDH-mutant glioblastomas, typically do not have *TERT* promoter mutation but an α-thalassemia/mental retardation syndrome X-linked (*ATRX*) mutation resulting in alternative lengthening of telomeres (**Fig. 3**).

Approximately 40% of all IDH-wildtype glioblastomas show amplification of the *EGFR* gene, and one-third to half of these *EGFR*-amplified tumors additionally show the *EGFRvIII* mutation variant. In this variant, exons 2–7 are deleted, and the consecutive loss of part of the extracellular domain of the receptor tyrosine kinase causes constitutive autophosphorylation and permanent activation of, for example, the PIK3 pathway, resulting in increased cell proliferation and resistance to apoptosis. Although the *EGFRvIII* mutation variant is often present in only a subset of the tumor cells, it has been demonstrated that cells expressing this variant can stimulate neighboring tumor cells by a paracrine mechanism. Furthermore, the in-frame deletion of the *EGFRvIII* mutation generates a neoepitope that can be detected with specific antibodies. Unfortunately, attempts to use this neoepitope for therapeutic vaccination have so far failed. In the TCGA RNAseq analysis of *EGFR*, glioblastomas with deletions of exons 12–13 and 14–15 were also found, but it remains to be seen if these variants are the consequence of aberrant splicing. In about 10% of the glioblastomas in the

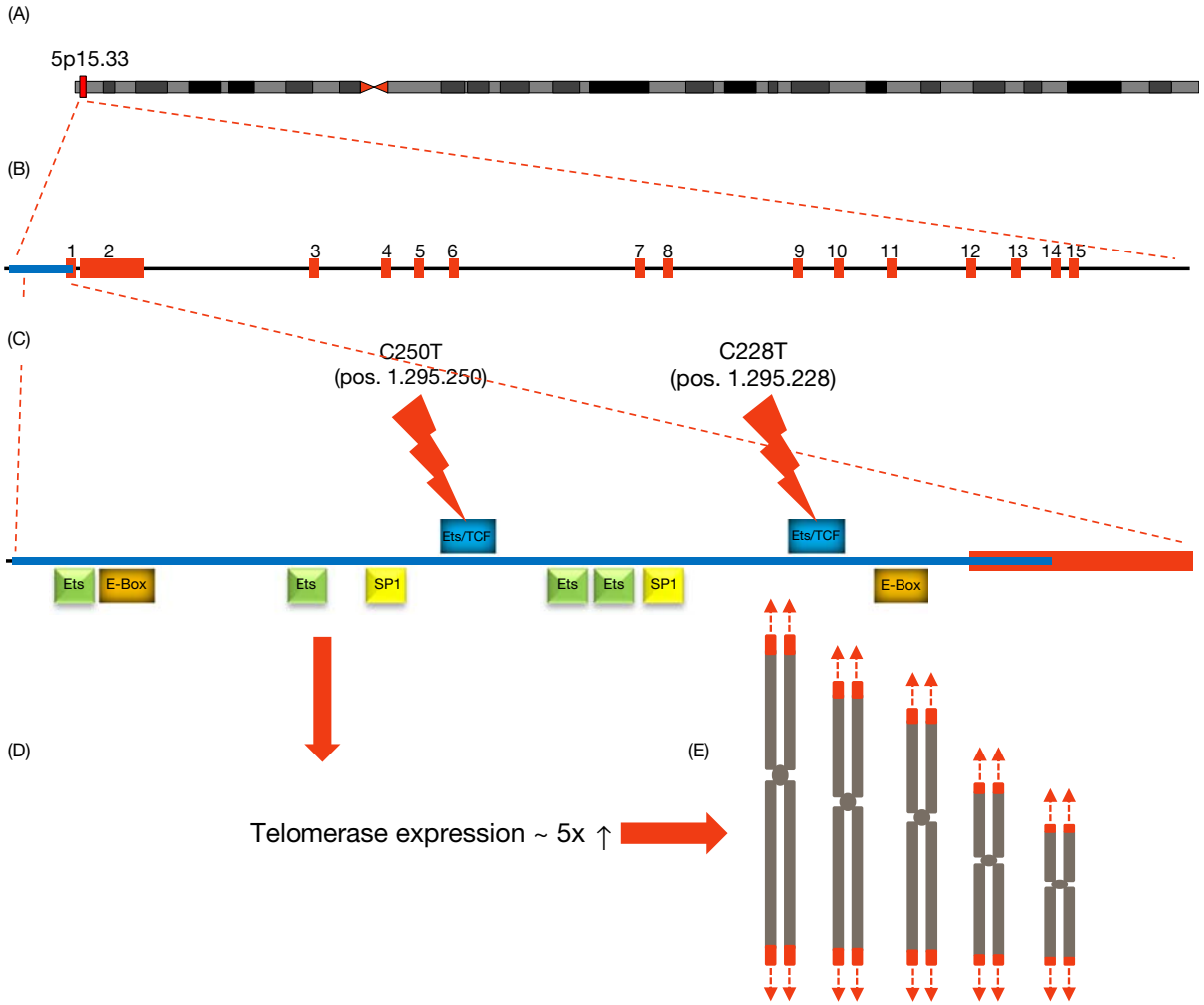

Fig. 3 Schematic illustration of *TERT* promoter mutation and its consequences. (A) The *TERT* gene is located on chromosome 5p15.33. (B) The *TERT* gene has 15 exons and a promoter (blue line), which partially extends into exon 1. (C) The *TERT* promoter has various transcription factor binding motifs (below the blue line); in IDH-wildtype glioblastomas, an additional Ets/TCF motif is frequently inserted by a C250T or a C228T mutation in the *TERT* promoter region. (D) this mutation results in increased translation of telomerase. (E) As a consequence, tumor cells are able to continue proliferating without critical telomere shortening forcing them into senescence or apoptosis.

TCGA series the mutation variant *EGFRvIV* (affecting exon 25–27) was found, and sporadically yet another mutation variant occurred as fusion between *EGFR* and adjacent genes. In somewhat over 10% of glioblastomas *EGFR* point mutations occur. In contrast to pulmonary cancer, *EGFR* point mutations in glioblastomas usually do not involve the kinase domain but the extracellular domain of the receptor tyrosine kinase. (**Fig. 4**) This difference may explain why studies with receptor tyrosine kinase inhibitors were negative in patients with glioblastoma. Neither *EGFR* amplification nor the combination with the *EGFRvIII* mutation variant has a clear prognostic significance in IDH-wildtype glioblastomas. However, demonstration of *EGFR* amplification, as well as of +7/−10 and/or *TERT* promoter mutation is a serious indication of high-grade malignancy in adults with IDH-wildtype diffuse astrocytic tumors to which based on histologic examination not (yet) a WHO grade IV can be assigned.

The *MGMT* gene encodes the O^6-methylguanine-DNA methyltransferase, a DNA repair enzyme which can transfer a methyl group from the O^6 position of a guanine nucleotide irreversibly to itself, thereby repairing the mutated DNA. The O^6-methylation or alkylation of guanine is a typical DNA mutation event induced by various mutagenic events. Application of alkylating drugs like temozolomide induces such O^6-guanine methylation as well, the therapeutic intention being to induce DNA mismatch, DNA-double-strand breakage and ultimately apoptosis in glioblastoma cells. MGMT expression is under control of a large CpG island in the gene promoter. In case of methylation of these CpG sites, MGMT expression is strongly reduced and cells have a limited capacity to repair drug-induced DNA damage. In approximately 40% of patients with IDH-wildtype glioblastomas the tumors demonstrate *MGMT* promoter methylation, thereby rendering this subgroup of patients relatively responsive to treatment with alkylating drugs. Because of this predictive value, recent guidelines of the European Association for Neuro-Oncology (EANO) recommend *MGMT* promoter methylation testing for certain groups of patients with high-grade gliomas.

Diffuse gliomas with a *H3F3A*, *HIST1H3B/C*, or *HIST2H3C* K27M mutation are typically found in midline structures in children (see separate paragraph below). Since the first description of these mutations it became obvious that such histone H3 mutations not affect only codon 27 but also codon 34. Glioblastomas with a H3 G34R or, less frequently, H3 G34V mutation are rare, occur mostly in the cerebral hemispheres, affect predominantly older children and young adults, and are associated with a somewhat better prognosis than patients with ordinary glioblastomas. H3F3A G34R/V-mutant tumors can also have the phenotype of a CNS embryonal

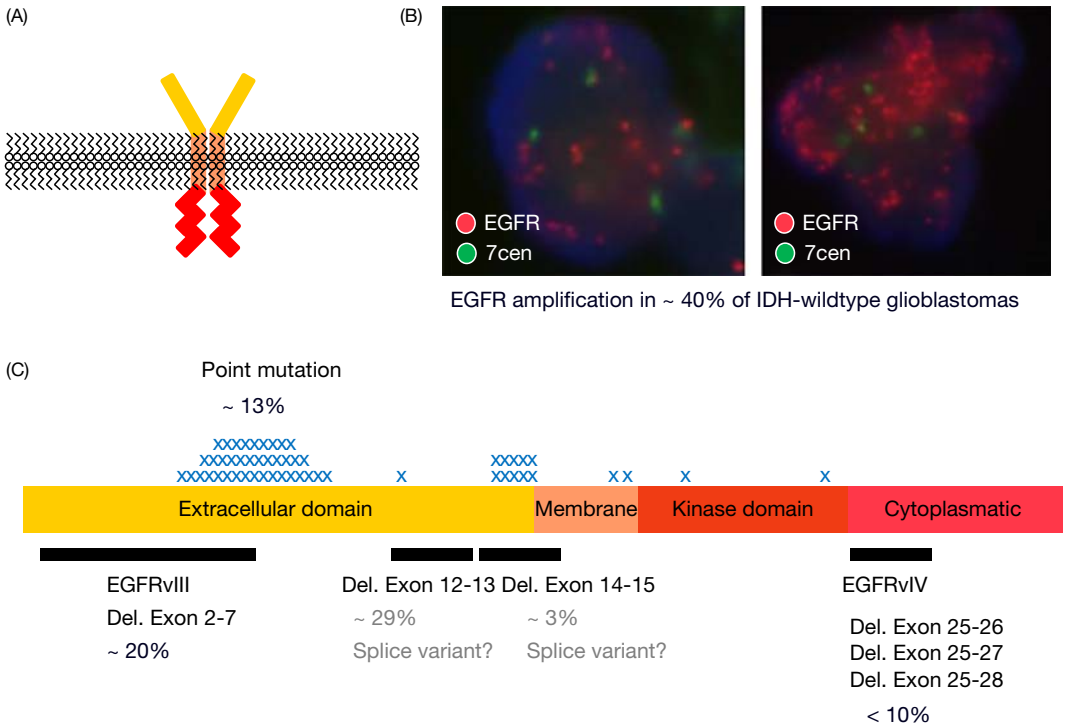

Fig. 4 Schematic illustration of involvement of the epidermal growth factor receptor (EGFR) in glioblastomas. (A) The EGFR protein normally has an extracellular domain (yellow), a domain in the membrane (orange), and an intracellular part (kinase domain and cytoplasmic end). (B) About 40% of all IDH-wildtype glioblastomas show high copy amplification of *EGFR*, here illustrated by FISH images of two different tumors; the centromere of chromosome 7 is marked by a green signal, and the fact that more than two green signals are visible indicates polyploidy of chromosome 7; the multitude of red dots represent the signal of the *EGFR* probe and reflect high copy amplification of this gene. (C) Schematic illustration of other mutation variants affecting the *EGFR* gene. Up to half of all glioblastomas with *EGFR* amplification additionally have a deletion of exon 2–7, leading to the *EGFRvIII* mutation variant which is constitutively active. The mutation variant *EGFRvIV* affects exon 25–27 and is found in about 10% of cases. The importance of the potential deletions of exons 12–13 and 14–15 is still unclear, these may in fact be mRNA splicing variants rather than genetic deletions. Approximately 13% of IDH-wildtype glioblastomas show point mutations in *EGFR*, mainly in the extracellular domain, and partly in combination with *EGFR* amplification.

tumor (formerly known as (supratentorial) CNS PNET), but no prognostic difference was found between high grade glioma versus embryonal histology. In contrast, the lack of amplification of oncogenes, and the presence of *MGMT* promoter methylation in such tumors was reported to be associated with better outcome. As these tumors seem to represent a discrete group, one can expect that they will be listed as a separate entity in a next edition of the WHO classification of CNS tumors.

Glioblastoma, IDH-Mutant

The establishment of IDH-mutant glioblastomas as a separate entity by the WHO classification was significantly influenced by the clinical concept of the existence of primary ("de novo") glioblastomas (diagnosed in patients without clinical, radiological and/or pathological evidence for a lower grade precursor lesion) and secondary glioblastomas (that apparently evolved from such a lower grade precursor lesion). After it was shown that the vast majority of primary glioblastomas are IDH-wildtype, while most secondary glioblastomas are IDH-mutant, and given the fact that "primary" glioblastomas with IDH mutation might represent cases in which the lower grade precursor lesion was missed, nowadays the terms primary and secondary glioblastoma are considered to be largely synonymous with IDH-wildtype and IDH-mutant glioblastoma, respectively.

In different studies the relative frequency of IDH-mutant glioblastomas varies between 6% and 13%. Patients with an IDH-mutant glioblastoma are on average significantly younger than patients with an IDH-wildtype glioblastoma, in a population-based study the mean age was 48 versus 61 years, respectively. In MRI imaging, patients IDH-mutant glioblastomas showed more rarely large central necrosis, less pronounced edema, more frequently cystic changes and more diffuse tumor infiltration than patients without this genetic alteration. IDH-mutant glioblastomas usually show the same genetic alterations as diffuse, lower grade (WHO grade II and III) astrocytomas, with especially *TP53* and *ATRX* mutations being present in a high percentage of cases. While *TP53* mutations also occur quite frequently in IDH-wildtype glioblastomas, *ATRX* mutations are typical for IDH-mutant astrocytomas/glioblastomas and functionally partially substitute *TERT* promoter mutations. IDH-mutant glioblastomas typically do not exhibit +7/−10 or EGFR amplification.

The gene *IDH1* (located on chromosome 2q33.3) encodes the cytoplasmatic (type 1) form of isocitrate dehydrogenase (IDH1), while the *IDH2* gene (located on 15q26.1) encodes IDH2 that is located in the mitochondria. Both proteins catalyze as homodimers isocitrate and NADP by an oxidative decarboxylation to α-ketoglutarate (α-KG) and NADPH. Only the much more complex IDH3, but not IDH1 and IDH2, has a functional role in the tricarboxylic acid cycle. IDH mutations only affect one of the two alleles (heterozygous deletion) and are generally "hotspot" mutations of *IDH1* codon 132 or of *IDH2* codon 172. *IDH1* mutations account for over 90% of the IDH mutations found in diffuse gliomas, with *IDH1 R132H* representing up to 90% of all *IDH1* mutations. The availability of a specific antibody against the IDH1 R132H mutant protein that can be applied on formalin-fixed, paraffin-embedded tissue thus enables reliable detection of the vast majority of IDH-mutant gliomas/glioblastomas by a simple immunohistochemical procedure in routinely processed glioma tissue (**Fig. 1**C).

Mutant IDH protein utilizes the physiological product α-KG as an educt and catalyzes it to 2-hydroxyglutarate (2-HG). As a result, the so-called "oncometabolite" 2-HG is present in excessive concentrations in affected tumor cells and induces a competitive inhibition of a large group of more than 60 different α-KG-dependent dioxygenases. From this point on, various dysfunctional pathways result of which the individual significance for neoplastic transformation is unclear (**Fig. 5**). The physiological function of TET2 comprises a large-scale demethylation of CpG sites in promoter regions of various genes, and impaired function due to IDH-mutant status results in a global hypermethylation of the genome, also described as the glioma CpG island methylator phenotype (g-CIMP). This situation may explain why the vast majority of IDH-mutant gliomas show hypermethylation of the *MGMT* promoter. The genomic hypermethylation also affects cohesin and CCCTC-binding factor (CTCF)-binding sites, thereby compromising binding of this methylation-sensitive insulator protein. This in turn leads to a disrupted chromosomal topology with enhanced PDGFRA activity. Furthermore, excessively increased 2-HG results in inhibition of KDM4A, whose functional failure leads to an increase in DEPTOR activity, which in turn causes mTOR pathway activation. As a further consequence of massively increased concentrations of 2-HG, competitive inhibition of the α-KG-dependent prolylhydroxylase was demonstrated, resulting in inhibition of the degradation of HIF1α.

Diffuse Midline Glioma, H3 K27M-Mutant

While in adult patients glioblastomas mostly are located in supratentorial parts of the CNS, in children glioblastomas occur relatively frequently infratentorially. A particular subset of such (mainly) pediatric tumors, the diffuse midline glioma, H3 K27M-mutant, was added to the list of diffuse gliomas in the WHO 2016 classification. Diffuse midline glioma, H3 K27M-mutant carries a mutation in either the *H3F3A*, *HIST1H3B/C*, or *HIST2H3C* gene and represents a WHO grade IV tumor. By definition, these tumors with a H3 K27M mutation should be (a) gliomas with (b) diffuse growth and (c) located in the midline (brain stem, thalamus, cerebellum, and/or spinal cord). Tumors that were previously diagnosed as diffuse intrinsic pontine glioma (DIPG) are a frequent representative of this group, with about 80% of the previously diagnosed DIPGs indeed carrying the H3 K27M mutation. Patients with a diffuse midline glioma, H3 K27M-mutant in the pons are on average younger than those with such a tumor in the thalamus (around 7 years and 11 years, respectively). In both children and adults, diffuse midline gliomas, H3 K27M-mutant that show WHO grade II or III phenotype generally still carry a grim (WHO grade IV-like) prognosis. Importantly, other glial CNS tumors (especially nondiffuse

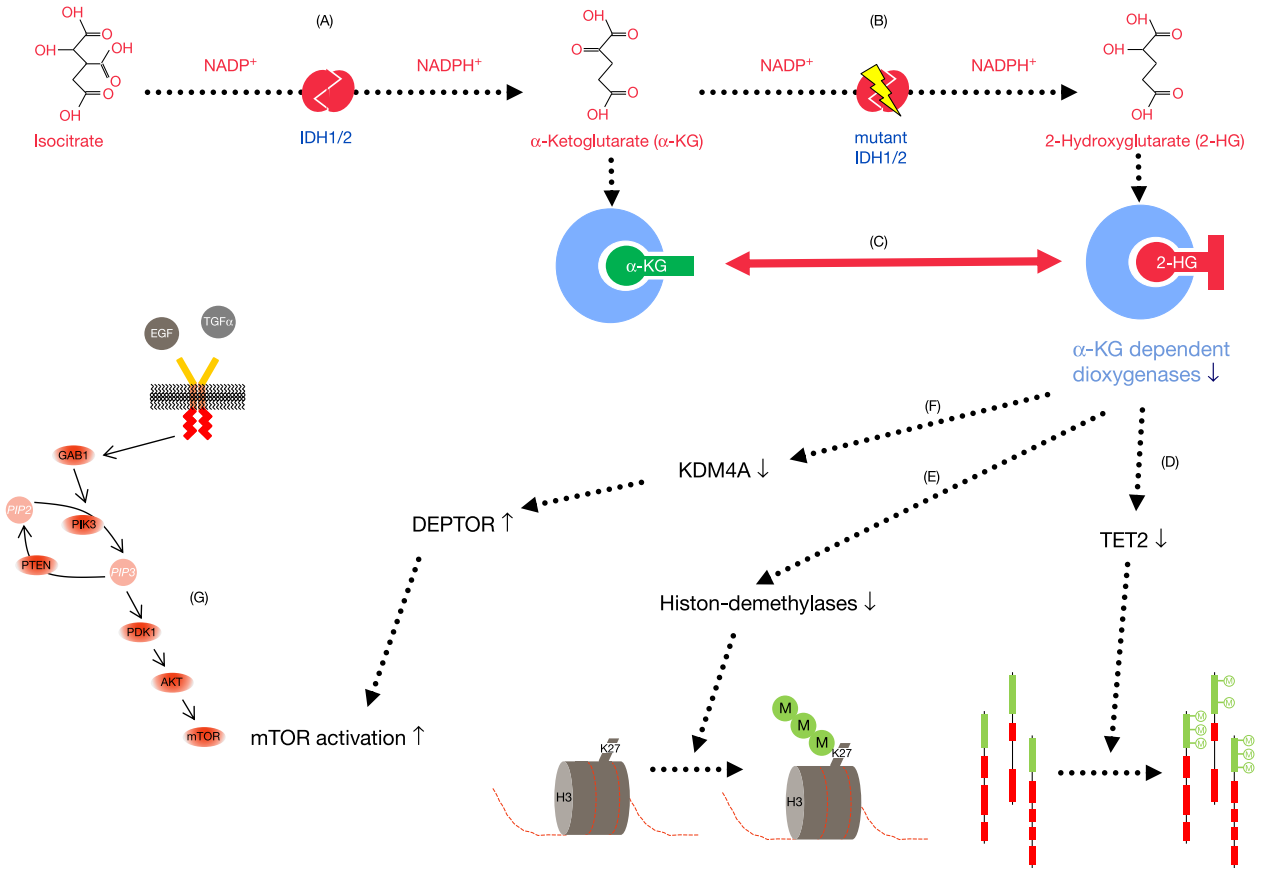

Fig. 5 Schematic drawing of mechanisms involved in the isocitrate dehydrogenase (IDH) pathway. (A) Normal IDH protein catalyzes isocitrate to α-ketoglutarate (α-KG); (B) mutant IDH protein utilizes the physiological product α-KG as educt and catalyzes it to 2-hydroxyglutarate (2-HG); (C) 2-HG occurs in excessive concentrations in affected tumor cells and is competitively inhibiting a large number of different α-KG-dependent dioxygenases (D–F); (D) by blocking the activity of the DNA demethylase TET2 a global CpG island methylator phenotype (g-CIMP) results; (E) by inhibiting the activity of histone demethylases for example H3 histone protein becomes trimethylated; (F) by inhibition of KDM4A, the activity of DEPTOR increases, which in turn causes mTOR pathway activation; (G) frequently occurring alternative route for mTOR pathway activation in cancer.

gliomas such as ependymomas and pilocytic astrocytomas, and nonmidline gliomas) may occasionally carry the H3 K27M mutation as well, but the prognostic impact of this mutation in such a nondiffuse and/or nonmidline glioma is unclear.

Up to now four different histone-encoding genes carrying H3 K27M mutations have been identified. By far the most frequently mutated gene is *H3F3A* (mutant in about 80% of H3 K27M-mutant diffuse midline gliomas and encoding histone H3.3), followed by *HIST1H3B* (mutant in about 20% of these cases and encoding histone H3.1). In less than 1% of the H3 K27M-mutant diffuse midline gliomas a mutation is found in *HIST1H3C* (encoding histone H3.1) or *HIST2H3C* (encoding histone H3.2). The functional consequences of H3 K27M mutations are not yet completely understood. A H3 K27M mutation results in an altered histone protein that cannot become trimethylated at position 27 anymore. When the DNA-trimethylating polycomb repression complex 2 (PCR2) encounters such a mutant oncohistone, it stops functioning and detaches itself from the DNA. As a result, there is a global reduction in histone trimethylation (loss of H3K27me3) in tumor cells with a H3 K27M mutation. A global change of the epigenome then results, with as a consequence abnormal cell cycle control, inhibition of autophagy and presumably also an increased resistance of the tumor cells to radiotherapy (Fig. 6). Like for the IDH R132H mutant protein, a highly specific antibody is available for H3 K27M mutant protein that allows for detection of the H3 K27M-mutant status of a (glial) tumor by a simple immunohistochemical test on formalin-fixed, paraffin-embedded tumor tissue. In this staining a H3 K27M-mutant glioma shows positive staining of tumor cell nuclei (with the negative nuclei of nonneoplastic (e.g., vascular) cells representing a negative internal control in the same slide).

Interestingly, *H3F3A* K27M mutations were reported to occur in diffuse midline gliomas in all anatomical structures of the midline, while *HIST1H3B* K27M mutations occur mainly in those originating in the pons. First data indicate that patients with *H3F3A* K27M mutation in their diffuse midline glioma usually have a worse prognosis than those with an *HIST1H3B* mutation. Furthermore, *HIST1H3B* mutations are very often combined with *ACVR1* mutations which have been reported as a slightly better prognostic factor as well. *PDGFRA* and *PIK3CA* mutations are interpreted as progression-associated genetic changes in diffuse midline gliomas with a *H3F3A* K27M mutation. *PIK3CA* mutations were reported as progression-associated changes in *HIST1H3B*-mutant tumors as well.

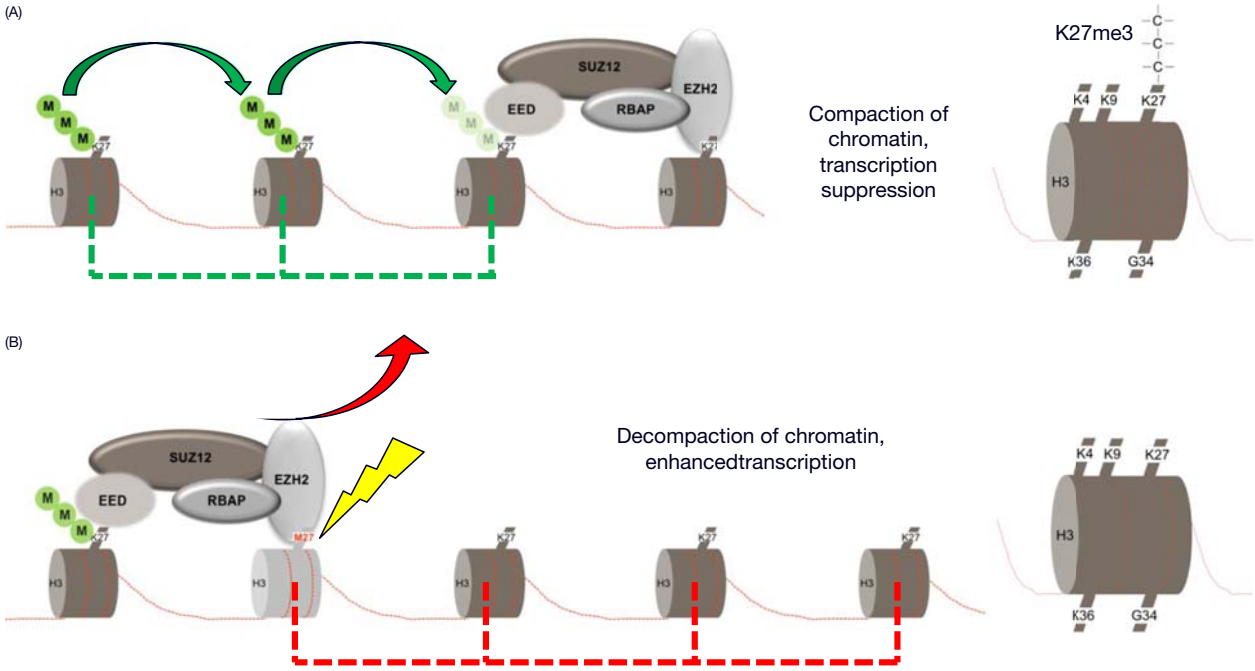

Fig. 6 H3 K27M mutation results in decompaction of DNA and promotion of transcription. (A) The polycomb repression complex 2 (PRC2, with EZH2, SUZ12, EED, RBAP as components) moves gradually along histones associated with DNA and establishes tri-methylation at the H3 K27 site of the histone tail (H3 K27me3), resulting in DNA compaction and transcription suppression. Presence of a H3 K27M mutation in the *H3F3A* or *HIST1H3A/B* gene obstructs this effect of PRC2 not only at the site of the mutant histone, but also at subsequent histones as further movement of the PRC2 complex is impaired. The focal presence of H3 K27M may thus cause more widespread blockage of H3 K27 trimethylation and, consequently, more widespread decompaction of DNA and promotion of transcription.

Glioblastoma Classification by Next Generation Molecular Technologies

Especially in the last two decades, technologies have become increasingly developed that not only enabled the investigation of individual molecular markers, but also the analysis of complex correlations on a molecular basis. In the field of neurooncology, this was achieved for the first time with the comparative genomic hybridization (CGH) technology, which was significantly improved by the array-CGH (aCGH) method. These assays allowed the detection of chromosomal gains or losses in gliomas. Another step forward was expression array analysis, for which RNA is extracted from tumor tissue and converted into cDNA. Subsequently, the expression pattern of a large number of genes can be determined on this basis.

Furthermore, different methods for next-generation sequencing (NGS) were increasingly used in neuro-oncology. While initially only small panels of genes were investigated, further development of the technique allowed for wider availability and higher efficiency of NGS and now analysis of entire (glioma) exomes and genomes are performed. The NGS technology also has been extended to the transcriptome of gliomas (whole transcriptome shotgun sequencing; RNAseq). Using RNAseq it is possible to determine both the expression and the mutations of the (glioma) genome. Another advantage of RNAseq is that fusion genes can be detected more effectively than with any other technology. Probably the most productive technology for classifying gliomas at present is the 450 K, or more recently, 850 K BeadChip methylation analysis. This platform generates detailed information on the epigenetic profile of a tumor as well as on chromosomal gains and losses. On the basis of these molecular high-throughput techniques, new subgroups of glioblastomas were identified. The following paragraphs present some examples of such newly recognized glioblastoma subgroups (Fig. 7).

Expression Profiling

Initial expression profiling studies revealed that glioblastomas can be distinguished from pilocytic astrocytomas, anaplastic astrocytomas or oligodendrogliomas, and that "primary" glioblastomas can be discriminated from "secondary" glioblastomas. Ordering the results of expression profiling using unsupervised clustering algorithms indicated patterns that correlated with diagnoses and malignancy grades assigned to the tumors based on histopathologic analysis. Meanwhile, several studies reported that the prognosis of patients with (astrocytic) gliomas could be more accurately predicted based on expression profiling than on morphologic evaluation.

Various attempts have been made to use expression profiling for the recognition of clinically relevant subgroups of histopathologically diagnosed glioblastomas. At first, three groups were described: proneural, proliferative and mesenchymal. In

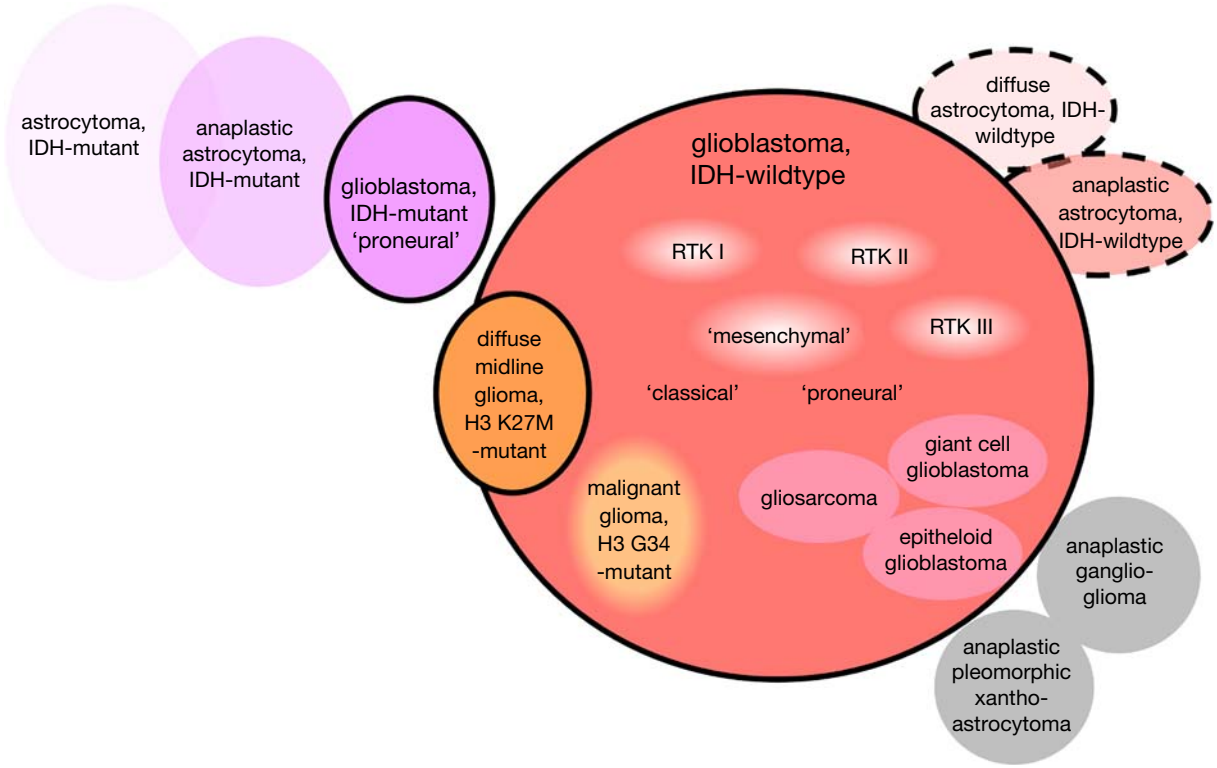

Fig. 7 Scheme of histologic and molecular categories of glioblastoma. The current WHO classification of CNS tumors lists three, partly molecularly defined groups of grade IV diffuse gliomas/glioblastomas (encircled by a solid line): (A) IDH-wildtype, by far the most frequent group in adults; (B) IDH-mutant, generally occurring in younger adults than IDH-wildtype glioblastomas and often the result of malignant progression of a previously diagnosed lower grade IDH-mutant astrocytoma; (C) H3 K27M-mutant diffuse midline gliomas (that are IDH-wildtype as well), typically but not only occurring in children. Of note, many tumors that are histologically diagnosed as lower grade (WHO grade II and III) IDH-wildtype diffuse astrocytomas (encircled by the dashed line in this figure) have molecular characteristics of and in fact clinically behave like IDH-wildtype glioblastomas (WHO grade IV). Meanwhile, the IDH-wildtype glioblastoma group is still a very heterogeneous one with different subgroups that can be recognized based on histology (e.g., gliosarcoma, giant cell glioblastoma, epitheloid glioblastoma), expression profiling (classical, mesenchymal, or proneural subtype), or methylation profiling (e.g., receptor tyrosine kinase type I (RTK I), RTK II, RTK III, mesenchymal). In the IDH-wildtype glioblastoma group, the H3 G34-mutant tumors already stand out as a distinct subgroup that may well be designated as a separate entity in the next WHO classification. Discrimination of high-grade "nondiffuse" gliomas like anaplastic pleomorphic xanthoastrocytoma or anaplastic ganglioglioma from especially the epitheloid variant of glioblastoma may be difficult not only because of histopathologic similarities but also because these tumors share the frequent occurrence of *BRAF* V600E mutation.

the proneural group, patients were generally younger and the tumors lacked *EGFR* amplification and *PTEN* mutation. In the proliferative and mesenchymal groups, on the other hand, the tumors typically showed the genetic profile of glioblastoma in (older) adult patients, including gain of chromosome 7 and loss of chromosome 10. Later on, based on in depth analysis of the large and complex data sets of the TCGA project, modification of expression profiling-based subgrouping of glioblastomas was suggested with recognition of four transcription types: proneural, neural, classic, and mesenchymal. These four subtypes were associated with different frequencies of mutations in especially *TP53*, *EGFR*, and *NF1*. More than 90% of IDH-mutant glioblastomas belonged to the proneural expression signature, and about 30% of glioblastomas with a proneural expression signature were IDH-mutant. Diffuse lower grade astrocytomas and oligodendrogliomas also typically had a proneural signature.

Later on, the neural transcription profile could at least partly be explained by the presence of relatively large amounts of non-neoplastic CNS tissue in the glioblastoma samples in this group. In other words, identification of this neural group may partly have been based on "selection-bias" towards the use of samples with relatively low tumor cell percentage, for example, from the periphery of a glioblastoma, rather than have a basis in a real profile of the tumor itself. Furthermore, using single cell analysis it was demonstrated that different transcription types can occur within one and the same glioblastoma. Also in a human glioblastoma xenograft model cells could be transformed from a proneural to a mesenchymal expression type by defined conditions. Such observations raised doubts about the stability of classification of glioblastomas by RNA-based transcriptional profiling. Yet another problem with expression profiling as a diagnostic tool in clinical practice is that in routinely processed, formalin-fixed, paraffin-embedded tumor tissue the integrity of RNA is significantly impaired. Ideally, for expression profiling tumor tissue should thus be fresh frozen.

Methylation Profiling

Analyzing the epigenome, and in particular the methylome of tumor cells has proved to be a robust and reproducible technology allowing for the analysis of even small, formalin-fixed, paraffin-embedded tissue samples of morphologically poor quality. The analysis of the methylome can be used to evaluate which epigenetic changes are evolving due to the induction and progression of tumors. On the other hand, part of the epigenetic imprinting remains stable from the original cell to the tumor cell, so that the analysis of the methylome provides information on the cell of origin of the tumor and can be used to classify tumors. Various technologies for global methylation analysis are available. The commercial Infinium BeadChip 450 K assay, later on replaced by the Infinium MethylationEPIC BeadChip 850 K assay, has found the broadest distribution and has been successfully used for subclassification of glioblastomas as well. With 850 K analysis, over 850,000 CpG sites are examined, which corresponds to about 3.5% genomic CpG coverage. An alternative approach is whole genome bisulfite sequencing (WBGS), allowing investigation of more than 26 million CpG sites, corresponding to a CpG coverage of more than 90%. The results of tumor DNA analysis using the 450 K and the WBGS method showed comparable results, indicating that different methods can be applied for robust characterization of the tumor methylome.

One of the first observations obtained with methylation profiling analysis of gliomas was the existence of a glioma CpG island methylator phenotype (g-CIMP) cluster. This "cluster #1" with g-CIMP signature encompassed most of the lower-grade diffuse infiltrating gliomas as well as a fraction of glioblastomas and was strongly associated with presence of an IDH mutation. For example, in the TCGA dataset 30 of the total number of analyzed glioblastomas (6%) carried an IDH mutation, largely overlapping with the 42 tumors that exhibited a g-CIMP phenotype (7.9%). In addition, two other methylation clusters of glioblastoma were found (#2 and #3) in which IDH-wildtype glioblastomas were grouped. Comparison of these three methylation clusters with the four established expression profiles of glioblastomas revealed that most glioblastomas with a proneural expression signature were found in methylation cluster #1 (g-CIMP), while glioblastomas with a neural, mesenchymal or classical expression signature were assigned to the other two methylation clusters without a particularly clear distribution pattern. Furthermore, tumors with a 1p/19q-codeletion ("g-CIMP-A") had a clearly different methylation profile compared to *TP53*-mutant gliomas ("g-CIMP-B").

A systematic classification of CNS tumors including glioblastomas on the basis of their methylation profile became possible due to the availability of the 450 K/850 K assay in combination with newly developed software, which is capable of unsupervised analysis of the complex results. Several closely collaborating research groups from Heidelberg have now established a comprehensive CNS tumor classifier based on methylation profiling and thereby became leaders in this field. In a first publication six glioblastoma subgroups were reported: (1) the IDH subtype, characterized by IDH mutations with a g-CIMP phenotype; (2) the H3 K27 subtype, consisting of tumors with, for example, an *H3F3A K27M* mutation; (3) the H3 G34 subtype of tumors with an *H3F3A G34R/V* mutation; (4) the RTK I or *PDGFRA* subtype; (5) the mesenchymal subtype; and (6) the classic, RTK II subtype. This RTK II subtype overlapped with methylation cluster #3 as described in the previous paragraph, subtypes 2–5 with methylation cluster #2, and subtype 1 with methylation cluster #1. The CNS tumor classifier was further refined by the analysis of a large number of additional tumors, and the current classifier (02/18) with version number 11b4 (https://www.molecularneuropathology.org/mnp/classifier/2) now lists the following nine glioblastoma methylation classes (ordered from highest to lowest median age of the patients in these classes):

- *Glioblastoma, IDH-wildtype, subclass RTK I PDGFRA*: these tumors are typically found in the cerebral hemisphere; the patients have a median age of 64 years; genetically, gain of chromosome 7 with or without *EGFR* amplification, loss of chromosome 10, and homozygous deletions of *CDKN2A* are typically present; also, amplification of *PDGFRA* is more common in this methylation class and the expression profile corresponds to the proneural subtype.
- *Glioblastoma, IDH-wildtype, subclass RTK II classic*: in this methylation class the tumors have a supratentorial location; the patients have a median age of 61 years; some gliosarcomas are grouped in this class; genetically, like in the previous methylation class, these tumors also show gain of chromosome 7 with or without *EGFR* amplification, loss of chromosome 10, and homozygous deletions of CDKN2A; additionally, around 40% of these tumors also have gains of chromosome 19 and 20.
- *Glioblastoma, IDH-wildtype, subclass mesenchymal*: these tumors typically grow supratentorially and the patients have a median age of 59 years; histologically this group includes gliosarcomas; genetically, gain of chromosome 7 with or without amplification of *EGFR*, loss of chromosome 10, and homozygous deletions of CDKN2A are observed; *NF1* mutations are enriched in this methylation class, which overlaps with the mesenchymal expression profile.
- *IDH glioma, subclass high-grade astrocytoma*: this class encompasses supratentorially located astrocytomas with an IDH mutation, which are often derived from a low-grade lesion and are associated with the g-CIMP phenotype; the median age of the patients in this class is 38 years; this methylation class corresponds to glioblastoma, IDH-mutant (and partially anaplastic astrocytoma, IDH-mutant) in the WHO classification.
- *Glioblastoma, IDH-wildtype, H3.3 G34-mutant*: this typically concerns supratentorial tumors with the morphological appearance of a glioblastoma or an embryonal tumor; the median age of the patient is 20 years; the tumors are *H3F3A* G34R/V-mutant.
- *Glioblastoma, IDH-wildtype, subclass midline*: this class represents glioblastomas growing in the midline regions of the brain and showing an epigenetic overlap with the methylation class diffuse midline glioma, H3 K27M-mutant but the tumors do not have the H3 K27M mutation; the median age of the patients in this group is 13 years.
- *Diffuse midline glioma, H3 K27M-mutant*: this class overlaps with diffuse midline gliomas, H3 K27M mutantas listed in the WHO 2016 classification; the median age of patients in this class is 12 years.

- *Glioblastoma, IDH-wildtype, subclass MYCN*: these tumors are mostly located supratentorially or in the posterior fossa; the median age of the patients is 11 years, and amplification of *MYCN* can be detected in 20%–30% of these tumors;
- *Glioblastoma, IDH-wildtype, subclass RTK III*: these tumors are found mainly in the cerebral hemisphere; the median age of the patients in this class is 9 years, and in this group *EGFR* amplification and deletions of chromosome 10 are frequent.

Further analysis of diffuse infiltrating gliomas in children with the morphological appearance of a glioblastoma allowed the identification of two further subtypes: tumors with the epigenetic profile pleomorphic xanthoastrocytoma (PXA)-like and low-grade glioma (LGG)-like. The tumors in this latter class are characterized by the absence of an IDH mutation. Patients from both subgroups showed a significantly better prognosis. A further analysis of supratentorial pediatric glioblastomas without H3 and without IDH mutation resulted in the identification of three methylation classes: (1) pediatric glioblastoma MYCN; (2) pediatric glioblastoma RTK1; and (3) pediatric glioblastoma RTK2. Patients with tumors in methylation class RTK1 and RTK2 showed a relatively good prognosis. The epigenetic pattern of the pediatric methylation classes RTK1 and RTK2 was different from the profile of the adult methylation classes RTK I and RTK II. Pediatric patients in methylation class MYCN, on the other hand, showed an exceptionally poor prognosis. *MYCN* amplification was relatively frequently found in this methylation class, but the majority of tumors did not exhibit such genetic alteration.

In addition to the actual classification as part of a methylation analysis, information on chromosomal gains and losses (e.g., complete 1p/19q codeletion versus partial deletion of (one of) these chromosome arms) and on the methylation profile of (the promoters of) individual genes such as *MGMT* is also provided. Methylation analysis also facilitates that different neuropathologists generate identical diagnoses, and it was reported that compared to traditional histopathologic evaluation the classification by methylation analysis enabled a better estimation of the prognosis associated with the investigated tumors. As the "Heidelberg CNS tumor classifier" is a self-learning system, identification of new, rare subgroups can be expected by adding methylation data of additional tumors. In other words: The more CNS tumors are examined by methylation profiling in this system, the better the classification of these tumors will be. Disadvantages of this approach are, however, that the necessary laboratory infrastructure is costly and that processing individual samples is relatively expensive as well. Furthermore, using the 450 or 850 K technology as described above creates a dependence on a single manufacturer, as it has not yet been conclusively proven whether transfer to another global methylation assay is possible without problems with preserving the established classifier.

Integrated TCGA Approach

As the Cancer Genome Atlas research network (TCGA) focused already in an early phase on glioblastomas, most of the data sets on these tumors are now completely available. An essential concept of the TCGA network was the integrated analysis of glioblastomas using various high-throughput assays. For example, many hundreds of clinically annotated glioblastomas were examined with regard to their protein expression, mRNA expression profile, methylation profile, mutation status and chromosomal alterations and the resulting data were combined. Fortunately, the results confirmed data from many previous studies with regard to the molecular characteristics of glioblastomas. Potential downsides of the TCGA approach are the retrospective approach, the heterogeneous therapy of the patients of which material was included, the potential bias resulting from the inclusion criterion of sufficient tissue for performing multiple high throughput analyses, the focus on adult patients and the bias towards patients that were treated predominantly in academic centers.

Fusion of the TCGA datasets from different high-throughput assays resulted in only a few relevant and new association patterns. It was not possible to identify individual molecular markers that could clearly be assigned to one of the different subgroups. At best, singular molecular markers were more common in one subgroup than in the other. For example, *NF1* mutations were frequently observed in glioblastomas of the mesenchymal expression profile. *PDGFRA* amplifications were found mainly in glioblastomas of the proneural signature, which did not correspond to the g-CIMP methylation profile. *ATRX* mutations and *MYC* amplifications were detected mainly in glioblastomas of the g-CIMP methylation profile, *CDK4* and *SOX2* amplifications in tumors of the proneural subtype, and amplifications of chromosomes 19 and 20 in the classical subtype.

Also, within the TCGA project, an attempt was made to characterize epigenetic glioblastoma subtypes at the methylation level. Six methylation groups of glioblastomas were identified (M1–6, with M5 being the g-CIMP subtype). In comparison to the Heidelberg glioblastoma methylation classes discussed above, the Heidelberg IDH-mutant subtype was found in group M5, the mesenchymal subtype especially in groups M1 and M2, the RTK II subtype in groups M3 and M4, and the RTK I subtype in group M6. Since only adult glioblastoma patients were included in the TCGA cohort, the Heidelberg subgroups K27 and G34, which mainly affect children and young adults, could not be identified.

When considering the frequencies of molecular aberrations in the cohort of glioblastoma patients investigated by the TCGA project, the *TERT* promoter mutation is the most frequent aberration (present in about 80% of the glioblastomas analyzed). A homozygous deletion of *CDKN2A* and an *EGFR* alteration were found in about 60% of the tumors, *PTEN* was altered in about 40%, and *TP53* in 35% of cases. Furthermore, amplifications in *CDK4* and mutations in *NF1* were found in about 14%, *PIK3CA* and *PIK3R1* mutations as well as *PDGFRA* amplifications in slightly more than 10% of the tumors. A surprisingly broad spectrum of *EGFR* alterations was detected (amplification, deletion, point mutation, fusion). Furthermore, it was noted that frequently several genes for tyrosine kinase receptors (*EGFR*, *PDGFRA*, *MET*, *FGFR*) were altered in a single tumor. While most of these observations confirmed the knowledge obtained in previous studies on the genetic changes in glioblastomas, the relatively high frequency of *NF1*

mutations came as a sort of surprise. However, since *NF1* is a very large gene, complete analysis of this gene using classic Sanger sequencing is complex, explaining that until the TCGA data became available limited reliable data on the involvement of *NF1* in glioblastomas were published.

When mapping the broad spectrum of mutations identified in the TCGA glioblastoma cohort into different tumor-relevant signaling pathways, it appears that these pathways are all frequently affected. Alterations in the PIK3CA/AKT and the MAPK signaling pathway were found in 90% of the investigated glioblastomas. In 66% of the tumors, combined alterations at the upper end of the signaling pathways were found by alteration of the receptor tyrosine kinase, and in almost 70% of the tumors either a *PTEN* or a *PIK3CA/PIK3R1* mutation was detected. Senescence/apoptosis regulation was affected in 86% of glioblastomas. The homozygous deletion of *CDKN2A/B* was predominant, followed by *TP53* mutations and deletions as well as amplification of *MDM2* and *MDM4*. In 79% of the investigated glioblastomas, the genes involved in cell cycle control were also impaired. Again, *CDKN2A/B* proved to be the leader in this respect, while other relevant genes of this signaling pathway were altered at significantly lower frequencies (**Fig. 8**).

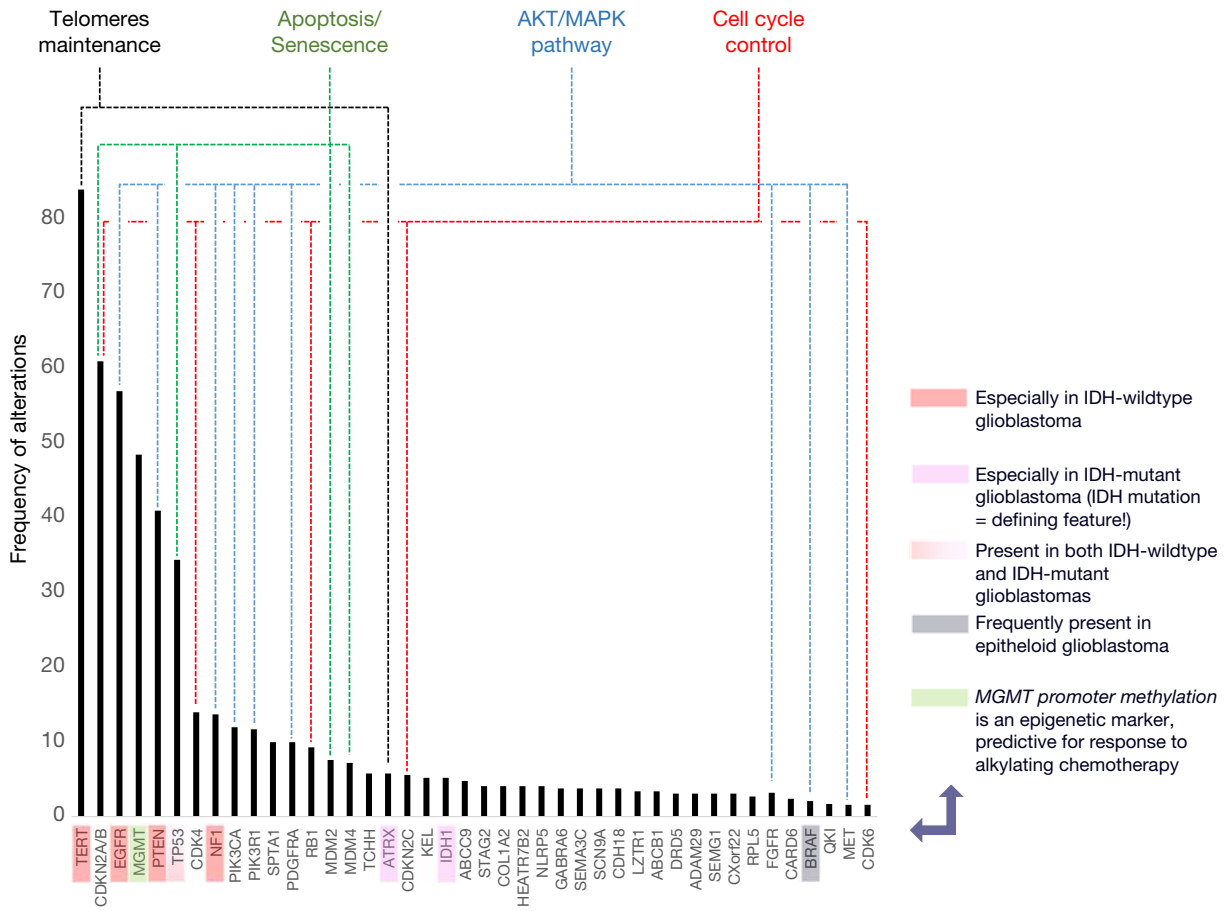

Fig. 8 Genes most frequently showing aberrations (mutation, loss, amplification, fusion) in glioblastomas according to The Cancer Genome Atlas consortium (TCGA; see https://cancergenome.nih.gov). Because the TCGA study focused on glioblastomas in adult patients, the H3 K27- and H3 G34-mutant tumors are not represented in this graph. Examples of genes (almost) exclusively affected in the IDH-wildtype versus IDH-mutant category of glioblastoma in adults are indicated by a light red versus light pink square (with presence versus absence of an IDH mutation in fact being the criterion based on which these two groups are discriminated). *TP53* is frequently involved in both molecular groups of glioblastomas (indicated by square showing both light red and light pink color). *BRAF V600E* mutation (highlighted by a gray square) is frequent in epitheloid glioblastoma as well as in histologic look-alikes such as anaplastic pleomorphic xanthoastrocytoma and anaplastic ganglioglioma (present in about 50% of these neoplasms). Many of the genes listed in this graph can be assigned to the cellular systems of telomere maintenance (black dashed lines), apoptosis/senescence (green dashed lines), the AKT/MAPK pathways (blue dashed lines) and the cell cycle control pathway (red dashed lines). *CDKN2A/B* belongs functionally to both the apoptosis/senescence and cell cycle control pathway. *MGMT* is also also included in this figure (highlighted in light green); about 50% of glioblastomas in the TCGA cohort showed *MGMT* promoter (hyper)methylation which serves as a marker indicating better response to alkylating chemotherapy; as a result of their glioma CpG-island methylator phenotype (g-CIMP) status, IDH-mutant glioblastomas generally have a methylated MGMT promoter, while in IDH-wildtype glioblastomas this is found in about 40% of the cases.

Prospective Vision

The introduction of molecular markers in the WHO 2016 definition of CNS tumors is a paradigm shift that allows for a more robust recognition of clinically relevant subgroups of glioblastoma. This change necessitates further study of what exactly the clinical behavior of these subgroups is and how additional molecular markers can be optimally used for the diagnosis of these newly defined categories. It is already evident that apart from assessment of IDH and H3 K27M mutation status, which are part of the definition of glioblastoma subgroups, analysis of other molecular markers such as mutation of *ATRX* and/or *TP53* (especially present in adult IDH-mutant glioblastomas in adults) or *TERT* promoter mutation (present in a large percentage of adult IDH-wildtype glioblastomas) may be helpful as well. For instance, in a situation where molecular tools for further characterization of glioblastomas are lacking, many cases can still be put in a particular molecular category based on immunohistochemical analysis of (surrogate) markers. Cytoplasmic staining of glioblastoma cells for IDH1 R132H mutant protein is, if the procedure was adequately performed, proof of an IDH1-mutant tumor. Obviously, lack of IDH1 R132H staining does not completely rule out an IDH-mutant tumor as other IDH1 and IDH2 mutant proteins (i.e., about 10% of all IDH-mutant gliomas) are not recognized by this antibody. Similarly, in the right context H3 K27M-mutant protein staining in tumor cell nuclei (with negative nuclei of non-neoplastic cells as an internal control) allows for unequivocal identification of a diffuse midline glioma, H3 K27M-mutant. As *ATRX*-mutant and *TP53*-mutant diffuse gliomas generally show lack of ATRX staining of tumor cell nuclei versus strong and extensive p53 staining of the nuclei these immunohistochemical markers may help to put a glioblastoma in a particular, molecular subgroup as well. It can be expected that the development of novel immunohistochemical (surrogate) markers will further facilitate making a "molecular diagnosis" without performing genetic testing.

Meanwhile, in many centers high-throughput technologies for sophisticated molecular classification become increasingly available in a routine diagnostic setting. Improved methods for extraction of RNA in sufficient quality and quantity from formalin-fixed, paraffin-embedded tissue may help to create a niche for expression profiling as a routine tool for the neuropathological diagnosis of glioblastomas. Application of such tools will provide more information on the presence and clinical importance of fusion genes (i.e., chromosomal alterations that are easily "lost" in DNA NGS). With *TERT* promoter mutations as a first example, another aspect that needs further elucidation is the importance of mutations in noncoding DNA segments. For some tumors there is already data on mutated super enhancers which may be located on chromosomes other than the relevant gene. Furthermore, it can be assumed that the classification of CNS tumors including glioblastomas will become increasingly better the more such tumors are examined at methylation level. As the "Heidelberg CNS tumor classifier" is a self-learning system, identification of new, very rare subgroups can be expected by adding further methylation data of additional tumors. Time will tell how exactly this classifier will eventually be used in the neurooncology community as a support tool for (or even alternative for) a histomolecular CNS tumor classification.

Much more information on the (epi)genetic, molecular, and protein characteristics of glioblastomas will certainly be acquired in the years to come and will help to acquire a more complete understanding of these tumors. For instance, it is now possible to interrogate individual tumor cells for their DNA, RNA and methylome characteristics, and the results so far underscore a high level of intratumoral heterogeneity. Another aspect that requires a better understanding is the relationship between tumor cells and their microenvironment (or of the host more in general), not in the least because of the so far disappointing results of antiangiogenic and immune-therapy approaches in patients with glioblastoma. Elucidation of how tumor cells change under pressure of (chemo-, radio-, immuno-)therapy may help to improve the efficacy of treatment of recurrent glioblastomas.

One can expect that sooner or later a plateau phase will be reached in our understanding of glioblastoma pathobiology. Meanwhile, it is increasingly possible to make a precise, sometimes even molecular diagnosis based on minimally invasive diagnostic approaches such as innovative imaging and liquid biopsies. Unfortunately, against the background of all the progress that has been made in elucidating the pathology and genetics of glioblastomas, the sobering reality so far is that the prognosis for glioblastoma patients has not substantially improved. It is thus time that the increased knowledge described in this chapter can be translated in therapeutic strategies that do improve the outcome for patients suffering from these tumors.

See also: Glioblastoma: Biology Diagnosis, and Treatment.

Further Reading

Barthel, F.P., Wesseling, P., Verhaak, R., 2018. Reconstructing the molecular life history of gliomas. Acta Neuropathologica 135, 649–670.

Cancer Genome Atlas Research Network, Brat, D.J., Verhaak, R.G, et al., 2015. Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. New England Journal of Medicine 372, 2481–2498.

Capper, D., Jones, D.T.W., Sill, M., et al., 2018. DNA methylation-based classification of central nervous system tumours. Nature 555, 469–474.

Ceccarelli, M., Barthel, F.P., Malta, T.M., et al., 2016. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. Cell 164, 550–563.

Eckel-Passow, J.E., Lachance, D.H., Molinaro, A.M., et al., 2015. Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. New England Journal of Medicine 372, 2499–2508.

Korshunov, A., Ryzhova, M., Hovestadt, V., et al., 2015. Integrated analysis of pediatric glioblastoma reveals a subset of biologically favorable tumors with associated molecular prognostic markers. Acta Neuropathologica 129, 669–678.

Louis, D.N., Perry, A., Burger, P., et al., 2014. International Society of Neuropathology-Haarlem consensus guidelines for nervous system tumor classification and grading. Brain Pathology 24, 429–435.
Louis, D.N., Ohgaki, H., Wiestler, O.D., et al. (Eds.), 2016. WHO classification of tumours of the central nervous system. IARC Press, Lyon.
Louis, D.N., Aldape, K., Brat, D.J., et al., 2017. Announcing cIMPACT-NOW: The consortium to inform molecular and practical approaches to CNS tumor taxonomy. Acta Neuropathologica 133, 1–3.
Ostrom, Q.T., Gittleman, H., Liao, P., et al., 2017. CBTRUS statistical report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010–2014. Neuro Oncology 19 (Suppl. 5), v1–v88.
Perry, A., Wesseling, P., 2016. Histologic classification of gliomas. Handbook of Clinical Neurology 134, 71–95.
Reuss, D.E., Sahm, F., Schrimpf, D., et al., 2015. ATRX and IDH1-R132H immunohistochemistry with subsequent copy number analysis and IDH sequencing as a basis for an "integrated" diagnostic approach for adult astrocytoma, oligodendroglioma and glioblastoma. Acta Neuropathologica 129, 133–146.
Schwartzentruber, J., Korshunov, A., Liu, X.Y., et al., 2014. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. Nature 482, 226–231.
Sturm, D., Witt, H., Hovestadt, V., et al., 2012. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. Cancer Cell 22, 425–437.
Wesseling, P., Capper, D., 2018. WHO 2016 classification of gliomas. Neuropathology Applied Neurobiology 44, 139–150.
Yan, H., Parsons, D.W., Jin, G., et al., 2009. IDH1 and IDH2 mutations in gliomas. New England Journal of Medicine 360, 765–773.

Relevant Websites

https://icgc.org—International Cancer Genome Consortium.
https://cancergenome.nih.gov—The Cancer Genome Atlas/TCGA.
http://cancerdb.genomics.org.cn—Chinese Cancer Genome Consortium/CCGC.
https://www.molecularneuropathology.org—Methylation profiling of CNS tumors.
https://www.eortc.org—European Organization for Research and Treatment of Cancer.
https://www.rtog.org—Radiation Therapy Oncology Group.
http://www.cbtrus.org—Central Brain Tumor Registry of the United States.
http://www.pedbraintumor.org—ICGC PedBrainTumor.

Glutamine Metabolism and Cancer

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Glossary

Anabolism The biochemical processes in living systems to synthesize complex molecules from simpler molecules. An anabolic process usually requires energy input in the form of ATP or GTP.

Anaplerosis The biochemical processes that provides or replenish intermediate metabolites into a metabolic pathway. A common example is TCA cycle. During active biosynthesis, intermediate metabolites of the TCA cycle may be utilized and consumed for biosynthesis. Various mechanisms exist to replenish the metabolites of the TCA cycle.

Catabolism The biochemical processes in living system that break down of complex molecules to form simpler molecules. Catabolic reaction usually releases energy, which may be used to generate ATP and heat.

Chirality of amino acids Most amino acids assume either L- or D- chirality. Except for glycine which is achiral, all other proteinogenic amino acids are L-α-amino acids. To simply the discussion, we will omit the L-α prefix in the text unless other configuration is involved.

Cystine A dimer made of two cysteine molecules which form a disulfide bond between the sufhydro groups. This amino acid is usually imported into the cell by the cystine/glutamate antiporter (known as system Xc- or xCT) encoded by the *SLC7A11* gene in humans. After being uptaken into cells, a cystine molecule is converted into two cysteine molecules by reductive reactions.

Endoplasmic reticulum stress response An evolutionarily conserved cell response to the disturbances of the normal functions of the endoplasmic reticulum. The most common causes and consequences are accumulation of unfolded proteins in the ER lumen, also known as the unfolded protein response, and a disruption of calcium homeostasis.

Essential amino acids The amino acids that are required to support the metabolism of the living system, but the living system cannot synthesize them. Accordingly, essential amino acids have to be obtained by diet.

Homeostasis The ability of the living system to maintain a condition of equilibrium or stability within its internal environment when dealing with external changes.

mTOR Mechanistic target of rapamycin, also known as mammalian target of rapamycin, is the catalytic subunit of two protein kinase complexes (mTORC1 and mTORC2) that serve as the central sensors and integrators of multiple stimuli inputs. The best known stimuli are extracellular nutrient status, intracellular energy status and availability of growth factors. Based on the integration of the stimuli, mTORCs may regulate a variety of biological processes including cell survival, metabolism, proliferation, migration, autophagy and apoptosis.

Nonessential amino acids The amino acids that can be made by a living system, thus not being required to present in the diet.

Oncometabolite A molecule or metabolite produced by mutated metabolic enzymes and contribute to oncogenesis and tumor progression. An oncometabolite is commonly resulted from gain of function mutations which alter the normal function of a metabolic enzyme.

Abbreviations

AARE Amino acid responsive element
ALT Alanine aminotransferase
AST Aspartate aminotransferase
ATF4 Activating transcription factor 4
BCAA Branched chain amino acids
ER Endoplasmic reticulum
GCN2 General Control Non-derepressible 2
GDH Glutamate dehydrogenase
GLS Glutaminase
GSH Glutathione
IDH Isocitrate dehydrogenase
LAT1 Large neutral amino acid transporter 1

NF-κB Nuclear factor kappa B
TCA Tricarboxylic acid cycle
α-KG α-ketoglutarate

Introduction

Glutamine is one of the 20 amino acids used in protein translation processes. In plants, glutamine can be synthesized by glutamine synthetase, which uses glutamate and free ammonia as substrates and ATP as the energy source. Biochemically, the glutamine synthesis process is the major way to introduce inorganic nitrogen into organic molecules in the form of amino acids, which eventually are converted into proteins and other nitrogenous molecules and flux into higher organisms through food-chain or food-web. In addition to the dietary sources, in animals, multiple tissues can synthesize glutamine, and often use this synthesis as a way to dispose free ammonium ion, a metabolic waste. The most relevant glutamine-producing tissue is the skeletal muscle, accounting for about 90% of all glutamine synthesized. As one of the three amino acids that shuttle the toxic ammonium ion from extrahepatic tissues to hepatocytes, glutamine serves a special role in disposal of the nitrogenous waste through urea cycle. Since glutamine can be synthesized, at organism level glutamine is classified as a nonessential amino acid, and as such, its importance in anabolic pathways other than protein translation and nucleotide biosynthesis are often overlooked.

However, rapid proliferating cells including cancer cells, pathogen stimulated immune cells and various progenitor or stem cells have increased demand for glutamine, and often show glutamine dependent growth and proliferation. This increased demand usually excesses the needs for protein translation and nucleotide synthesis. In fact, most types of cancer cells cultured in vitro depend on high levels of supplemented glutamine. In those cells, most of the glutamine uptaken by cells are converted into glutamate through glutaminolysis catalyzed by glutaminase. More and more published studies support the notion that proliferating cancer cells use glutaminolysis to maintain the intracellular glutamate homeostasis to support a metabolic reprogramming associated with malignant transformation. The amido and amino groups from glutamine are crucial for the active nitrogen anabolism in proliferating cells, and the carbon skeleton from glutamine catabolism eventually contributes to the carbon pool, supporting ATP production, anaplerosis and lipid biosynthesis.

In this article, we will summarize the glutamine metabolism in cancer cells, focusing on biosynthetic roles of glutamine in proliferating cells as both a nitrogen source and a carbon source and the regulatory roles of oncogenic signaling.

Direct Roles of Glutamine and Major Enzymes

Glutamine is the most abundant one among all amino acids in circulation, including the proteinogenic or nonproteinogenic amino acids. Cells uptake glutamine through glutamine transporters, a transmembrane protein family consisting of multiple members. The structural–functional relationships and specific roles of these transporters under defined physiological or pathological conditions remain not well studied. However, SLC1A5 (ASCT2) may have special importance for cancer cells to uptake glutamine. Upon uptaken by cells, glutamine can be directly utilized for protein translation and nucleotides biosynthesis, two processes are described in details in most molecular biology textbooks. Intracellular accumulation of glutamine also provides a chemical potential for cells to uptake other nutrients. For example, leucine is an essential amino acid, and the uptake of leucine by the large neutral amino acid transporter 1 (LAT1) antiporter requires the simultaneous release of glutamine to the outside of cells. A summary of glutamine utilization in cells are outlined in Fig. 1.

Importantly, a major portion of intracellular glutamine will be converted into glutamate and an ammonium ion primarily in the mitochondria by the enzyme glutaminase. Part of the mitochondrial glutamate will be exported to cytosol to join the glutamate pool, and the other portion of glutamate is further converted into α-ketoglutarate by glutamate dehydrogenase and release ammonia. This interconversion between glutamate and α-ketoglutarate can also be catalyzed by aspartate transaminase (AST), but instead of releasing ammonia, this reaction transfers the amino group from glutamate to oxaloacetate and generates aspartate, another amino acid with special importance in nucleotide biosynthesis.

There are two genes encoding glutaminase, *GLS* and *GLS2*. *GLS* encodes for the kidney-type glutaminase (KGA), which is primarily expressed in the brain and kidney and plays a key role in synthesizing the neurotransmitter glutamate and maintaining acid–base balance in the kidney. *GLS2* encodes for the liver type glutaminase (LGA), which was originally considered liver-specific, but later found to be expressed in other tissues and cancer cells as well. Alternative splicing may result in multiple transcript variants which generate various glutaminase isoforms in different tissues and cancer cells.

Aspartate transaminases, better known clinically as glutamate-oxaloacetate transaminase (GOT), also have two isoenzymes in human: the cytosolic isoenzyme (cAST or GOT1) which primarily expresses in muscle and red blood cells, and the mitochondrial AST (mAST or GOT2) which primarily expresses in hepatocytes. The expression of both isoenzymes have been reported in different types of cancers.

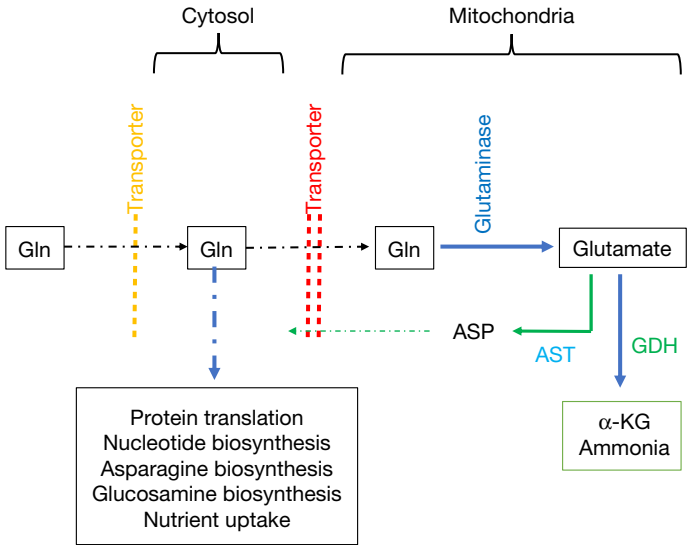

Fig. 1 Schematic drawing shows glutamine uptake, intracellular dynamics and direct roles in cell metabolism. Transporters and enzymes involved are marked with color text. *Dotted fine lines* show intracellular diamics or flux, and *solid lines* indicate biochemical conversion. *Gln*, glutamine; *Asp*, aspartate; *AST*, aspartate transaminase; *GDH*, glutamate dehydrogenase.

Indirect But Important Roles of Glutamine in Cancer Metabolism

Replenishing the pool of cytoplasmic glutamate by glutaminolysis is the most crucial role of glutaminolysis in cancer metabolism, particularly in nitrogen anabolism. In normal cells, various vitamin B6-dependent transaminases operate a glutamate-α-KG cycle, in which α-KG collects amino groups from excess amino acids doomed to be catabolized; on the other hand, when an NAEE becomes scarce, glutamate may donate the amino group to synthesize it (Fig. 2A). In cancer cells and other proliferating cells, to support proliferation, active biosynthesis, including many nitrogen anabolic processes in addition to protein translation, is necessary and sometimes can be rate-limiting for cell division. Particularly, these nitrogen-dependent biosynthetic processes consume large quantity of several amino acids that provide the nitrogen source, or carbon

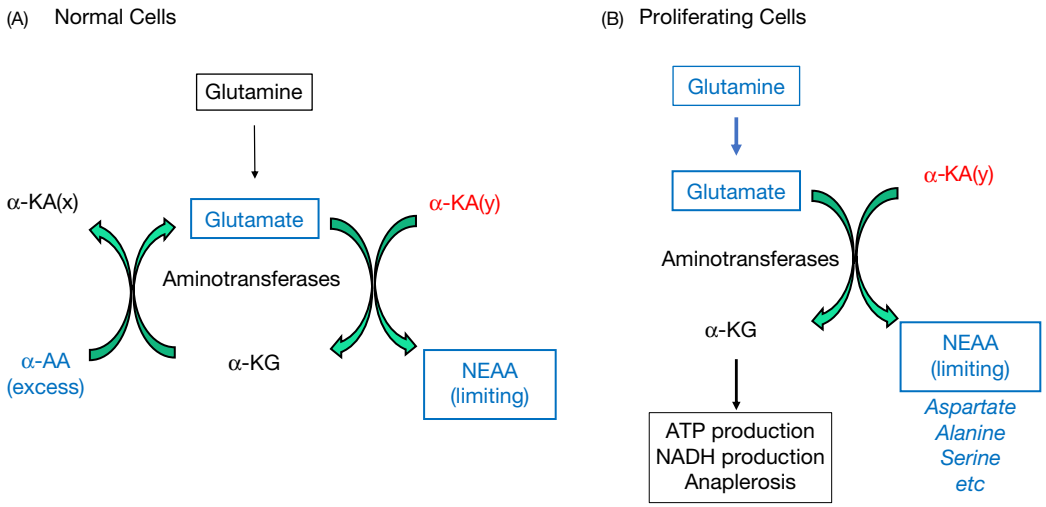

Fig. 2 Central role of glutamate in the homeostasis of nonessential amino acids in normal cells and proliferating cells. (A) In normal cells, the glutamate and α-KG cycle plays the key role to maintain a dynamic homeostasis of the intracellular pool of amino acids. Any amino acids in excess will be catabolized by transamination with α-KG first prior to feeding the carbon skeletons (ketoacids) into TCA cycle. Glutamate then can be used to synthesize any nonessential amino acid in need. (B) In rapid proliferating cells, active biosynthesis will consume most of the amino acids, particularly, some amino acids are consumed in large quantity. In this case, glutamine plays the important role in replenishing the glutamate pool consumed directly or indirectly in biosynthesis of nitrogenous molecules. Metabolites in blue indicate the flux of nitrogen.

source as well in some cases. Glutamate derived from glutaminolysis either directly participates in the biosynthesis, or indirectly provides the nitrogen source or carbon source by synthesizing these amino acids required in large quantity for the active biosynthesis (Fig. 2B). In turn, glutaminolysis provides the ultimate mechanism to maintain the cytosolic homeostasis of glutamate.

First of all, in addition to glutamine, glutamate, aspartate and glycine are well-known for their roles in de novo biosynthesis of nucleotides. Moreover, glycine is an important substrate for biosynthesis of heme, a prosthetic factor required for a variety of redox enzymes; and glutathione, an important molecule in maintaining the intracellular redox homeostasis. Similarly, serine plays multiple roles in proliferating cells, thus being required in large quantity. Serine cleavage provides important source of one carbon units for various reactions, including nucleotide synthesis, protein methylation, epigenetic control of gene expression etc. Serine also is a substrate for the biosynthesis of biomembrane by either serving as an alcohol head of phospholids, or the core structure of sphinosine (2-amino-4-octadecene-1, 3-diol). Collectively, to meet the needs for cell growth and division will consume considerable amount of serine in biosynthesis based on the stoichiometry. As such, glutamate is usually consumed as a substrate in large quantity through transamination to synthesize aspartate, glycine, serine and other nonessential amino acids actively participating in proliferative biosynthesis (Fig. 2B).

Most human cells maintain high levels of intracellular glutamate, and the cross-membrane glutamate gradient serves as a chemical potential to facilitate the uptake of key metabolites. The human *SLC7A11* gene encodes for a sodium-independent but chloride dependent cystine-glutamate antiporter well-known as system Xc- or xCT. SLC7A11 is highly expressed in astrocytes as well as in some types of cancer cells including lung, head and neck carcinomas. Cystine-glutamate antiporter uses the cross-membrane gradient of glutamate as an energy source, and couples the uptake of one molecule of cystine with the release of one molecule of glutamate to the outside of cells. The uptaken cystine in cells will be converted to two molecules of cysteine. In addition to participate in protein translation, as a special amino acid with thiol group, cysteine can be converted into methionine and participate in one carbon unit metabolism and in the biosynthesis of polyamines, a group of amines interacting with newly synthesized DNA and facilitating chromatin condensation. More importantly, cysteine is one of the critical substrate for the biosynthesis of glutathione, participating in the maintenance of redox homeostasis. The important role of glutamine metabolism in supporting glutathione biosynthesis is summarized in Fig. 3.

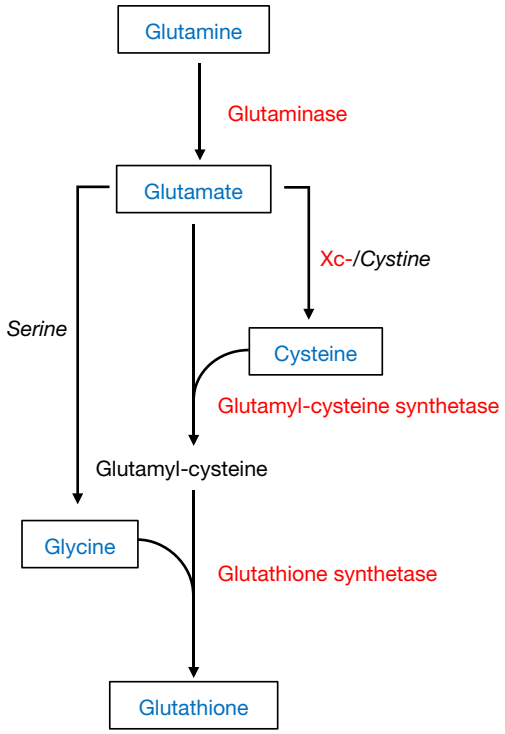

Fig. 3 Biochemical importance of glutamine in the biosynthesis of glutathione. Glutathione (GSH) is a tripeptide plays important role in protecting cells from damage caused by reactive oxygen or reactive nitrogen species, which may arise from cell metabolic processes, radiation or therapeutic drugs. Rapid proliferating cells require the biosynthesis of a large quantity of GSH to prepare for the cell division. The biosynthesis of GSH requires glutamate, cysteine and glycine as substrates. Glutamine plays important roles in maintaining the intracellular homeostasis of all three amino acids. Key amino acids are *boxed*. Enzymes and transporters involved are in *red*. Intermediate metabolites are indicated in *italics*.

Glutamine, Glutamate and α-Ketoglutarate Pathway in Carbon Metabolism

As the carbon skeleton derived from glutamine catabolism, α-KG joins the pool of reduced carbons and contributes to the carbon metabolism. As one of the metabolites of the TCA cycle, α-KG can be oxidized in TCA cycle as a fuel to generate NADH and FADH2, which transfers the electrons to electron transfer chain for ATP production. Moreover, α-KG also plays roles in anaplerosis to support the synthetic function of TCA cycle. Three metabolites of the TCA cycle have particular importance in supporting the proliferative biosynthesis thus being commonly withdrawn from the TCA cycle. Succinyl CoA is required as a substrate for the biosynthesis of Heme, an important cofactor needed for a variety of oxidoreductases. Oxaloacetate can be transaminated into aspartate in the mitochondria and exported to cytosol to support active protein translation and nucleotide biosynthesis. The citrate can be exported to the cytosol to replenish the cytosolic pool of acetyl-CoA and malate. Malic enzyme-catalyzed subsequent reaction utilizes malate as a substrate to produce cytosolic NADPH. Thereafter, both acetyl-CoA and NADPH are utilized for the biosynthesis of fatty acids, cholesterol and other molecules derived from the mevalonate pathway. In addition, α-KG can also directly undergo reductive carboxylation in the cytosol to generate citrate by the cytosolic $NADP^+$-dependent isocitrate dehydrogenase 1 (IDH1), providing another metabolic pathway generating acetyl-CoA and NADPH for lipogenesis in the cytosol.

As an excitatory neurotransmitter and the precursor of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), glutamate is the most abundant amino acids in human brains. Although the role of glutamate in the physiology of central nerve system is apparently beyond the scope of this article, glutamate metabolism has a special importance in astrocytoma, glioma or glioblastoma, the major forms of brain malignancies derived from astrocytes or glial cells. These tumors frequently carry mutations in the gene encoding IDH1 or the mitochondrial IDH2, which render new enzymatic activity; for example, a common gain of function of IDH1 is to convert α-KG into D-2-hydroxoglutarate, an oncometabolite which competitively inhibits α-KG-dependent enzymes. Similar IDH1/2 mutations and oncogenic metabolism were found in other types of malignancies, including sarcomas, hematologic malignancies and colon cancers, adding a new level of twist of glutamine metabolism in cancer cells.

Glutamine in Maintaining mTORC Activity and Active Biosynthesis

As a crucial nutrient, glutamine maintains the activity of mTORC signaling pathways. It is well-known that the mTORC signaling pathways serve as master regulators of cell survival, proliferation and proper functioning. Proper mTOR signaling is important to suppress degradative processes such as autophagy and apoptosis. The mTOR kinase forms two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). In addition to upstream regulators, the availability of nutrients and growth factor signaling are crucial to maintain mTORC activity. mTORC1 is required for various biosynthetic activities including protein translation and lipogenesis, whereas mTORC2 facilitates cell survival, migration and invasion. Adequate mTORC activity is also important to limit degradative processes including autophagy. According to the above discussion of the metabolic roles of glutamine, it is obvious that glutamine is required in multiple aspects to maintain the mTORC1 activity; particularly glutamine utilization is critical to ensure the homeostasis of the intracellular amino acids pool, redox status and energy which are monitored by mTORC1. Knocking down glutamine transporter SLC1A5 not only inhibits glutamine uptake, but also suppresses mTORC1 signaling.

Glutamine Utilization Promoted by Oncogenic Activation and Loss of Tumor Suppressors

In cancer cells, active anabolism requires increased glutamine utilization, which is driven by the activation of oncogenes, the loss of tumor suppressors or other genetic alterations (Fig. 4). Altered expression of glutamine transporters, GLS and glutamate dehydrogenase has been linked to tumor cell growth and proliferation.

The first oncogene demonstrated to promote glutamine utilization is c-Myc. *MYC* encodes a protein containing the basic helix-loop-helix leucine zipper (bHLHZip) motifs. With this motif, c-Myc contributes to the genesis of human malignancy via a network of protein–protein interaction and DNA binding. In this network, c-Myc binds to Max, another bHLHZip family member, forming a heterodimer. The structure of Myc protein contains an unstructured N-terminal region which functions as transcriptional regulatory domain, and the C-terminal bHLHZip domain, which dimerizes with Max. The Myc-Max heterodimer recognizes the DNA sequence CACGTG which belongs to the *E*-box sites. The DNA binding recruits coactivator or corepressor complexes that modify the chromatin, thus regulating the expression of the downstream genes with E-box in their promoters. These genes regulate a wide range of cellular processes, including cell growth, proliferation, and tumorigenesis. Importantly, c-Myc lies at the downstream of, and is regulated in response to some upstream oncogenic signals. Studies showed that the upregulation of MYC drives glutamine utilization via upregulating glutamine transporters and metabolism enzymes such as GLS. The second reported oncogenic signaling pathway that drives glutamine utilization is HER2 (ErbB2). Activation of ErbB2 is frequently involved in breast cancer, ovarian cancer, gastric cancer and other cancers. The formation of homodimer or heterodimer of ErbB2 with other forms of ErbBs triggers its downstream signaling including MAPK/ERK, PI3K-Akt pathway, or nuclear factor kappa B (NF-κB). It has been demonstrated that ErbB2 drives glutamine utilization by activating GLS in breast cancers. Finally, AP1 (c-Jun/Fos) has been reported to promote glutaminolysis. c-Jun, as a transcription regulator, is derived from the proto-oncogene *JUN*. c-Jun modulates oncogenic Rho-GTPase signaling to enhance the expression and enzymatic activity of mitochondrial GLS in breast cancer, which facilitates cancer cell

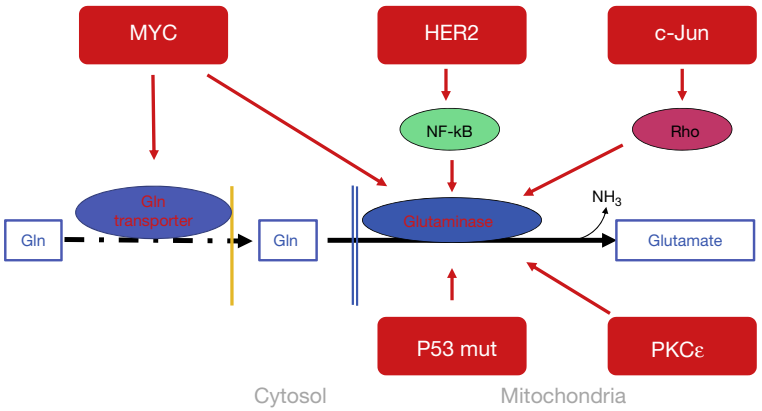

Fig. 4 Common oncogenic signaling pathways that promote glutamine utilization in cancer cells. Major oncogenic signaling pathways that promote glutamine utilization in cancer cells are shown schematically. Oncogenes or tumor suppressors were shown in *red boxes*.

metabolism and progression. More recently, a truncated isoform of glutaminase (GAC) expressed in some cancer cells was found to be activated by phosphorylation catalyzed by PKCε, providing a posttranslational mechanism to enhance glutamine utilization in cancer cells.

As a tumor suppressor, p53 suppresses the production of reactive oxygen species (ROS) through upregulating GLS2, which enhances the production of reduced glutathione. Exogenous GLS2 expression inhibits tumor growth in vivo, consistent with the tumor suppressive activity of p53. However, in some tumor cells, GLS2 is upregulated to promote tumor growth. In addition, another tumor suppressor, retinoblastoma protein (Rb) also regulates glutamine metabolism, and the loss of Rb drives tumor cells' dependency on glutamine, which also exemplifies a proliferation enhanced utilization of glutamine.

Responses of Cancer Cells to Glutamine Depletion

During tumorigenesis and tumor progression, cancer cells are frequently exposed to a poorly vascularized microenvironment which is often characterized by the lack of oxygen, glucose and glutamine. To survive in such a stressful microenvironment, tumor cells have obtained strategies to sense the lack of a specific nutrient and respond to the status by reprogramming cellular processes and metabolism. The general responses to stresses usually include the induction of autophagy, inhibition of mTOR activity, cell cycle arrest, endoplasmic reticulum (ER) stress response and activation of the heat shock system. Like other stress conditions, glutamine depletion triggers the above general responses as well (Fig. 5).

In addition to these general responses to stresses, cancer cells also develop metabolic reprogramming more specific to glutamine depletion (Fig. 5). These reprogramming include the decreased utilization of glucose, increased uptaking and utilization of other amino acids and enhanced recycling of ammonia. The decreased utilization of glucose is manifested by the decreased expression and activity of enzymes involving in both glycolytic and pentose pathways. The increased uptaking and utilization of other amino acids is highlighted by the enhanced expression of transporters for amino acids, such as branched chain amino acids (BCAA). Moreover, glutamine depletion also promotes the expression of transaminases for alanine (ALT) and branched chain amino acids. Recycling of ammonia in cancer cells is catalyzed by glutamate dehydrogenase, whose activity can be easily reversed by the alteration of substrate and/or product concentrations. Collectively, those metabolic reprogramming events triggered by glutamine depletion may either utilize alternative sources of nitrogen, or slow down global cell metabolism to conserve the nitrogen sources.

How cells monitor the glutamine availability remain illusive. Initially, the lack of essential or conditionally essential amino acids including cysteine and leucine induces an "integrated stress response" to amino acid depletion and redox stress, in which GCN2 senses the increased amount of nonaminoacylated tRNA. Nonaminoacylated tRNA binds with and activates GCN2. Like PERK in the ER lumen, activated GCN2 phosphorylates eIF2α, attenuating general protein synthesis but selectively translates ATF4, a transcription factor that binds to amino acid responsive elements (AARE) and regulates gene transcription involved in relieving the stresses caused by amino acid depletion. Later, it is found that glutamine depletion is sufficient to induce eIF2α phosphorylation and thus activates ATF4. This process may involve both GCN2 activation and the PERK kinase activity as a part of the ER stress responses. Considering glutamine's crucial role in biosynthesis of nonessential amino acids, glutamine depletion could lead to insufficiency of nonessential amino acids such as serine, glycine, aspartate which are required in large quantity in proliferating cells. In addition, glutamine depletion disrupted glutamate homeostasis may directly compromise GSH synthesis, and thereafter, the accumulation of redox stress facilitates the activation of ER stress, providing additional mechanism to activate ATF4. Activation of ATF4 may lead to transcriptional reprogramming and the relieving of stresses including nutrient depletion. In this aspect, it seems that the availability of multiple amino acids as a pool, instead of glutamine alone, is collectively sensed by multiple cellular mechanisms.

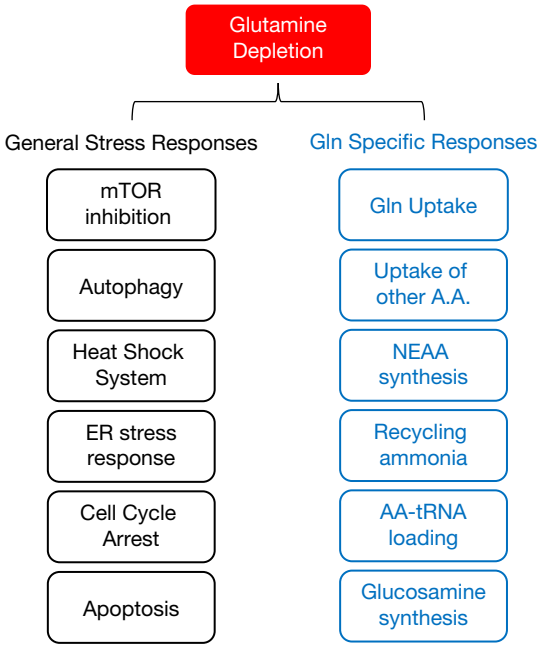

Fig. 5 Glutamine depletion triggeres general stress responses and glutamine depletion specific, adaptive metabolism. Glutamine depletion is a common condition that cancer cells may frequently encounter in vivo. Current studies indicate that glutamine depletion triggered cellular responses can be classified into two major categories: those not specific to defined stresses, and those specific to glutamine depletion. The responses specific to glutamine depletion generally tend to increase the ability or enzyme activities to reach a new homeostasis of a metabolite in scarce supply, indicative of compensatory strategies of cancer cells.

Prospective Vision

Cancer metabolism is characterized by increased glycolysis and active glutaminolysis, representing the enhanced utilization of glucose and glutamine respectively. Targeting cancer metabolism as potential novel therapies has been explored at various stages including basic research, preclinical and clinical stages in recent years. The initial success has been demonstrated by various trials targeting the glucose utilization pathways in cancers. Preclinical studies on the use of inhibitors of glutamine transporters or metabolic enzymes in order to block glutamine utilization also result in initial excitement. However, cancer cells may usually evolve the ability to adapt to nutrient depletion, which negatively affects the efficacy of various strategies to "starve" cancer cells. Accordingly, identification of critical adaptive strategies of cancer cells induced by nutrient depletion will potentially provide novel therapeutic targets. A combination of targeting both cancer metabolism and its adaptive strategy may provide therapeutic advantages and improve the efficacy.

Existing data from cultured cells indicate that whereas glutamine depletion suppresses glucose utilization, and that glutamine utilization may be limited by the availability of other nutrients such as glucose and oxygen, indicating a coordinated utilization of glutamine, glucose and molecular oxygen. Our unpublished observations show that glucose depletion suppresses c-Myc/Max, which indicates limited glucose supply may suppress glutamine utilization driven by Myc oncogene. Hypoxia-inducible factors (HIF-1, 2, collectively HIFs) are heterodimeric transcription factors which are activated primarily by hypoxia and serve as drivers of ATP production. Accordingly, HIFs are often activated by hypoxia and other biological stresses such as injury and infection to promote cell repair and defense. It has been reported that there is a functional interaction between the c-Myc and HIF pathways, and HIF activation counteracts the cell cycle promoting effect of c-Myc. A better understanding of the coordination of cellular response to nutrient depletion may provide new insight into the molecular and biochemical mechanisms of cancer cells' adaptation to a combined nutrient depletion which is more relevant to cancer metabolism in real patients.

Mechanistically, while the enzymes involved in glutamine utilization are well studied, and the metabolic flux of glutamine also has been extensively studied by combined isotope-tracking and mass-spectrometric analyses, the expression and regulation of glutamine transporters in cancers remain to be further clarified. Altered metabolic pathways or metabolites in cancers caused by gain-of-function mutations of metabolic enzymes remain to be further elucidated.

Acknowledgments

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Further Reading

Coloff, J.L., Murphy, J.P., Braun, C.R., Harris, I.S., Shelton, L.M., Kami, K., Gygi, S.P., Selfors, L.M., Brugge, J.S., 2016. Differential glutamate metabolism in proliferating and quiescent mammary epithelial cells. Cell Metabolism 23, 867–880.

Gao, P., Tchernyshyov, I., Chang, T.C., Lee, Y.S., Kita, K., Ochi, T., Zeller, K.I., De Marzo, A.M., Van Eyk, J.E., Mendell, J.T., Dang, C.V., 2009. C-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 8, 762–765.

Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D.F., 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Molecular Cell 11, 619–633.

Locasale, J.W., 2013. Serine, glycine and one'carbon units: Cancer metabolism in full circle. Nature Reviews. Cancer 13, 572–583.

Lukey, M.J., Greene, K.S., Erickson, J.W., Wilson, K.F., Cerione, R.A., 2016. The oncogenic transcription factor c-Jun regulates glutaminase expression and sensitizes cells to glutaminase-targeted therapy. Nature Communications 7, 11321.

Medina, M.A., 2001. Glutamine and cancer. The Journal of Nutrition 131, 2539S–2542S.

Meng, M., Chen, S., Lao, T., Liang, D., Sang, N., 2010. Nitrogen anabolism underlies the importance of glutaminolysis in proliferating cells. Cell Cycle 9, 3921–3932.

Possemato, R., Marks, K.M., Shaul, Y.D., Pacold, M.E., Kim, D., Birsoy, K., Sethumadhavan, S., Woo, H.K., Jang, H.G., Jha, A.K., Chen, W.W., Sabatini, D.M., 2011. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature 476, 346–350.

Qie, S., Liang, D., Yin, C., Gu, W., Meng, M., Wang, C., Sang, N., 2012. Glutamine depletion and glucose depletion trigger growth inhibition via distinctive gene expression reprogramming. Cell Cycle 11, 3679–3690.

Qie, S., Chu, C., Li, W., Wang, C., Sang, N., 2014. ErbB2 activation upregulates glutaminase 1 expression which promotes breast cancer cell proliferation. Journal of Cellular Biochemistry 115, 498–509.

Spinelli, J.B., Yoon, H., Ringel, A.E., Jeanfavre, S., Clish, C.B., Haigis, M.C., 2017. Metabolic recycling of ammonia via glutamate dehydrogenase supports breast cancer biomass. Science 358, 941–946.

Wise, D.R., DeBerardinis, R.J., Mancuso, A., Sayed, N., Zhang, X.Y., Pfeiffer, H.K., Nissim, I., Daikhin, E., Yudkoff, M., McMahon, S.B., Thompson, C.B., 2008. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proceedings of the National Academy of Sciences of the United States of America 105, 18782–18787.

Helicobacter Pylori-Mediated Carcinogenesis

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The Discovery of *H. pylori* as an Infectious Agent Within the Stomach

Helicobacter pylori has colonized and coevolved with its human host for millennia. The earliest direct evidence of *H. pylori* colonization can be seen from gastric biopsies taken from the Iceman, a 5300-year old mummy from the Copper Age, yet *H. pylori* colonization dates back at least 100,000 years. Indeed, conserved domains within the *H. pylori* genome can be used to mirror the paths of human migration out of Africa.

Although *H. pylori* was not officially discovered until 1983, spiral-shaped bacteria had been observed in human stomachs by German physicians as early as 1875. In 1899, a Polish physician named Walery Jaworski observed spiral shaped bacteria in sediments from gastric washings and named the organism *Vibrio rugula*. In his book entitled "*Handbook of Gastric Diseases*", Jaworski even implicated *V. rugula* as a causative agent of gastric disease. However, the observations of these scientists were forgotten until 1939–40 when physicians again observed "spirochaetes" within the human gastric mucosa of patients with and without gastric disease. Finally, in 1954 Palmer et al. examined over 1000 gastric biopsies and found no bacteria with spirochetal structure from their samples. They concluded that the stomach was a sterile organ and that any observed bacteria were oral contaminants that multiplied in postmortem specimens. Nonetheless, observations of bacteria within the human stomach persisted into 1975 when Steer and Colin-Jones observed Gram-negative bacilli in 80% of patients with gastric ulcer, yet they dismissed their observation as a possible contaminant of *Pseudomonas* (Fig. 1).

In 1983 Barry J. Marshall and John Robin Warren were the first to both observe and culture back an unspecified bacteria from the gastric epithelium among patients with chronic gastritis. To accomplish what their predecessors could not, Marshall and Warren had used a silver stain, which was unconventional for gastric specimens at the time, to observe the spiral-shaped bacteria. Furthermore, the conventional amount of time for bacteria recovered from the gastrointestinal tract to grow in the lab was only 2 days, after which the agar plates would be discarded if there was no bacterial growth. However, due to a fortunate accident whereby Marshall and Warren had accidentally left their agar plates in the incubator for 6 days over the Easter weekend, they were then able to recover their unspecified bacterium. They classified the bacterium not as a spirochete as others had described, but rather a species of *Campylobacter* due to its morphology. In 1984, they again published that this unspecified bacterium was recoverable from chronic active gastritis as well as peptic ulcers. The pair named this unspecified organism *Campylobacter pyloridis* due to its predominance within the gastric antrum. In 1989, 16S ribosomal RNA sequencing of *Campylobacter pyloridis* revealed that it was not a *Campylobacter* species, but rather a new bacterial species and subsequently renamed to *Helicobacter pylori*.

In 1983, the hypothesis that *H. pylori* could be the causative agent of peptic ulcer was not well received in the medical community despite a clinical trial demonstrating gastritis could be healed after bacterial eradication. One of the main obstacles in proving that *H. pylori* was the etiological agent of gastritis was the lack of a proper animal model to prove Koch's postulates. Therefore, after multiple failures in trying to infect rats, mice, and pigs, Barry Marshall chose to drink a pure culture of the bacterium and track his disease progression. Within 5 days he developed acute gastritis associated with the presence of many *H. pylori* until day 14 when his symptoms and the *Helicobacter* spontaneously subsided. By 1988, two independent follow-up studies published outside

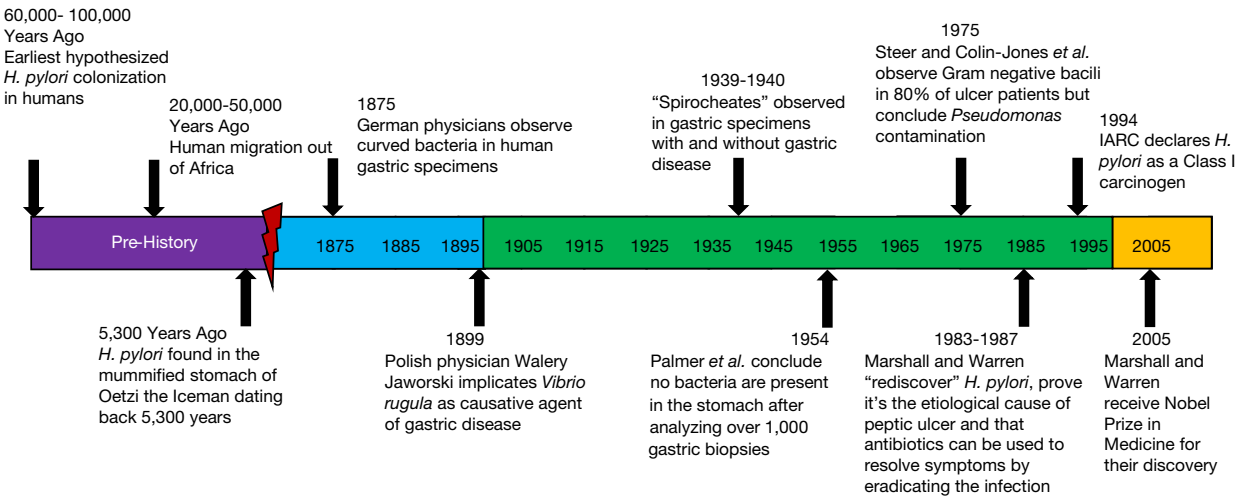

Fig. 1 Timeline of major events in the history of *H. pylori* from its coevolution with humans in prehistory to the revelation of its contribution to peptic ulcer disease and gastric adenocarcinoma.

the United States had shown that patients with duodenal ulcer and concurrent *H. pylori* infection could be cured with a regimen of antibiotics combined with bismuth. By 1991 the first study demonstrating the cure of duodenal ulcer was published in the United States. These clinical trials, combined with Marshall's proof of Koch's postulates, proved *H. pylori* as a causative infectious agent leading to gastroduodenal disease.

Since Marshall and Warren's discovery, *H. pylori* has been associated with a range of gastroduodenal disorders including gastric and duodenal ulcers, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma, as well as non-Hodgkin lymphoma of the stomach. In 1994, the International Agency for Research on Cancer officially classified *H. pylori* as a class I carcinogen. Additionally, as a result of their work and subsequent scientific advancement of the field of gastroenterology, Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine in 2005.

Bacteriology of *H. pylori*

Helicobacter pylori is the archetypal species of its genus. *H. pylori* is a Gram-negative, curved-rod or spiral-shaped microaerophilic organism that resides in the mucus gel layer above the gastric epithelium (Fig. 2). The genome size is approximately 1.7 Mbp with a G+C content of 35%–40%. Every strain of *H. pylori* is genetically distinct, possibly as an adaptation to the unique gastric conditions and immune response of each human host. In vitro, *H. pylori* colonies are translucent and uniformly sized (~1 mm). Each bacterium measures approximately 2–4 μm in length 0.5–1 μm in width, however in response to environmental stress, *H. pylori* may temporarily assume a smaller, coccoid shape until ideal growth conditions are restored. *H. pylori* harbor two to seven unipolar, sheathed flagella that measure approximately 3 μm in length and typically carry a distinctive bulb at the terminus. *H. pylori* strains are characteristically catalase, oxidase, and urease positive organisms. Urease productivity is critical for *H. pylori* survival, representing nearly 15% of the total expressed proteome. Urease is of particular importance because it is the foundation of both noninvasive and invasive clinical diagnostics for active *H. pylori* infection.

Virulence Factors

In order to sustain a persistent infection, *H. pylori* must liberate nutrients from the host while also evading the host immune response. To accomplish this task, *H. pylori* maintains an armamentarium of virulence factors which serve to both strategically

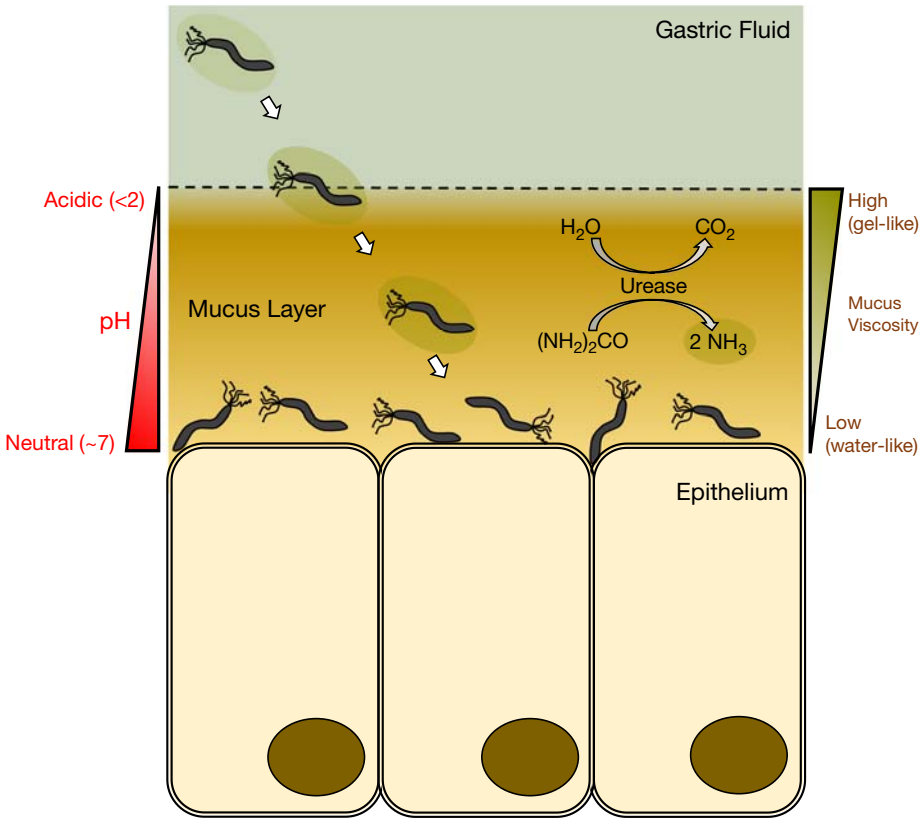

Fig. 2 Schematic representation of the natural niche of *H. pylori*. Upon initial colonization, *H. pylori* utilizes it flagella and urease to survive the acidity of the gastric lumen. Through chemotaxis, the bacteria migrate through the mucus gel layer to the border with the gastric epithelium where it can establish infection. The majority of colonizing bacteria are free swimming, while some will adhere to the gastric epithelium, inducing proinflammatory immune responses.

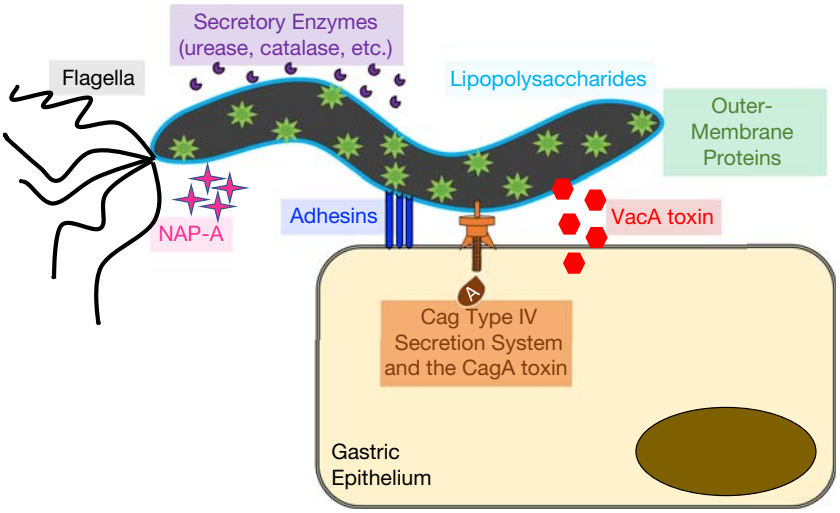

Fig. 3 Summary of *H. pylori* virulence factors that contribute to oncogenesis.

induce an inflammatory response to free nutrients sequestered by host cells while simultaneously shielding it from clearance by the immune response (Fig. 3).

Cag pathogenicity island

The cytotoxin associated gene pathogenicity island (*cag*PAI) is a highly proinflammatory, strain-specific virulence factor that significantly augments risk for gastric cancer and ulceration. Nearly 50% of all strains in the United Sates and 90% of stains from East Asia are cag^{+}. Genes within the *cag* pathogenicity island encode the structural components of a bacterial type IV secretion system (T4SS), a needle-like appendage that delivers microbial effectors into host cells (Fig. 4). The protein product of the terminal gene in the *cag* PAI is the CagA toxin, which is delivered into host cells following bacterial attachment. Once inside host cells, CagA can become tyrosine-phosphorylated at EPIYA amino acid sequence motifs and subsequently induce proinflammatory signaling pathways and cytoskeletal rearrangements. Intracellular CagA may also remain unphosphorylated, thereby inducing aberrant β-catenin activation, disruption of apical junction complexes, and a loss of cellular polarity. In vitro and in vivo experiments have also demonstrated that CagA can inhibit apoptosis and induce cellular proliferation, thus designating this toxin as a bacterial oncoprotein. The Cag T4SS can also deliver other effector molecules such as peptidoglycan from the bacterial cell wall, subsequently activating the innate immune receptor NOD1 and triggering further downstream proinflammatory signaling cascades. Lastly, nascent and ongoing studies have shown that *H. pylori* may also deliver its genomic DNA via the Cag T4SS into host cells to modulate innate immune responses.

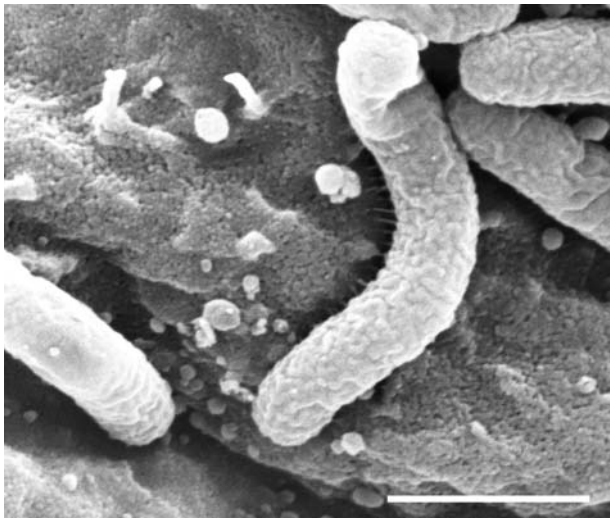

Fig. 4 Electron micrograph of *H. pylori* strain 26695 in coculture with human gastric epithelial cells. Numerous cag T4SS pili are visible at the bacteria–host cell interface. Scale bar represents 1 μm. Image courtesy of Dr. Carrie Shaffer, University of Kentucky.

Vacuolating toxin A

Vacuolating toxin A (VacA) is an *H. pylori* secreted protein that assembles into oligomers when in contact with host cell membranes. These oligomers assemble into selective anion channels that classically define VacA as a pore forming toxin. In addition to causing vacuolation, VacA pores can increase paracellular permeability to organic molecules, iron, and nickel within gastric epithelial cells, thereby liberating vital nutrients for *H. pylori* survival. The toxin can also damage mitochondria and induce apoptosis. To evade the adaptive immune response, VacA can also inhibit the expansion of bacterial protein-detecting T-cells and promote the differentiation of dendritic cells into a tolerogenic phenotype which subsequently trigger the development of regulatory T-cells. All *H. pylori* strains harbor *vacA* genes, however there is significant genetic variability within the *vacA* gene that influences its toxicity. The more virulent form of the toxin (s1m1 allelic variant) is associated with increased risk for both peptic ulcer as well as gastric adenocarcinoma.

Neutrophil activating protein A

The *H. pylori* NAP-A (synonymously called HP-NAP) is a highly conserved, secreted protein which acts as a chemoattractant for neutrophils. In response to NapA, these recruited neutrophils then produce oxygen radicals, cytokines, and chemokines involved in a strong T_H1 immune response.

Outer membrane proteins and adhesins

H. pylori adhesins decorate the outer membrane and facilitate a closer association between the bacterium and the gastric epithelial cell. This interaction is essential for successful colonization and allows for enhanced host cell exposure to bacterial virulence factors, resulting in greater inflammation and mucosal damage. Several of the major *H. pylori* adhesins include BabA, SabA, and OipA. BabA binds difucosylated $Lewis^b$ blood group antigens found on epithelial cells and mucins, and is the major adhesin involved in facilitating *H. pylori* colonization. It has two allelic forms, *babA1* and *babA2*; strains expressing *babA2* have been associated with increased *H. pylori* colonization density, higher levels of inflammation, and more severe gastric disease, particularly when coexpressed with CagA and VacA. SabA binds sialyated $Lewis^x$ antigens and is predominantly expressed in response to a chronically inflamed stomach. Strains expressing SabA are associated with increased risk for gastric cancer. OipA expressing strains have been associated with increased risk of both peptic ulcer and gastric cancer. Finally, from studies of serum antibody titers, two other uncharacterized *H. pylori* proteins, an outer membrane protein, Omp (*HP1564*) and the hypothetical protein encoded by the gene *HP0305* have been consistently associated with gastric cancer incidence.

DupA and tfs4

H. pylori species can encode multiple type IV secretion systems in addition to the *cag* system. These include the *comB*, *tfs3*, and *tfs4* systems. However, the *comB and tfs3* systems are involved in horizontal gene transfer between *H. pylori* strains and are thus not directly involved in *H. pylori* virulence. Similar to the *cag*PAI, the *tfs4* gene cluster encodes a T4SS and the duodenal ulcer promoting gene A (*dupA*). As the name suggests, $DupA^+$ strains were initially associated with duodenal ulcer disease, however not all studies have been able to demonstrate a positive association. Discrepancies may be due to differences in the expression of DupA alone compared to the expression of the entire *tfs4* gene cluster.

Strategies of Persistence and Immune Evasion

Although *H. pylori* engenders a robust immune response, it can maintain colonization for the lifetime of the host. To accomplish this feat, *H. pylori* employs a multitude of strategies to survive the hostile conditions of the human stomach and escape the immune response.

Physical barriers

Immediately after infecting its host, *H. pylori* must overcome multiple host defenses to initiate colonization in the human stomach. The basal pH of the gastric lumen is typically less than pH 2.0 and can rise to pH 5.5 after a meal in adults. However, *H. pylori* is not an acidophile and thus cannot survive long under this pH stress. To combat these conditions, nearly 15% of the total expressed *H. pylori* proteins are devoted to manufacturing enzymes designed to buffer the acidic environment. Below pH 6.5, *H. pylori* opens a channel in its cytoplasmic membrane to allow for urea produced by the host to enter the cytosol. *H. pylori* urease then catalyzes host urea into ammonia and carbon dioxide which act as buffers in the bacterial cytoplasm. Subsequently, the periplasmic enzyme α′carbonic anhydrase catalyzes the reversible reaction of hydrating the carbon dioxide released via cytoplasmic urease activity into bicarbonate, which then buffers the *H. pylori* periplasm.

Despite the capacity to buffer the acidic conditions found within the gastric lumen, *H. pylori* must also remain in the stomach after gastric emptying. Gastric epithelial cells are protected from acid in the gastric lumen by a mucus layer. The mucus layer is thick and gel-like nearest the lumen and gradually increases in viscosity as well as pH until it reaches the epithelial cell surface where it is highly viscous and has near-neutral pH. Through the use of chemotaxis and its unipolar flagella, *H. pylori* can identify this pH gradient and navigate away from the acidic lumen and toward the epithelial cell surface. *H. pylori* then resides within this viscous, pH neutral niche adjacent to the gastric epithelium where the majority are free swimming, while others adhere to gastric epithelial cells via adhesins and secrete effectors to induce the release of host nutrients (**Fig. 2**).

Innate immune response

Whilst the colonization of the mucus layer and gastric epithelium helps *H. pylori*, the interaction with the epithelial surface also triggers inflammatory responses designed to clear pathogens. However, *H. pylori* has evolved to limit its activation of the host immune response and even alter the response to become more tolerogenic.

Toll-like receptors (TLRs) orchestrate immune responses targeting invading pathogens and bridge innate with adaptive immunity via the detection of conserved pathogen associated molecular patterns (PAMPs). These PAMPs can originate from a wide array of molecules such as lipids, nucleic acids, and specific proteins that can be derived from organisms of bacterial, viral, or fungal origin. As TLRs are integral to the innate immune response, *H. pylori* has developed highly specific adaptations to its PAMPs such that it can avoid or reduce the intensity of their activation. *H. pylori* can activate TLR2 to subsequently trigger the expression of many antiinflammatory cytokines. Bacterial lipopolysaccharides (LPS) are recognized by TLR4 and can be highly immunogenic, yet *H. pylori* has subtle modifications within the structure of the lipid A core of its LPS that render it nearly undetectable. Furthermore, additional adaptations to *H. pylori* LPS make it less susceptible to destruction by cationic antimicrobial peptides. The natural ligand of TLR5 is flagellin; however, *H. pylori* flagellin is not recognized by TLR5 due to a mutation in the conserved domain of FlaA, which alters the domain where TLR5 binds. Recent studies have also shown that *H. pylori* DNA activates TLR9 to induce antiinflammatory responses during the initial stages of infection, however *H. pylori*-induced chronic activation of this receptor can eventually yield a proinflammatory response.

Phagocytosis is a key component of the host response to clear bacterial pathogens. Infection with *H. pylori* can recruit neutrophils, polymorphonuclear lymphocytes (PMNs), and monocytes. *H. pylori* can evade phagocytosis by PMNs and monocytes and even if engulfed, can use its virulence factors CagA and VacA to survive phagocytosis. Furthermore, phagocytes release reactive oxygen species (ROS) and nitric oxide to facilitate bacterial killing. Yet, *H. pylori* possess the enzymes catalase and superoxide dismutase to detoxify ROSs while *H. pylori* arginase protects it from the effects of nitric oxide.

Dendritic cells link innate and adaptive immunity. The c-type lectin receptors (CLRs) are a class of innate immune receptors expressed by dendritic cells that also recognize conserved bacterial motifs. DC-SIGN is the predominant CLR involved in detecting *H. pylori*, specifically the surface proteins Le^x and Le^y antigens. However, DC-SIGN engagement of *H. pylori* Le^x or Le^y antigens can induce antiinflammatory signaling cascades and can also block proinflammatory T_H1 cell recruitment. The *H. pylori* virulence factor CagA can further regulate DCs to favor a tolerogenic T_{Reg} immune response and also block $CD4^+$ T-cell differentiation into T_H1 types. The VacA toxin can also inhibit DC maturation by inducing cell cycle arrest. The *H. pylori* secreted virulence factor γ-glutamyl-transferase (GGT) blocks IL-6 production and favors IL-10 and IL-18 production in DCs, subsequently favoring the expansion of naive T cells into T_{Reg} cells to further suppress T_H1 and T_H17 effector functions.

Adaptive immune response

The adaptive immune response to *H. pylori* is predominantly mediated by $CD4^+$ polarized T-cells, namely T_H1 and T_H17 immune responses. As DCs bridge the innate and adaptive immune responses, the previously mentioned mechanisms by which *H. pylori* manipulates DC responses are also attributable to the manipulation of the adaptive immune response. However, *H. pylori* may use its virulence factors VacA and GGT to also directly inhibit T-cell functions as a means of persistence. VacA can traverse T-cell membranes where, once in the cytoplasm, it can impair T-cell activation and proliferation through a variety of independent mechanisms. GGT can inhibit T-cell proliferation through disruption of the Ras signaling pathway. GGT can also reduce the efficacy of T-cell responses by depriving cells of glutamine, which is required for the synthesis of the proinflammatory effector cytokines such as IL-2, IL-17, and IFNγ. Similarly, the *H. pylori* enzyme arginase can deplete levels of available L-arginine, which is required by T-cells for proliferation. Although *H. pylori* elicits strong T_H1 and T_H17 adaptive immune responses, it can persist within its human host by actively preventing these T cell responses from clearing the infection while also promoting the recruitment of antiinflammatory T_{Reg} cells.

The humoral immune response to *H. pylori* is robust yet mostly ineffective. Multiple in vivo murine studies have demonstrated that B-cell responses are entirely dispensable for bacterial clearance and that they may even be harmful to the host. Most infected individuals will mount IgG and IgA antibody responses typically directed toward membrane proteins, flagellin, urease, LPS, and chaperonin GroEL.

Epidemiology of *H. pylori* Infection

Transmission and Colonization

H. pylori almost exclusively colonizes the gastric mucosa of humans and is typically the only *Helicobacter* species found within the human stomach. Greater than half of the world's population is colonized with *H. pylori.* Infection is typically acquired during early childhood and the infection usually persists for the lifetime of the host if left untreated. Reinfection after successful eradication is rare among adults, occurring in less than 2% of patients per year. Increased risk for acquiring an *H. pylori* infection has been related to a mother with an *H. pylori* infection, non-white race, lower socioeconomic status, poor hygiene, residing in an area of high population density, greater number of siblings, and a lack of running water.

H. pylori strains can be recovered from saliva, gastric reflux fluid, diarrhea, and vomitus yet *H. pylori* does not survive passage through the intestine into normal feces. The mode of transmission remains unclear; intrafamilial case clustering as well as the

lack of a nonhuman reservoir suggest that close person–person contact is required for transmission. Therefore, the prevailing hypotheses posit that fecal–oral and/or oral–oral routes may be the most likely mode(s) of transmission, with children generally infected by their mother, older siblings, or classmates.

Geographical Variation

Currently, the global prevalence of *H. pylori* infection is approximately 50% where more-affluent countries generally have a lower prevalence (approximately 20%–40%) compared to low and middle-income countries where the prevalence can reach upwards of 80%. However, one notable exception is the East Asian region, where the more-developed countries of Korea, Japan, and China have historically maintained near developing-country levels of *H. pylori* prevalence (Fig. 5). Even within countries, racial and ethnic differences and variation in socio-economic status also influence the prevalence of infection. In the United States, for example, individuals of non-white race, including Asian Americans, African Americans, American Indians, and Hispanic Americans, have been found to have significantly higher prevalence of *H. pylori* than white Americans. Additionally, in both high- and low-*H. pylori*-prevalent countries, individuals of lower socioeconomic status are more likely to harbor the bacteria.

Temporal Trends

Over time, *H. pylori* prevalence has remained fairly stable and at a high level in developing and newly industrialized countries. In contrast, decreasing trends have been observed in many more-developed countries as living conditions have improved. In established industrialized nations, including the United States, Japan, and China, the declines in prevalence are beginning to slow and stabilize. Moreover, the decline of *H. pylori* prevalence in the United States appears limited to the white population.

The Role of *H. pylori* in Disease

Gastritis

H. pylori infection results in a robust, localized, immune response that activates proinflammatory signaling cascades in the gastric mucosa (gastritis). Nearly every person infected with *H. pylori* develops superficial atrophic gastritis that will last throughout the duration of their infection. The level of gastritis varies significantly per person, ultimately influencing the risk of disease progression.

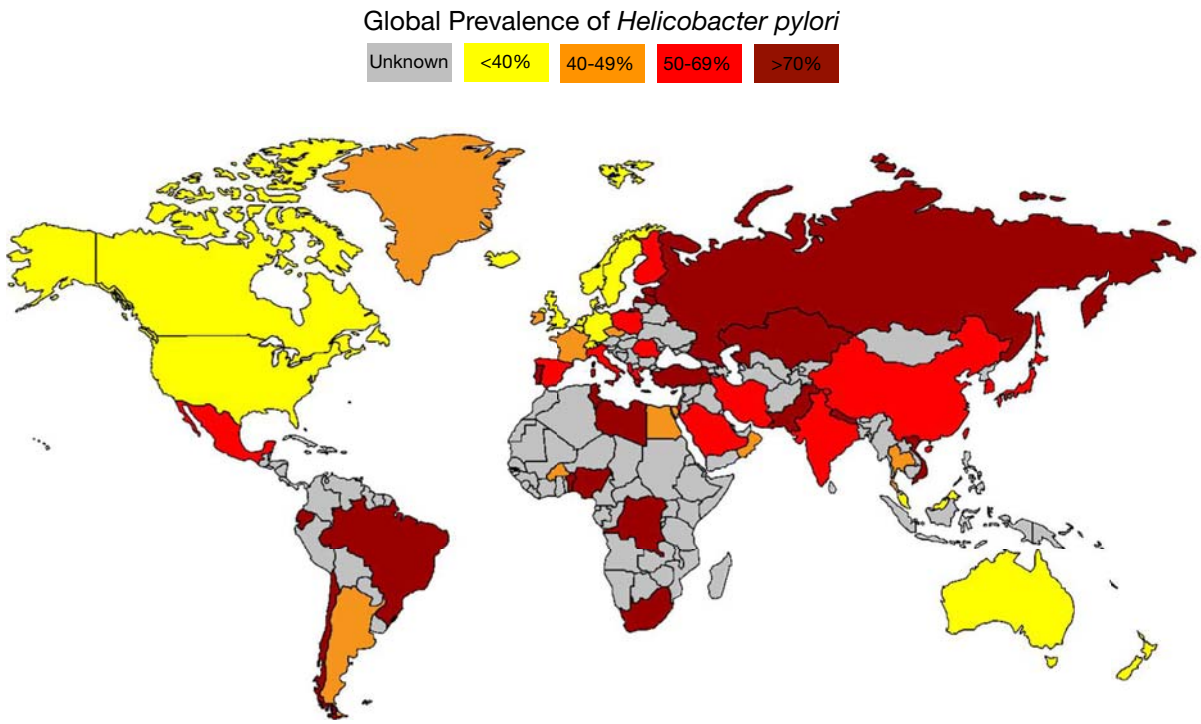

Fig. 5 Map demonstrating global prevalence of *H. pylori*. Prevalence estimates are based on data collected from January 1, 1970 to January 1, 2016. Adapted from Hooi, J. K. Y., Lai, W. Y., Ng, W. K., Suen, M. M. Y., Underwood, F. E., Tanyingoh, D., Malfertheiner, P., Graham, D. Y., Wong, V. W. S., Wu, J. C. Y., Chan, F. K. L., Sung, J. J. Y., Kaplan, G. G., Ng, S. C. (2017) Global prevalence of *Helicobacter pylori* infection: Systematic review and meta-analysis. *Gastroenterology***153**(2), 420–429. doi: 10.1053/j.gastro.2017.04.022. PubMed PMID: 28456631.

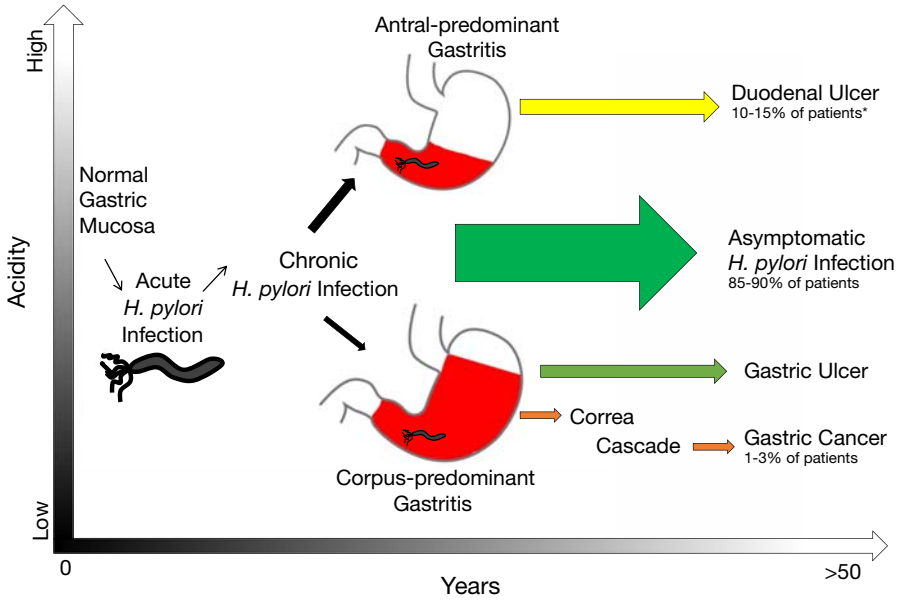

Fig. 6 Potential outcomes of *H. pylori* infection. *H. pylori* infection can persist for the lifetime of the host, causing alterations in the gastric environment and histology. Approximately 85%–90% of infected individuals will remain asymptomatic, only developing nonatrophic gastritis. However, a minority of infected patients can progress over the course of decades to either antral- or corpus-predominant gastritis, which can lead to peptic ulcer disease or gastric cancer. *Percent representative of patients progressing to peptic ulcer disease, including both gastric and duodenal ulcer.

While the majority of infected individuals remain asymptomatic with only chronic gastritis, some may progress further to a permanent loss of epithelial glands termed atrophic gastritis, a recognized risk factor for gastric cancer. Another determinant of disease outcome involves the pattern of gastritis within the stomach, although the drivers of each particular pattern remain unknown. Antral-predominant gastritis is associated with duodenal ulceration in contrast to corpus-predominant or pan-gastritis which are both associated with gastric ulcer and gastric adenocarcinoma (Fig. 6).

Peptic Ulcer Disease

Gastric and duodenal ulcers are mucosal defects that penetrate the muscularis mucosa and typically appear in areas where mucosal inflammation is most severe. Peptic ulcer disease is a chronic, relapsing disease that causes significant morbidity and mortality due to pain, bleeding and perforation of the gastric mucosa. Nearly 70% of all gastric ulcers and 95% of duodenal ulcers are attributable to *H. pylori* infection. However, eradication of *H. pylori* allows most peptic ulcers to heal and prevents further relapse.

Mechanistically, gastric ulcers originate from prolonged, intimate contact between *H. pylori* and the gastric epithelium. This interaction leads to continuous inflammation in the gastric antrum, thereby perpetuating mucosal breakdown, erosive gastritis, and eventually gastric ulceration. Conversely, the mechanism by which *H. pylori* promotes duodenal ulceration is not completely understood. The leading hypothesis is that *H. pylori*-induced high levels of gastric acid secretion in the antrum can eventually lead to replacement of intestinal-type tissue in the duodenum with gastric tissue. Subsequently, *H. pylori* can then colonize the areas of gastric metaplasia and together with elevated acid levels, ultimately promote ulceration in this locale.

MALT Lymphoma

Normal gastric mucosa does not typically contain lymphoid tissue, however colonization with *H. pylori* recruits mucosa associated lymphoid tissue (MALT). In very rare instances, the chronic expression of cytokines released by T-cells involved in combating *H. pylori* infection can ultimately support the uncontrolled growth and proliferation of a monoclonal population of B lymphocytes leading to MALT lymphoma. Almost all MALT lymphoma patients are *H. pylori*-positive, however this disease manifests in less than 1% of *H. pylori* infected individuals. If found early, *H. pylori* eradication can lead to complete remission in more than 80% of patients.

Gastric Adenocarcinoma

Infection with *H. pylori* is the single greatest risk factor for the development of noncardia gastric adenocarcinoma, with nearly 89% of cases attributable to infection. Due to its association with gastric carcinogenesis, the World Health Organization has classified *H. pylori* as a type I carcinogen. Among infected individuals, the risk of developing gastric cancer is between 1% and 2%. The

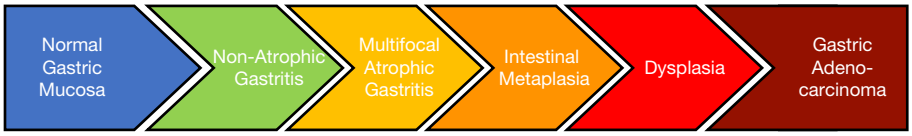

Fig. 7 Schematic of the Correa Cascade representing the histological progression from normal gastric mucosa to gastric adenocarcinoma.

progression from superficial gastritis to adenocarcinoma is a lengthy process taking 15–20 years to move through a series of progressive histological steps termed the Correa Cascade (Fig. 7). *H. pylori* eradication can reduce the risk for gastric cancer if therapy occurs prior to the onset of premalignant histological changes.

The chronic inflammatory process resultant from infection is the primary mechanism of *H. pylori*-induced carcinogenesis. *H. pylori* can decrease apoptosis and promote cell survival, which, in a highly proinflammatory environment rich in free radicals, can subsequently increase the likelihood of DNA damage and DNA mutations within host cells. In addition, the T_H17 immune response elicited by *H. pylori* can enhance carcinogenesis because the secreted cytokine profile favors angiogenesis and tumor invasiveness. *H. pylori* also induces a robust T_H1 response which does have antitumorigenic properties, however the tolerogenic T_{Reg} response also elicited by *H. pylori* to dampen the proinflammatory response can simultaneously inhibit antitumor activity.

Extragastric Diseases

Helicobacter pylori has been implicated in a variety of extragastric diseases due to its coevolutionary history with humans, its immunomodulatory properties, and its antigenic molecular mimicry. As of 2017, studies have been conducted investigating the role of *H. pylori* infection in diseases ranging from neurological disorders (including Alzheimer's and Parkinson's diseases) cardiovascular diseases, skin diseases, metabolic disorders (including type II diabetes, obesity and nonalcoholic fatty liver disease), and cancers occurring outside of the stomach (including colorectal and pancreatic), as well as a potential protective effect of *H. pylori* infection against allergy and autoimmune diseases (including inflammatory bowel disease and asthma) and esophageal diseases (including gastroesophageal reflux disease and esophageal adenocarcinoma). The epidemiological evidence linking the role of *H. pylori* to these diseases is not yet definitive and requires significant further investigation.

Treatment and Prevention Strategies

H. pylori Detection Strategies

The diagnosis of *H. pylori* was pioneered by Marshall and Warren in the 1980s, including serology and urease testing. Since then further tests have been developed and refined, each with specific advantages and disadvantages. These tests can be categorized into two groups: invasive tests based upon gastric biopsies collected during endoscopy to be used for histological analysis, bacterial culture, or molecular/biochemical assays; and noninvasive tests based on peripheral samples such as blood, stool, or saliva (Table 1). Typically in clinical practice one type of test is sufficient for diagnosis, however in laboratory research-based settings a combination of at least two tests are used to determine the status of *H. pylori* infection.

Noninvasive tests

Noninvasive tests for *H. pylori* include serology, urea breath test, and stool antigen testing. Serological methods are preferred for large epidemiological studies and can be used clinically for primary diagnosis of *H. pylori* infection due to its high sensitivity and specificity. However, serological methods are not useful in assessing successful eradication as *H. pylori* antibody titers fall gradually and can remain long after the infection is cleared.

In contrast, the urea breath test (UBT) can be used to detect primary infection and follow-up testing for successful eradication. The UBT involves ingestion of carbon labeled (^{13}C or radio-labeled ^{14}C) urea, and if *H. pylori* are present, the urea will be hydrolyzed yielding labeled carbon dioxide which can be detected in breath samples. The sensitivity and specificity range from 88% to 95% and 95% to 100%, respectively. False negatives can arise in patients who have recently taken proton pump inhibitors (PPIs), bismuth, or antibiotics or patients with active ulcer bleeding.

Stool antigen testing (SAT) is based upon an enzymatic monoclonal immunoassay that detects *H. pylori* antigens in feces. The detection of *H. pylori* antigens is representative of an active infection and can thus be used as tool for both primary diagnosis as well as confirmation of successful eradication. Similar to the UBT, the sensitivity and specificity are high (94% and 97%, respectively) and the results can be complicated by use of PPIs, bismuth, antibiotics, and peptic ulcer bleeds. In regions of low to intermediate *H. pylori* prevalence, the SAT is the most cost-effective diagnostic tool compared to serological assays and the UBT.

Invasive (endoscopy-based) tests

Endoscopy is not required solely for the purpose of *H. pylori* diagnosis, however if a patient has undergone an upper endoscopy and gastric biopsies retrieved, the biopsies can be used to assess *H. pylori* infection. Clinical diagnostic tests for *H. pylori* infection based on biopsies include urease testing, histology, or bacterial culture. Similar to the UBT, biopsy samples can be placed in medium

Table 1 Summary of diagnostic methods for the detection of *Helicobacter pylori* infection

Diagnostic method	*Specimen type*	*Application*	*Remarks*
Invasive methods			
Histology	Mucosal biopsy (at least 2)	Primary diagnosis	Pros: Also provides patient histology data (i.e., Inflammation, atrophy) Cons: Requires high expertise, multiple biopsies, high intra-observer variability
Culture	Mucosal biopsy	Primary diagnosis	Pros: Can be used to quantify antibiotic resistance, high specificity Cons: Low sensitivity and slow detection time
Rapid urease test	Mucosal biopsy	Primary diagnosis	Pros: Rapid results (~1 h), least expensive endoscopy-based method, kits commercially available Cons: Requires additional tests to confirm *H. pylori*
Noninvasive methods			
Urea breath test (UBT)	Breath sample	Primary and follow-up diagnoses	Pros: "Gold standard" of noninvasive tests. Cons: False negatives frequently occur in patients who have recently taken antibiotics or PPIs, or those who have an ulcer bleed.
Stool antigen test (SAT)	Stool	Primary and follow-up diagnoses	Pros: Most cost-effective option in populations with low to intermediate *H. pylori* prevalence Cons: False negatives frequently occur in patients who have recently taken antibiotics or PPIs, or those who have an ulcer bleed.
Serology	Serum	Primary diagnosis or indication of ever infection	Pros: Most cost effective for large population (epidemiological) studies Cons: Cannot discriminate between active and prior infection
Research-based methods			
PCR	Gastric juice, stool sample, mucosal biopsy	Research only	Pros: Highly specific, diversity of samples available for analysis, yields high amounts of microbe-specific data (i.e., Virulence factors, antibiotic resistance) Cons: Cost prohibitive for clinical use

containing urea and a pH reagent. The enzymatic breakdown of urea by *H. pylori* urease yields ammonia and bicarbonate resulting in a change in pH that is visualized by a color change of the pH reagent. Commercially available test kits yield a sensitivity and specificity of 90% and 95% respectively with results in about an hour. This test is the least expensive compared to other invasive tests and is thus the preferred method when endoscopy is used. Alternatively, biopsies can be assessed by histological examination for *H. pylori* with a hematoxylin and eosin stain (H&E) however the implementation of Giemsa, Steiner, or Warthin-Starry stains or through specific immunohistochemical means can make detection easier. Multiple biopsies from both the antrum and corpus should be examined as the density of *H. pylori* can be highly variable at different sites. Lastly, biopsies may be used for direct bacterial culture to diagnose *H. pylori* infection. Although this modality is highly specific and allows for simultaneous antibiotic resistance measurements, it is the least common diagnostic test because of its low sensitivity. The low sensitivity is attributable to the highly fastidious nature of *H. pylori*, which makes it difficult to recover and grow from biopsy specimens. Other uncommonly used diagnostic tests include PCR-based strategies on biopsy tissue or stool samples, however those are typically limited to research-only settings due to the high cost of the assay.

Treatment Strategies

Current guidelines in the United States state that when a physician chooses to test a patient for *H. pylori*, they should be unequivocally committed to eradication therapy if the patient tests positive. Although *H. pylori* is susceptible to many antibiotics in vitro, the gastric niche protects the bacteria from efficacious single-treatment therapies due to the low pH and the high viscosity of the mucus layer. In general, treatments for *H. pylori* involve an acid reducer such as a proton pump inhibitor (PPI) to reduce acid output with the addition of two antibiotics. First line treatment options for *H. pylori* include (1) clarithromycin triple therapy, (2) levofloxacin triple therapy, (3) Bismuth quadruple therapy (4) concomitant therapy (5) sequential therapy, (6) fluoroquinolone sequential therapy, or (7) hybrid therapy (**Table 2**). The most important predictors of successful *H. pylori* eradication are the choice of treatment regimen, patient adherence, and the sensitivity of the *H. pylori* strain to the combination of antibiotics. Unfortunately, the frequency of treatment failures is growing due to increasing antimicrobial resistance to clarithromycin and metronidazole. Four to six weeks after treatment, patients should be retested for *H. pylori* via UBT, SAT, or endoscopy-based methods. If the *H. pylori* infection is not successfully eradicated, second line therapies should be selected based upon which antibiotics were used in first-line therapies, as well as the local antibiotic resistances in the area. Metronidazole resistance is the most common, with 75% of strains in Africa and 46% in Asia and 30% of strains in Western countries demonstrating resistance. Recently, a new class of acid reducers termed potassium competitive acid blockers (P-CAB) have emerged as a more potent alternative to PPIs and may benefit

Table 2 Summary of *Helicobacter pylori* treatment regimens

H. pylori *treatment regimen*	*Drug(s)*	*Duration*
Triple therapy	(1) Either clarithromycin or levofloxacin (2) Amoxicillin (or other metronidazole) (3) PPI	10–14 days
Bismuth quadruple therapy	(1) Bismuth (2) Tetracycline (3) A Nitroimidazole (4) PPI	10–14 days
Concomitant therapy	(1) Clarithromycin (2) Amoxicillin (3) A Nitroimidazole (4) PPI	10–14 days
Sequential therapy	(1) Amoxicillin (2) PPI Followed by: (1) Amoxicillin (or fluoroquinolone) (2) Clarithromycin (3) A Nitromidazole (4) PPI	5–7 days 5–7 days
Hybrid therapy	(1) Amoxicillin (2) PPI Followed by: (1) Amoxicillin (2) Clarithromycin (3) A Nitromidazole (4) PPI	7 days 5–7 days

H. pylori eradication strategies. To date, P-CAB has been tested in triple therapy regimens and has shown to increase *H. pylori* eradication rates compared to PPIs, however its efficacy in quadruple, sequential or bismuth-containing therapies have not yet been studied.

Prospects of a Vaccine

As the complexity of *H. pylori* treatment increases and antimicrobial resistance continues to grow, a therapeutic vaccine could be a possible long-term solution to manage an *H. pylori* infection. Since *H. pylori* infection is acquired in early childhood and the possibility exists that infection may actually be beneficial in childhood, a therapeutic vaccine to eradicate the bacteria is the preferred strategy as opposed to prophylaxis. Formulations for vaccines have been attempted using whole cell lysates, outer membrane vesicles, as well as single or multiple purified antigens such as CagA, VacA, NapA, urease, and catalase. Some studies conducted in animals have shown that *H. pylori* vaccines can cure infection, while other studies have shown that *H. pylori* vaccination can augment the efficacy of antibiotic therapy and prevent recurrence. To date, progress on a human vaccine has been slow. The most recent study involved a phase 3 trial in China which demonstrated an approximately 70% protective effect of an orally administered *H. pylori* vaccine (based upon a recombinant urease B protein) in children aged 6–15 years, however the efficacy began to deteriorate after one year.

Population Screening

Interventional strategies such as eradication therapy, in conjunction with improved living conditions and hygiene across much of the world, have led to a significant decline in *H. pylori* prevalence over the recent decades. However, despite the decreasing prevalence of *H. pylori*, gastric cancer incidence is projected to rise as a result of changing population demographics. As such, there remains a need to reduce the number of future gastric cancer cases through eradication of *H. pylori*. Universal test and treat strategies among healthy, asymptomatic individuals within high gastric cancer-risk populations could reduce the incidence of gastric cancer in a cost-effective way. Furthermore, the cost-benefit of *H. pylori* eradication is increased when considering the benefit of reducing the incidence of other diseases such as peptic ulcer and dyspepsia. However, arguments against universal test and treat strategies include: (1) findings that *H. pylori* infection has been associated with other gastrointestinal and nongastrointestinal diseases where it may serve a beneficial role to the host; and (2) the impact of large-scale eradication therapy on antimicrobial stewardship remains unknown. Nonetheless, the growing consensus in the field suggests that population screening for *H. pylori* is a worthwhile endeavor to prevent premalignant and malignant gastric lesions.

Prospective Vision

H. pylori is the most successful bacterial pathogen worldwide, coevolving with its human host for millennia and infecting over 50% of the global population. Yet, it was not identified as a stomach pathogen until the late 20th century by the Nobel Prize-winning physicians Barry Marshall and Robin Warren. Their landmark discovery ultimately revolutionized the field of gastroenterology, providing an etiological cause for both peptic ulcer disease and gastric cancer. Laboratory investigations of this Gram-negative, spiral-shaped, microaerophilic organism have yielded the identification of numerous and highly specific virulence factors that enable its initiation and colonization in the mucus gel layer between the gastric lumen and the epithelial cell surface. Research has also illuminated many of the strategies employed by *H. pylori* to both persist in the highly inhospitable environment of the human stomach and to evade innate and adaptive immune responses. Simultaneously, in concert with improved hygiene and sanitary conditions around the world, clinical investigations have borne effective detection and treatment strategies that have contributed to the declining prevalence of this bacterium over the past several decades. Despite these advancements, *H. pylori* prevalence remains very high in developing nations and, as the global population continues to age, it is expected that we may still see an increase in lives lost from *H. pylori*-induced gastric diseases in the years ahead. As the diseases that result from *H. pylori* infection include not only those of the stomach but also potentially numerous extragastric diseases, noninvasive testing followed by eradication therapy for those in high-risk populations could significantly reduce the morbidity and mortality from this common bacterial pathogen.

See also: Cancer Disparities. Gastric Cancer: Pathology and Genetics.

Further Reading

Backert, S., Yamaoka, Y. (Eds.), 2016. *Helicobacter pylori* research: From bench to bedside. Springer, Tokyo.
Marshall, B.J. (Ed.), 2002. *Helicobacter* pioneers: Firsthand accounts from the scientists who discovered *Helicobacters*, 1892–1982. Blackwell, Victoria, Australia/Malden, MA.
Tegtmeyer, N., Backert, S. (Eds.), 2017. Molecular pathogenesis and signal transduction by *Helicobacter pylori*. Springer, Cham.

Hepatocellular Carcinoma: Pathology and Genetics

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Glossary

Cell cycle checkpoints Regulatory mechanisms at various phases of the cell cycle to ensure the correct completion of cellular processes crucial to the survival of a cell. Checkpoints are important for faithful DNA replication and the prevention of uncontrolled cell division.

Chromosomal instability A higher than normal rate of missegregation of chromosomes or parts of chromosomes during mitosis due to defective cell cycle quality control mechanisms.

Dysplastic nodule A focal nodular region (≥ 1 mm) showing nuclear atypia with increased fat or glycogen in a cluster of dysplastic cells. Dysplastic nodules are often associated with cirrhosis but without definite evidence of malignancy.

Edmondson-Steiner grading A 4-point scale describing the extent of differentiation of hepatocellular carcinoma cells and their resemblance of normal hepatocytes. It is the most widely used system for histologic grading of hepatocellular carcinoma.

Telomerase reverse-transcriptase A catalytic subunit of the enzyme telomerase, which maintains telomere ends by the addition of the telomere repeat TTAGGG. Reactivation of telomerase activity allows cells to overcome replicative senescence and to escape apoptosis.

WNT signaling pathways A series of signal transduction pathways responsible for passing signals into a cell through cell surface receptors. The three WNT signaling pathways described are the canonical WNT pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway.

Introduction

Liver cancer was responsible for an estimated 782,000 new cancer cases and nearly 746,000 deaths in 2012. Unlike most other malignancies, mortality from liver cancer has increased significantly over the past 20 years and the medical and economic burden of liver cancer will likely increase significantly in Western populations over the next decades. Of primary liver cancer cases, hepatocellular carcinoma (HCC) accounts for >90%. HCC is the sixth most common cause of cancer and third most common cause of death worldwide, accounting for nearly 10% of cancer deaths worldwide. Currently, curative treatment options for early-stage HCC detected by screening (one lesion <5 cm or up to three lesions <3 cm) involve resection and/or liver transplantation, with a >50% 5-year survival. Prognosis for patients with late-stage, inoperable HCC is poor with >90% mortality, particularly for cases associated with high serum α-fetoprotein (AFP) levels or *TP53* mutation. Until 2016, sorafenib, a multi-kinase inhibitor, remained the only approved systemic agent for HCC but only prolongs patient survival by 2.8 months. In 2017, regorafenib, a multi-kinase inhibitor, and nivolumab, a PD-1 inhibitor, were approved as second-line treatments following sorafenib, bringing the total number of approved systemic agents to three, far fewer than most other cancer types.

HCC shows great variation in incidence according to geographical regions. Around 80%–85% of HCCs are related to hepatitis B (HBV) and/or hepatitis C virus (HCV) chronic infection. In sub-Saharan Africa and South-East Asia, HBV chronic infection is endemic and accounts for the majority of HCCs diagnosed. In Western populations, however, the rising incidence of HCC is attributed to the increasing prevalence of chronic liver diseases associated with HCV infection, alcohol consumption, obesity and non-alcoholic fatty liver disease (NAFLD). Patients with hereditary diseases such as hemochromatosis or α-1-antitrypsin deficiency are also at increased risk of cirrhosis and fibrosis and, thus, of HCC. The male gender, exposure to aflatoxin B1 through dietary consumption and smoking are also known risk factors.

In about 80% of cases, HCCs arise in cirrhotic livers or in livers with advanced fibrosis. In fact, regardless of etiologies, liver cirrhosis is considered the major clinical risk factor. The 5-year cumulative risk for HCC in patients with cirrhosis ranges between 5% and 30%, depending on the cause (with the highest risk among those infected with HCV), region or ethnic group (17% in the United States and 30% in Japan) and stage of cirrhosis (with the highest risk among patients with decompensated disease). Although liver cirrhosis itself is not considered pre-malignant per se, there is increasing evidence to support a multistep model of hepatocarcinogenesis. Following hepatic injury resulting from one or more of HBV, HCV, excessive alcohol, exposure to aflatoxin B1 and/or other agents or conditions, necrosis develops followed by hepatocyte proliferation. Repeated cycles of this destructive-regenerative process lead to chronic liver condition that culminates in liver cirrhosis. Subsequently, hyperplastic nodules arise, followed by dysplastic nodules, resulting in the development of HCC. HCCs that occur in the absence of cirrhosis may present with unique clinicopathologic features, such as the fibrolamellar subtype that is predominantly diagnosed in young female patients.

Traditional pathology analyses have revealed the extraordinary histologic and phenotypic heterogeneity of HCCs. In fact, our understanding of HCC pathology is still evolving, with the last update of the WHO classification including substantial updates on the classification and diagnosis of early HCCs and lesions thought to derive from progenitor cells. From a diagnostic point

of view, the increased detection of small and early lesions in screening programs has highlighted the difficulties in distinguishing benign, precursor, and early HCC lesions. In addition, the advances in next-generation sequencing in recent years have also revealed the startling molecular heterogeneity of HCCs, with many deregulated pathways through an accumulation of somatic genetic and epigenetic changes in the cells.

Pathology of HCC

Precursor Lesions of HCC

Hepatocellular adenoma

Hepatocellular adenoma (HCA) is a benign neoplasm that sometimes undergoes malignant transformation into HCC. HCA is diagnosed predominantly in young women using oral contraceptives. Less commonly, it has been described in female patients with maturity onset diabetes of the young type 3, and in men using anabolic steroids, with glycogen storage disorders or receiving androgen treatment. Recently, metabolic syndrome, obesity and alcohol have also described as likely risk factors of HCA, in particular of the inflammatory subtype. Patients with HCA have normal liver function, but may occasionally have elevated serum AFP levels. Unlike other precursor lesions of HCCs, HCA is uncommonly associated with cirrhosis.

HCA is typically a large (up to 30 cm in diameter) tumor that resembles normal liver tissue microscopically but may display a pseudoglandular architecture. Large cell and fatty changes are often seen, but the cells have regular nuclei without atypia and mitoses are almost never present. In 20%–30% of cases, multiple HCAs may be present. The reticulin framework is usually present, bile ducts are absent and the sinusoids are usually compressed.

In general, the risk of transformation to HCC has been reported to be between 4% and 8%. Increased risk has been reported in male patients, patients presenting with large HCA (but not the number of HCAs), with pseudogland formation.

Molecular classification has divided HCA into several subgroups linked with distinct risk factors, clinical behaviors, histologic as well as imaging features: HNF1A-inactivated HCA, inflammatory HCA, *CTNNB1* exon 3-mutated HCA, *CTNNB1* exons 7 or 8-mutated HCA, sonic hedgehog HCA and unclassified HCA. *CTNNB1* exon 3-mutated HCA and sonic hedgehog HCA have been linked to high risk of malignant transformation and bleeding, respectively. Liver fatty acid-binding protein, serum amyloid A, C-reactive protein, prostaglandin D2 synthetase, glutamine synthetase, and β-catenin can be used either on biopsy or surgical specimen to classify HCA into these subclasses.

Transformed HCA typically appears as well differentiated HCC within the HCA with vascular invasion.

Dysplastic foci

Dysplastic foci are microscopic (<1 mm in diameter), uniform non-invasive lesions that differ from surrounding liver tissue based on morphology, cytoplasmic staining, nuclear size and cellular atypia. Dysplastic nodules are usually incidental findings and are usually found within cirrhotic nodules. Dysplastic hepatocytes within these foci may display large cell change, small cell change or iron-free foci.

Large cell change refers to hepatocytes with nuclear and cytoplasmic enlargement (thus retaining the nuclear/cytoplasmic ratio), associated with nuclear pleomorphism, multinucleation, and hyperchromasia. Large cell change may encompass dysplasia as well as reactive changes, and its precise role as an HCC precursor lesion is unclear. Studies reporting low cell proliferation, increased apoptosis and association with cholestasis support the notion that large cell changes are reactive changes. On the other hand, other studies have reported increased proliferation, telomere shortening and abnormal DNA content, including chromosomal copy number aberrations, leading to the hypothesis that large cell changes are indeed precursor lesions, especially in cirrhosis associated with HBV or HCV.

By contrast, small cell change refers to hepatocytes with decreased cell volume, mild nuclear pleomorphism, increased nuclear/cytoplasmic ratio, basophilic cytoplasm and hyperchromasia. Hepatocytes with small cell change typically show increased cell proliferation, inactivation of cell cycle checkpoint and telomere shortening. Morphologically, small cell change resembles well differentiated HCC. Importantly, while expansile small cell changes foci are considered dysplastic foci, a diffuse pattern is considered regenerative changes. Compared to large cell changes, small cell changes are considered more advanced.

Iron-free foci are proliferative lesions consisting of clusters of hepatocytes devoid of or low in iron content. Large or small cell change may be present. The precise clinical significance of iron-free foci is not clear, but it has been suggested that iron-free foci arising in livers with hereditary hemochromatosis are likely HCC precursor lesions.

Dysplastic nodules

Dysplastic nodules are larger than dysplastic foci and are defined as those ≥ 1 mm in diameter, usually around 1 cm in diameter. Dysplastic nodules are detectable macroscopically and sometimes radiologically. Similar to dysplastic foci, dysplastic nodules are also usually found on a cirrhotic background and may occur as single or multiple nodules. Macroscopically, dysplastic nodules are distinctly nodular lesions that differ from surrounding liver tissue based on color and texture, and may bulge from the surface of the liver (Fig. 1).

Dysplastic nodules are subdivided into low and high grades (LGDN and HGDN), with HGDNs associated with an increased risk of malignant transformation. LGDN shows minimal architectural atypia, mildly increased cell density, with normal to slightly elevated nuclear/cytoplasmic ratio, no mitoses and 1–2 cell wide cell plates. The borders of LGDN may be rounded

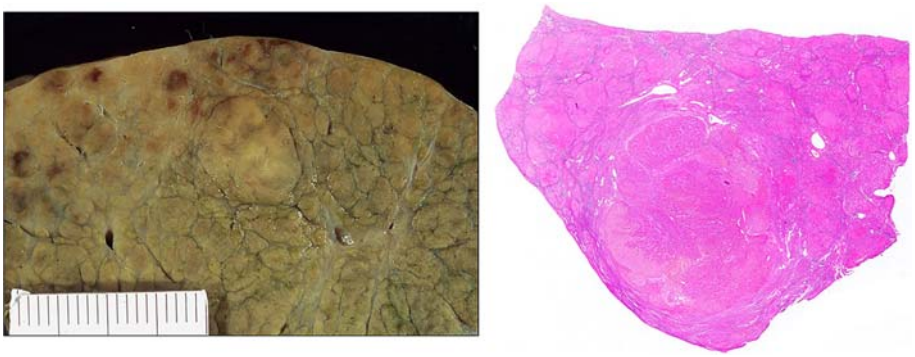

Fig. 1 Macroscopic and microscopic images of a dysplastic nodule. Macroscopic image of a liver dysplastic nodule taken during a routine procedure (*left*) and micrograph of hematoxylin and eosin stain (*right*).

but do not usually compress the adjacent liver tissue. Portal tracts and the reticulin network are retained. Steatosis and Mallory bodies may be present. Large cell change, but not small cell change, may also be present. However, histologic diagnosis of LGDN has not been found to be reproducible in practice, because of significant overlapping features with large regenerative nodule. By contrast, HGDN histologically and molecularly resembles HCC. In particular, HGDN displays significant architectural and cytologic atypia, including enlarged and irregular nuclei, nuclear hyperchromasia, peripheral location of the nucleus, occasional mitosis, thickening of hepatic cell plates (>2 cells), basophilic cytoplasm, formation of pseudoglands, focal loss of the reticulin network and resistance to iron accumulation. Small cell change is frequently seen in HGDN. Some of these features may be confined to foci within the HGDN. In HGDN, portal tracts may be few and sinusoidal capillarization is increased. Neoangiogenesis may be seen in dysplastic nodules, in particular in HGDNs, including the formation of unpaired arteries with no accompanying bile ducts. Dysplastic nodules usually show an isovascular or hypovascular appearance compared to surrounding tissues (Fig. 1).

Macroscopic Features of HCC

HCCs are highly heterogeneous macroscopically. HCC typically forms soft nodular masses that vary in color from gray to light brown to yellowish-green and are often punctuated by foci of hemorrhage and necrosis. HCC associated with cirrhosis typically presents as fibrous, encapsulated nodules while HCC not associated with cirrhosis is usually unencapsulated. HCC may also present as a single or as multiple nodules, with sizes from <1 to >20 cm. Indeed, compared to other tumor types, one of the distinguishing features of HCC is the frequent occurrence of multiple nodules. The multiple nodules of HCC may represent intrahepatic metastasis, in which malignant cells are disseminated from a single primary tumor to form additional tumor nodules, or may represent independent tumors. The distinction between these two scenarios is not trivial but has important clinical implications, given that intrahepatic metastases are likely to be more poorly differentiated and aggressive than independent, single nodules that do not disseminate. Extrahepatic metastases are most frequently identified in the lungs, the lymph nodes, bone and the adrenal glands.

The WHO 2010 guidelines divide HCCs into early HCCs and progressed HCCs. Early HCC refers to single, well differentiated, vaguely nodular tumor with poorly defined margins and of <2 cm in diameter, whereas progressed HCC refers to a single tumor >2 cm in diameter, or small (<2 cm), moderately differentiated HCC of distinctly nodular type or multiple tumors. Both vaguely and distinctly nodular HCCs usually occur in a cirrhotic liver, but vaguely nodular HCCs do not have well defined margins, whereas distinctly nodular HCCs are encapsulated. Compared to the distinctly nodular type, vaguely nodular type HCCs are usually smaller in size, represent a biologically earlier stage with more favorable prognosis and are rarely associated with intrahepatic metastasis.

Macroscopically, progressed HCC may be nodular, massive or diffuse. Nodular HCC may involve one or more nodules. Single nodular HCC is usually encapsulated and may show extracapsular growth around the nodule. Multinodular (or expansive) HCC is defined by the presence of multiple nodules throughout the cirrhotic tissues, usually with one dominant nodule, with satellite nodules in the proximity. Multinodular HCC is the most common type and are typically seen in association with cirrhosis. Massive (or infiltrative) HCC is a large dominant, poorly circumscribed tumor with ill-defined invasive borders and may be associated with smaller satellite nodules. Massive HCCs tend to be larger, are more frequently seen in non-cirrhotic livers and are associated with a poor prognosis. HCC can also be mixed nodular and massive. The less frequent diffuse (or cirrhotomimetic) HCC consists of many small nodules in a growth pattern that resembles regenerative nodules. Diffuse HCC can involve either a liver lobe or the entire liver, may be associated with macroscopic portal vein and/or hepatic vein invasion and is associated with a poor prognosis. On rare occasions, HCCs may display a pedunculated or protruded growth pattern, referring to extrahepatic growth with and without a peduncle, respectively.

Additional macroscopic features of HCC include the presence of necrotic areas, involvement of portal and hepatic veins and bile or fat production.

Microscopic Features of HCC

The most distinctive features of HCC are that HCC cells resemble hepatocytes and attempt to mimic the growth pattern of normal liver cells.

Early HCC is well differentiated and consists of tumor cells with increased nuclear/cytoplasmic ratio. Early HCC typically displays trabecular or pseudoglandular, or admixed, growth pattern. Relative to surrounding liver tissues, increased cell density is usually observed. Early HCC tends to be isovascular or hypovascular with varying numbers of portal tracts, and does not show vascular invasion. The reticulin framework is reduced but not lost.

The most common presentation of progressed HCC includes the following features: one or more well vascularized tumors with vascular invasion, prominent trabecular and pseudoglandular growth pattern with small cell change, cytologic atypia with increased mitotic activity, a lack of fibrous connective tissue between tumor cords and cells, fatty change, loss of the reticulin network, and an absence of Kupffer cells. Bile canaliculi are present and bile production is frequently observed. In progressed HCC, complete neovascularization with vascular infiltration is not uncommonly seen. However, there is substantial heterogeneity between and within HCCs in terms of their architecture, cytologic features and histologic grades.

HCC may display multiple architectural patterns: (1) trabecular (or sinusoidal) pattern where tumor cells grow in cords of variable thickness separated by prominent sinusoids lined by flat endothelial cells, with the trabeculae becoming thicker and contorted with dedifferentiation; (2) pseudoglandular (or acinar) pattern with a variety of abnormal, dilated bile canaliculi forming between tumor cells, often admixed with the trabecular pattern; (3) compact (or solid pattern) where tumor displays a trabecular phenotype but the tumor cells apparently grow in solid masses compressing the sinusoids and rendering the thick trabeculae inconspicuous by compression. Progressed HCC, in particular, sometimes display all three architectural patterns. Trabecular and pseudoglandular patterns are more frequently seen in well to moderately differentiated HCCs, whereas compact is more common in poorly differentiated HCCs (Fig. 2).

HCC may also display the following cytologic variants: (1) pleomorphic cells that vary in size, shape, nuclei and/or staining patterns and are often associated with the presence of multinucleated or mononuclear giant cells; (2) clear cells resulting from the accumulation of glycogen; (3) spindle cells with sarcomatoid differentiation; (4) fatty change, most frequently observed in early HCCs; (5) bile production seen as plugs in pseudoglands; (6) hyaline (Mallory-Denk) bodies with aggregation of intermediate filaments within hepatocytes; (7) pale bodies which are round to ovoid cells with cystically dilated endoplasmic reticulum containing amorphous and lightly eosinophilic accumulations; (8) ground glass inclusions which are hepatocytes with granular, eosinophilic cytoplasm mainly resulting from HBsAg positivity.

Histologic grade

HCC cells show variable degree of resemblance to normal hepatocytes, depending on the extent of differentiation. The most widely used system for histologic grading is the Edmondson-Steiner grading. Grade I tumors display abundant cytoplasm and minimal nuclear atypia that resembles normal liver. Grade II tumors show mild nuclear atypia with prominent nucleoli and hyperchromasia. Grade III tumors show moderate nuclear atypia with greater hyperchromasia, nuclear irregularity and granular cytoplasm. Grade IV tumors show marked nuclear pleomorphism, marked hyperchromasia, loss of trabecular pattern and are often associated with anaplastic giant cells. Most HCCs are of grades II or III.

It should be noted that HCCs often vary histologically even within a single nodule. Most primary cancer nodules < 1 cm are uniformly well differentiated (grades I/II). On the other hand, around 40% of nodules between 1 and 3 cm in diameter contain tumor cells of different histologic grades, with the less differentiated tumor cells concentrated in the central regions of the tumors. Although histologic grading in liver biopsies may be subjected to tumor heterogeneity, it has been shown that tumor grade in preoperative biopsy is correlated with the final grade on resection and predicts survival of the patients. Histologic grade in preoperative biopsy tends to be lower than the grade on resection. However, compared to several other clinicopathologic factors such as tumor size, tumor stage, vascular invasion and liver function, histologic grade is a relatively weak independent prognostic indicator.

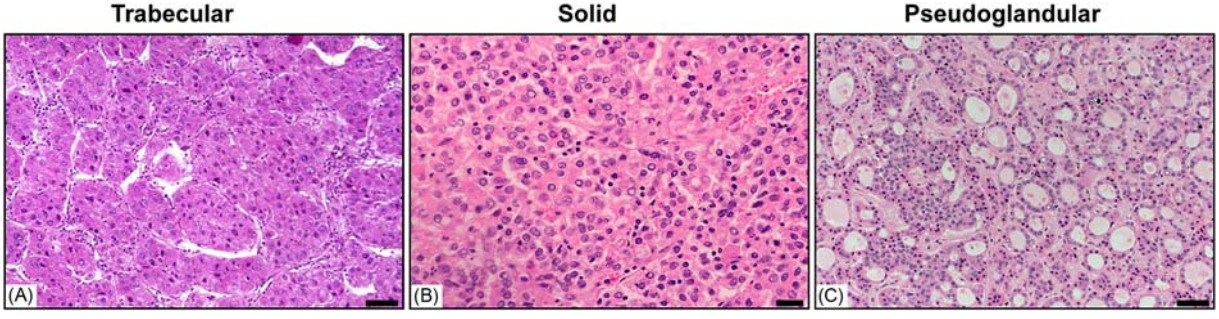

Fig. 2 Architectural patterns of HCC. Representative micrographs of the most common architectural patterns of HCC: (A) trabecular pattern (scale bar 50 μm), (B) solid pattern (scale bar 25 μm) and (C) pseudoglandular pattern (scale bar 50 μm).

Histologic variants of HCC

Fibrolamellar HCC is a rare variant of HCC, accounting for $<1\%$ of liver tumors. The majority of fibrolamellar HCC ($\sim 85\%$) is diagnosed in children and young adults <35 years old. Fibrolamellar HCC is characterized by well differentiated and eosinophilic tumor cells with prominent nucleoli growing between laminated collagen fibrous layers that show a lamellar pattern. This type of HCC is typically large and well circumscribed, grows with pushing borders and is often hyalinized. In some cases, fibrolamellar HCC resembles nodular hyperplasia, with a central scarred zone that is often calcified. Cytoplasmic inclusions are common, and may include Mallory bodies, pale bodies and/or cytoplasmic globules of variable positivity to PAS and immunoreactivity to anti-fibrinogen. In contrast to classical HCC, fibrolamellar HCC is reported to show abundant cytokeratin (CK) 7 and focal CK19 expression. Among the histologic variants of HCC, fibrolamellar HCC is unique as it is usually diagnosed in the absence of cirrhosis. Despite the usually large tumor size at diagnosis, fibrolamellar HCC has better prognosis than classical HCC, in part due to the fact that it is usually diagnosed in young patients not associated with primary risk factors and that the tumor can usually be resected surgically. Metastases are sometimes seen and most frequently seen in the peritoneum, the lungs and abdominal lymph nodes.

Sarcomatoid HCC is seen in $\sim 2\%$ in HCCs, alone or within classical HCC. Sarcomatoid HCC is fully or partially composed of spindle-shaped cells and shows bizarre anaplastic features that resemble those seen in leiomyosarcoma and fibrosarcoma. Giant cells may also be present. Patients with sarcomatoid HCC have been reported to show low serum AFP levels and frequently present with distant metastases. Sarcomatoid HCC has been associated with repeated chemotherapy and/or transarterial chemoembolization. Other clinical features are similar to classical HCC.

Scirrhous HCC accounts for $<5\%$ of HCCs and is characterized by the presence of diffuse fibrotic changes, alone or in conjunction with classical HCC. The tumor is usually large and unencapsulated with serrated border. Microscopically, the fibrosis is usually seen along the sinusoid-like blood spaces separating trabecular cell plates, often of >3 cells thick. Scirrhous HCC typically does not display prominent nucleoli and has granular eosinophilic cytoplasm. Scirrhous HCC is associated with hypercalcemia but not with bone metastasis. Scirrhous HCC is often misdiagnosed as post-therapy fibrosis, cholangiocarcinoma or metastatic tumors due to the presence of diffuse fibrosis. Scirrhous HCC differs from fibrolamellar HCC in terms of its lack of lamellar fibrosis and central scar, and the presence of cirrhosis.

The clear cell variant of HCC is characterized by the presence of tumor cells with clear cytoplasm due to cytoplasmic fat or glycogen. Predominant appearance of clear cell change is seen in up to 20% of HCCs and some degree of clear cell change can be found in up to 40% of HCC. The clear cell variant of HCC may display trabecular, pseudoglandular, solid or mixed architectural pattern. The clear cell variant is reported to be more frequently diagnosed in male patients.

Steatohepatic-type HCC is more frequently seen in patients suffering from non-alcoholic steatohepatitis and is characterized by the steatotic appearance in $>5\%$ of the tumor, including the presence of Mallory bodies, fibrosis, inflammation and the ballooning of hepatocytes. If present, the fibrosis usually displays a trabecular or a pericellular pattern. Inflammatory infiltrates, if present, usually consist of neutrophils, plasma cells and lymphocytes.

HCC with lymphoid stroma has been described in a few case reports and is defined by the presence of prominent inflammatory infiltrates. Lymphocytes make up the majority of the stroma, with the presence of some macrophages, plasma cells, neutrophils and/or giant cells. The T-cells are mostly CD4+. The existence of HCC with lymphoid stroma as a distinct entity is controversial, with some suggesting that the lymphoid stroma merely represents a regression phenomenon.

Differential Diagnosis of HCC

While the diagnosis of poorly to moderately differentiated HCC is usually straightforward, differentiating HCA and well differentiated HCC, or HGDN and well differentiated HCC presents significant diagnostic difficulty. The identification of surrogate markers to distinguish early HCC from pre-malignant lesions is also a major issue in clinical management. Additionally, metastasis from other organs may resemble primary HCC. Differential diagnosis of HCC involves the use of hematoxylin & eosin (H&E) stain, reticulin stain (loss of reticulin), and immunohistochemistry (IHC).

HCA versus well-differentiated HCC

Differential diagnosis of well differentiated HCC from HCA is based on a combination of increased mitotic activity, nuclei atypia, trabecular growth with thickened cell plates, vascular invasion, loss of the reticulin framework and the presence of bile ducts. HCA rarely occurs on a background of cirrhosis and are negative for glypican 3 (GPC3) and AFP. Mitoses are extremely rare in HCA. If mitoses are present, a diagnosis of HCC should be considered. CD34+ arterialized sinusoids are preferentially found in HCC.

High-grade dysplastic nodule versus HCC

Differential diagnosis between HGDN and well differentiated HCC is sometimes very difficult, given that they display very similar histologic features. Microscopically, stromal invasion is an important distinguishing feature of HCC but its identification is especially challenging in small biopsies. In a cirrhotic liver, stromal invasion may be difficult to distinguish from small clusters of hepatocytes within fibrous septae. Immunostains of CK7/9 that show a ductular reaction favors pseudoinvasion and therefore a diagnosis of HGDN. Elevated nuclear/cytoplasmic ratio, even when the nuclei do not show atypia, together with trabecular or pseudoglandular growth strongly suggest a diagnosis of well differentiated HCC. The complete loss of the reticulin framework is also considered one of the strongest indicators for a diagnosis of HCC.

Several IHC markers are used to distinguish HGDN and well differentiated HCC in cirrhotic livers. A panel of three markers GPC3, glutamine synthetase and heat shock protein 70 (HSP70) has been employed. GPC3 is an oncofetal protein that is not expressed in human livers but frequently reactivated in HCC, with cytoplasmic/membranous staining in >5%–10% of cells. Positivity of glutamine synthetase, a target of β-catenin signaling, increases from LGDN to HGDN to HCC. In HCC, glutamine synthetase may show diffuse cytoplasmic positivity in >50% of cells unrelated to vessels. HSP70 is negative in non-tumor hepatocytes and its positivity correlates with the grade of the HCC, in which it shows cytoplasmic or nuclear stain in >5%–10% of tumor cells. When two of the three markers are positive, the sensitivity and specificity for detecting well differentiated HCC in resection specimens are 72% and 100%, respectively. For the detection of small (<2 cm) early well differentiated HCC by core biopsy using a 20-21G needle, the sensitivity and specificity for 2/3 markers are 50% and 100%, respectively. Adding clathrin heavy chain to the panel of three markers increases the sensitivity and specificity for the detection of small early HCCs by core biopsy to 67% and 100%, respectively.

HCC versus metastasis

In patients without underlying chronic liver disease, the vast majority of malignant tumors in the liver is from non-liver origin, particularly in Western countries. The most common sites of origin are the breasts, the lungs, colon, and pancreas. While most metastases resemble their primary tumors histologically, certain types of tumors from non-liver origin, such as clear cell adenocarcinoma, clear cell renal cell carcinoma, melanoma, neuroendocrine tumor of the gastrointestinal tract and gastrointestinal adenocarcinoma with hepatoid features, may be difficult to differentiate from primary HCC. HCC typically lacks a desmoplastic stroma, the presence of which is typical of metastatic adenocarcinomas, although HCCs of the fibrolamellar and scirrhous subtypes may contain abundant stroma. The presence of bile production, the presence of multiple types of cytologic variants usually seen in classical HCC, and/or an absence of mucin in pseudoglands would suggest predominantly hepatic phenotype.

Immunostains of arginase 1, hepatocyte paraffin-1 antigen (HepPar-1, also known as hepatocyte specific antigen), polyclonal CEA (pCEA), CD10 and various cytokeratins may help determine if the tumors show hepatocellular differentiation. Arginase-1, HepPar-1, and pCEA are positive in normal liver and almost all well differentiated HCCs. HepPar-1 shows a granular, cytoplasmic (mitochondrial) pattern of staining in ~90% of HCC. HepPar-1 may also be expressed by tumors of the gastrointestinal tract, lung, pancreas and biliary tract but in these tumors, staining is usually weak or focal. pCEA shows a distinctive canalicular pattern of staining in HCC. A diffuse cytoplasmic pCEA staining pattern would favor a diagnosis of tumors other than HCC. However, sensitivity and specificity for both markers decline with the dedifferentiation of the tumor. CD10 is negative in adenocarcinomas but shows similar staining patterns to pCEA in HCCs. AFP shows a patchy and cytoplasmic staining pattern in ~50% of HCC and its staining increases with dedifferentiation. Cytokeratins CK7, CK19, and CK20 are usually negative in HCCs and one or more is frequently positive in adenocarcinomas. The presence of liver-specific proteins such as albumin would also suggest a liver origin. Interestingly, CK19 positivity in HCC seems to correlate with clinicopathologic features of tumor aggressiveness, more invasive characteristics, compared with CK19-negative HCCs through the upregulation of epithelial-to-mesenchymal transition-associated genes.

Combined hepatocellular-cholangiocarcinoma

Combined HCC-cholangiocarcinoma (CHC) is rare and accounts for <1% of malignant liver tumors. However, unlike HCC that is believed to have arisen from mature hepatocytes through a multistep process of hepatocarcinogenesis, CHC is thought to derive from a progenitor cell that differentiates into both hepatocytes and cholangiocytes. As its name suggests, combined HCC–CHC contains intimate mixtures of HCC and cholangiocarcinoma cells and includes tumors of transitional type in which the tumor cells display morphologic features intermediate of HCC and cholangiocarcinoma. CHC is usually positive for CK7 and CK19 although positivity is also occasionally seen in HCC. CHC must be also distinguished from collision tumors.

The WHO subclassifies CHC into the classical subtype and the subtype with stem cell features. The more common classical subtype consists of tumor cells with classical features of HCC and cholangiocarcinoma, in which the HCC component may be identified by cytoplasmic staining of HepPar-1 and canalicular staining of pCEA and CD10, and the cholangiocarcinoma component with CK7/19 and epithelial membrane antigen (EMA). Additionally, the cholangiocarcinoma component may be identified using D-PAS or mucicarmine stain to show mucin production and is usually associated with abundant desmoplastic stroma. CD133 and vimentin are usually negative. Both components can be well, moderately or poorly differentiated. Foci of intermediate HCC–cholangiocarcinoma phenotype may be present. However, we have to keep in mind that the concept of stem cell features in HCC is controversial and that the definitions in the 2010 WHO classification are not precise, leading to variable interpretations. Moreover, there are no reliable immunohistochemical markers to identify stem cells.

CHC with stem cell features does not produce mucin, shows high expression of CD133, EpCAM and vimentin, but less frequent expression of HepPar-1, thus recapitulating the features of progenitor cells. The subtype with stem cell features is further subclassified into typical, intermediate and cholangiolocellular subtypes, although the subclassification remains controversial. The typical subtype consists of mature hepatocytes with high nuclear/cytoplasmic ratio and hyperchromatic nuclei on the periphery. The peripheral cells stain positive for CK7/19, CD56, EMA, c-KIT, and EpCAM. The hepatocytes in the center of the tumor may show clear cell change. The intermediate subtype displays features intermediate between hepatocytes and cholangiocytes, consisting of small cells with hyperchromatic nuclei and scant cytoplasm. The cells are arranged in trabecular, solid nests or strands. CK7/19 and EMA positivity is frequent. The cholangiolocellular subtype consists of small cells with high nuclear/cytoplasmic ratio, hyperchromasia, and oval nuclei embedded in a fibrous stroma, and grows in an antler-like intersection pattern. Cellular atypia is usually mild. CK7/9 and EMA are positive and EpCAM and CD133 expression may be increased. The cholangiolocellular subtype was

previously considered a subtype of cholangiocarcinoma but at the moment is considered part of the spectrum of CHC with stem cell features.

Genetic Alterations in HCC

HBV Integration

Recent reports attribute over 50% of HCC cases worldwide to HBV infection, which is the major cause of HCC in East Asian and sub-Saharan countries. Compared with uninfected individuals, HBV carriers have 10- to 25-fold greater risk to develop HCC.

Integration of the HBV genome into the host genomic DNA occurs randomly in infected regenerating hepatocytes, is believed to be an early event in HBV infection and is involved in the development of HBV-related HCC. Whole genome sequencing studies of HBV-related HCCs have demonstrated that HBV genome integration occurs in approximately 80% of cases with a mean of 2.5 HBV integration sites per tumor. The HBx gene (encoding for the hepatitis B viral protein) is the HBV gene most frequently integrated into the human genome and the expression of the trans-acting wild-type and truncated HBx chimeric transcript has been shown to enhance transforming potential.

The first two integration sites in the human genome were described in the early 1990s when chimera sequences formed by the fusion of the HBV genome with the *RARB* or *CCNA* genes were identified. With the advancement of sequencing technologies, various additional integration sites have been described. The most recurrent HBV integration site is in the promoter region and within the gene body of the *TERT* gene, observed in ~20% of the HBV-associated HCC. HBV has also been reported to recurrently integrate into other cancer-related genes such as *SENP5*, *ROCK1*, *MLL4*, *CCNE1*, and *SOX2*. Furthermore, HBV integration sites within repetitive or non-coding sequences such as long interspersed nuclear elements (LINEs), Alu and the long terminal repeats of endogenous retroviruses have also been reported.

The precise mechanism of HBV genome integration in the development and the progression of HCC is under active investigation. Molecular studies have demonstrated that although HBV integration does not promote HBV replication, its integration into coding genes or their promoter regions may alter the expression of the target genes and increase genomic instability. Interestingly, HBV integration into the LINE1 region results in the generation of a chimeric transcript HBx-LINE1 detected in 21 of 90 (23%) tumors of HBV-related HCC patients and is significantly associated with poor survival of HCC patients.

Chromosomal Instability

Chromosomal instability (CIN) refers to a higher than normal rate of missegregation of chromosomes or parts of chromosomes during mitosis due to defective cell cycle quality control mechanisms, resulting in copy number alterations (CNAs) or aneuploidy. CIN is frequently a consequence of genetic or epigenetic alterations in genes involved in telomere maintenance, DNA replication, chromosome segregation or cell cycle checkpoints. In the context of HCC, the most frequent alterations that result in CIN include alterations in *TERT*, *TP53*, and *CDKN2A*.

CNAs cause dosage effects on the genes located in the affected genomic regions. The non-random distribution of CNAs in HCC suggests these CNAs, in particular focal amplifications and homozygous deletions, may lead to the overexpression of oncogenes or the loss of tumor suppressor genes. Indeed, profiling HCC using comparative genomic hybridization or single nucleotide polymorphism arrays has revealed recurrent chromosomal gains of 1q, 5p, 6p, 7q, 8q, 17q, and 20q and losses of 1p, 4q, 6q, 8p, 9p, 13q, 14q, 16p-q, 17p, 21p-q, and 22q. Analyses of focal amplifications have demonstrated recurrent amplifications targeting genomic regions of *TERT* (5p15.33), *VEGFA* (6p21.1), *MYC* (8q24.21), *CCND1* and *FGF19* (11q13.3), and *CCNE1* (19q12). On the other hand, genes reported to be homozygously deleted include *IRF2* (4q35.1), *CDKN2A* (9p21.3), *PTEN* (10q23.31), *RB1* (13q14.2), and *AXIN1* (16p13.3).

Among the most commonly gained regions in HCC are copy number gains of 1q and 8q, both in around 60%–70% of HCC. The minimal amplified region 1q21 has been linked to the early development of HCC (1q21-23) and to advanced metastatic HCC cases (1q21-q22). The region has been shown to contain potential oncogenes that when overexpressed may be involved in hepatocarcinogenesis. For instance, the gene *CHD1L* has been shown to be amplified and overexpressed in HCC but not in matching normal parenchyma. Additionally, expression of *CHD1L* is positively associated with vascular invasion and has been found to be an independent predictor of decreased disease-free survival (DFS) in HCC patients after surgical resection. Chromosome arm 8q contains the well-known oncogene *MYC*, a central regulator of malignant transformation in early hepatocarcinogenesis and has also been found to be significantly correlated with DFS and overall survival (OS) in patients with HCC.

On the other hand, 4q, 8p, and 17p are the chromosome arms most frequently lost. While how 4q loss contributes to hepatocarcinogenesis is not clear, it has been linked to high levels of serum AFP and is more frequently seen in poorly differentiated HCC. The loss of chromosome arm 8p have been extensively described in the HCC. A cluster of tumor suppressor genes on 8p have been identified, including the *DLC1*, *CCDC25*, *ELP3*, *SH2D4A*, and *SORBS3* genes, and the loss of these genes has been shown to be associated with metastasis and poor prognoses for HCC patients. On chromosome 17p is the *TP53* gene, one of the most frequently mutated genes in HCC.

Compared to focal amplifications and homozygous deletions, the biological effects of gains or losses of whole chromosomes or large genomic regions are more difficult to pinpoint. There remain many important questions regarding the identification of the genes in these large CNAs that are involved in hepatocarcinogenesis and their biological and clinical implications.

Genes and Pathways Altered by Somatic Genetic Alterations

Activation of the WNT/β-catenin pathway

The WNT/β-catenin pathway is of great importance in physiologic embryogenesis, zonation, and metabolic control in the liver. The WNT/β-catenin pathway can be classified into canonical and non-canonical pathways (based on the dependency of β-catenin). β-catenin, encoded by the *CTNNB1* gene, plays a pivotal role in the regulation of the canonical WNT signaling pathway. In the absence of WNT, cytoplasmic β-catenin protein is phosphorylated at serines 33, 37, and 45, and threonine 41 residues by the Axin complex (composed of Axin, APC, CK1, and GSK3) and then ubiquitinated and degraded by β-Trcp ubiquitin ligase. Activation of Wnt signaling induces the stabilization and translocation of β-catenin to the nucleus, where it associates with transcription factors of the TCF family and generates a functional complex able to transactivate several genes involved in the cell cycle control and proliferation, such as *MYC*, *CCND1*, *MT-CO*$_2$, and *MMP7*.

Dysregulation of the WNT pathway has been observed in 40%–70% of HCCs suggesting a major role of this pathway in HCC development. Processes WNT signaling regulates include angiogenesis, infiltration and metastasis by regulating the expression of angiogenic factors such as MMP-2, MMP-9, VEGF-A, and VEGF-C. The most common mechanism of β-catenin activation in HCC is through mutation of the *CTNNB1* gene, identified in about 40% of HCC. The majority of the *CTNNB1* mutations occurs at mutation "hotspots" in exon 3, specifically at the four phosphorylation sites. Mutation of serine/threonine residues results in impaired Axin/APC/GSK3b mediated degradation of β-catenin and the gain of oncogenic activity. HCCs with *CTNNB1* mutation display a specific transcriptomic profile with the overexpression of glutamine synthetase (encoded by *GLUL*) and leucine rich repeat containing G protein-coupled receptor 5 (encoded by *LGR5*), as well as increased expression of glutamine synthetase on the protein level. Other alterations that lead to an upregulation of WNT signaling include mutations or homozygous deletions of *AXIN1* (5%–15%), *AXIN2* (3%), and *APC* (1%–2%).

Several studies demonstrated a critical role of the deregulation of WNT/β-catenin signaling in hepatocarcinogenesis. HCV-positive HCC tends to be associated with frequent *CTNNB1* mutations. *CTNNB1* mutations have been found to be associated with macrovascular and microvascular invasion and increased tumor size. At the same time, HCCs characterized by the activation of the Wnt/β-catenin pathway exhibit specific features such as high differentiation associated with a homogeneous microtrabeculo-acinar pattern, low-grade cellular atypia, and cholestasis. Moreover, WNT/β-catenin signaling has been shown to play an important role in the activation of oval cells, considered to be hepatic stem cells, and HCCs with stem cell signatures have a more aggressive behavior. However, the clinical implications of *CTNNB1* activating mutations in terms of overall survival and disease recurrence have been contradictory. These findings demonstrate an essential role of the Wnt signaling in hepatocarcinogenesis and suggest that targeting this pathway may be promising for therapeutic options (**Figs. 3** and **4**).

Alterations in TP53 and cell cycle signaling pathway

TP53 is the most frequently mutated gene in human cancers. The p53 protein modulates multiple cellular functions, including transcription, DNA synthesis and repair, cell cycle arrest, senescence and apoptosis. Mutations in *TP53* can abrogate these functions, leading to genomic instability and progression to cancer. In HCC, *TP53* is one of the most frequent mutated genes and its

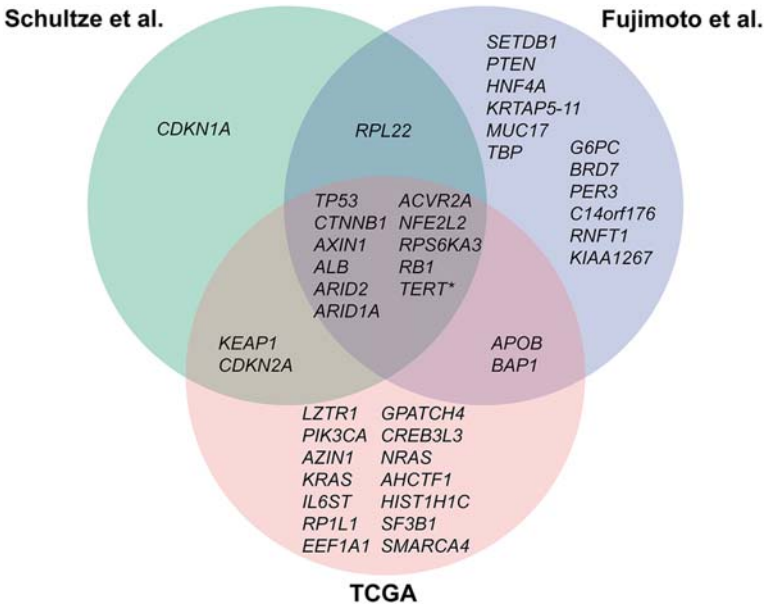

Fig. 3 Significantly mutated genes in HCC. Venn diagram illustrates the significantly mutated genes in HCC identified in three seminal studies that described in the genomic landscape of HCC. *Promoter region.

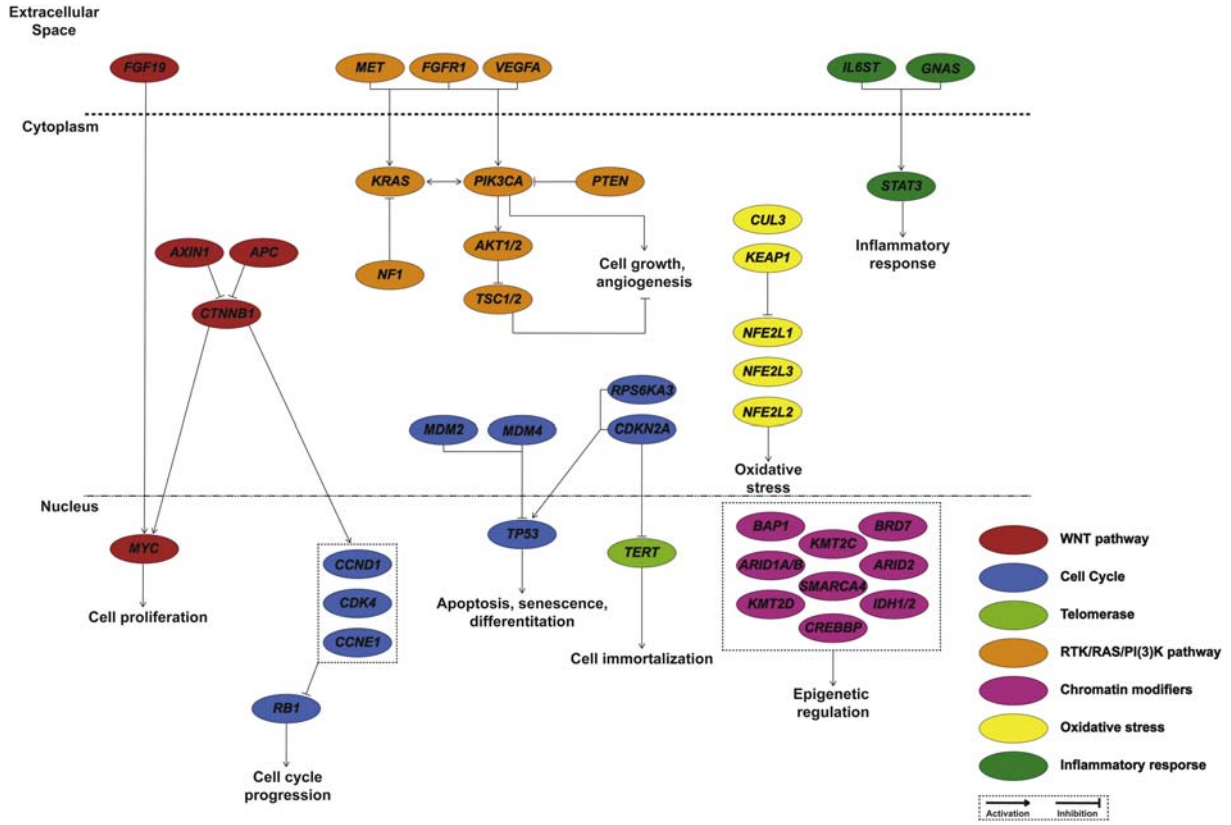

Fig. 4 Altered signaling pathways in HCC. Schematic representation of the major signaling pathways recurrently altered in HCC. Genes belonging to each pathway are represented and activating or inhibitory interactions between pathways are indicated with lines according to the legend.

mutational frequency is between 18% and 50%, depending on the geographic regions, etiological factors and carcinogen exposure, with more frequent *TP53* mutations in HBV-associated HCCs. Inactivating mutations in the *TP53* gene, or other components in the p53 pathway, may render hepatocytes susceptible to the effects of other carcinogens that activate oncogenic pathways and may also predispose to the development of HCCs with a more aggressive phenotype. For instance, *TP53*-mutant HCCs have been associated with features linked to poor prognosis, including high levels of serum AFP, high Edmondson grade, expression of stem-like markers, and activation of pro-oncogenic signaling pathways. Indeed, patients with *TP53*-mutant HCCs tend to have shorter OS and DFS.

The G1 to S phase of the cell cycle (retinoblastoma pathway) is often deregulated via homozygous deletion of *CDKN2A* (p21, 2%–12%) or *RB1* mutations (3%–8%) and is often associated with poor prognosis suggesting a role of the related pathway inactivation in tumor aggressiveness. Additionally, recurrent HBV integration into the *CCNE1* locus (5%) and amplification of the *CCND1/FGF19* locus (5%–14%) have been reported in HCC, both of which have implications in the regulation of cell cycle.

Not all *TP53* mutations in HCCs are equal. Dietary exposure to fungal aflatoxin B1 results in a specific *TP53* mutation that is rarely found in other cancers, namely the R249S mutation resulting from G>T transversion; this is considered to be a driver mutation since it is also found in the normal livers of patients exposed to aflatoxin B1. There is strong epidemiologic synergism between aflatoxin B1 exposure and chronic HBV infection in the induction of HCC, and it has been shown that, in patients infected with HBV, expression of HBx is associated with an approximately twofold increase in the incidence of G/C-to-T/A transversion mutations following aflatoxin B1 exposure (Figs. 3 and 4).

Alterations in the human telomerase reverse-transcriptase

The human telomerase reverse-transcriptase (*TERT*) gene encodes a rate-limiting catalytic subunit of telomerase, which maintains the length of telomeric DNA and chromosomal stability. Thus, *TERT* plays a pivotal role in cellular immortalization, cancer development and progression. Reactivation of telomerase activity allows cells to overcome replicative senescence and to escape apoptosis, both of which are fundamental steps in the initiation of malignant transformation. The interests in the role of telomerase maintenance in the development of HCC have grown since the discovery of *TERT* promoter hotspot mutations in HCC and other cancers. While telomere shortening is a feature of chronic liver diseases and cirrhosis, telomerase reactivation through *TERT* promoter mutations (30%–60%), amplifications (5%–6%) and HBV integration into the *TERT* promoter or the gene body of *TERT* (10%–15%) is associated with hepatocarcinogenesis. Two hotspot mutations c.-124C>T and c.-150C>T have been reported in HCC, and in particular the c.-124C>T mutation has now been shown to be the most common mutation in HCC, observed in

30%–60% of HCC. This mutation is in fact the most frequent mechanism of telomerase activation. The c.-124C>T and c.-150C>T mutations result in the formation of novel ETS transcription factor binding sites upstream of the *TERT* transcription start site, which leads to increased *TERT* transcript expression. *TERT* promoter mutations have been reported to be more frequent in HCV-associated HCC. On the other hand, compared to HCCs of other etiologies, HBV-induced HCCs are less likely to harbor *TERT* promoter mutations but may be deregulated by integration of HBV sequences into the *TERT* gene locus, which serves as a complementary mechanism for telomerase activation.

Deregulation of *TERT* has been suggested to be an early driver event in hepatocarcinogenesis. *TERT mRNA* has been reported to be detectable in the serum of patients with HCC with higher sensitivity and specificity than serum AFP, AFP-L3, and des-gamma-carboxy prothrombin (DCP) in the diagnosis of early stage HCC. Thus, measuring serum *TERT* mRNA levels may potentially be used as diagnostic tool for HCC detection at early stage. In fact, *TERT* promoter mutations have been suggested to be early driver mutations, given that *TERT* promoter mutations have been found in 30% of cirrhotic preneoplastic lesions. These findings suggest that *TERT* promoter mutations are among the earliest genetic alterations in hepatocarcinogenesis, occurring at preneoplastic stages and behaving as a "gatekeeper" during the malignant transformation sequence. It has also been suggested that telomerase inhibition may be a potential therapeutic target in treating HCC (**Figs. 3 and 4**).

Alterations in chromatin modifiers

Chromatin remodelers are epigenetic modifiers that play important roles in maintaining nucleosome positioning and thus in transcriptional regulation. Alterations in ATP-dependent chromatin remodeling complexes have been implicated in carcinogenesis, tumor heterogeneity and cellular response to therapy. Among others, the most frequently altered chromatin remodelers in human cancer are part of the SWI/SNF complex (20%). In fact, this complex has been associated with epigenetic modification including roles in maintaining nucleosome positioning and interacting with other chromatin modifiers. The SWI/SNF complex contains two main subunits, namely the BAF and the PBAF complexes. In HCC, inactivating mutations are frequently found in the *ARID1A* and *ARID1B* genes (4%–17%), encoding the AT-rich interacting domain containing protein 1A-B which are components of the BAF complex, and in *ARID2* (3%–18%), encoding the AT-rich interacting domain 2, a component of the PBAF complex. The frequent occurrence of inactivating mutations in the SWI/SNF complex suggests that the complex acts as a tumor suppressor. Although it has been suggested that alterations in the SWI/SNF complex modify chromatin structure and nucleosome position, the functional role of the mutations and the molecular mechanisms that result in the initiation and progression of HCC are not yet fully understood.

In addition to the SWI/SNF complex, recurrent somatic genetic alterations in the histone methylation family members *MLL* (3%–4%), *MLL2* (2%–3%), *MLL3* (3%–6%), and *MLL4* (2%–3%) or HBV insertions into the *MLL4* gene locus (10%) are also frequent in HCC. The MLL gene family encodes for proteins that modify histone methylation by adding and removing H3K4 methyl groups. As with alterations in the SWI/SNF complex, the functional significance of alterations in the *MLL* genes in hepatocarcinogenesis remains to be further elucidated (**Figs. 3** and **4**).

Alterations in genes involved in oxidative stress response

Oxidative stress pathway dysfunctions have been linked to carcinogenesis and the progression of cancer via the production of reactive oxygen species (ROS) that cause damage to proteins, DNA and lipids. One of the major oxidative stress pathways is the NRF2-KEAP1 signaling pathway, which is a regulator of cytoprotective responses to endogenous and exogenous stresses caused by ROS and electrophiles. In this pathway, NRF2 is the mediator of the oxidative stress response while KEAP1 is the negative regulator of NRF2 activity. Deregulation of the NRF2-KEAP1 signaling pathway prevents proteasome degradation of NRF2, which is physiologically induced by KEAP1/CUL3 complex ubiquitinylation. This results in epigenetic instability or altered chromatin status, leading to abnormal methylation of tumor suppressor genes.

In HCC, the NRF2-KEAP1 signaling pathway is altered by activating mutations of NRF2 (encoded by *NFE2L2*) or inactivating *KEAP1* mutations in 5%–15% of the cases. The role of the somatic mutations in these genes in cancer formation/development is, however, controversial. Studies carried out in rats have reported the presence of these somatic alterations in preneoplastic lesions and early HCCs. By contrast, mutations in *NFE2L2* and *KEAP1* in humans have only been observed in advanced HCC and not in premalignant nodules or early HCC, suggesting that these mutations are late genetic events in hepatocarcinogenesis in humans. Furthermore, mutations in *NFE2L2* or *KEAP1* are significantly correlated with the deregulation of the WNT/β-catenin pathway via *CTNNB1* or *AXIN1* mutations, suggesting that the NRF2/KEAP1 pathway may interact with WNT/β-catenin signaling to promote hepatocarcinogenesis (**Figs. 3 and 4**).

Deregulation of PI3K-AKT-mTOR and RAS/RAF/MAPK signaling pathways

The PI3K/AKT/mTOR pathway is an intracellular signaling pathway that plays a pivotal role in the cell cycle control. Phosphorylation of PI3K activates AKT which regulates several downstream molecules, including mTOR. Several factors may constitutively activate the PI3K/AKT/mTOR signaling pathway, such as EGF, shh, IGF-1, insulin, and CaM. On the other hand, PTEN, GSK3B and HB9 are inhibitors of the pathway. In many cancers including HCC, the PI3K/AKT/mTOR pathway is hyperactive, thus reducing apoptosis and promoting cellular proliferation.

Activation of the PI3K/AKT/mTOR pathway is a common feature of HCC and has been reported in 40%–50% of HCC. Pathway activation may be achieved through several distinct mechanisms. Activating mutations of *PIK3CA* are observed in 0%–2% of patients and inactivating mutations of *TSC1* or *TSC2* are observed in 3%–8% of patients. Furthermore, homozygous deletion of *PTEN* has been identified in 1%–3% of the HCCs. However, some HCCs with activation of PI3K/AKT/mTOR signaling have no

genetic alterations in the pathway. Indirect activation of the upstream insulin growth factor pathway has been proposed as an alternative mechanism to active the PI3K/AKT/mTOR pathway.

Genetic alterations in the RAS/RAF/MAPK pathway have also been implicated in the development of HCC, albeit rarer. Activating mutations in the RAS family genes (e.g., *KRAS*, *NRAS*) are rare (<2%). More common are inactivating mutations in *RP6SKA3*, encoding for the RAS inhibitor RSK2, identified in 2%–9% of HCCs. RSK2 is downstream of MAPK and is a negative regulator of RAS signaling. Inactivating RSK2 disrupts the negative feedback loop and results in the constitutive activation of the RAS pathway (**Figs. 3 and 4**).

Alterations in liver metabolic pathways

Liver is unique organ and has very different gene expression patterns compared to other organs. A number of genes highly expressed and/or only expressed in the liver have been found to be frequently mutated in HCC. Of these, *ALB* (encoding for albumin) and *APOB* (encoding for apolipoprotein B) have been found to be affected by mutations in 10%–15% of HCC. Mutations in *ALB* and *APOB* are dominated by inactivating mutations (i.e., nonsense, splice site and insertions/deletions), strongly suggesting a tumor suppressive role for these genes in liver. Indeed, *ALB* and *APOB*-mutant HCCs have been found to have reduced expression of the respective gene. It has been suggested that *ALB* and *APOB* mutations may be under positive selection to divert energy for other cancer-associated metabolic requirements. *HNF1A* and *HNF4A* (encoding for hepatocyte nuclear factors 1-alpha and 4-alpha) are each mutated in ~2% of HCC. Both encode for transcription factors essential for the transcription of a number of genes required for hepatocyte differentiation, with *HNF4A* regulating the transcription of *HNF1A*. Alterations in *HNF1A* and *HNF4A* are also preferentially loss-of-function alterations (**Figs. 3 and 4**).

Alterations in non-coding elements

Considering that protein-coding regions only account for 1%–2% of the human genome, it is not surprising that recent whole-genome sequencing studies of HCC and other cancers have revealed that the number of somatic mutations in non-coding elements far outnumber that in coding regions. The significance of the vast majority of the non-coding mutations is still unknown but several non-coding regions/genes have been identified to harbor elevated number of mutations. Besides the hotspot mutations in the promoter regions of *TERT*, an additional 10 promoter regions, including those of *TFPI2*, *MED16* and *WDR74*, and nine untranslated regions, including those of *BCL6* and *AFF4*, have also been identified as regions with significantly elevated number of mutations. Among these, mutations in the *TFPI2* (encoding for tissue factor pathway inhibitor 2, a serine proteinase inhibitor) promoter have been linked to lower expression of *TFPI2*, a gene reported to have tumor suppressive roles in several cancers. Finally, the long non-coding genes *NEAT1* (~20%) and *MALAT1* (~5%) are recurrently mutated in HCC. Both *NEAT1* and *MALAT1* encode for transcripts that are subnuclear structural components and regulate the transcription of cancer genes. In particular, *MALAT1* is upstream of p53 and is required for G1/S cell cycle progression. On the other hand, *NEAT1* expression is reported to be influenced by p53. Our understanding of the precise effect of mutations in non-coding elements is very preliminary and the functional impact of these alterations is currently under active investigation.

DNAJB1-PRKACA fusion gene in fibrolamellar HCC

Fibrolamellar HCC is a clinically and morphologically distinctive subtype that accounts for <1% of HCC. Unlike classical HCC, fibrolamellar HCC is usually diagnosed in young adults and is rarely associated with pre-existing liver disease or cirrhosis. Whole genome and transcriptome sequencing studies have identified a recurrent fusion gene resulting from a 400 kb deletion on chromosome 19 in 100% of fibrolamellar HCC. This in-frame chimeric transcript fuses exon 1 of the *DNAJB1* gene, which encodes for a member of the heat shock 40 protein family, to exons 2–10 of the *PRKACA* gene, which encodes for the adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase A (PKA) catalytic subunit alpha. Additional sequencing studies revealed no other highly recurrent genetic lesions in fibrolamellar HCC and the absence of the fusion in non-fibrolamellar HCC. These observations suggest that the fusion is pathognomonic of fibrolamellar HCC.

The gene fusion leads to the formation of a chimeric protein that differs from wild-type PKA in size. The functional catalytic domain and kinase activity are retained but the fusion protein lacks the domain that binds to the regulatory subunit of PKA. The functional role of this fusion transcript is still under investigation (**Fig. 5**).

HCC Susceptibility Loci

Germline polymorphisms module HCC risk. Genome-wide association studies have identified susceptibility loci in genes associated with signaling pathways known to be involved in carcinogenesis. These include *SOD2* and *MPO* involved in oxidative stress response, *TNFA*, *IL1B*, and *TGFB1* in inflammatory response, *MTHFR* and *XRCC3* in DNA repair, *MDM2* and *TP53* in cell cycle regulation, and *EGF* (epidermal growth factor).

The majority of the susceptibility loci associated with HCC development are linked to specific risk factors. For instance, single nucleotide polymorphisms (SNPs) of *GTSM1* and *GSTT1* are linked to aflatoxin B1 exposure and HBV infection. Also in HBV-positive patients, SNPs in *STAT4*, *TPTE2*, *DCL1* are associated with increased risk of HCC. In HCV-positive patients, however, polymorphisms in *MICA* (a gene involved in regulating immune response), *DEPDC5* (a gene in the mTOR pathway) and in the 5′ UTR of *EGF* have been reported as susceptibility loci. A *PNPLA3* polymorphism has been linked to HCC associated with fatty and alcoholic liver diseases. These associations suggest interaction between these SNPs, environmental factors and chronic infection.

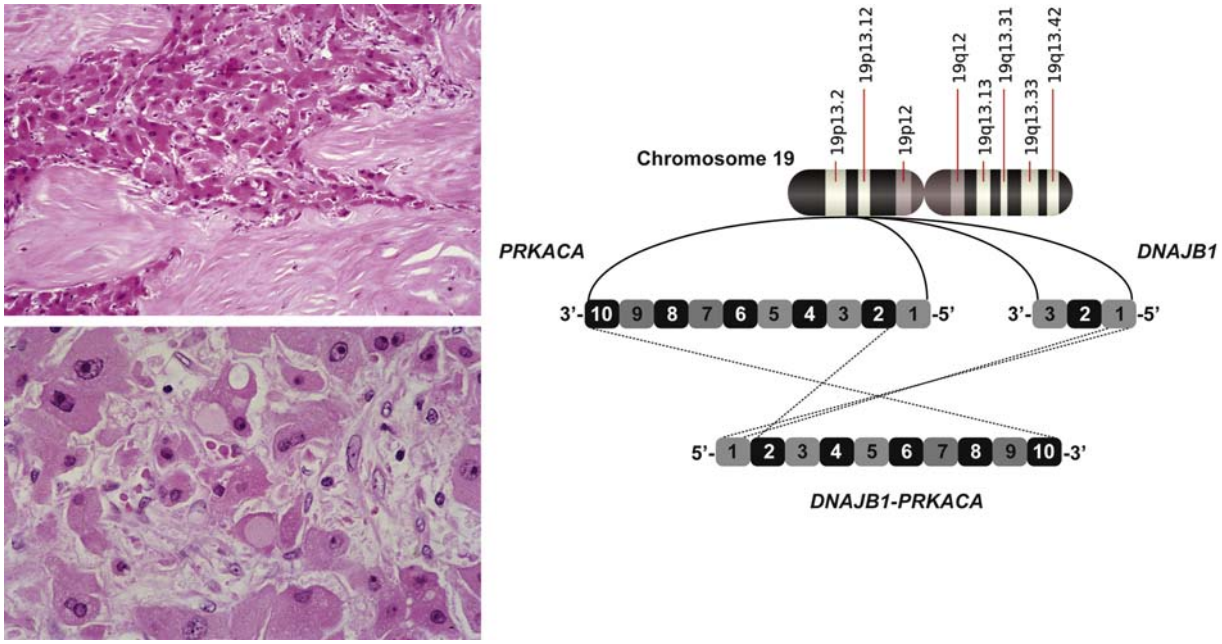

Fig. 5 Recurrent molecular alteration in fibrolamellar HCC. High power and low power micrographs of a fibrolamellar HCC (*left*) and a schematic representation of the major recurrent fusion gene *DNAJB1-PRKACA* present in nearly 100% of fibrolamellar HCCs (*right*).

Genetic Syndromes Associated With Increased Risk of HCC

Several rare monogenic syndromes have been linked to increased risks of hepatocarcinogenesis. The autosomal recessive α-1-antitrypsin deficiency results from mutations in the *SERPINA1* gene, a serine protease synthesized at high levels in the liver and responsible for the inhibition of neutrophil elastase. Glycogen storage disease type I, caused by the deficiency of glucose-6-phosphatase (G6Pase) activity results in excess glycogen storage in the liver and causes hepatomegaly, fasting hypoglycemia, lactic acidosis, hyperlipidemia, hyperuricemia, and growth retardation. Hemochromatosis is caused by a mutation in the *HFE* gene (most commonly single base mutation C282Y) and leads to the development of fibrous tissue at the site of iron deposition, causing liver dysfunction. Patients with hemochromatosis are at increased risk of developing hepatic cirrhosis followed by HCC. Acute intermittent porphyria (AIP) is a low penetrant autosomal dominant disease caused by mutation in enzymatic activity of hydroxymethylbilane synthase (*HMBS*), one of the enzymes in the heme biosynthesis pathway. AIP patients have an >30-fold increase in the risk of HCC compared to the general population. HCC in AIP patients are rarely associated with cirrhosis. Tyrosinemia type I is an autosomal recessive disorder caused by biallelic pathogenic variants in the *FAH* gene. The deficiency of the fumarylacetoacetate hydrolase enzyme leads to an accumulation of tyrosine catabolic intermediates, acute hepatic failure in infancy or chronic liver disease associated with cirrhosis and HCC development. Finally, HCC has also been reported in patients with familial adenomatous polyposis associated with *APC* germline mutation.

Conclusions

With sorafenib, regorafenib, and nivolumab still the only approved systemic therapies for HCC, the identification of additional therapeutic targets is crucial to improve the prognosis of HCC patients. On the one hand, the consensus on the classification between early and late HCCs is having an impact in the clinic to optimize clinical management. On the other hand, our evolving understanding of the genetic heterogeneity of HCC is beginning to reveal additional pathways and potential vulnerabilities that may be exploited in future studies. Integration of histopathologic and molecular analyses will provide further insight into the pathogenesis and genotype–phenotype interactions important in HCC.

See also: Cholangiocarcinoma: Diagnosis and Treatment.

Further Reading

Atlas, C.G., 2017. Comprehensive and integrative genomic characterization of hepatocellular carcinoma. Cell 169, 1327–1341.
Di Tommaso, L., Destro, A., Seok, J.Y., et al., 2009. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. Journal of Hepatology 50, 746–754.
Fujimoto, A., Furuta, M., Totoki, Y., et al., 2016. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. Nature Genetics 48, 500–509.
Schlageter, M., Terracciano, L.M., D'Angelo, S., et al., 2014. Histopathology of hepatocellular carcinoma. World Journal of Gastroenterology 20, 15955–15964.
Schulze, K., Imbeaud, S., Letouzé, E., et al., 2015. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. Nature Genetics 47, 505–511.

Hereditary Cancer Syndromes: Identification and Management

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Cancer Control From a Public Health Perspective

Incidence and prevalence of cancer have been increasing for most neoplastic diseases in the past decades, due to different causes, including the progressive aging of the population and the continuous improvement in diagnostic and treatment strategies, that, if not always able to cure, at least make people live longer and with an acceptable quality of life.

In this context, the healthcare assistance related to cancer, from screening to diagnosis, to cure or treatment, has become a very crucial, but challenging, public health topic.

Cancer is a multifactorial disease, whose pathogenesis is complex, but strongly driven by genetic modifications in the genome DNA. This makes cancer field of study of Public Health genomics, the field in epidemiology wherein molecular data at population scale are integrated into new strategies both from a personalized medicine and a public health perspective.

The completion of the human genome-sequencing project in 2003, and the further advances in numerous sectors of biotechnology, created great expectations about health benefits for several diseases, including cancer. Moreover, the decrease of the costs related to the DNA sequencing techniques has contributed to the spread of genetic testing in healthcare.

The genomic approach to cancer means a consistent reduction in terms of mortality, morbidity, and, therefore, a possible reduction of costs for the healthcare systems. Genetic testing may contribute to cancer control by identifying individuals at high risk of disease. Some monogenic subtypes of common cancers such as *BRCA*-related breast cancer and Lynch syndrome (LS) colorectal cancer (CRC) are autosomal dominant disorders with a risk of 50% for first-degree relatives of the index case to be affected. The identification of these hereditary forms of cancer that show high incidence, mortality rate, and that are potentially preventable or efficiently treatable is of critical public health importance because it changes cancer risk management options and enables patients and their at-risk family relatives to benefit from targeted intervention measures.

Hereditary Breast Cancer

Breast cancer is the most common malignancy in women, and one of the three most common cancers worldwide, with around 1,671,000 new cases diagnosed in 2012.

The majority of breast cancers show a multifactorial pathogenesis; however, approximately 30% of breast cancer cases occur in family clusters and up to 7% have a strong hereditary component.

Among affected women, the genetic variants of *BRCA1* and *BRCA2* (breast cancer susceptibility genes) account for up to the 30% of the genetic predisposition to breast cancer and show a high-penetrance pattern of hereditary predisposition. The prevalence of pathogenic variants of *BRCA* genes has been estimated as 1/800; however, a number of founder effects with higher prevalence have been observed in different populations as Ashkenazi Jewish, Swedish, Dutch, Hungarian, and Icelandic.

Mutations in these genes predispose women to develop cancer at a younger age than general population. In women, *BRCA1* or *BRCA2* mutations result in a 57%–65% or 45%–55% risk of developing breast cancer by age 70 years, and a lifetime risk of 39%–44% or 11%–18% of developing ovarian cancer (includes fallopian tube and primary peritoneal cancers), respectively. In men, the lifetime risk of breast cancer is 1.2% for *BRCA1* mutation carriers and 6.8% for *BRCA2* mutation carriers. Besides, carriers have a considerable risk to develop a contralateral breast cancer, after the first one.

In 2003 the European Council recommended the implementation of cancer screening programs for women aged 50–69 years. Also the U.S. Preventive Services Task Force and the American Cancer Society recommend that women aged over 50 years undergo regular screening mammography for the early detection of breast cancer. However, women with a *BRCA1* or *BRCA2* mutation may develop cancer at a younger age.

Identifying *BRCA* mutated women is important not only for cancer-affected women because of the prognosis and the related treatment choices but also for healthy carriers because of the different options in terms of primary and secondary prevention. According to international guidelines, surveillance of breast cancer in *BRCA* carriers includes monthly self-examinations, clinical breast examinations twice a year, and yearly mammograms and magnetic resonance imaging of breasts starting at age 25 or younger based on the earliest age at which breast cancer was diagnosed in the family. Screening for ovarian cancer is also recommended and preventive surgery (mastectomy and salpingo-oophorectomy) can be considered.

Considering the relevance of breast cancer, international communities are strengthening the efforts in order to implement adequate integrated diagnostic-therapeutic pathways for the affected women. In this context, breast units have a key role, coordinating high quality and appropriate assistance, taking care of patients in all the stages of their disease.

To date, according to the most recent international guidelines it is not indicated to perform a screening program for genetic testing of *BRCA* genes, neither in the general population nor in all women with breast cancer. In fact, the U.S. Preventive Services Task Force recommends to perform the genetic testing only in the presence of specific anamnestic criteria that identify the high-risk woman that might carry the *BRCA* mutations. Women with positive screening results should receive genetic counseling and, if indicated after counseling, *BRCA* testing.

Considering economic evaluations, there are good evidences in support of the implementation of dedicated screening pathways to identify high-risk women. According to a recent systematic review of the economical evaluations of *BRCA* screening strategies, family history-based genetic testing is potentially very cost-effective, but there was no evidence of cost-effectiveness for testing all the newly diagnosed cases of cancers.

Hereditary breast cancer is addressed as one of the main topics in cancer control policies and the implementation of prevention programs would reduce the burden of the inherited disease. In some countries public health genomics policies have already been formulated; however, the implementation of integrated pathways for the identification and follow-up of *BRCA* carriers is still at the beginning.

Hereditary Colorectal Cancer

CRC is the third leading cause of cancer death worldwide, accounting for 774,000 deaths in 2015, and with an estimated annual worldwide incidence of 1.4 million new cases. CRC ranked third in prevalence, after lung and prostate cancers, for men, and second, after breast cancer, for women.

Due to its high incidence rate, its long preclinical phase, its recognizable and tractable precursor, and the association between the tumor stage and mortality rate, CRC is a condition suitable for screening according to the criteria established by Wilson and Jungner as the "gold standard of screening assessment." The recommended screening can be performed through an annual or biennial fecal immunochemical test, or sigmoidoscopy every 5 years, or colonoscopy every 10 years. The burden of this disease could be dramatically reduced by screening, but CRC screening programs in many countries start at an age when the neoplastic process has already gone in hereditary forms of CRC, leading to a late-stage diagnosis for those patients.

Between 5% and 10% of CRC are hereditary forms, of which the most common is LS, that is associated with approximately 3%–5% of all CRCs.

LS, previously known as hereditary nonpolyposis CRC, is an autosomal dominant disorder caused by mutations in DNA mismatch repair (MMR) genes that have the role to maintain genomic integrity during DNA replication. Inactivation of MMR genes causes an increased mutation rate (genomic instability) and a microsatellite instability.

In individuals with LS, current CRC screening recommendations include colonoscopy every 1–2 years beginning at age 20–25 years. Annual transvaginal ultrasound of the uterus and ovaries and endometrial sampling are also recommended although efficacy is still debated as well as the utility of screening for other LS cancers. Furthermore, prophylactic surgery can be considered as an effective option for women with LS. Additionally, regular, long-term aspirin use is reported as a preventive measure to reduce incidence and mortality due to CRC and the most relevant impact of chemopreventive strategies is expected in patients with an hereditary predisposition as LS.

Hence, the identification of LS-related CRC can lead to beneficial outcomes in terms of survival for the patients, and in terms of prevention for their affected family members.

Several international guidelines recommend to screen for LS all newly diagnosed CRC patients, regardless the age or the clinical criteria. The first step of the screening is performed on tumor tissue through immunohistochemistry staining or the microsatellite instability molecular testing, two laboratory procedures with comparable sensitivity and specificity. In case of positive tumor test, the patient is referred to genetic services for counseling and germline testing for MMR mutations to establish the diagnosis of LS.

Moreover, economic evaluations documented the cost-effectiveness of the universal tumor screening strategy versus no screening because it reduces the costs related to morbidity and mortality from CRC from the early identification of LS-carriers among family members.

However, in resource-limited settings, a universal tumor screening could be hardly achievable. This strategy requires a multidisciplinary team (involving pathologists, surgeons, gastroenterologists, oncologists, geneticists, genetic counselors) that might be not available in all clinical contexts. Furthermore, genetic services are not always available in all clinical settings, and some laboratories could lack in resources, expertise, and quality assessment to perform all the required tests for tumor screening.

Implementation of Genetic Testing Programs in Health Care: Barriers and Future Challenges

The increasing evidence of genomic contribution to cancer onset and progression makes it a relevant part of research and public health which can no longer be overlooked.

The awareness of the health benefits of using genetic tests and family health history in clinical practice has been increasing, as underscored in the agenda items in Healthy People 2020: "Increase the proportion of women with a family history of breast and/or ovarian cancer who receive genetic counseling" and "Increase the proportion of persons with newly diagnosed colorectal cancer who receive genetic testing to identify Lynch syndrome."

In this framework, to make genetic testing for hereditary forms of cancer accessible to all the patients and their family members, when appropriate, as part of the routine diagnostic and therapeutic pathways, many barriers still need to be overcome.

The health professionals' lack of knowledge about genetic testing and their lack of confidence in interpreting familial patterns of disease are some of the most significant barriers to integrate genetic testing into cancer prevention. The lack of consensus and providers' awareness as well as specific guidelines for every country, that take into account the feasibility of different approaches in different contexts, have been associated with divergent referral and testing practices. The miscommunication between patient and physicians and the refusal of genetic counseling by the patients, due to a perceived lack of benefit, have to be also considered.

Therefore, further efforts are necessary to overcome the gap between expectations on genetics and clinical results. A promotion of genetic education and training for healthcare professionals is crucial to assure awareness of emerging issues and appropriate utilization of new genetic technologies, as well as measures to foster the public understanding of scientific developments in human genetics and associated ethical, legal, and social issues. Moreover, a reduction in financial, geographic, and cultural barriers to access genetic services, an optimal management of test-positive individuals, and a family support through integrated services will be crucial. A multidisciplinary team is usually involved in the screening pathways; therefore, a standardized plan should be created to drive their efforts in order to provide effective and equitable cancer assistance to all patients.

In conclusion, genetic research is rapidly increasing the opportunities for the detection of inherited cancer risk, but coordinated strategies in educating clinicians and policy makers and addressing public concerns are necessary to ensure that public health needs are satisfied through appropriate and sustainable healthcare services.

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See also: Genetic and Epigenetic Deregulation of Enhancers in Cancer. Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2. Li–Fraumeni Syndrome. Lynch Syndrome. TP53.

Further Reading

Boccia, S., Michelazzo, M.B., Tognetto, A., Del Sole, A.M., 2016. The prevention of hereditary breast cancer in Italy: towards the implementation of the national prevention plan in the Italian regions. Epidemiology Biostatistics and Public Health 13 (2), 119431–119432. Editorial.

Burke, W., Khoury, M., Stewart, A., Zimmern, R.L., for the Bellagio Group, 2006. The path from genome-based research to population health: development of an international public health genomics network. Genetics in Medicine 8 (7), 451–458.

D'Andrea, E., Marzuillo, C., De Vito, C., et al., 2016. Which BRCA genetic testing programs are ready for implementation in health care? A systematic review of economic evaluations. Genetics in Medicine 18 (12), 1171–1180.

Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group, 2009. Recommendations from the EGAPP Working Group: Genetic testing strategies in newly diagnosed individuals with colorectal cancer aimed at reducing morbidity and mortality from Lynch syndrome in relatives. Genetics in Medicine 11, 35–41.

Giardiello, F.M., Allen, J.I., Axilbund, J.E., et al., 2014. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on colorectal cancer. The American Journal of Gastroenterology 109 (8), 1159–1179.

Hampel, H., Frankel, W.L., Martin, E., et al., 2008. Feasibility of screening for Lynch syndrome among patients with colorectal cancer. Journal of Clinical Oncology 26, 5783–5788.

Hampel, H., Bennett, R.L., Buchanan, A., Guideline Development Group, American College of Medical Genetics and Genomics Professional Practice and Guidelines Committee and National Society of Genetic Counselors Practice Guidelines Committee, 2015. A practice guideline from the American College of Medical Genetics and Genomics and the National Society of Genetic Counselors: referral indications for cancer predisposition assessment. Genetics in Medicine 17, 70–87.

Lerner-Ellis, J., Khalouei, S., Sopik, V., Narod, S.A., 2015. Genetic risk assessment and prevention: the role of genetic testing panels in breast cancer. Expert Review of Anticancer Therapy 15 (11).

Moyer, V.A., 2014. Risk assessment, genetic counseling, and genetic testing for BRCA-related cancer in women: U.S. Preventive Services Task Force recommendation statement. Annals of Internal Medicine 160 (4), 271–281.

Pastorino, R., Tognetto, A., Boccia, S., 2017. Screening programs for Lynch syndrome in Italy: state of the art and future challenges. Epidemiology Biostatistics and Public Health 14 (2), 126151–126153. Editorial.

Rubenstein, J.H., Enns, R., Heidelbaugh, J., et al., 2015. Clinical Guidelines C. American Gastroenterological Association Institute. Guideline on the diagnosis and management of Lynch syndrome. Gastroenterology 149 (3), 777–782.

Tognetto, A., Michelazzo, M.B., Calabrò, G.E., et al., 2017. A systematic review on the existing screening pathways for Lynch syndrome identification. Front. Public Health 5, 243.

Relevant Websites

https://www.cancer.org/health-care-professionals/american-cancer-society-prevention-early-detection-guidelines/breast-cancer-screening-guidelines.html.
http://globocan.iarc.fr—International Agency for Research on Cancer; 2013. GLOBOCAN.
https://www.healthypeople.gov/2020/topics-objectives/topic/genomics/objectives—Healthy People 2020.
https://www.nice.org.uk/guidance/DG27—NICE guidelines. Molecular testing strategies for Lynch syndrome in people with colorectal cancer.
https://www.uspreventiveservicestaskforce.org/.

Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2☆

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Glossary

BRCA1 gene A breast cancer susceptibility gene located on chromosome 17q21, known to be mutated in families prone to a high incidence of early onset breast cancers and also ovarian cancers.

BRCA2 gene A gene mapped to chromosome 13q12-13; known to be mutated in families prone to a high incidence of early onset breast cancers and also ovarian cancers.

HBOC Hereditary breast and ovarian cancer (HBOC).

Homologous recombination repair (HRR) A DNA repair pathway acting on DNA double-strand breaks that uses the undamaged sister chromatid as template for error-free repair.

Locus heterogeneity A genetic term describing how variations in different genes cause the same disorder. The genes are not linked physically; examples are hereditary breast and ovarian cancer predisposition by BRCA1, BRCA2, partner and localizer of BRCA2 (PALB2) and TP53.

Variant of unknown significance (VUS) Variants in genes are classified according to their impact on the protein function, for example, BRCA1 function. A variant with an unknown clinical function owing to lack of functional or clinical data is classified as a VUS.

Characterization of BRCA1 and BRCA2

Fifteen years of analysis of large multigenerational families with a strong history of breast cancer led in 1990 to the identification of a gene on chromosome 17q12–21 that conferred a greatly increased risk of breast cancer in an autosomal-dominant manner. The gene itself, BRCA1, was cloned in 1994. The discovery of the complete sequence of a second such gene, BRCA2 on 13q12-13, was reported in 1995.

The protein-coding region of BRCA1 consists of 5592 bp in 22 exons that encode a protein of 1863 amino acids, and the protein-coding region of *BRCA2* consists of 10,254 bp in 26 exons that encode a protein of 3418 amino acids. Studies of the interactions of these proteins led to the conclusion that they are involved in the regulation of genomic stability and DNA repair. The BRCA1 and BRCA2 proteins interact through a third breast cancer tumor suppressor, PALB2 (partner and localizer of BRCA2) that serves as a major link. A key function of the BRCA1–PALB2–BRCA2 pathway is to secure proper and stable loading of the RAD51 protein at sites of DNA lesions such as DNA double-strand breaks. RAD51 is crucial for homologous recombination repair as well as for the protection of exposed DNA from undergoing excessive degradation during DNA replication. Mice lacking Brca1 and Brca2 (the murine homologues of *BRCA1* and *BRCA2*) undergo developmental arrest during embryogenesis. Furthermore, the modeling of mice with mutations in Brca1 and Brca2 revealed that such genetic changes display enhanced tumorigenesis. This can be modeled to take place in the murine breast tissue, particularly when combined with the TP53 loss. Mice homozygous for a truncating mutation of Brca2 that survive to adulthood have a wide range of defects, including improper tissue differentiation, absence of germ cells, and the development of lethal thymomas, and cultured embryonic fibroblasts from these mice are unable to repair radiation-induced DNA damage. Other studies suggest that mouse embryonic stem cells deficient in Brca1 are unable to carry out a transcription-coupled repair of oxidative DNA damage and are hypersensitive to ionizing radiation and hydrogen peroxide. Human *BRCA2*-defective cancer cells are also deficient in the repair of double-strand DNA breaks induced by ionizing radiation, although individuals with germline mutations in *BRCA1* and *BRCA2* do not appear to be hypersensitive to ionizing radiation.

Cancer Risk Due to Mutations in BRCA1 and BRCA2

Mutations in *BRCA1* and *BRCA2* are responsible for increasing the risk of breast cancer to between 56% and 87% for carriers by the age 70. The lower estimate of risk was derived from an analysis of a general population with little or no family history of cancer, and the higher estimate was derived from families with multiple women affected by breast cancer. The increased risk of breast cancer is observed not only over a woman's lifetime, but particularly at a young age, to 33%–50% before age 50 compared to the general population risk of 2%.

☆*Change History*: August 2017. Claus Sørensen has updated the text and references.
This is an update of Thomas S. Frank, Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2 in Encyclopedia of Cancer (Second Edition), edited by Joseph R. Bertino, Academic Press, 2002, Pages 381–385.

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Mutations in *BRCA1* also confer a greatly increased risk of ovarian cancer of up to 44%, by age 70, whereas mutations in *BRCA2* increase this risk to approximately 27% compared to 1% for the general population of women. The age of onset of hereditary ovarian cancer is typically later than that of hereditary breast cancer, especially when associated with a *BRCA2* mutation.

Women already diagnosed with breast cancer are at a greatly increased risk of a second cancer of breast and ovary if they carry a mutation in *BRCA1* or *BRCA2*. Mutations in *BRCA1* confer a 64% risk of contralateral breast cancer by age 70, or 20% within 5 years of the initial diagnosis, whereas mutations in *BRCA2* increase these risks to about 50% by age 70, or 12% within 5 years of the first breast cancer. The risk of developing ovarian cancer after breast cancer is increased 10-fold to at least 16% compared to women with early onset breast cancer without such mutations. Mutations in both *BRCA1* and *BRCA2* also confer increased (albeit low) risks of some cancers of men, particularly male breast cancer. Mutations in *BRCA2* are responsible for an increased risk of prostate cancer (8% by age 70 and 20% by age 80), as well as an elevated but still low (2%–3%) risk of pancreatic cancer.

There is also a familial component in "triple-negative" breast cancer where cancer cells do not express the receptors estrogen, progesterone, and HER2/neu. About 10%–30% of women under the age of 60 diagnosed with "triple-negative" breast cancer will have a gene mutation in *BRCA1*. Triple-negative breast cancer has relatively poor prognosis, hence these women are also included in clinical genetic programs.

Assessment of Hereditary Risk of Breast and Ovarian Cancer

Genetic testing for mutations in *BRCA1* and *BRCA2* genes is no longer confined to research protocols, but is now clinically available to healthcare professionals. Mutations in the *BRCA1* or *BRCA2* genes can be identified through a blood or saliva test. DNA sequence analysis is generally acknowledged as the most sensitive method of analyzing these genes because more than thousand mutations have been described throughout the lengths of each. Sequence analysis detects the majority of clinically significant abnormalities in *BRCA1* and *BRCA2*. Large duplications and deletions in the respective genomic regions have also been reported for which clinical testing has become available.

Prior to genetic testing, a patient is generally provided pretest education and counseling regarding hereditary risk and genetic testing, including consideration of which relatives with whom the patient would share test results. Individuals who choose to be tested are asked to sign an "informed consent" form indicating that they understand the benefits and limitations of the test that they have chosen. An identified mutation in either *BRCA1* or *BRCA2* can usually be assumed to have been inherited from one parent or the other, as spontaneous germline mutations in either gene are rare. An individual who does not have a germline mutation in *BRCA1* or *BRCA2* at the time of birth cannot acquire one later in life so tests for mutations in these genes are normally performed only once in a person's lifetime. If a mutation is identified in HBOC predisposing genes, a tailored surveillance plan is developed based on the pattern of cancers associated with the specific genes and family history of cancer. This plan must be developed by a team of healthcare professionals and must include expertise in clinical cancer genetics.

Testing for mutations in the *BRCA1* or *BRCA2* genes may not be beneficial for the average woman since most breast and ovarian cancers are sporadic. Genetic testing for hereditary cancer risk is generally preceded by thorough assessment of an individual's family history in order to determine whether she (or he) is likely to carry a mutation responsible for hereditary cancer risk. The hallmarks of mutations in *BRCA1* and *BRCA2* include two or more family members on the same side of the family with breast cancer at an early age of onset (usually before age 50) or ovarian cancer at any age. Breast and ovarian cancer in the same individual or male breast cancer at any age also indicates the possibility of HBOC. In evaluating a family history for the possibility of hereditary cancer risk, it is important to equally assess the father's side of the family as well as the mother's, as half of women with a hereditary risk of breast and ovarian cancer inherited it from their father.

Mutations in *BRCA1*and *BRCA2* have been described throughout the world but are more prevalent in some populations than others, such as individuals of Ashkenazi Jewish descent (i.e., central and eastern European origin). Evaluation of hereditary cancer risk may be warranted for any Ashkenazi Jewish woman with early onset breast cancer or ovarian cancer at any age regardless of family history. Notably, identifying a *BRCA1* and *BRCA2* variant does not immediately suggest that the individual is a carrier of a pathogenic variant. Indeed, many variants of *BRCA1* and *BRCA2* have no clear disease linkage, which can be due to a documented lack of cancer predisposition, or, the individual may carry a rare or newly discovered variant. These variants of unknown significance (VUS) can constitute a major issue for the genetic counseling, and such result generally indicate that the individual should be monitored more carefully.

BRCA1 and *BRCA2* are not the only HBOC predisposing genes. Mutations in other genes may be associated with an increased risk of developing breast cancers, including mutations in the *PALB2*, *ATM*, and *CHEK2* genes. Another set of genes are involved in syndromes with more varied pattern of cancer types, which is including the Li–Fraumeni syndrome (*TP53* gene), Cowden syndrome (*PTEN* gene), and others. Thus, the pattern of cancers in a family can indicate the specific gene predisposing to hereditary cancer for that family.

To aid in optimal genetic analysis, new panels of multiple genes have been developed for testing in patients with a suggestive personal and family history. These panel tests include *BRCA1* and *BRCA2* and other genes that increase the risk of breast, ovarian, and other cancers. Panels may include 6, 20, 40, or more genes depending on the personal and family history. Thus, even if an individual has a negative test result for *BRCA1* and *BRCA2*, mutations in other genes may still be elucidated. These mutations can be relevant as BRCA1 and BRCA2 operate in complex pathways where locus heterogeneity can be observed. Thus, several gene products collaborate to maintain genome stability, and mutations in several such factors can lead to a BRCA1/2-like phenotype. Because these non-BRCA1/2 mutations are rare, accurate risk assessments may not be possible. However, if a genetic analysis indicates a potential pathogenic variant, prevention and screening strategies may still be initiated.

It is in principle possible to use massive in-depth sequencing (next-generation sequencing) of blood samples from individuals with suspected family history. However, this leads to the inclusion of vast number of genes with no clear link to HBOC, which makes it virtually impossible to use such massive data in the clinical genetic setting.

Hereditary Risk of Breast and Ovarian Cancer: Implications for Medical Care

Evaluation for hereditary cancer risk should occur in the context of the medical interventions available to address that risk. Management options generally include increased surveillance, prophylactic surgery, and chemoprevention (medications that reduce the risk of cancer). The risks and benefits of these options should each be considered not only for women who do not themselves have cancer, but also women with breast cancer who are at the risk of subsequent malignancy of the breast and ovary.

Elaborate guidelines have been developed to establish standardized and adequate clinical practices. These are based on expert opinions and are from organizations such as the National Comprehensive Cancer Network (NCCN), the National Institutes of Health and Clinical Excellence (NICE), and the European Society for Molecular Oncology (ESMO). The reader is referred to these (see references) for comprehensive and recently updated guidelines on initial risk assessment, genetic counseling, and testing.

Specifically, it is recommended that women with mutations in *BRCA1*or *BRCA2* perform monthly breast self-examinations, beginning at age 18. The female carrier then undergoes annual or semiannual clinician breast examinations from the age of 25. Annual screening MRI should be started from age 25 as well as annual mammography to commence from age 30. Increased surveillance may be implemented in association with chemoprevention. Tamoxifen, a selective estrogen receptor modulator, may reduce the risk of breast cancer in high-risk women and specifically reduces the risk of contralateral breast cancer in women with *BRCA1*or *BRCA2* mutations. It is thus likely, although not yet specifically demonstrated, that tamoxifen will reduce the occurrence of primary cancer in such women.

Several risk-reducing breast surgical techniques exist ranging from total to nipple-sparing mastectomy (NSM). Prophylactic mastectomy has been shown to reduce the risk of breast cancer in "high-risk" women by at least 90% with mutations in *BRCA1* or *BRCA2*. Bilateral risk-reducing total mastectomy is an option, however, this procedure is not the sole approach because of the efficacy of surveillance in detecting most early stage breast cancer and the high cure rate of breast cancers detected at an early stage. Prophylactic total mastectomy may be considered by women whose mammographic assessment is compromised by extensive fibrocystic change or by women whose perception of breast cancer has been affected by relatives or friends with the disease. Following NSM, there might be somewhat elevated residual risk due to breast tissue being left behind after surgery, however, such elevated risk is not clearly established. In any case, all aspects of surgical procedures should be carefully discussed with the individual carrier or patient.

Oral contraceptives reduce the risk of ovarian carcinoma by 50% in the general population and have also been associated with a 60% reduction in the risk of ovarian cancer in women with mutations in *BRCA1* and *BRCA2*. It should be noted that oral contraceptives may increase the risk of breast cancer for carriers.

Unfortunately, surveillance for ovarian cancer is often ineffective. Measurement of serum CA-125 is regarded as a quite unreliable screen for ovarian cancer and transvaginal ultrasound lacks specificity. Prophylactic oophorectomy should therefore be considered by women around age 35–40 with mutations in *BRCA1*or *BRCA2* as recommended by the clinical practice guidelines. Prophylactic oophorectomy is believed to reduce the risk of ovarian cancer in women with mutations in *BRCA1* and *BRCA2* by as much as 95% and also reduces their risk of breast cancer by nearly 50%.

A negative as well as a positive test result can have significant implications for patient care. If a mutation in *BRCA1* or *BRCA2* has previously been characterized in a relative, a negative result indicates that a woman is at the population risk of breast and ovarian cancer, despite having a strong family history of either or both diseases. It has also been shown that individuals who learn through genetic testing that they do not carry the mutation identified in their family have significant reductions in depressive symptoms compared with untested individuals.

Options exist for couples interested in having a child when they know that one of them carries a gene mutation that increases the risk for this or any other hereditary cancer syndrome. Preimplantation genetic diagnosis done in conjunction with in vitro fertilization is used to avoid the carrier chromosome. Thus, it allows both women and men who carry a specific known genetic mutation to have children who do not carry the mutation.

Summary

Inherited mutations in the genes *BRCA1* and *BRCA2* are associated with a significantly increased risk of breast cancer, particularly before age 50, as well as an increased risk of ovarian cancer. Laboratory analysis of these genes can determine whether a woman has inherited increased risks of breast and ovarian cancer. Identification of a hereditary risk of breast and ovarian cancer can facilitate the medical care of healthy mutation carriers, as well as those already diagnosed with cancer who are at risk of a second malignancy. Once a mutation has been characterized in a family, a family member whose test indicates that he or she did not inherit the mutation has no elevated risk of cancer despite the strong family history, and can therefore avoid unnecessary interventions that might have previously been considered appropriate. Clinical laboratory analysis of *BRCA1* and *BRCA2* can be used to assist in the identification and management of individuals with a hereditary risk of breast and ovarian cancer.

Prospective Vision

The identification of BRCA1 and BRCA2 as major HBOC factors has been crucial in the development of tailored clinical approaches to improve counseling and treatment of affected individuals. However, a number of unresolved issues still remain, including the detailed analysis and validation of VUS as well as the elucidation of non-BRCA1/2 genes contributing to a substantial number of HBOCs. The latter may even operate in the same functional pathways as BRCA1/2. Thus further understanding of genetic risk factors as well as nongenetic risk contributors are important to further advance the field. In this regard, ongoing international large-scale efforts aim at tackling these issues, and it is likely that comprehensive whole exome/genome studies will contribute in the near future. When coupled with functional assays to characterize and validate potential variant dysfunction, it is conceivable that HBOC clinical practices will be markedly improved within the coming decade.

See also: Breast Cancer: Pathology and Genetics. Hereditary Cancer Syndromes: Identification and Management. Ovarian Cancer: Diagnosis and Treatment. Ovarian Cancer: Pathology and Genetics. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics.

Further Reading

COMPLEXO, Southey, M.C., Park, D.J., et al., 2013. COMPLEXO: identifying the missing heritability of breast cancer via next generation collaboration. Breast Cancer Research 15, 402.

Easton, D.F., Pharoah, P.D., Antoniou, A.C., et al., 2015. Gene-panel sequencing and the prediction of breast-cancer risk. New England Journal of Medicine 372, 2243–2257.

Hartmann, L.C., Lindor, N.M., 2016. The role of risk reducing surgery in hereditary breast and ovarian cancer. New England Journal of Medicine 374, 454–468.

Kuchenbaecker, K.B., Hopper, J.L., Barnes, D.R., et al., 2017. Risks of breast, ovarian, and contralateral breast cancer for BRCA1 and BRCA2 mutation carriers. Journal of the American Medical Association 317 (23), 2402–2416.

Lord, C.J., Ashworth, A., 2016. BRCAness revisited. Nature Reviews Cancer 16, 110–120.

Millot, G.A., Carvalho, M.A., Caputo, S.M., et al., 2012. A guide for functional analysis of BRCA1 variants of uncertain significance. Human Mutation 33, 1526–1537.

National Collaborating Centre for Cancer (UK), 2013. Familial breast cancer: classification and care of people at risk of familial breast cancer and management of breast cancer and related risks in people with a family history of breast cancer. NICE Clinical Guidelines, No. 164. NICE, Cardiff.

NCCN, 2016. Clinical practice guidelines in oncology: genetic/familial high risk assessment: breast & ovarian. Version 2.2016. nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf (Version 2.2017 – December 7, 2016).

Nielsen, F.C., van Overeem Hansen, T., Sørensen, C.S., 2016. Hereditary breast and ovarian cancer: new genes in confined pathways. Nature Reviews Cancer 16 (9), 599–612.

Nik-Zainal, S., Davies, H., Staaf, J., et al., 2016. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature 534, 47–54.

Paluch-Shimon, S., Cardoso, F., Sessa, C., et al., 2016. Prevention and screening in BRCA mutation carriers and other breast/ovarian hereditary cancer syndromes: ESMO Clinical Practice Guidelines for cancer prevention and screening. Annals of Oncology 27 (Suppl. 5), v103–v110.

Spurdle, A.B., Healey, S., Devereau, A., et al., 2012. ENIGMA—evidence-based network for the interpretation of germline mutant alleles: an international initiative to evaluate risk and clinical significance associated with sequence variation in BRCA1 and BRCA2 genes. Human Mutation 33, 2–7.

HIV (Human Immunodeficiency Virus)☆

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Glossary

Acquired immunodeficiency syndrome (AIDS) A disease of the immune system caused by HIV infection and characterized by increased susceptibility to opportunistic infections, certain cancers, and neurological disorders.

CD4 A cellular surface protein found on T cells as well as other cells. It functions as a receptor for HIV binding and facilitates recognition of the T-cell receptor to antigens bound to MHC class II complexes.

Cellular immunity The immune system response by a number of cellular components such as CD8 + T cells, CD4+ T cells, NK cells, neutrophils, macrophages, and dendritic cells. The effector function involves contact of the immune cells with infected cells or cancer cells.

Chemokines A class of proinflammatory cytokines that have the ability to attract and activate leukocytes. They can be divided into at least three structural branches—C, CC, and CXC—according to variations in a shared cysteine motif.

Cytokines Proteins secreted by leukocytes and some nonleukocytic cells that act as intercellular mediators. They are also produced by a number of tissue or cell types and generally act locally in a paracrine or autocrine manner.

Dendritic cells Immune cells, derived from bone marrow, with long, tentacle-like branches called dendrites. Among dendritic cells are the Langerhans cells of the skin and follicular dendritic cells in the lymph nodes. Most dendritic cells function as antigen-presenting cells that digest extracellular pathogens and present segments of protein (antigen) on the cell surface to induce a primary immune response.

Humoral immunity An antibody-mediated immunity involving secreted products of B cells that interact and neutralize extracellular pathogens.

Innate immunity A nonantigen specific immune system that is the first line of host defense against incoming pathogens and foreign substances. Unlike the adaptive immune system, the innate immune system response is not to a specific antigen nor does it usually have memory of previous exposure to an antigen. This immune system acts within hours to days whereas the adaptive immune system, reflected by humoral and cellular immunity, responds after days to weeks. The innate immunity plays a role in activating the adaptive immune system.

Lentivirus A genus of the family Retroviridae consisting of nononcogenic retroviruses that produce multiorgan diseases characterized by long incubation periods and persistent infection. Lentiviruses are unique in that they contain open reading frames between *pol* and *env* genes and in the 3′ *env* region.

Long terminal repeats (LTR) Identical DNA sequences, several hundred nucleotides long, found at either end of transposons and proviral DNA. LTRs are formed by reverse transcription of retroviral RNA. In proviruses, the upstream LTR acts as a promoter and enhancer, whereas the downstream LTR acts as a polyadenylation site.

Long-term survivors Individuals infected by HIV for many years who, without therapy, do not show any signs of the infection. They have a normal immune system reflected by normal CD4+ cell counts and low virus levels in the blood. Some are elite controllers who have had undetectable HIV in their plasma for several years.

Macrophages Large white blood cells found mainly in connective tissue and in the bloodstream that ingest foreign particles and infectious microorganisms by phagocytosis.

Retroviridae A family of viruses with a single-stranded RNA that, upon infection, generates a DNA copy via a viral reverse transcriptase; lentivirus is a genus in a subfamily.

Simian immunodeficiency virus (SIV) A member of the genus lentivirus that induces acquired immunodeficiency syndrome in monkeys and apes. SIV and HIV-2 exhibit close structural and immunologic properties and are 75% homologous.

T lymphocyte A subset of lymphocytes that develop in the thymus and circulate in the blood and lymphoid tissue. They orchestrate the response of the immune system to infected or malignant cells, either by lymphokine secretions or by direct contact. Helper T cells recognize foreign antigen on the surfaces of other cells, thus causing the stimulation of B cells to produce antibody and to cytotoxic T cells to destroy antigen-displaying cells.

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The human immunodeficiency virus (HIV), is a retrovirus that gradually destroys the immune system and has caused the worldwide epidemic of acquired immunodeficiency syndrome (AIDS). In 1983, HIV was first isolated from AIDS patients and was later identified as the infectious agent responsible for the disease. Other reports indicated the presence of this agent in many populations, including healthy individuals. HIV is cytotoxic to CD4+ cells and does not transform them into malignant cells characteristic of HTLV infection. This infection of CD4+ cells appeared to contribute to the dramatic decrease in CD4+ cell numbers observed in AIDS patients. HIV can be transmitted to individuals through sexual contact, blood products, or via mother-to-child transmission before or shortly after birth. Consistent with this observation, the levels of infectious HIV virions are substantially higher in the peripheral blood and genital fluids of individuals with the greatest chance of transmitting the virus.

Stages of HIV Infection

The typical course of HIV infection begins with an acute infection characterized by the presence of increasing levels of infectious viral RNA in plasma and a transient decrease in CD4+ T lymphocytes. Within weeks to a few months, the amount of virus detected in the peripheral blood decreases as a result of immune responses, while the CD4+ T lymphocyte count returns to a near-normal level. The initial infection is often associated with a flu-like syndrome that occurs within weeks and is followed by a quiescent period characterized by a healthy clinical state. The length of this asymptomatic latent period is influenced by environmental, genetic, and immunologic factors as well as by the predominant virus type. In the final stages of the infection, which takes place after an average of 10 years, the viral load increases in the peripheral blood and lymphoid tissues. This phenomenon is accompanied by a major decrease in CD4+ cell numbers and the occurrence of opportunistic infection or cancer leading to a fatal outcome.

Genetic Structure and Products

HIV-1 and HIV-2 are grouped within the lentivirus genus as members of the Retroviridae family. The two types of AIDS viruses, HIV-1 and HIV-2, share up to 50% overall nucleotide homology and are similar in structure and cellular tropism. Based on virus genetic similarities, four major groups of HIV-1 (M, N, O, P) have been identified. Each group differs genetically by up to 30%. Within the HIV-1 group M there are at least 9 subtypes or clades that vary in sequence by about 15%. Most HIV infections worldwide involve HIV-1 clade C. For HIV-2, there are 2 groups and no clades. Most evidence suggests that the difference in pathogenicity of these various HIV isolates is determined by host specific factors (intrinsic or immune) rather than the particularly virus isolate.

The genomes of HIV-1 and HIV-2 consist of two identical single RNA strands enclosed within a cone-shaped core, surrounded by a glycosylated outer virion surface (Fig. 1). The 9.8-kb RNA genome contains a 5′ cap (Gppp), a 3′ poly(A) tail, and several open reading frames (ORFs). This genome codes for 15 major molecular products (Table 1). The longest ORF encodes for viral structural proteins, whereas smaller ORFs encode regulators of viral binding, fusion, replication, and assembly.

Structural Proteins

Env

The Env structural polyprotein, gp160, is produced from a long ORF transcript. Gp160 is cleaved into gp120 and gp41 in the endoplasmic reticulum. These proteins are highly glycosylated and can be expressed on either the cell or the virion surface. Interaction of the viral gp120 with the cellular receptor CD4 causes a conformational change that facilitates virus binding and entry. The gp120 protein forms trimeric structures that are anchored to the membrane of the virion by the viral transmembrane protein gp41. The

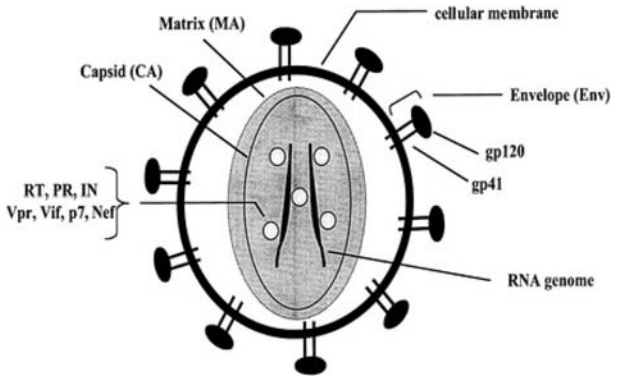

Fig. 1 Structure of the HIV virion. The HIV RNA genome is encapsulated in a capsid (CA) composed of p24 and is stabilized by a matrix protein, MA. The cellular lipid bilayer containing envelope glycoproteins completes the outer structure. Several proteins located inside the capsid are required for maturation and infectivity of HIV.

Table 1 HIV proteins and their functions

Protein	*Size (kDa)*	*Function*
Gag	p24	Capsid (CA) structural protein
	p17	Matrix (MA) protein-myristoylated
	p6	Role in budding
	p7	Nucleocapsid (NC) protein; helps in reverse transcription
Polymerase (Pol)	p66, p51	Reverse transcriptase (RT); RNase H–inside core
Protease (PR)	p10	Posttranslational processing of viral proteins
Integrase (IN)	p32	Viral cDNA integration
Envelope	gp120	Envelope surface (SU) protein
	gp41	Envelope transmembrane (TM) protein
Tat	p14	Transactivation
Rev	p19	Regulation of viral mRNA expression
Nef	p27	Pleiotropic, can increase or decrease virus replication
Vif	p23	Increases virus infectivity and cell-to-cell transmission; helps in proviral DNA synthesis and/or in virion assembly
Vpr	p15	Helps in virus replication; transactivation
Vpu	p16	Helps in virus release; disrupts gp160–CD4 complexes
Vpx	p15	Helps in infectivity

Adapted from Levy, J. A. (2007). HIV and the pathogenesis of AIDS (3rd ed.). Washington, DC: ASM Press.

gp120 surface protein has several loops highly variable in amino acid sequence (V1–V5) among viral strains. These regions primarily regulate the efficiency of virus entry and cellular tropism. The envelope also contains five constant regions (C1–C5) interspersed within its structure. Mutations in the V3 loop can decrease or alter infectivity and the fusion capabilities of HIV. One of the conserved regions of gp120, C4, has a complex folded structure that, if mutated, can affect conformational changes and thus the efficiency of virus binding to CD4. The C2 region of gp120 is important for interactions with CD4 and with gp41, to which gp120 binds by hydrophobic interaction. The gp41 protein mediates fusion and, along with gp120, cytotoxicity (see Section IVB).

PR, RT, and IN

Another long viral ORF generates precursor Gag and Gag-Pol transcripts, which can be transported to the cytoplasm for translation by free ribosomes into polyproteins. A ribosomal frameshifting event produces the Gag-Pol polyprotein $Pr160^{Gag\text{-}Pol}$ that can be proteolytically processed by the viral protease, PR. The autocatalysis of PR, a product of Pol, is activated in the presence of high $Pr160^{Gag\text{-}Pol}$ protein concentrations. The PR-mediated cleavage of $Pr55^{gag}$ and $Pr160^{Gag\text{-}Pol}$, during assembly of the virion, is necessary for the generation of infectious particles. In addition, PR processes the reverse transcriptase (RT) and integrase (IN) proteins from Pol and matrix (MA), p24, p9, and p6 proteins from Gag. Reverse transcriptase generates the complementary strand from the RNA genome producing the provirus cDNA form. Because RT is colocalized with the RNA genome in the capsid, some reverse transcription can occur in the viral particle before infection of the target cell. However, the completion of provirus production requires infection of the host cell and uncoating of the virion. Within the capsid, viral IN proteins are found along with MA and the accessory viral protein, Vpr. IN is required for integration of the provirus into the host genome.

MA, p24, p9, and p6

The Gag precursor protein $Pr55^{gag}$ is responsible for generating structural proteins required for virus assembly. Due to myristoylation of $Pr55^{gag}$, the protein can be targeted to the plasma membrane for the regulation of viral assembly at the cell surface. Proteolytic processing of $Pr55^{gag}$ by PR generates MA, p24, p9, and p6. MA is a myristoylated protein (p17) that forms a matrix assuring the structural integrity of the virion. The p24 protein is the major structural component of the cone-shaped core or capsid (CA) and can influence infectivity of the virion through interaction with cyclophilin A. The p9 protein is an RNA-binding protein in the core that protects viral RNA from nucleases and transports full-length viral RNA to the assembly complex. The p6 protein can assist in budding of the virion and affect infectivity.

Regulatory Proteins

Tat and Rev

The smaller ORFs generate viral regulatory and accessory proteins. These viral gene products, Tat, Rev, Nef, Vpu, Vpr, and Vif, can be spliced in the nucleus by cellular spliceosomes and transported to the cytoplasm for translation by membrane-bound ribosomes. Tat regulates viral transcription through interactions with specific cellular factors and TAR, the transactivation response element in the 5′ stem–loop region of the HIV RNA genome. Tat can also enhance, but is not required for, virus replication. The Rev protein regulates nuclear export of transcripts by interacting with a Rev responsive element (RRE) located in the viral *env*-coding region. Rev controls the ratio of unspliced to spliced transcripts, which is important for initiating virus assembly.

Accessory Proteins

Vif, Vpu, Vpr, Nef

In addition to regulatory proteins, several accessory proteins have important functions in the HIV replicative cycle. Vif increases virus infectivity, and Vpu, found in HIV-1 only, mediates downregulation of the CD4 molecule and enhances infectious HIV virion release from the cell surface. Vpr, a nuclear protein found in most HIV subtypes, regulates transport of the preintegration complex. Both Vpr and Vpx, a cytoplasmic protein found only in HIV-2 and SIV, affect assembly and budding, as well as enhance viral replication. Nef is a pleiotropic protein that can positively regulate virus replication in quiescent cells and mediate pathogenicity. Nef appears to affect T-cell signaling activity, resulting in altered or lower levels of cytokines, particularly IL-2. The role for Nef in the regulation of viral infectivity and pathogenesis is supported by the recovery of Nef mutants of HIV-1 from infected individuals with no evidence of disease progression. However, the full mechanism of Nef activity remains unclear.

HIV Replication Cycle

The infection cycle of HIV can be divided into four major stages: binding and entry, reverse transcription and integration, transcription and translation, and assembly and budding (Fig. 2). (Step 1) In the binding and entry stage, the HIV envelope protein, gp120, has a high-affinity interaction with the cellular surface protein, CD4, and specific cellular coreceptors, which facilitate fusion with the cellular membrane. Once inside the cell, viral CA protein p24 and cyclophilins mediate the uncoating of the HIV core and release of the RNA genome. (Step 2) In association with several structural proteins, the RNA genome is primed by viral tRNA-lysine and is reverse transcribed into a double-stranded cDNA. The MA and Vpr viral proteins target the preintegration complex, composed of viral cDNA and MA, Vpr, and IN, to the nucleus. Integration of the provirus occurs randomly in the cellular genome and is regulated by IN, which is capable of cleaving chromosomal DNA and mediating ligation of the provirus.

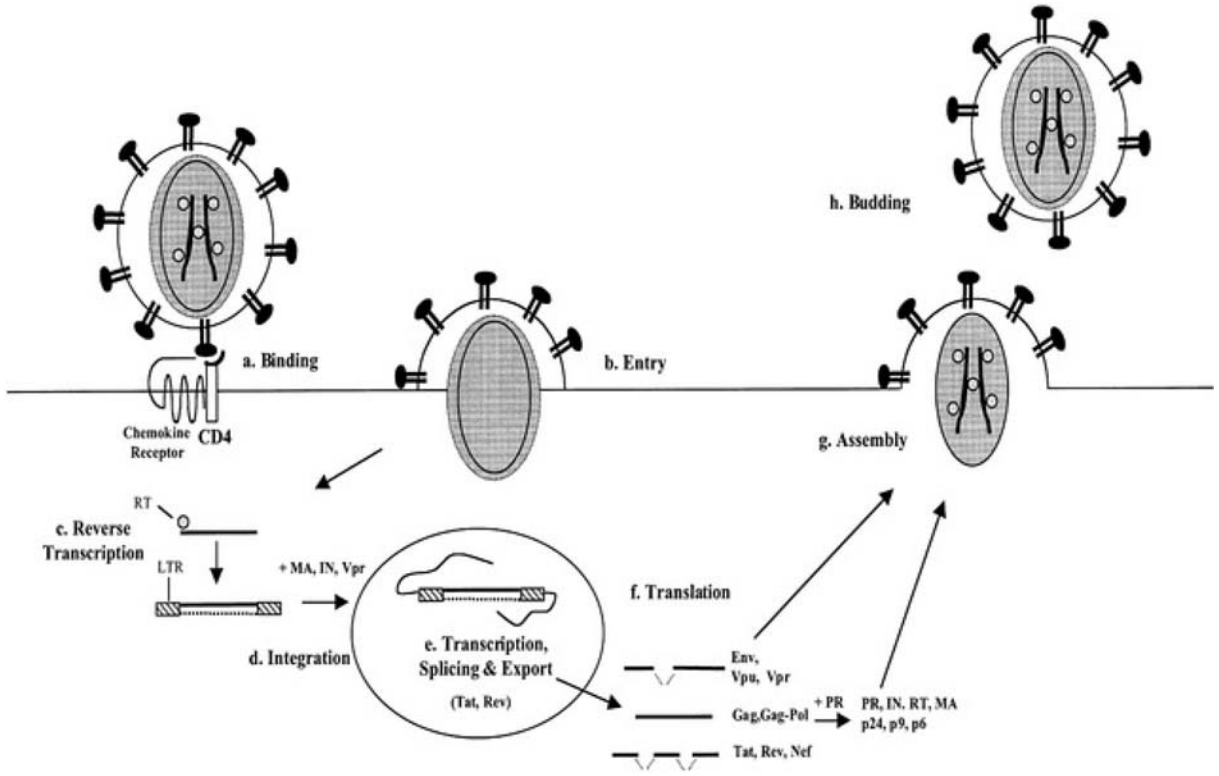

Fig. 2 Steps in the HIV replication cycle. HIV infection proceeds through several stages involving binding, entry, reverse transcription, integration, transcription, translation, assembly, and budding. (A) The HIV gp120 envelope protein binds CD4 on the cellular surface and specific chemokine receptors facilitate fusion with the cellular membrane. (B) Entry involves uncoating of the virion, releasing the capsid containing the viral RNA genome and viral proteins into the cytoplasm. (C) Virally encoded reverse transcriptase generates the RNA genome into double-stranded cDNA. (D) MA, IN, and Vpr mediate nuclear transport of the cDNA preintegration complex, and IN completes integration of the HIV cDNA into random sites of the host genome. (E) Transcription of viral genes is initiated at the 5′ LTR and requires Tat for optimal transcription. Splicing occurs in the nucleus, and the export of long viral transcripts is mediated by Rev. (F) Translation of viral transcripts in the cytoplasm produces viral products that assist in (G) assembly of the virion at the cell surface and the subsequent (H) budding from the cellular surface.

The HIV proviral cDNA is flanked by a 5′ LTR and 3′ LTR that regulate initiation of transcription and RNA termination, respectively, of regulatory enzymatic and structural genes. (Step 3) The 5′ LTR is composed of several regulatory sites for the transcription of viral genes by RNA polymerase II. NF-κB, Sp1, and TATA binding protein (TBP) are among some of the cellular factors that bind upstream of the 5′ LTR and assist in initiating viral transcription. The viral regulatory protein Tat is essential for increasing the rate of transcription. The regulation of splicing events, directed by the Rev protein, allows for the proper translation of many of the viral RNAs. Unspliced viral RNA can incorporate, at the cell membrane, into virus capsids that subsequently bud from the cell surface, incorporating the virus envelope expressed on the cell surface. (Step 4) During the budding process, proteolytic processing (mediated by the viral protease) brings about the maturation of the viral particle into an infectious virion.

Cellular Binding and Tropism

Cellular determinants of the host range include receptors for HIV binding and entry, such as the CD4 surface molecule, chemokine coreceptors, galactosyl ceramide, complement, and Fc receptors used when virus is complexed with antibody. However, high levels of productive infection are predominantly observed in CD4+ hematopoietic cells. The CD4 surface protein expressed by mononuclear phagocytes and helper T lymphocytes contains region D1 important for viral entry and cellular tropism. This CD4 molecule provides a docking site for, and induces a conformational change in, the envelope protein gp120, which exposes a site(s) for virus binding to coreceptors on the cell and fusion with the cell.

HIV: Cell Interactions

Coreceptors are necessary for efficient HIV virion attachment that leads to viral fusion and entry. Two such receptors are the G protein-coupled seven transmembrane chemokine receptors CXCR-4 and CCR5. Chemokine receptors have important roles in the trafficking of T lymphocytes and macrophages. In HIV infection, chemokine receptors are essential for HIV entry after initial binding to the CD4 surface molecule and are determinants of cellular tropism.

A morphologic hallmark of productive HIV infection in cultured cells is the presence of syncytia, a fusion of infected cells resulting in multinucleated cellular complexes, and subsequent cell death. All HIV isolates can replicate in CD4+ T lymphocytes and some are more cytopathic than others, as observed by the rapid formation of syncytia. Those HIV strains that replicate in established T-cell lines, as well as CD4+ lymphocytes, are the syncytia-inducing (SI) strains and utilize the CXCR-4 coreceptor, expressed on T lymphocytes. In contrast, non-syncytia-inducing (NSI) strains that also infect CD4+ lymphocytes grow well in macrophages using the CCR5 coreceptor. This receptor is expressed on monocytes, dendritic cells, T cells, and microglia. Additionally, some isolates are dual-tropic and can infect both cell types. Therefore, the diversity of HIV isolates and coreceptor expression can affect cellular tropism.

Evidence has shown that SDF-1, the natural ligand to CXCR-4, and the β-chemokines that bind to CCR-5 can suppress HIV replication in cell culture by blocking entry. These findings further support the importance of chemokine receptors in mediating HIV infection. Moreover, at the genetic level, homozygosity for a CCR5 allele with a 32-bp deletion, which affects CCR5 surface expression, confers resistance in some HIV-infected individuals to the NSI virus strains.

HIV can infect CD4 negative cells, although with less replication capacity, through alternate receptors. In brain cells and bowel epithelium, gp120 interacts with surface galactosyl ceramide at a site distinct from its CD4 receptor-binding region. Another mode of entry may involve Fc and complement receptors. These receptors can interact with the Fc portion of nonneutralizing antibodies that attach to HIV and can transport the virus into the cell.

Role of HIV Envelope Protein

HIV envelope proteins act as viral determinants of cellular binding and host range. Both gp120 and gp41 appear important in regulating the rate and efficiency of virus attachment and entry through defined conformational changes or glycosylation patterns. Specific regions in the V3 loop also influence the pathogenicity or syncytia-inducing capability of the virion. The V3 loop is particularly important for determining the host range. Additionally, cleavage of gp120 by cellular proteases may be an essential step for viral entry and facilitate gp41 fusion with the cellular membrane.

Virus Replication and Cytopathicity

The difference in virus production and cytopathogenicity in T cells and macrophages may correlate with the state of activation. Quiescent T lymphocytes expressing CD4 on the surface are very susceptible to HIV infection. However, until activated, they cannot elicit a productive infection or permit integration of viral DNA. In nondividing CD4+ macrophages, HIV infection can be productive with integration of viral DNA. Levels of virus replication are generally lower than in CD4+ lymphocytes but some NSI strains can be produced to high levels in macrophages. In some CD4 negative cells, HIV can enter but generally replicates to low levels. Differences in the intracellular factors influencing transcription, reverse transcription and virus production may account for the incapacity of some cell types to produce substantial amounts of virus (see below). In addition, virus entry or envelope processing can vary among cell types and affect the extent of viral replication.

Cytopathic Effects

The loss of CD4+ cells could be due to a variety of mechanisms, including syncytia formation, antibody-dependent cellular cytotoxicity, gp120-induced apoptosis, cytotoxic T lymphocyte (CTL) lysis of infected cells, or death of uninfected bystander cells by apoptosis, or necrosis. Syncytia formation (or cell:cell fusion) is followed by membrane damage, leading to "balloon cell" degeneration, or necrosis. As noted earlier, SI viruses are generally more cytopathic than NSI viruses, and the appearance of SI isolates in infected individuals strongly correlates with progression to AIDS. Regions on CD4 and gp120 can regulate syncytia formation and, therefore, toxicity to CD4+ cells. Programmed cell death, or apoptosis, in response to virus infection, appears to account in part for the decline in CD4+ cell numbers in individuals as they progress to disease. This process is increased with inflammation and cell activation. Direct infection of the cells is not needed to observe this effect. Many reports suggest that alterations in cytokine production can influence the rate of cell death by inhibiting survival cytokines (e.g., IL-2) or by directly inducing the death of uninfected bystander lymphocytes as well as infected cells. However, the pathway(s) leading to apoptosis in vivo is still unclear.

Cellular Latency

Cellular latency occurs following HIV provirus integration and is characterized by minimal transcriptional or translational activity of viral genes. Virus activation from a latent state is often the result of stimulation by mitogens, cytokines, or DNA-damaging agents. The regulation of viral latency remains elusive. Some of the cellular factors recruited to the LTR for the active transcription of viral genes include NF-κB, Sp-1, and TBP However, Rev, Tat, Vpu, and Nef have been implicated in mediating latency through their interactions with the LTR and replication cycle. Further understanding of the factors preventing induction of and reactivation from viral latency would be helpful for developing approaches to inhibit progression to disease. Activation of HIV from a latent state is a recent approach at bringing about a cure (see below).

Host Immune Responses to HIV Infection

The effect of HIV on the immune system heavily influences the pathogenesis of the infection. Some of the differences observed in immune responses to HIV infection can be associated with small genetic changes in the virus or differences in the host genome. Certain HLA genotypes (e.g., HLA-B57 B27 and B51) have been associated with long term survival and slow disease progression. Also HLA-KIR interactions can influence the natural killer (NK) cell anti-HIV activity. A humoral response to the initial acute infection is reflected by the production by B cells of anti-HIV antibodies. As a host response to HIV infection, some of these antibodies are able to neutralize the virus, generally blocking the epitopes responsible for binding and/or fusion of gp120 or gp41 to the target cell. Antibody-dependent cellular cytotoxicity (ADCC) is another host humoral antiviral response that targets specific HIV epitopes for destruction by interactions of antibody-coated infected cells with effector cells such as NK cells, neutrophils, or macrophages. In contrast, antibody-dependent enhancement (ADE) can potentiate HIV infection through complement or Fc receptors. A decreased concentration of inhibitors of complement in infected individuals suggests that the antibody-independent complement cascade pathway can be activated to lyse either the virus or the virus-infected cell. This and other innate immune responses may play a role in controlling HIV infection.

Natural Killer Cells

A component of the innate immune system, NK cells, can directly eliminate virus-infected cells. The cytotoxic mechanism is not MHC-dependent and potentially involves ADCC. In individuals progressing to disease, a loss in NK cell number and function, partly reflecting direct infection of these cells by HIV, alters the production of proinflammatory cytokines such as interferon-γ. A decrease in interferon-γ production can also interfere with CD8+ cell function.

CD4 + T Lymphocytes

The CD4+ cell number and proliferative responses are dramatically reduced during disease progression. Their loss is caused by several processes including direct infection, apoptosis, and immune cell killing. Dysfunctional T helper cells and a loss of CD4+ cells are characteristically observed during infection. Pronounced deficits in CD4+ cell function are manifested by a decreased proliferative response first to recall antigens, then alloantigens, and, in later stages, mitogenic stimulation. Lower levels of IL-2 production in addition to decreased IL-2 receptor expression are observed with progression to disease. Generally, CD4+ T helper cells produce higher levels of type 2 cytokines (IL-4, IL-5, IL-13) than type 1 cytokines (e.g., IL-2) during the course of symptomatic infection.

CD8 + T Lymphocytes

Unlike NK cells, CD8+ cells with cytotoxic function require MHC restriction and are antigen-specific. These cytotoxic T lymphocytes (CTL) eliminate virus-infected cells through perforin or Fas/FasL-mediated pathways. CD8+ T lymphocytes are critical for

controlling the dissemination of HIV in stages early in infection and before the appearance of antibodies. Importantly, an increase of CD8+ cells is observed during the asymptomatic stage of the infection, reflecting a response to infection as well as a compensatory reaction to the loss of CD4+ T helper cells.

Both transient and long-lived CTL populations, specific for viral proteins, such as Env, Nef, RT, and Gag gene products, are found in vivo. However, during the progression to disease, a decrease in the frequency of HIV-specific CTLs has been observed along with a decline in cytotoxic responses. Proposed mechanisms for the loss of function include the generation of viral mutants that escape CTL recognition, downregulation of MHC I molecules (potentially through viral *tat* or *nef* gene products), and decreased T helper cell function.

CD8+ cells can also exhibit noncytotoxic, non-MHC-dependent anti-HIV activity. This innate CD8+ cell noncytotoxic antiviral response (CNAR), which is not HIV isolate-specific, acts on all strains of HIV-1 and HIV-2, and is mediated, at least in part, by a factor secreted by CD8+ cells. This CD8+ cell antiviral factor (CAF) decreases HIV replication in CD4+ cells and in macrophages by acting on the HIV LTR to block virus transcription. Lower levels of viral RNA and protein are observed in infected cells exposed to CAF. The activity of CAF is distinct from chemokines and other known cytokines; its identity remains unknown. CNAR activity correlates directly with a healthy clinical state; CD8+ cells from asymptomatic individuals exhibit a greater capacity to suppress HIV replication than individuals progressing to disease. People infected for more than 10 years who are healthy and without therapy have a strong CNAR. Thus, this host innate immune response appears to be very important in controlling HIV infection.

Dendritic Cells

Dendritic cells and other antigen-presenting cells (APC) migrate in the peripheral blood and line mucosal surfaces. They are necessary, after interacting with infectious virus, for triggering subsets of antiviral effector cells. The replication of virus in these cell types is relatively low and stable and therefore they can remain for long periods of time as reservoirs releasing virus in the body. Because certain dendritic cells are believed to harbor virus, they can readily transfer HIV to T lymphocytes and contribute to the loss of CD4+ cells. Many of the functions of APCs, including presentation capabilities and cytokine regulation for the differentiation, survival, and maintenance of memory and naive T cells, are compromised in HIV-infected individuals. Because dendritic cells are among the first cell subset to encounter infectious HIV and are capable of modulating a broad range of lymphocyte functions, a further understanding of their role in HIV pathogenesis is of particular importance.

Intrinsic Anti-HIV Cellular Factors

When HIV infects a cell there can be differences in the extent of virus replication depending on intracellular intrinsic factors that have been identified. These include APOBEC3 G/F, Trim 5α, LD2, tetherin, MURR-1, and SAMHD1. APOBEC, a cytidine deaminase, causes mutations in the HIV genome affecting virus replication. The viral protein Vif can counter this anti-viral response by inducing APOBEC degradation. Trim 5α, a cytoplasmic protein, prevents the uncoating of the virus by binding to the capsid. LD2 has a similar effect in blocking HIV-2 replication after entry. Tetherin, a membrane protein, prevents budding of virus particles brought to the cell surface and can be countered by HIV Vpu. MURR-1 can affect viral transcription. Finally SAMHD1 blocks proviral synthesis during reverse transcription, particularly in monocytes and dendritic cells. This antiviral intrinsic effect can be countered by HIV Vpx which induces proteolytic degradation.

Cancers Associated with HIV Infection

HIV-infected patients and recipients of organ transplants show an increased prevalence of cancer particularly those associated with a viral infection (**Table 2**).

As seen in transplant patients, HIV infection leads to an immune deficiency that can decrease immune responses against cancer or increase immune responses associated with inflammation that are associated with cancer development (**Fig. 3**). It is estimated that about 40% of cancers in HIV-infected subjects can be attributed to viral infections. Herpes and papilloma viruses are responsible for cancers that were initially linked to the diagnosis of AIDS. The mechanism for the induction of malignancy involves initiation by the virus, promotion of cell proliferation, and cytokine production that leads to cellular karyotypic and genomic mutational events. Obviously the current vaccines against known viruses such as Hepatitis B and HPV should reduce the prevalence of these cancers.

Anti-HIV Approaches

Current therapeutic approaches to block HIV replication involve targeting the three viral enzymes reverse transcriptase, integrase and protease. These treatments have decreased plasma viral loads dramatically and increased the life expectancy for symptomatic individuals infected with HIV. Recent combination therapy approaches (using three drugs) have given infected people the possibility of living a near normal lifetime, although some treated subjects develop resistance to the antiviral drugs or toxic side effects

Table 2 Cancers associated with HIV infection

Cancer	*Virus*
AIDS-defining cancers	
Kaposi's sarcoma	KSHV (HHV8)
Non-Hodgkin's lymphoma	EBV
Invasive cervical cancer	HPV
Non-AIDS-defining cancers	
Anal Cancer	HPV
Hodgkin's disease (Hodgkin's lymphoma)	EBV
Adult T cell leukemia/lymphoma	HTLV
Melanoma skin cancer	
Liver cancer	Hep B; Hep C
Lung cancer	
Mouth and throat cancers	HPV
Testicular cancer	
Squamous cell and basal cell skin cancers	HPV?

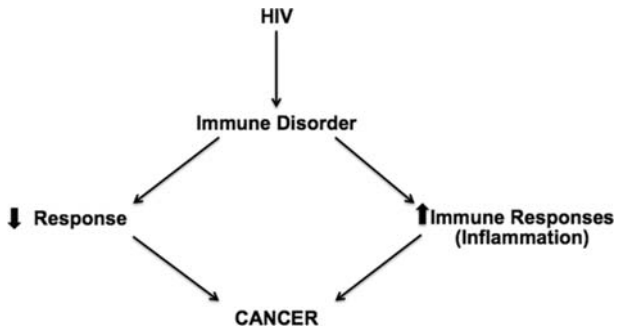

Fig. 3 HIV Infection and Cancer Development.

affecting tissues such as the kidney, liver, and pancreas. Most recently, combination HIV therapy can be delivered as one pill taken daily. That has reduced resistance by helping increase adherence. Also, because HIV infection often leads to the development of B-cell lymphomas and several central nervous system disorders, the extended life expectancies as a result of therapeutics could increase the prevalence of HIV-associated cancers and neuropathies.

With the success of antiviral drugs, many in the HIV field are advocating therapy for all infected individuals. Reducing the viral load by using anti-viral drugs can also markedly decrease the transmission rate. Thus the aim in some communities is to establish universal anti-HIV drug administration that could reduce transmission rates and overall prevalence of the infection.

In addition, the success of antiretroviral therapy (ART) has led to strategies to prevent HIV infection by preexposure therapy and to effect a cure. In the latter case, the drugs are used in attempts to rid the body of HIV completely or reduce HIV presence to a state in which there are no signs of the infection. Attempts at establishing a cure involve activation of HIV from latent cellular reservoirs so that the immune system can eliminate them, gene therapy in which the CCR 5 gene and other host genes required for HIV infection are deleted, as well as approaches to boost the immune system to control virus-infected cells and prevent HIV spread in the host. Short-term advances toward a cure have been reported but elimination of the virus that integrates into several different cells in the body presents a notable challenge. Many researchers are suggesting that combination antiviral therapies and immune enhancing drugs could lead to a "functional" cure in which ART will not be needed for a lifetime. The host immune system would control the virus as seen in healthy long-term infected individuals who have no detectable circulating virus or very low viraemia. Innovative approaches at enhancing antiviral immunologic responses can offer advantages to individuals infected with HIV, as the immune system can recognize a variety of viral subtypes and control infection in many different tissues of the body (e.g., bowel, brain).

Finally, the development of vaccines using antigenic components of HIV delivered through a variety of different modalities and with different adjuvants will be necessary for the prevention of infection. HIV is very variable and can be transmitted by infected cells as well as by free virus. The approaches needed for inhibiting all HIV isolates and for blocking an incoming infected cell, therefore, raise major challenges to the development of an effective vaccine.

It is hoped that by studying the cellular, molecular, and biochemical pathways involved in HIV replication and the effective immune responses that can control the virus, particularly in long-term survivors, optimal therapies and strategies for the prevention of HIV and AIDS can be developed.

See also: Non-Hodgkin Lymphoma: Diagnosis and Treatment.

Further Reading

Allers, K., Hütter, G., Hofmann, J., Loddenkemper, C., Rieger, K., Thiel, E., Schneider, T., 2011. Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. Blood 117 (10), 2791–2799.

Altfeld, M., Fadda, L., Frleta, D., Bhardwaj, N., 2011. DCs and NK cells: Critical effectors in the immune response to HIV-1. Nature Reviews. Immunology 11 (3), 176–186.

Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W., Montagnier, L., 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220 (4599), 868–871.

Berger, E.A., Murphy, P.M., Farber, J.M., 1999. Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. Annual Review of Immunology 17, 657–700.

Cheng-Mayer, C., Seto, D., Tateno, M., Levy, J.A., 1988. Biologic features of HIV-1 that correlate with virulence in the host. Science 240 (4848), 80–82.

Clerici, M., Stocks, N.I., Zajac, R.A., Boswell, R.N., Lucey, D.R., Via, C.S., Shearer, G.M., 1989. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging. The Journal of Clinical Investigation 84 (6), 1892–1899.

Cohen, M.S., Smith, M.K., Muessig, K.E., Hallett, T.B., Powers, K.A., Kashuba, A.D., 2013. Antiretroviral treatment of HIV-1 prevents transmission of HIV-1: Where do we go from here? Lancet 382 (9903), 1515–1524.

Deeks, S.G., Tracy, R., Douek, D.C., 2013. Systemic effects of inflammation on health during chronic HIV infection. Immunity 39 (4), 633–645.

Frankel, A.D., Young, J.A.T., 1998. HIV-1: Fifteen proteins and an RNA. Annual Review of Biochemistry 67, 1–25. Review.

Grant, R.M., Lama, J.R., Anderson, P.L., McMahan, V., Liu, A.Y., Vargas, L., Goicochea, P., Casapía, M., Guanira-Carranza, J.V., Ramirez-Cardich, M.E., Montoya-Herrera, O., Fernández, T., Veloso, V.G., Buchbinder, S.P., Chariyalertsak, S., Schechter, M., Bekker, L.G., Mayer, K.H., Kallás, E.G., Amico, K.R., Mulligan, K., Bushman, L.R., Hance, R.J., Ganoza, C., Defechereux, P., Postle, B., Wang, F., McConnell, J.J., Zheng, J.H., Lee, J., Rooney, J.F., Jaffe, H.S., Martinez, A.I., Burns, D.N., Glidden, D.V., iPrEx Study Team, 2010. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. The New England Journal of Medicine 363 (27), 2587–2599.

Homsy, J., Meyer, M., Tateno, M., Clarkson, S., Levy, J.A., 1989. The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells. Science 244 (4910), 1357–1360.

Jones, K.A., Peterlin, B.M., 1994. Control of RNA initiation and elongation at the HIV-1 promoter. Annual Review of Biochemistry 63, 717–743.

Killian, M.S., Levy, J.A., 2011. HIV/AIDS: 30 years of progress and future challenges. European Journal of Immunology 41 (12), 3401–3411.

Levy, J.A., 2015. Dispelling myths and focusing on notable concepts in HIV pathogenesis. Trends in Molecular Medicine 21 (6), 341–353.

Levy, J.A., 2007. HIV and the pathogenesis of AIDS, 3rd ed. ASM Press, Washington, DC.

Levy, J.A., Scott, I., Mackewicz, C., 2003. Protection from HIV/AIDS: The importance of innate immunity. Clinical Immunology 108 (3), 167–174.

Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M., Oshiro, L.S., 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 225 (4664), 840–842.

Maartens, G., Celum, C., Lewin, S.R., 2014. HIV infection: Epidemiology, pathogenesis, treatment, and prevention. Lancet 384 (9939), 258–271.

Margolis, D.M., 2010. Mechanisms of HIV latency: An emerging picture of complexity. Current HIV/AIDS Reports 7 (1), 37–43.

Neil, S., Bieniasz, P., 2009. Human immunodeficiency virus, restriction factors, and interferon. Journal of Interferon & Cytokine Research 29 (9), 569–580.

Silverberg, M.J., Chao, C., Leyden, W.A., Xu, L., Horberg, M.A., Klein, D., Towner, W.J., Dubrow, R., Quesenberry Jr., C.P., Neugebauer, R.S., Abrams, D.I., 2011. HIV infection, immunodeficiency, viral replication, and the risk of cancer. Cancer Epidemiology, Biomarkers & Prevention 20 (12), 2551–2559.

Hodgkin Lymphoma: Pathology and Genetics

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Glossary

The HUGO (Human Genome Organization) Committee nomenclature is used throughout: genes are quoted in italics and capital letters, except for those encoding for immunoglobulins (IG) or the variable region of their heavy chains (IGVH) and T-cell receptor (TR), which are reported in ordinary capital letters. The gene products are quoted in ordinary capital letters.
The cluster of differentiation (CD) nomenclature of human leukocyte antigens is used through the text.
The terminology of the Revised 4th Edition of the World Health Organization: WHO Classification of tumors of hematopoietic and lymphoid tissues is used throughout.

Abbreviations

ABVD Adriamycin, bleomycin, vinblastine, dacarbazine
ALK Anaplastic large cell lymphoma kinase
CD Cluster of differentiation
EBER Epstein–Barr encoding region
HIV Human immunodeficiency virus
MW Molecular weight
OS Overall survival
PCR Polymerase chain reaction
PET Positron emission tomography
(R-)CHOP (Rituximab-)cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone
WHO World Health Organization

Definition

Hodgkin lymphoma (HL) is usually characterized by the presence of a low number of neoplastic cells, either multinucleated (Reed–Sternberg cells, RSCs) or mononucleated (Hodgkin cells and variants, HCs), which are comprised in an overwhelming inflammatory background with variable cytological composition. RSCs, which measure about 60 μm in diameter, display a large rim of cytoplasm and two or more huge nuclei with central, inclusion-like nucleoli, often acidophilic at hematoxylin and eosin (H&E) (Fig. 1). HCs differ from RSCs by the presence of a single nucleus.

Historical Annotations

The tumor was first described by Sir Thomas Hodgkin in 1832 and subsequently named "Hodgkin's Disease" by Samuel Wilks. The histogenesis of the condition was a matter of debate for several decades, until the use of single cell micro-dissection allowed establishment of the monoclonal B-cell nature of the neoplastic cells. As a result of this finding the term "Hodgkin's disease" was abandoned in favor of "Hodgkin lymphoma," with as implication its inclusion among lymphoid neoplasms. On histological grounds, the Lukes and Butler classification became the reference in the mid-1960s, by distinguishing four varieties of HL (lymphocyte predominant, nodular sclerosing, mixed cellularity, and lymphocyte depleted), mainly based on the composition of the microenvironment. This classification still represents the basis for the diagnosis of HL, although some refinements have been introduced meanwhile. Nowadays, a major distinction is made between nodular lymphocyte predominant HL (NLPHL) and classical HL (CHL), each characterized by particular clinical, morphologic, immunophenotypic and molecular features. Accordingly, the Revised Edition of the WHO Classification has abandoned the unifying term "Hodgkin lymphoma" in favor of "Hodgkin lymphomas." CHL is further subdivided into four types: lymphocyte-rich (LR), nodular sclerosing (NS), mixed cellularity (MC), and lymphocyte depleted (LD). LRCHL was in the past lumped with NLPHL. However, although it shares architectural and microenvironmental features with the latter, it shows the morphologic and immunophenotypic profile of CHL.

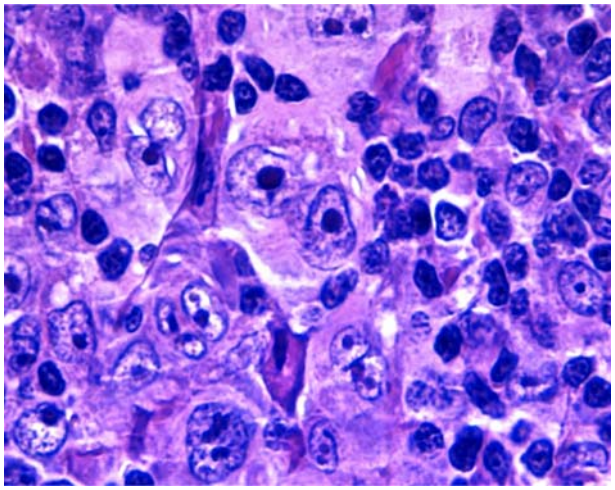

Fig. 1 Example of a binucleated Reed–Sternberg cell (center) surrounded by some mononucleated Hodgkin cells.

Epidemiological Notes

HL usually affects lymph nodes, except for NSCHL that can develop in the thymus. About 95% of HL cases belong to the classical category, the remaining 5% corresponding to NLPHL. In resource-rich countries, the latter more often occurs in males in the fourth decade of life. It has a rather indolent course with possible late recurrences and a spread that is closer to that of non-Hodgkin lymphomas. NLPHL can be concomitant with or preceded or followed by a peculiar modality of lymph node regression, known as progressively transformed germinal centers (PTGC). The latter, however, is not necessarily associated with NLPHL. CHL shows a bimodal age distribution with peaks in the second and seventh decades of life. It is more common in males, except for NSCHL that is more predominant among females. It has a distinct pattern of extension in the body, starting from a single node or group of adjacent nodes; this pattern represents the rationale for the staging procedures.

Lymphocyte Predominant HL

This form of HL has unique morphologic and immunophenotypic features. In 80% of cases it is characterized by the presence of nodules mostly composed of small B-lymphocytes (CD20+, CD79a+, PAX5+) with some epithelioid elements (CD68+/CD163+). Inside the nodules, which tend to expand and coalesce through time, producing a nodular and diffuse growth pattern, there are mononucleated cells measuring about 60 μm in diameter, which represent 1%–2% of the cell population (Fig. 2A–C). They show polylobated nuclei, with dispersed chromatin and multiple small nucleoli at times adjacent to the nuclear membrane, and a narrow rim of slightly basophilic cytoplasm. Because of the nuclear morphology, they were called "pop-corn" cells, a term that has been almost completely abandoned in favor of "lymphocyte predominant" cells (LPCs). RSCs are exceedingly rare and are usually detected only when serial sections are examined. LPCs express a complete B-cell phenotype (CD20+, CD75+, CD79a+, PAX5+, BOB.1+, OCT2+) as well as the leukocyte common antigen CD45 and—in most cases—epithelial membrane-antigen (EMA) (Fig. 3A–G). They also stain positive for BCL6 and IRF4 (Fig. 2J and K), which supports their derivation from a cell still residing in the germinal centers along with the presence of a high mutational load of the variable region of the immunoglobulin heavy chain genes (IGVH). In 10%–20% of cases, they express IgD and CD38, but not IgM or CD27 (Fig. 3G). This peculiar pattern is usually observed in young males and is associated with a more favorable course. A meshwork of follicular dendritic cells (FDCs) (CD21+, CD23+) is detected within the nodules. Importantly, LPCs lack expression of CD30 and CD15, which are characteristic of HRSCs of CHL. Furthermore, LPCs are typically surrounded by rosettes of follicular T-helper (FTH) lymphocytes (CD3+, CD279/PD1+, BCL6+, CD57+) (Fig. 3H and I). A few scattered CD30+ elements may be observed, which usually correspond to activated B-immunoblasts and only exceptionally have the typical LP morphology. The regular CD20 positivity of LPCs represents the rationale for treatment with Rituximab, either as monotherapy in low stage disease, which may be followed by maintenance therapy, or in combination with CHOP or ABVD in more advanced stages. Interestingly, the administration of Rituximab might prevent disease transformation.

Besides this classic nodular, B-cell rich appearance, LPHL can display some variant patterns: serpiginous/interconnected nodular, nodular with prominent extra-nodular LPCs, nodular with T-cell rich background, diffuse (T-cell rich B-cell lymphoma-like), and diffuse "moth-eaten" with B-cell rich background. The latter two variants correspond to the 20% of cases in which no nodular pattern is observed and may be difficult to distinguish from T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL), also due to the absence of FDCs and the similarity in terms of gene expression profile between the two neoplasms. The following elements can help under these circumstances to reach a diagnosis: (1) the clinical presentation, which is aggressive with widespread disease

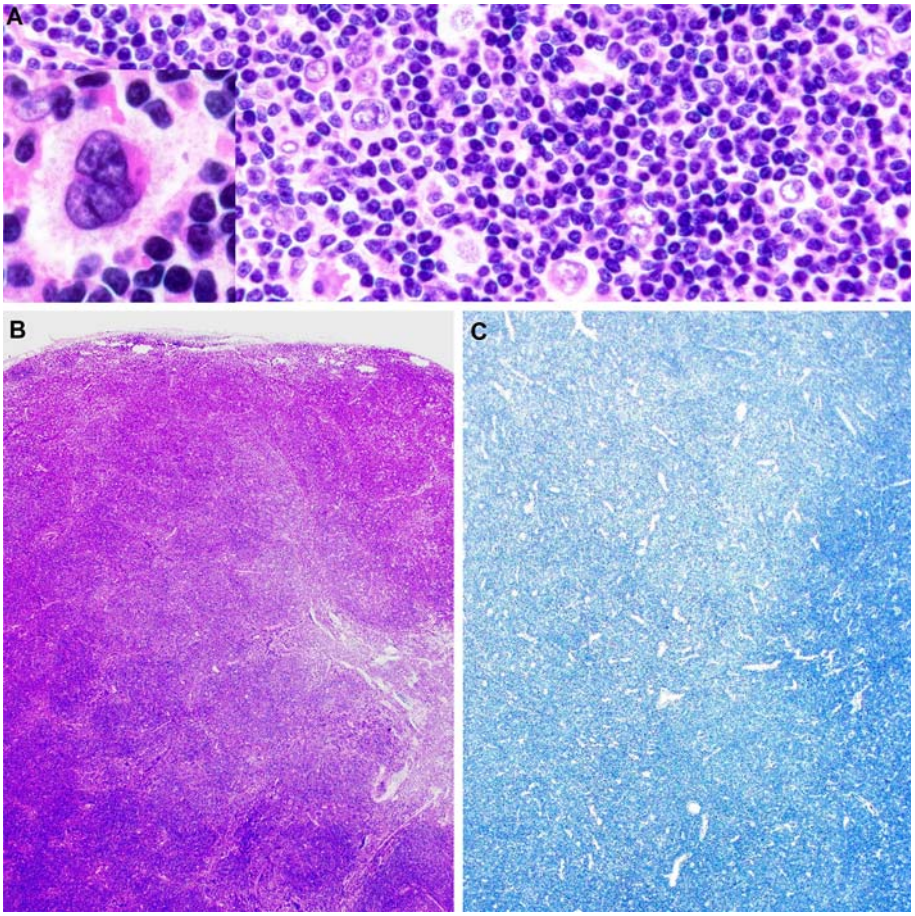

Fig. 2 So-called "lymphocyte predominant cells" (LPCs), scattered in a background rich in small lymphocytes (A, inset: nuclear detail of a LPC). Nodular lymphocyte predominant Hodgkin lymphoma may show a nodular (B) or diffuse growth pattern (C). These patterns are frequently coexistent in the same lymph node.

and systemic symptoms in THRLBCL; (2) the presence of CD279/PD1+ T-cell rosettes around otherwise canonical LPCs; (3) the expression of PU.1 and the negativity for LSP-1 in LPCs, the opposite pattern being observed in the neoplastic elements of THRLBCL; and (4) the presence of a residual nodule with the typical characteristics of NLPHL.

Molecular studies have shown that LPCs harbor clonally rearranged IGVH. As mentioned above, the clonal rearrangements can be easily detected in the DNA of single microdissected neoplastic cells, and the variable regions of IGVH carry a high load of somatic mutations indicative of ongoing mutational activity. Epstein-Barr virus (EBV) infection detected by EBER 1/2 in situ hybridization may be found in LPCs in 3%–5% of cases in both children and adults. EBV may be also present in bystander lymphocytes. *BCL6* rearrangements (involving IG, *IKAROS*, *ABR* and other partner genes) are present in about half NLPHL cases. Aberrant somatic hypermutations are found in 80% of cases, most frequently in *PAX5*, but also in *PIM1*, *RHOH/TTF*, and *MYC*. Mutations of the genes *SGK1*, *DUSP2*, and *JUNB* are also reported in about half of NLPHL cases by targeted next-generation sequencing (NGS). An increased risk of NLPHL has been noted in some families. PHL has also been identified in patients affected with Hermansky-Pudlak type 2 syndrome or autoimmune lymphoproliferative syndrome (ALPS) with mutations in *FAS*.

As previously mentioned, nodular and nodular and diffuse forms of NLPHL develop slowly, with frequent late relapses. It usually remains responsive to therapy and thus is rarely fatal, although in an advanced stage and higher age group it may behave more aggressively, as do histopathologic variants characterized by the presence of LPCs outside B-cell nodules or B-cell depletion of the microenvironment or THRLBCL-like transformation. Therefore, it is useful to mention these variant features in the pathology report. Progression to diffuse large B-cell lymphoma (DLBCL) has been observed in approximately 3%–5% of cases. In such cases the neoplastic cells maintain their typical immunophenotype. If localized, DLBCL associated with NLPHL generally has a good prognosis. NLPHL and the associated DLBCL are clonally related. Bone marrow involvement is rare in NLPHL and raises the possibility of THRLBCL, when there are only PD1/CD57-negative T-cells and no small B-cells in the background. Cases of NLPHL with bone-marrow involvement show aggressive clinical behavior. Advanced stage NLPHL responds poorly to the chemotherapy regimens traditionally used for CHL treatment but responds better to R-CHOP or regimens used to treat aggressive B-cell lymphomas.

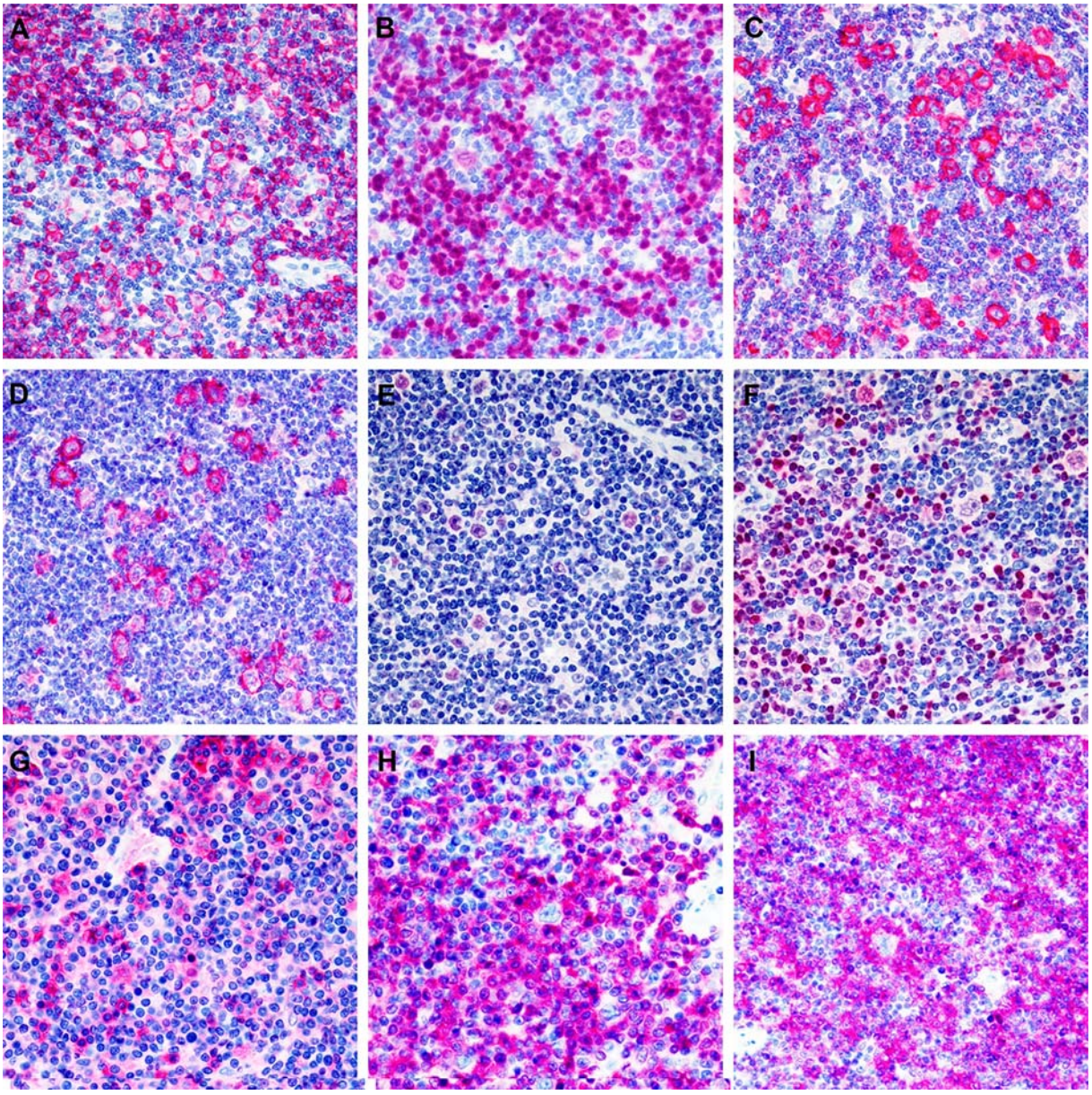

Fig. 3 Immunophenotypic features of nodular lymphocyte predominant Hodgkin lymphoma. LPCs are positive for CD20 (A), BSAP/PAX5 (B), CD75 (C), epithelial membrane antigen, EMA (D), BCL6 (E) IRF4/MUM1 (F), and, in a subset of cases, IgD (3G). LPCs are, as a rule, surrounded by small T-lymphocytes, which express CD3 (H) and PD-1 (I), with formation of rosette-like structures.

Classical HL

Morphology and Histotypes

Classical HL is characterized by the presence of typical HRSCs in a cellular milieu that varies depending on its histotype. Neoplastic cells can at times become smaller due to apoptotic changes: they are accordingly termed "dwarfs."

Lymphocyte-rich classical Hodgkin lymphoma

Lymphocyte-rich classical Hodgkin lymphoma (LRCHL) may proliferate in a nodular or, more rarely, diffuse pattern. The reactive component consists of small lymphocytes (mostly of B-cell origin) and some histiocytes with or without the formation of micro-granulomas. In this context, HRSCs are scattered through the nodules or more rarely located at their periphery (Fig. 4A). At times, residual germinal centers are seen within the nodules in an eccentric location. Neutrophils and eosinophils are absent. Based on morphological characteristics, in the past LRCHL was included in the setting of LPHL. With the advent of immunohistochemistry, it became clear that neoplastic cells had the typical phenotype of HRSCs (see below) and not that of LPCs. With the currently

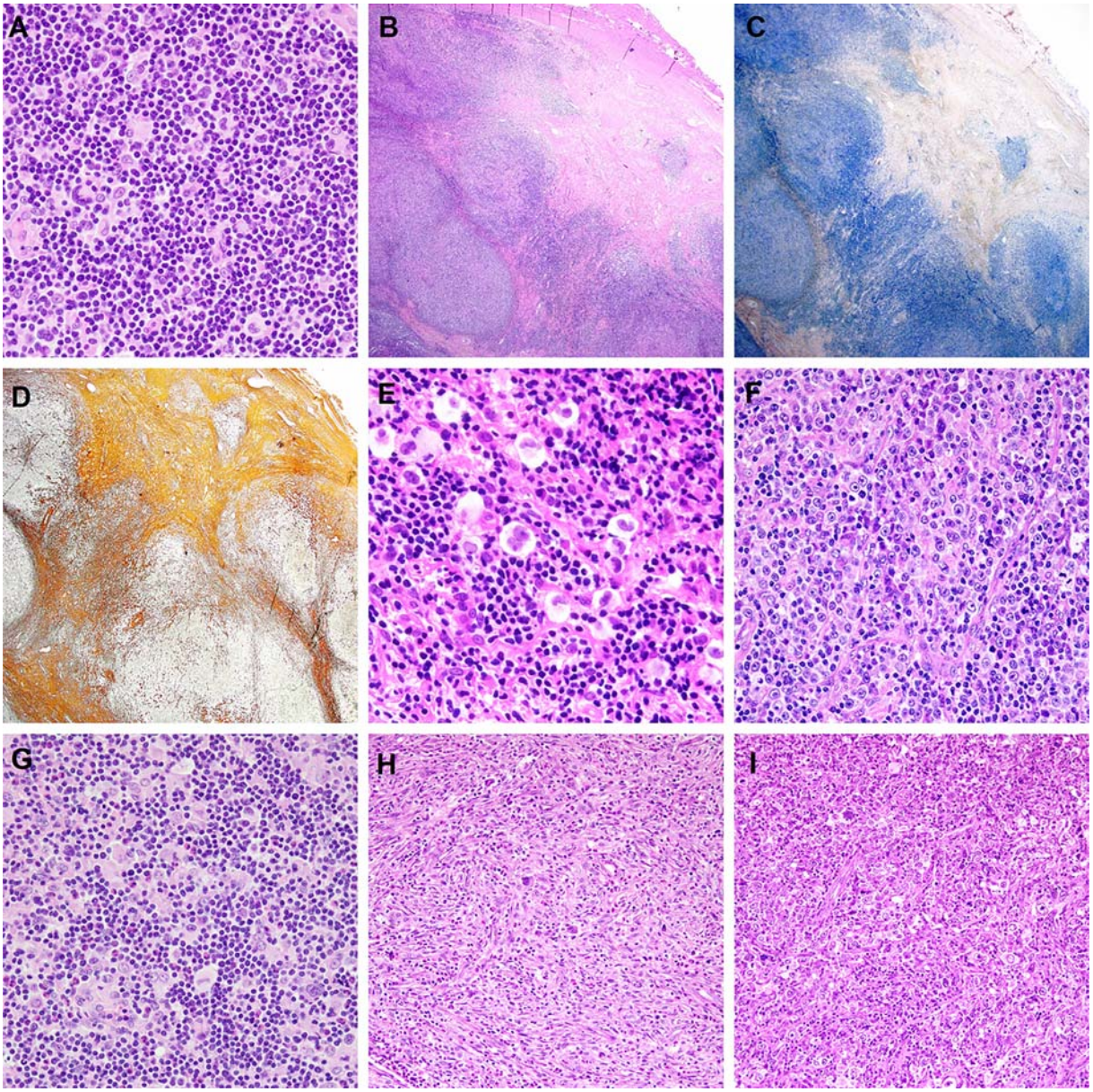

Fig. 4 Morphologic features of classical Hodgkin lymphoma (CHL). Lymphocyte rich CHL (A) displays scattered Hodgkin and Reed–Sternberg (HRS) cells in a background rich in small lymphocytes; eosinophils and sclerosis are not observed. Nodular sclerosis CHL is characterized by nodules surrounded by fibrous bands departing from a thickened lymph node capsule (B–D). The nodules contain variable amounts of small lymphocytes, eosinophils, macrophages, and plasma cells. HRS cells have a lacunar appearance and may be dispersed (BNLI type I, E) or form cohesive sheets (BNLI type II, F). Mixed cellularity CHL is distinguished by lack of sclerosis, frequent interfollicular growth and scattered classic HRS cells (G). Lymphocyte depletion CHL can show two different morphologic patterns: the fibro-histiocytic variant (H), that is characterized by a predominance of fibroblasts and histiocytes, with scattered HRS cells, and the sarcomatous variant (I) that is extremely rich in HRS cells. In both subtypes, the small lymphocytic component is extremely sparse.

available therapeutic approaches, LRCHL has a more favorable course than the other types of CHL, similar to that of LPHL. On the contrary, however, relapses are exceedingly rare.

Nodular sclerosing classical Hodgkin lymphoma

Nodular sclerosing classical Hodgkin lymphoma (NSCHL) represents about 70% of CHL cases in Western Countries, although its prevalence varies in different parts of the World. In contrast to the other types of CHL, it affects females more often than males and presents with a mediastinal mass (with possible features of bulky disease) in 80% of patients. At microscopic examination (Fig. 4B–D), NSCHL is characterized by nodules surrounded by collagen bands (birefringent on polarized light) that depart from a thickened lymph node capsule. The cellular composition of the nodules can significantly vary by mimicking the one of the other types of CHL

(LR, MC, and LD). Occasionally, only nodule formation is seen with minimal or absent fibrosis: this pattern is called the cellular phase of NSCHL. Neoplastic cells are mostly mononucleated and can acquire a "lacunar" appearance in improperly fixed material, consisting of the retraction of cytoplasm close to the nucleus with some thin connections to the cytoplasmic membrane, forming what look like small lacunae (Fig. 4E). The British National Lymphoma Investigation (BNLI) distinguishes two grades of NSCHL, which are potentially relevant for prognosis. Grade I corresponds to the presence of a small amount of scattered HRSCs in a composite cellular milieu, consisting of variable amounts of small lymphocytes, histiocytes, neutrophils, eosinophils, mastcells, and plasmacells. Grade II is assigned (Fig. 4F) when (1) >25% of the nodules show sarcomatous lymphocyte depletion, (2) >80% of the nodules show features of the fibro-histiocytic variant, or (3) >25% of the nodules show numerous bizarre, anaplastic-appearing Hodgkin cells without lymphocyte depletion. Following such criteria, approximately 15%–25% of NSCHL cases are classified as grade II. Grading is not mandatory for clinical purposes but has been applied in some clinical trials. However, it has become less relevant, due to advances in therapy which obscure differences seen in less effectively treated patients. Within the morphologic spectrum of NSCHL, the so-called "syncytial" variant merits to be mentioned. It is characterized by large aggregates of neoplastic cells within the nodules, resembling metastatic involvement by undifferentiated carcinoma. Immunohistochemistry is pivotal for the differential diagnosis as it is in the distinction between grade II NSCHL and ALK-negative anaplastic large cell lymphoma (ALCL) (see below).

Mixed cellularity classical Hodgkin lymphoma

In this variant, HRSCs can be easily found comprised in a mixed reactive population composed of lymphocytes (mainly T), histiocytes, plasma cells, neutrophils, and eosinophils (Fig. 4G). The normal lymph node structure is complete effaced, at times with sparing of some follicles with a germinal center. It should be differentiated from nodal peripheral T-cell lymphoma (PTCL), especially of the FTH-cell type, that can contain EBV-positive HRS-like cells. However, T-lymphocytes in mixed cellularity classical Hodgkin lymphoma (MCCHL) show a complete T-cell phenotype and do not homogeneously express FTH-related markers in contrast to those of FTH-PTCL. In addition, they do not display the large rim of clear cytoplasm that is characteristic of nodal FTH-PTCL.

Lymphocyte depleted classical Hodgkin lymphoma

Lymphocyte depleted classical Hodgkin lymphoma (LDCHL) includes two morphologic subtypes (Fig. 4H and I). The fibrohistiocytic subtype has a cellular milieu mostly consisting of fibroblasts and histiocytes. The sarcomatous subtype is extremely rich in neoplastic cells, which frequently form a palisade at the periphery of large necrotic areas but can also occur diffusely throughout sinuses. Immunophenotyping is pivotal to differentiate them from ALK-negative ALCL (see below).

Phenotype

HRSCs of CHL have a distinctive immunophenotypic profile. They strongly express CD30 in a characteristic dot-like and membrane-bound pattern, corresponding to the synthesis of the protein backbone (90 kDa MW) of the molecule, which is glycosylated in the Golgi apparatus (120 kDa MW) and then translocates to the cell membrane, where it acquires a transmembrane location (Fig. 5A). Ber-H2 is the reference antibody for immunohistochemical identification of CD30 and SGN30 is a humanized monoclonal antibody used for the construction of brentuximab-vedotin by conjugation with monomethyl auristatin E. The epitopes detected by these antibodies are located on the external domain of CD30, but are not shed in the circulation, in contrast to the CD30 epitopes that can be detected in peripheral blood. This is quite important, since CD30 is an important diagnostic and therapeutic target. CD30 is the target of specific immunoconjugates, administered either as monotherapy or in combination with chemotherapy with promising results, both in refractory cases or as first line treatment. In about 60% of cases HRSCs express CD15, also known as X-hapten, with a staining pattern similar to that of CD30 (Fig. 5B). This is one of the many molecules aberrantly expressed in at least 20% of HRSC cases, which include CD15, GATA3, TRAF1, ID2, ABF1, JUN, JUNB, AP-1, FLIP (CFLAR), JAK/STAT, STAT5, and the cytotoxic markers TIA-1, Granzyme B and perforin. It is assumed that these aberrant attributes facilitate the immune escape of neoplastic cells. A main feature of HRSCs is downregulation of the B-cell program and loss of most B-cell markers (see also section "**Molecular Characteristics**"), in spite of their B-cell derivation. In >90% of cases, they retain only the expression of the *PAX5* gene product, also known as BSAP (B-cell specific activator protein), although at a significantly lower intensity than normal B-cells (Fig. 5C). CD20 is expressed in about 30% of cases by a proportion of neoplastic cells with variable intensity (Fig. 5D). CD79a is detected in about 10% of cases with a staining pattern like CD20. The transcription factors BCL6, BOB.1, OCT2 and PU.1 are usually absent. At times, either BOB.1 or OCT2 may be expressed, which impairs the transcription of IG genes (Fig. 5E). CD45 and EMA are also defective (Fig. 5F and G). IRF4 and Ki-67 are expressed in most if not all cases of HRSC (Fig. 5H and I). Even though they express Ki-67, HRSC enter the cell cycle but do not proceed through due to abortive cytokinesis. BCL2 and TP53 are expressed by >50% and 25% of neoplastic cells in one third and 12% of cases respectively, which has been found of prognostic relevance (Fig. 6A and B). In 87% of cases, HRSCs express CD274/PD-L1 and CD273/PD-L2 a rationale for treatment with immune check-point inhibitors (e.g., nivolumab and pembrolizumab) (Fig. 6C). Copy number alterations of 9p24.1/*PDCD1/LG* contribute to robust PD-L1 and PD-L2 expression by HRSCs, although PD-L1 is also strongly expressed by reactive tissue macrophages. The combination of brentuximab-vedotin and nivolumab has recently been tested in relapsed or refractory CHL with promising results (82% overall response rate). In a small percentage of cases HRSCs may express one or more T-cell associated molecules. Intracytoplasmic and globular staining suggests passive absorption, but detection at the plasma

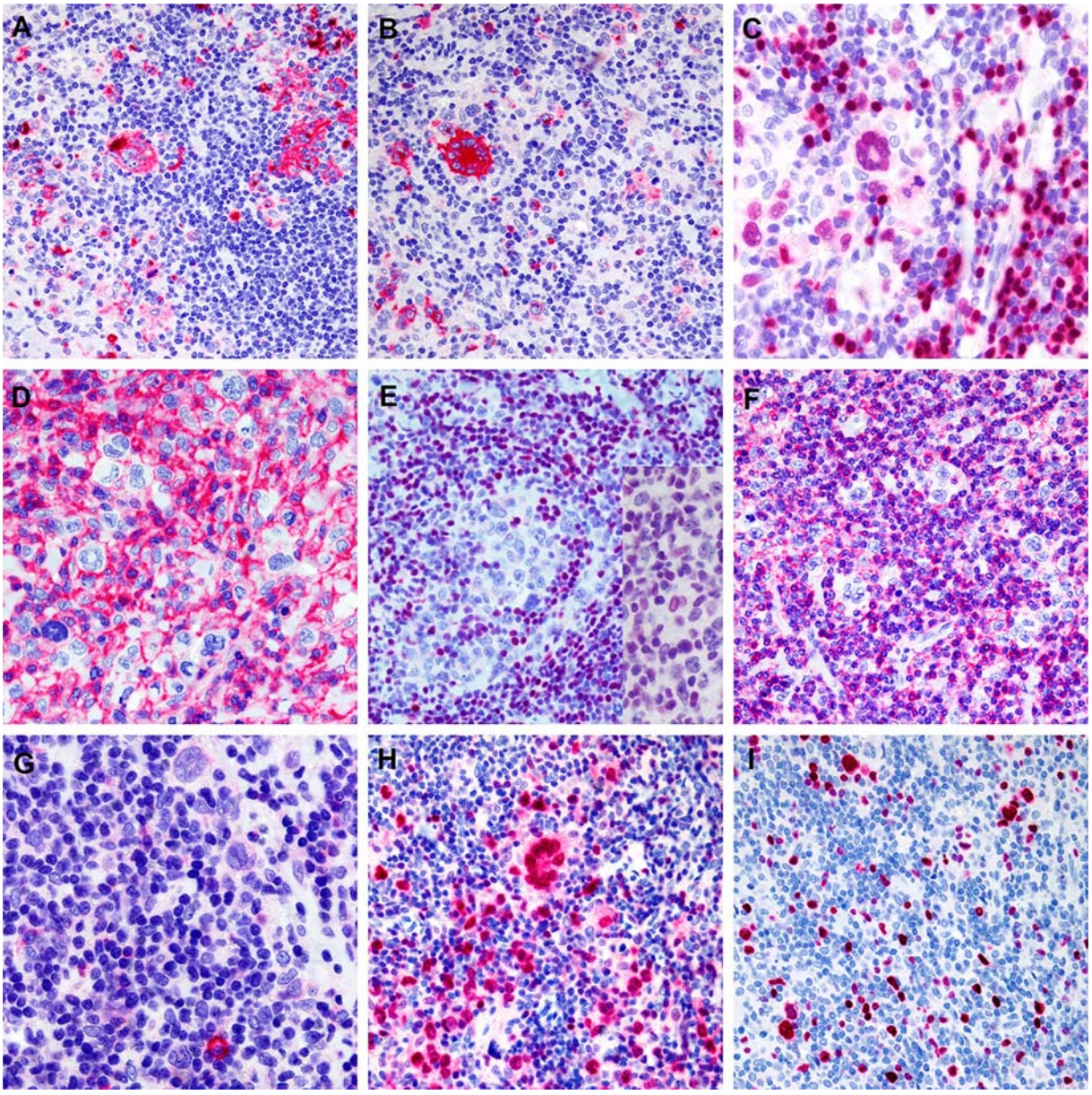

Fig. 5 Immunophenotypic features of classical Hodgkin lymphoma. HRS cells are positive for CD30 (A), CD15 (B) and, weakly, for BSAP/PAX5 (C). CD20 is negative or weakly expressed by a minority of cells (D). Furthermore, HRS cells are negative for CD45/LCA (E), OCT2 (F), BOB.1 (F inset) and EMA (G). Most cells are positive for IRF4/MUM1 (H) and for the proliferation marker KI-67 (I).

membrane level has been associated with IGVH gene rearrangements with polyclonal T-cell receptor (TR) (see section "**Molecular Characteristics**").

When a tumor is rich in neoplastic cells (NSCHL grade II or sarcomatous LDCHL), a differential diagnosis of ALK-negative ALCL should be considered. Expression of PAX5, IGVH clonal rearrangement, lack of TR clonality, and possible EBV infection will then favor a diagnosis of CHL.

Reactive cells in the microenvironment are important for cross-talk with neoplastic cells through cytokines and cytokine-receptors, and also convey prognostic information. The presence of macrophages (with cut-off values ranging from 5% to 25%), of T-lymphocyte subsets and of myeloid suppressor cells has been found of potential prognostic value, although conflicting data have been reported in the literature.

Numerous markers of HRSC or cells in the microenvironment have been proposed as prognostic indicators, and these have been compared with interim-PET for prediction of response to two cycles of ABVD treatment. In patients with a negative interim PET, expression of CD68 (>25%) and CD279/PD1 (diffuse or rosetting pattern) in microenvironmental cells, and STAT1 negativity in HRSC identify a subset of patients with a significantly lower 3-year progression-free survival (**Fig. 6**E and F).

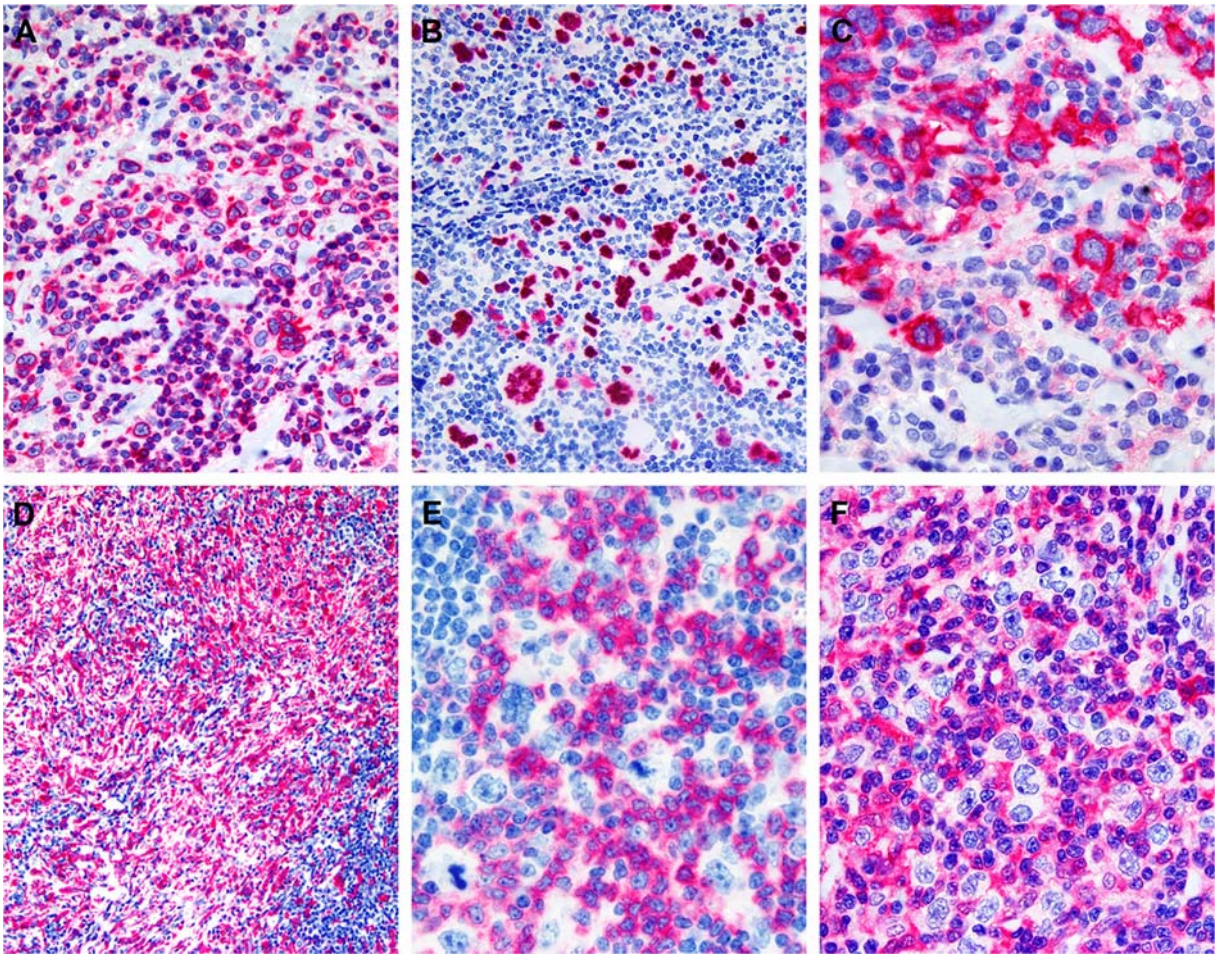

Fig. 6 Biologic prognostic markers in CHL. HRS cells can express BCL2 (A) and p53 (B). Expression above 50% and 25% of neoplastic cells represents an adverse prognostic factor. Positivity for PD1 ligand is found in HRS cells of most cases of CHL (C), and this can have impact on therapy choice. An increased macrophagic population above 25% of cellular background (D), as well as an increase of PD1-positive T-lymphocytes, with rosette formation (E) and down-regulation of STAT1 in HRS cells (F), are considered adverse prognostic factors.

EBV Infection

By in situ hybridization for EBER 1/2 and immunohistochemical detection of LMP1 and EBNA1 (latency II pattern) it has been convincingly shown that HRSC are EBV infected. LMP-1 expression is regarded as potentially implicated in the pathogenesis of the tumor because of its in vitro transforming capacity. Conceivably, EBV infection of a B-cell replaces one of the genetic alterations necessary for the development of CHL. The prevalence of EBV in HRSCs varies according to the histological subtype and epidemiologic factors. The highest frequency ($\sim$75%) is found in MCCHL, and the lowest (10%–40%) in NSCHL. In resource-poor regions and in patients infected with HIV, the rate of EBV infection is close to 100%. The type of EBV strain also varies between geographical areas. In resource-rich countries strain 1 prevails, and in resource-poor countries strain 2.

Cell of Origin

As previously mentioned, IGH gene rearrangement studies carried out by single cell microdissection provided convincing evidence that HRSCs of CHL are clonal and derived from germinal center B-cells, even if they lack morphologic or phenotypic similarities other than their PAX5/BSAP expression. In rare cases, HRSCs were reported to harbor clonally rearranged TR, indicating that cases with morphological features of CHL might be derived from T-cells. This issue remains unsolved, since differentiating between these exceptional cases and PTCLs, especially ALK-negative ALCL, can be very challenging.

Molecular Characteristics

HRSCs reveal clonal IGVH rearrangements in $>$98% of cases. As mentioned above, single cell microdissection is required to detect clonal rearrangements, as DNA extracted from the whole tissue specimens provides poor results. The rearranged IGVH of tumor cells

harbor a high load of somatic mutations (hypermutated), usually without signs of ongoing mutational activity. These findings support the view that in most if not all instances HRSCs are derived from a germinal center B-cell. Nonetheless, HRSCs have lost much of the B-cell specific expression program and have acquired B-cell inappropriate gene products, as described earlier. In addition, deregulated transcription factors in CHL promote proliferation and abrogate apoptosis of the neoplastic cells. The transcription factor NF-κB is constitutively activated in HRSCs, and there is altered activity of the NF-κB target genes, which regulate proliferation and survival, the AP-1 complex and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway. Mutations of the JAK regulator *SOC-1* are associated with nuclear STAT5 accumulation in HRSCs, indicating blocking of the negative feedback loop of the JAK/STAT5 pathway. Even though TP53 may be overexpressed, mutations of *TP53* are rare or absent in primary CHL.

Classical cytogenetic and fluorescence in situ hybridization (FISH) studies show aneuploidy and hypertetraploidy, consistent with the multinucleation of the neoplastic cells; however, these techniques fail to demonstrate recurrent and specific chromosomal changes in CHL. Comparative genomic hybridization, however, shows recurrent gains of sub-regions on chromosomal arms 2p, 9p, and 12q and distinct high-level amplifications on chromosomal bands 4p16, 4q23-q24, and 9p23-p24. Special attention deserves the alterations at 9p24.1, since this region includes the genes encoding for CD274/PD-L1, CD273/PD-L2, and JAK2, which herald sensitivity to specific targeted therapies as highlighted above in the "**Phenotype**" section.

Gene expression profiling (GEP) studies have confirmed all these findings, including global down-regulation of the B-cell program, and highlighted similarities between CHL and primary mediastinal large B-cell lymphoma (PMBL). PBML show overexpression of genes encoding for CD274/PD-L1, CD273/PD-L2 and JAK2, due to 9p24.1 alterations. The similarities between the signature of CHL and that of PMBL are not surprising, which is a reflection of the gray-zone between the two neoplasms. In the Revised WHO Classification, the latter condition for which the term "B-cell lymphoma unclassifiable with features intermediate between CHL and DLBCL" is used, corresponds to two different conditions: a tumor showing CHL morphology but complete B-cell immunophenotype or a tumor with the PMBL morphology but a CHL immunophenotype. By profiling HL cell lines and micro-dissected HRSCs two molecular subgroups of CHL were identified, associated with differences in activity of transcription factors of *NOTCH1*, *MYC*, and *IRF4*. Moreover, HRSCs displayed deregulated expression of several genes potentially highly relevant to lymphoma pathogenesis, including silencing of the apoptosis-inducer *BIK* and of *INPP5D*, an inhibitor of the PI3K-driven oncogenic pathway. In further GEP studies, using mRNA extracted from HRSCs micro-dissected from formalin-fixed, paraffin-embedded tissues, a macrophage-like signature in HRSCs significantly correlated with treatment failure. *CSF1R* is a representative of this signature, and its expression by mRNA in situ hybridization was significantly associated with progression-free and overall survival. A combined score of CSF1R in in situ hybridization and CD68 in immunohistochemistry was found to be independently predictive for progression-free survival in multivariate analysis. In one study, a 23-gene outcome predictor was generated, which identified a 29% difference in in 5-year overall survival between the high- and low-risk groups population in a validation cohort. The predictor was claimed to be superior to the International Prognostic Score and CD68 immunohistochemistry, but this was not confirmed in two later studies.

Whole exome sequencing on purified HRS cells has revealed inactivating *B2M* mutation as the most frequently detected gene mutation in CH. This leads to loss of major histocompatibility complex class I (MHC-I) expression. The absence of B2M protein in HRSCs has been associated with lower stage of disease, younger age at diagnosis, and better overall and progression-free survival. In a further study, fusions involving *CIIITA* (MHC II trans-activator) in 15% of CHL cases caused down-regulation of HLA class II expression and overexpression of CD274/PD-L1 and CD273/PD-L2.

Some novel gene loci have been identified as being associated with increased risk for the development of CHL. An association between rs6903608 and EBV-negative cHL was confined to the nodular sclerosis histological subtype. Other associations involving HLA Class I have been identified in EBV-positive CHL, mainly mixed cellularity subtype. These observations confirm the relevance of histological subtyping of CHL, and differences between EBV-positive and EBV-negative cases.

It has recently been reported that sequencing of circulating cell free (cf)DNA in CHL patients provides a surrogate for the mutational landscape of HRSCs obtained by micro-dissection. Genes recurrently mutated in $>20\%$ of CHL cases include *STAT6* (37.5%), *TNFAIP3* (35%), and *ITPKB* (27.5%), and are clustered in major pathways, including NF-κB, PI3K-AKT, cytokine and NOTCH signaling, and immune evasion. The mutational landscape of newly diagnosed and chemorefractory CHL largely overlaps. Refractory CHL does not show an increased rate of *TP53* mutation (see above). In contrast, *TET2* mutations, including newly acquired mutations, are more frequent in refractory CHL, which is potentially important for therapy choice. Longitudinal studies in patients relapsing under/after chemotherapy or brentuximab vedotin, of paired pre-treatment/relapse tumor tissue samples, have shown that recurrent tumor cell clones branch from ancestral clones through acquisition of specific mutations, while preserving the mutational landscape of the ancestral clone. Conversely, in patients under nivolumab maintenance therapy, ancestral clones were replaced by completely novel clones.

Conclusions

Only once the goal of curing all HL patients has been reached, the long journey begun by Sir Thomas Hodgkin can be concluded.

Acknowledgment

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Further Reading

Agostinelli, C., Gallamini, A., Stracqualursi, L., et al., 2016. The combined role of biomarkers and interim PET scan in prediction of treatment outcome in classical Hodgkin's lymphoma: A retrospective, European, multicentre cohort study. The Lancet Haematology 3, e467–e479.

Carey, C.D., Gusenleitner, D., Lipschitz, M., et al., 2017. Topological analysis reveals a PD-L1-associated microenvironmental niche for Reed–Sternberg cells in Hodgkin lymphoma. Blood 130, 2420–2430.

Cencini, E., Fabbri, A., Bocchia, M., 2016. Rituximab plus ABVD in newly diagnosed nodular lymphocyte-predominant Hodgkin lymphoma. British Journal of Haematology 176, 831–833.

Connors, J.M., Ansell, S.M., Fanale, M., et al., 2017. Five-year follow-up of brentuximab Vedotin combined with ABVD or AVD for advanced-stage classical Hodgkin lymphoma. Blood 130, 1375–1377.

Della Pepa, R., Picardi, M., Giordano, C., et al., 2017. Rituximab in a risk-adapted treatment strategy gives excellent therapeutic results in nodular lymphocyte-predominant Hodgkin lymphoma. British Journal of Haematology. https://doi.org/10.1111/bjh.14856.

Fan, Z., Natkunam, Y., Bair, E., et al., 2003. Characterization of variant patterns of nodular lymphocyte predominant Hodgkin lymphoma with immunohistologic and clinical correlation. The American Journal of Surgical Pathology 27, 1346–1356.

Fanale, M.A., Cheah, C.Y., Rich, A., et al., 2017. Encouraging activity for R-CHOP in advanced stage nodular lymphocyte-predominant Hodgkin lymphoma. Blood 130, 472–477.

Herrera, A.F., Moskowitz, A.J., Bartlett, N.L., et al., 2018. Interim results of brentuximab vedotin in combination with nivolumab in patients with relapsed or refractory Hodgkin lymphoma. Blood 131 (11), 1183–1194.

Hummel, M., Ziemann, K., Lammert, H., et al., 1995. Hodgkin's disease with monoclonal and polyclonal populations of Reed–Sternberg cells. New England Journal of Medicine 333, 901–905.

Küppers, R., Rajewsky, K., Zhao, M., et al., 1995. Hodgkin's disease: Clonal Ig gene rearrangements in Hodgkin and Reed–Sternberg cells picked from histological sections. Annals of the New York Academy of Sciences 764, 523–524.

Marafioti, T., Hummel, M., Anagnostopoulos, I., et al., 1997. Origin of nodular lymphocyte-predominant Hodgkin's disease from a clonal expansion of highly mutated germinal-center B cells. The New England Journal of Medicine 337, 453–458.

Shimabukuro-Vornhagen, A., Haverkamp, H., Engert, A., et al., 2005. Lymphocyte-rich classical Hodgkin's lymphoma: Clinical presentation and treatment outcome in 100 patients treated within German Hodgkin's Study Group trials. Journal of Clinical Oncology 23, 5739–5745.

Spina, V., Bruscaggin, A., Cuccaro, A., et al., 2017. Genotyping of classical Hodgkin lymphoma on the liquid biopsy. Blood 130, 307.

Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J., 2017. WHO Classification of tumours of haematopoietic and lymphoid tissues. Revised 4th edn. IARC Press, Lyon.

Tiacci, E., Döring, C., Brune, V., et al., 2012. Analyzing primary Hodgkin and Reed–Sternberg cells to capture the molecular and cellular pathogenesis of classical Hodgkin lymphoma. Blood 120, 4609–4620.

Hormones and Cancer

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Introduction

A range of biologic and epidemiologic evidence documents a key role of the hormonal milieu in the etiology of several cancers. Hormones regulate major physiological systems, playing key roles in metabolism, growth and development, reproduction, and neurological function. Produced in and secreted by endocrine glands, hormones enter circulation and target specific organs, where they bind receptors, producing downstream effects. In some cases, secretion may also occur locally, resulting in paracrine or autocrine effects.

Mechanistically, hormones may contribute to carcinogenesis through disruption of normal regulatory action. For example, estrogens and other sex-steroid hormones promote cell proliferation, which increases the opportunity for mutations to occur and can lead to increased tumor growth. Some hormones, including insulin-like growth factor I (IGF-I) and prolactin, have been shown to reduce apoptosis, promoting tumor development. In addition, hormones can affect one another to influence cancer progression; for example, gonadotropins may influence tumorigenesis by stimulating sex-steroid production. Antioxidant properties of certain hormones, such as melatonin, may prevent carcinogenesis. Dysregulation of broader functional processes including circadian rhythm and metabolic control, which is heavily influenced by insulin activity, may increase susceptibility to carcinogenesis through additional mechanistic pathways.

Hormonally related risk factors have long been associated with several cancer types, particularly breast cancer. These established breast cancer risk factors include parity and ages at menarche and menopause. Adulthood obesity is linked to as many as 13 cancer types. This association is mediated, at least in part, through hormonal pathways; for instance, most estrogen production among postmenopausal women takes place in adipose tissue, and obesity is also closely linked to insulin function. Diabetes, defined by insulin resistance, is also a moderate risk factor for several cancers, including breast and endometrial cancers.

Methodologic Considerations

To evaluate the role of hormones in cancer, measurement considerations in epidemiologic studies must be addressed. First, collection of blood or urine (or other tissue) must occur well before the cancer is clinically evident to establish temporality of the association between hormonal exposure and cancer development, given that the tumor and treatments may affect hormone levels.

It is rare for more than one blood or urine sample to be collected per participant in prospective studies, due to logistic and financial constraints. This increases the potential for misclassification of the participant's true long-term hormone levels (generally the exposure of interest), and precludes the ability to evaluate hormonal levels over time. Several hormone measurements show temporal variability, requiring careful consideration in study design and analysis (Fig. 1). However, single blood sample collection has been shown in multiple studies to reasonably reflect long-term hormone levels, with correlations over a 2-to-3-year period ranging between 0.5 and 0.9 for IGF-I, IGFBPs, gonadotropins, and sex-steroid hormones. Phase of the menstrual cycle influences some hormones, and correlations over time for estrogens and progesterone are substantially lower among premenopausal compared to postmenopausal women. Thus, use of a single blood measurement results in some attenuation of relative risk (RR) estimates. Overall, however, for many hormones of interest, reproducibility of hormone measures in blood are similar to reproducibility of serum cholesterol measures, and therefore can likewise be considered consistent predictors of disease.

Whether blood, urine, or both are collected varies based on the study goals and funding limitations. In either case, fasting samples are generally preferred to limit influence of metabolic effects on hormone levels, although exceptions exist, including insulin, where both fasting and nonfasting levels are potentially of scientific interest. Because serum is more proximal to tissues of interest, hormones measured in serum may be a more accurate surrogate marker for tissue exposure compared to urinary levels. Hormone levels tend to remain stable in serum over time, and storage at $-80°C$ or colder is acceptable to prevent degradation or dissociation over time. Urine collection is less invasive, and some urinary hormones are well correlated with serum levels. Urinary analysis is advantageous when breakdown products of hormones are of interest; evaluation of such products may provide insight into mechanistic pathways. However, the concentration of urine varies substantially within and among individuals, and correction for concentration must be considered when measuring urinary products. Moreover, it is less clear whether urine levels are representative of high tissue exposure, or if they simply represent enhanced excretion efficiency, making connections between urine measurements and cancer risk more difficult.

The ability to accurately represent tissue hormone levels via blood or urine measurements is a major consideration, although limited data exist because of challenges in obtaining tissues of interest and measuring hormone levels in these tissues. In a recent study, relatively low correlations were observed between circulating sex hormones and prostate tissue specific levels, while strong correlations were observed between plasma estradiol and expression of estrogen dependent genes in ER+ breast tumors, supporting use of blood biomarkers to evaluate the breast tissue milieu. In addition, external factors, such as exercise, alcohol intake, and

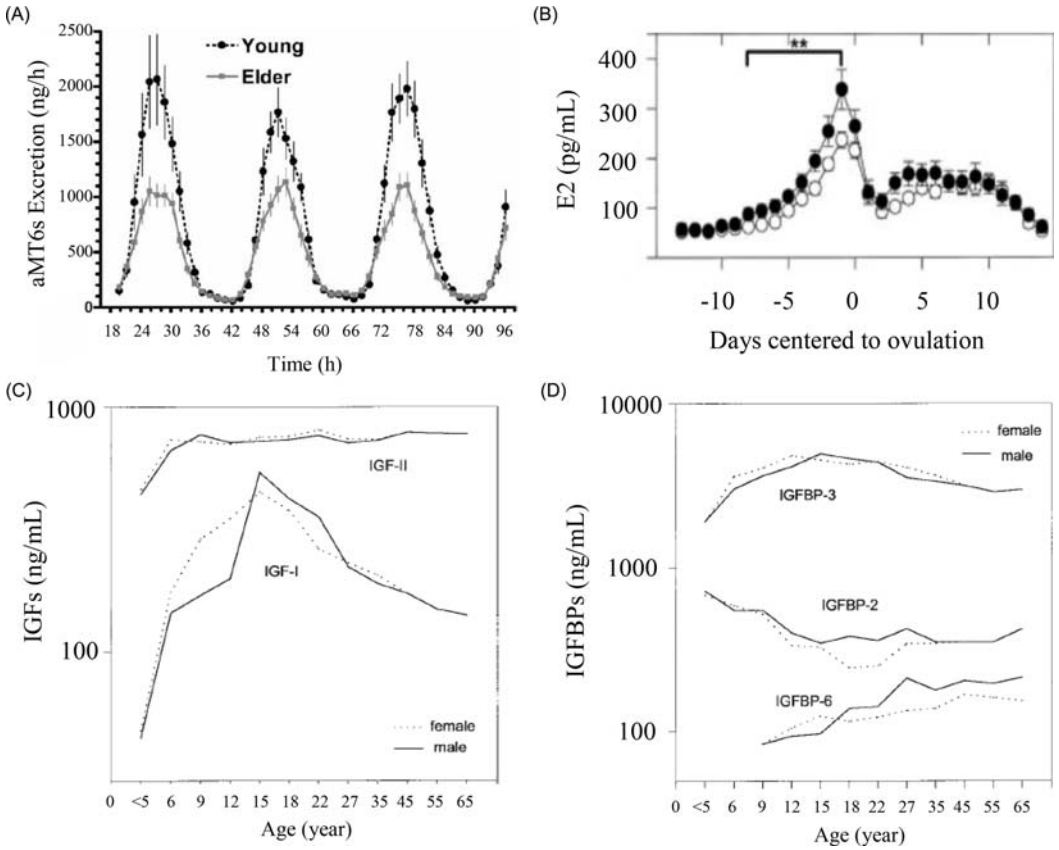

Fig. 1 Temporal variability of selected biomarkers. (A) Melatonin excretion by hour (aMT6s) for adults 19–40 years (young) or 58–84 years (elder). (B) Estradiol concentration by days centered to ovulation for women 19–42 years of age. (C) IGF-1 and IGF-II concentrations by age and sex. (D) IGFBP concentrations by age and sex. (A) Adapted from Kripke, D. F., Youngstedt, S. D., Elliot, J. A., et al. (2005). Circadian phase in adults of contrasting ages. *Chronobiology International***22** (4), 695–709. (B) Adapted from Welt, C. K., McNicholl, D. J., Taylor, A. E., et al. (1999). Female reproductive aging is marked by decreased secretion of dimeric inhibin. *The Journal of Clinical Endocrinology & Metabolism***84** (1), 105–111, Fig. 1. (C and D) Reprinted from Yu, H., Mistry, J., Nicar, M. J., et al. (1999). Insulin-like growth factors (IGF-I, free IGF-I, and IGF-II) and insulin-like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation. *Journal of Clinical Laboratory Analysis***13** (4), 166–172, Fig. 1 (C), Fig. 3 (D).

smoking, among others, can influence endogenous hormone production, and thus should be considered in epidemiologic analyses. Despite these concerns, consistency of associations between hormones measured in blood or urine and risk of specific cancers provides evidence for a connection between these measures and actual tissue exposure.

Overview

This article reviews endogenous hormones in relation to incident cancer risk. Data from biologic studies inform mechanistic actions, while data from prospective cohort studies (i.e., "nested" case–control studies) provide the strongest epidemiologic evidence for associations with cancer. The hormones included are sex steroids (estrogen and estrogen metabolites, progesterone, androgens), anti-Müllerian hormone (AMH), gonadotropins, prolactin, insulin/C-peptide, insulin-like growth factors (IGF), adiponectin, and melatonin.

Sex Steroids

Derivatives of cholesterol formed via steroidogenesis, sex-steroid hormones include estrogens, progesterone, and androgens. These hormones bind to their respective receptors, triggering multiple downstream gene pathways. The aromatase enzyme converts androgens to estrogens, representing the primary mode of estrogen production in males and postmenopausal females.

Sex steroids contribute to cancer risk primarily by promoting cellular proliferation, thereby increasing the chance of cellular mutations. Estrogens are thought to influence tumorigenesis via estrogen receptor-mediated transcriptional activation of estrogen-responsive genes, including proto-oncogenes (Fig. 2B). Estrogen also may be tumorigenic through genotoxic mechanisms. Androgens may increase cancer risk by directly influencing proliferation or via conversion to estrogens. Progesterone promotes

apoptotic signaling, and thus may balance the proliferative impact of estrogens; the absence of progesterone underlies the "unopposed estrogen" hypothesis.

Estrogens

Estrogens play an essential role in the development of female secondary sexual characteristics, and contribute to several biological systems, including reproductive, cardiovascular, and neuroendocrine. Major estrogens include estrone (E1), 17-β-estradiol (E2), the most biologically active estrogen, and estriol (E3). In premenopausal women, estrogen is primarily produced in the granulosa cells of ovarian follicles with assistance from follicle-stimulating hormone (FSH), and is produced in smaller amounts in the liver, adrenal glands, breasts, and adipose tissue. Estrone is a primary estrogen in postmenopausal women, produced from conversion of adrenal androgens. In males, estrogen production occurs in the Leydig cells of the testes, through conversion of testosterone to estradiol via the cytochrome p450 aromatase enzyme, and through conversion of androstenedione to estrone. Estrogens affect signaling pathways by binding to and activating one of two estrogen receptors, ER-α or ER-β, in target organs.

Parent estrogens, estradiol and estrone, can be metabolized via 2-, 4-, and 16α hydroxylation pathways. These oxidative metabolites of estrogen have been implicated in carcinogenesis (**Fig. 2A**). Catechol estrogens are formed following irreversible oxidation of estrone and estradiol at the C-2 or C-4 positions, while oxidation at the C-16 position yields 16α-hydroxyestrone. Catechol estrogens can be further methylated to 2-methoxyestrone and 4-methoxyestradiol, 2-hydroxyestrone-3-methyl ether, 4-methoxyestrone, and 4-methoxyestradiol, while 16α-hydroxyestrone can be metabolized to 17-epiestriol, 16-ketoestradiol, and 16-epistriol.

The estrogenic and proposed genotoxic activity of metabolites differs across pathways. The 4- and 16α- pathway metabolites have higher estrogenic activity than estrogen, and as such are thought to increase proliferation in the same manner as estradiol; in contrast, 2-catechol estrogen metabolites may inhibit or mitigate proliferation. These differences may be explained in part by each metabolites' binding affinity to ER: 2- and 4-catechol estrogen metabolites (EMs) bind with a similar affinity as estradiol, while 16α-hydroxyestrone binds with lower affinity, though covalently. Thus, bound 16α-hydroxyestrone results in a constitutively activated ER, whereas 2- and 4-catechol EM can dissociate. 4-Catechol EMs have lower dissociation rates than estradiol, so they tend to upregulate ER-dependent processes. In addition, the metabolism of 4-hydroxy catechols to reactive quinones leads to generation of reactive oxygen species and consequent oxidative damage which may contribute to tumorigenesis. While 2-catechol estrogens are similarly metabolized to quinones, they instead form stable, reversible, DNA adducts. In either case, the methylation of an adjacent hydroxyl group will block formation of quinones.

Women who have traits associated with high cumulative estrogen exposure, such early age at menarche or late age at natural menopause, and long menopausal hormone therapy use, are more likely to develop several cancers, including postmenopausal breast, ovarian, and endometrial cancers, supporting a role for estrogens in carcinogenesis. More recently, evidence has also emerged for estrogen pathways in premenopausal breast, prostate, and colorectal cancers.

Postmenopausal breast cancer

Increasing levels of circulating estrogens are consistently associated with increased risk of postmenopausal breast cancer. A 2002 pooled analysis of nine prospective studies by the Endogenous Hormones and Breast Cancer Collaborative Group included 663 women who developed breast cancer after blood collection and 1765 controls. Positive associations were observed between all measured estrogens and postmenopausal breast cancer (**Fig. 3**), with evident dose–response trends (5th vs. 1st quintile total estradiol relative risk (RR) = 2.00, 95% confidence interval [CI]: 1.47–2.71, p-trend < 0.001; free estradiol RR = 2.58, 95% CI: 1.76–3.78, p-trend < 0.001). Large prospective studies published subsequently confirmed these pooled findings. In a 20-year follow-up within the Nurses' Health Study (NHS), positive associations between estradiol and breast cancer risk were stronger among women with ER+/PR+ tumors (5th vs. 1st quintile RR = 2.8, 95% CI: 2.0–4.0), and for women with more aggressive disease (i.e., recurrent or fatal disease) (RR = 2.6, 95% CI: 1.3–5.1).

In an analysis investigating serum or urinary EMs in postmenopausal breast cancer, including 1298 cases from four studies, an inverse association was observed between 2-hydroxylation pathway metabolites and postmenopausal breast cancer, as well as an inverse association between the ratio of 2-hydroxylation: 16-hydroxylation pathway metabolites.

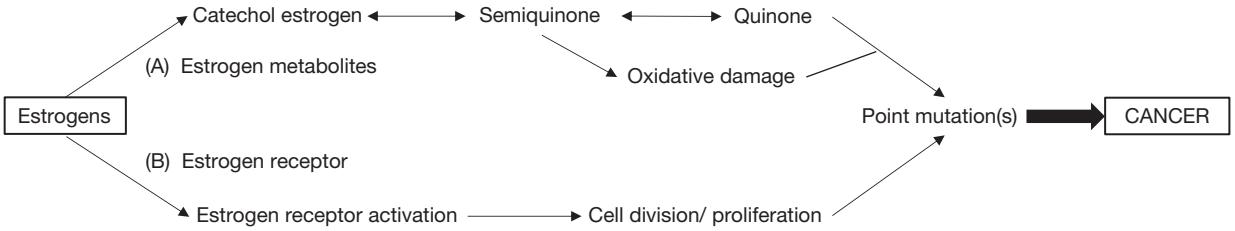

Fig. 2 Mechanisms of estrogens in carcinogenesis. (A) Catechol estrogens form semiquinones and quinones, that may lead to oxidative damage and genetic mutations. (B) Estrogen directly causes increased cell proliferation following ER activation, increasing likelihood of point mutations. Mutations via either mechanism promote carcinogenesis. Adapted from Fig. 1 in Bohra, A. and Bhateja, S. (2015). Carcinogenesis and sex hormones: A review. *Endocrinology & Metabolic Syndrome* **4**, 156. https://doi.org/10.4172/2161-1017.1000156.

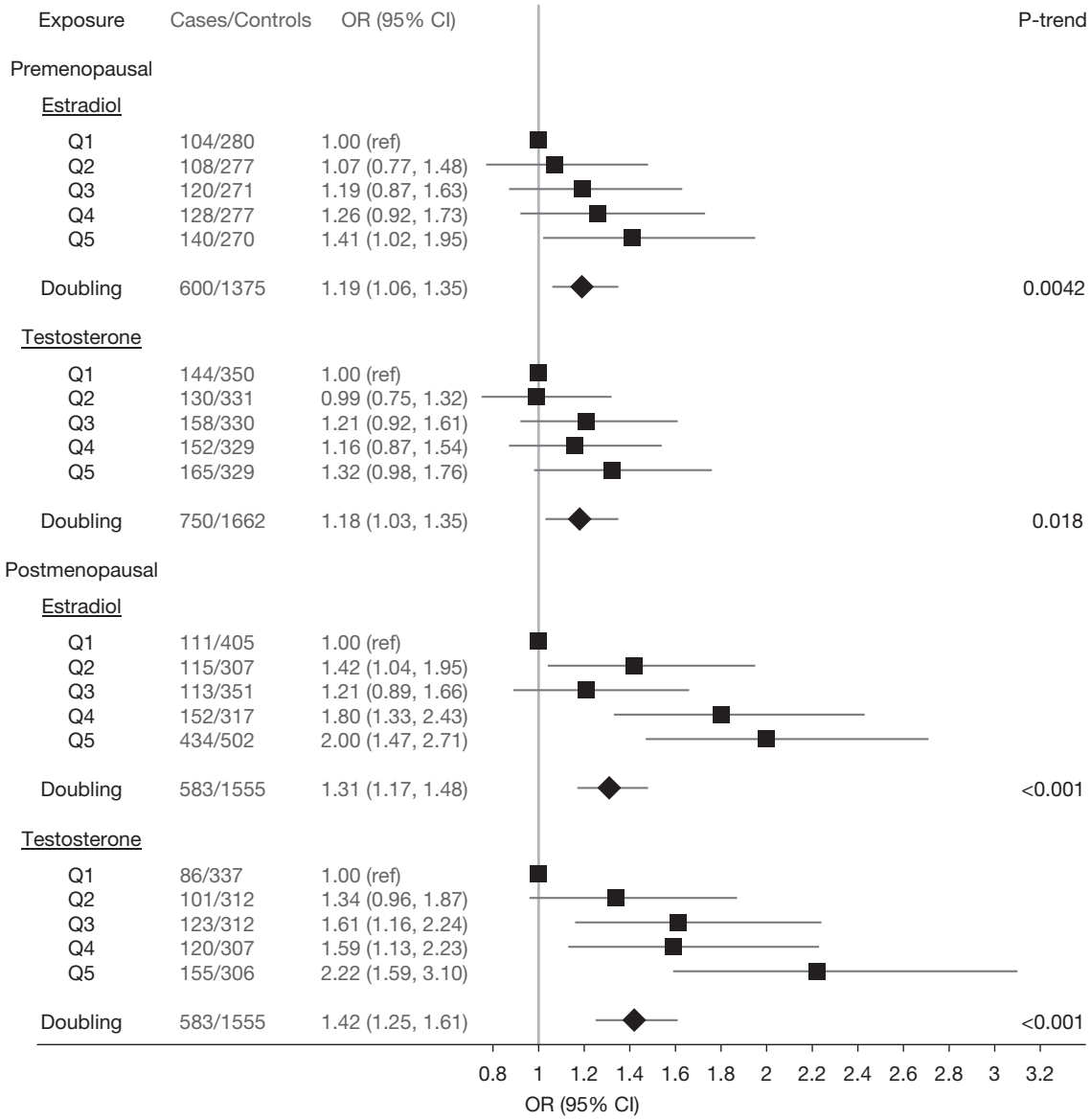

Fig. 3 Estradiol and testosterone levels and breast cancer risk, by menopausal status. Estimates are from conditional logistic regression on case–control sets matched within each study. For postmenopausal OR based on doubling of hormone, includes only those women with measurements for both estradiol and testosterone. Adapted from Key T, Appleby P, Barnes I, et al. (2002). Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *Journal of the National Cancer Institute* **94**(8), 606–616 (Fig. 1 and Table 4) (postmenopausal) and Key TJ, Appleby PN, Reeves GK, et al. (2013). Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies. *The Lancet Oncology* **14**(10), 1009–1019 (Fig. 1) (premenopausal).

Premenopausal breast cancer

Evaluating endogenous estrogens and risk of premenopausal breast cancer is difficult due to variation in concentrations across the menstrual cycle. A large pooled analysis of seven prospective studies by the Endogenous Hormones and Breast Cancer Collaborative Group evaluated this association with 767 cases and 1699 controls matched on day of menstrual cycle. A doubling of circulating estradiol was positively associated with premenopausal breast cancer risk (RR = 1.19, 95% CI: 1.06–1.35). Similar associations were observed for free estradiol, and estrone (Fig. 3). RRs were suggestively stronger for ER+/PR+ breast cancers, compared with ER−/PR− breast cancers.

While the pooled analysis supports a positive association between circulating premenopausal estrogen levels and risk of breast cancer, opposite associations have been observed with urinary premenopausal estrogens. Within the Nurses' Health Study II (NHSII), a cohort which enrolled majority premenopausal women at baseline, those with higher levels of urinary estrone and estradiol were at lower risk of breast cancer (4th vs. 1st quartile estradiol RR = 0.51; 95% CI: 0.30–0.86), possibly suggesting that higher estrogen excretion decreases risk.

In a large study ($n = 377$ cases) of premenopausal serum 2-hydroxyestrone and 16α-hydroxyestrone, no significant associations were observed with the metabolites or the ratio. However, a suggestive increased risk of ER+ premenopausal breast cancers was observed with a higher 2:16 metabolite ratio (top vs. bottom quartile RR = 2.15, 95% CI: 0.88–5.27). In the NHSII analysis of urinary EMs ($n = 247$ cases), suggestively inverse associations were observed for 2- and 4-hydroxylation pathway EMs, though a significant positive association was observed with a 16-pathway metabolite, 17-epiestriol (4th vs. 1st quartile RR = 1.74, 95% CI: 1.08–2.81).

Endometrial cancer

Strong positive associations of estrone and estradiol with overall endometrial cancer risk were observed in a nested case–control study within the Women's Health Initiative Observational Study (WHI-OS, $n = 313$ cases). The association with unconjugated estradiol was the strongest (5th vs. 1st quintile RR = 6.19, 95% CI: 2.95–13.03), though a positive association with estrone was observed even with adjustment for unconjugated estradiol (RR = 2.70, 95% CI: 1.34–5.45). Nearly all serum estrogen metabolites were associated with a two-to-threefold increased endometrial cancer risk as well. Together with a smaller study with similar findings, the evidence indicates a strong association between circulating estradiol and estrone and endometrial cancer, and potential role of estrogen metabolites in tumorigenesis.

Ovarian cancer

Relatively few epidemiologic studies have examined circulating estrogens and ovarian cancer risk, and results are inconsistent. In a case-cohort study of 67 ovarian cancer cases within the Breast and Bone Follow-up to the Fracture Intervention Trial (B-FIT), neither estrone nor estradiol were associated with ovarian cancer risk. Similarly, no association was observed between any of the 15 measured estrogen metabolites and risk. However, in a larger study of 169 epithelial ovarian cancer cases conducted within WHI-OS, a suggestive association was observed with circulating estrone (5th vs. 1st quintile RR = 1.54, 95% CI: 0.82–2.90), and positive associations were observed for 2- and 4-methoxyestrone metabolites (5th vs. 1st quintile RR = 2.03, 95% CI: 1.06–3.88). For each of these hormones, associations tended to be strongest for nonserous tumors.

Prostate cancer

Polymorphisms of genes in the estrogen metabolic pathway have been associated with prostate cancer risk, and because age is associated with prostate cancer risk this suggests that the natural lowering of the androgen to estrogen ratio with age may be a factor in carcinogenesis. However, a role for estrogen in prostate carcinogenesis is also seemingly paradoxical given the efficacy of estrogen therapy in androgen-dependent prostate cancer.

Despite biologic plausibility and some epidemiologic evidence suggesting an association between estradiol concentration and prostate cancer, the most recent evidence from a pooled analysis ($n = 3886$ cases) suggests endogenous estrogens are not importantly associated with prostate cancers.

Colorectal cancer

Epidemiologic evidence suggests a role of exogenous estrogens in lowering the risk of colorectal cancer among women. However, in an analysis of four prospective cohorts, circulating levels of estrone and estradiol were not associated with risk of colorectal cancer in either men or women.

Progesterone

Progesterone is an ovarian steroid hormone essential in breast and uterine development. Similar to estrogen, progesterone regulates gene transcription through one of two receptor isoforms, progesterone receptor (PR) -A and -B, located in the brain, breast, and reproductive organs. Once progesterone binds to the receptor, it becomes activated, binds to the DNA hormone response elements (HRE) in promoter regions of genes of interest, and, with assistance of coactivators, promotes transcription. Biologic hypotheses for this hormone suggest both pro- and antitumorigenic roles.

One hypothesis for progesterone-specific carcinogenic action in breast cancer is based on the proposed ability of ER+/PR+ cells to stimulate stem cell growth via growth promoters, spurring tumor development in normal breast epithelium. In addition, as most early breast cancer lesions express PR, it has been suggested that PR+ cells directly respond to progesterone to initiate PR-target gene transcription and subsequent proliferation. Progesterone levels are highest in the luteal phase, which corresponds with a period of high breast cell proliferation, suggesting a protumorigenic effect. However, progesterone also is hypothesized to decrease breast cancer risk by slowing estrogen-induced proliferation in breast epithelial cells.

Progesterone is particularly difficult to study separately from growth factors and prolactin in laboratory studies, and because mammary epithelial cells that express PR-A and PR-B also express ERs, the effect of estrogen versus progesterone on carcinoma development in breast tissue is often not easily distinguishable. A few epidemiologic studies have assessed progesterone levels with respect to post or premenopausal breast cancer, and have found null associations overall.

Androgens

Androgens are responsible for formation of male sex traits and play a role in female sexual processes both directly and through their conversion to estrogens. Adrenal androgens function as weak steroids, and include dehydroepiandrosterone (DHEA), produced from cholesterol in the adrenal cortex, dehydroepiandrosterone sulfate (DHEAS), and androstenedione, produced in the testes, adrenal cortex, and the ovaries, all of which can be converted to testosterone. DHEAS is converted to estrone following initial conversion to androstenedione, and is converted to testosterone after intermediate conversion to 5-androstenediol. Conversion of DHEA, DHEAS, and androstenedione to testosterone occurs in certain tissues including brain, skin, fat, and muscle.

Testosterone is one of the most bioactive androgens, produced by the male testes and female ovaries, and is further converted to its metabolite dihydrotestosterone (DHT), in the prostate, liver, brain, and skin; androgen effects are mediated through binding of testosterone or DHT to the androgen receptor (AR). The majority of testosterone is bound to sex-hormone-binding globulin (SHBG), or albumin, with approximately 2% of total testosterone freely circulating. Testosterone can be converted to estrogen via aromatase.

Androgens are hypothesized to increase risk of cancer by either increasing cell proliferation directly, or by their conversion to estrogens via aromatase.

Postmenopausal breast cancer

Approximately 60%–70% of breast tumors express androgen receptor (AR), indicating potential responsiveness to androgens. Moreover, normal and malignant breast tissues contain aromatase to convert androgens to estrogens. In the large pooled analysis of the Endogenous Hormones and Breast Cancer Collaborative Group mentioned above, testosterone concentration was associated with increased risk of breast cancer (5th vs. 1st quintile RR = 2.22, 95% CI: 1.59–3.10), with a similar association with androstenedione. DHEA and DHEAS associations with risk were slightly weaker than those of testosterone and androstenedione, likely due to the requirement of DHEA and DHEAS to be converted to androstenedione prior to estrone conversion. In a subsequent analysis within the NHS ($n = 707$ cases), stronger associations were observed for ER+/PR+ tumors (4th vs. 1st quartile overall RR = 1.5, 95% CI: 1.2–1.9; ER+/PR+ RR = 1.8, 95% CI: 1.3–2.5).

Premenopausal breast cancer

Significant positive associations between testosterone levels and risk of invasive breast cancer in premenopausal women have been consistent across several studies. In the same pooled analysis that examined estrogens and premenopausal breast cancer risk ($n = 750$ cases), androgens were associated with higher risk of premenopausal breast cancer (doubling testosterone RR = 1.18; 95% CI: 1.03–1.35; androstenedione RR = 1.30, 95% CI: 1.10–1.55; DHEAS RR = 1.17, 95% CI: 1.04–1.32).

Ovarian cancer

In vitro evidence suggests that androgens, along with estrogens and progesterone, help regulate the proliferation and invasion of ovarian cancer cells. It is hypothesized that, based on its strong ER-α agonist activity in other cell types, androstenedione may affect epithelial ovarian cancer (EOC) risk via interaction with ER.

In a nested case–control study within the large European Prospective Investigation into Cancer and Nutrition (EPIC) cohort ($n = 565$ cases), no association was observed between testosterone and EOC overall, though suggestively positive associations were observed with low-grade and type I tumors. Null associations were generally maintained across tumor characteristics for DHEAS and androstenedione, although a doubling of androstenedione was associated with higher risk of EOC for type I (RR = 1.99, 95% CI: 1.18–3.35), and low-grade tumors (RR = 1.99, 95% CI: 0.98–4.06). In contrast, in an earlier US study conducted within NHS, NHSII, and the Women's Health Study (WHS) cohorts, an inverse association was observed between androstenedione levels and ovarian cancer risk. Contradictory evidence may be due to differential inclusion criteria, or the heterogeneity of ovarian cancers.

Endometrial cancer

Within the EPIC cohort ($n = 247$ cases), higher risk of endometrial cancer among postmenopausal women was observed with increasing levels of total testosterone (3rd vs. 1st tertile RR = 1.44, 95% CI: 0.88–2.36), and free testosterone (RR = 2.05, 95% CI: 1.23–3.42), which was consistent across the eight included countries. These findings are consistent with smaller prospective cohort studies.

Prostate cancer

Given their critical role in normal growth and development of the prostate gland, androgens are hypothesized to increase risk of prostate cancer. Most prostate tumors initially respond to androgen deprivation therapy, and inhibition of testosterone to DHT conversion reduced incidence of prostate cancer by 25% in the Prostate Cancer Prevention Trial. Despite this plausibility, epidemiologic evidence to date does not support a role for testosterone in risk of prostate cancer. In the pooled analysis of 18 studies ($n = 3886$ cases), no associations were observed between testosterone and prostate cancer risk (5th vs. 1st quintile total testosterone RR = 0.94, 95% CI: 0.82–1.07; free testosterone RR = 1.11, 95% CI: 0.96–1.27). Consistent with this, no associations were observed for DHEA, DHEAS, and androstenedione.

Colorectal cancer

Within the pooled analysis of four male and female prospective cohort studies, higher levels of total testosterone were associated with a lower risk of colorectal cancer in men (4th vs. 1st quartile RR = 0.62, 95% CI: 0.40–0.96), but not women (4th vs. 1st quartile RR = 1.43, 95% CI: 0.82–2.50). The ratio of estradiol to testosterone was also positively associated with colorectal cancer in men (4th vs. 1st quartile RR = 2.63, 95% CI: 1.58–4.36) but inversely associated with risk among postmenopausal women (RR = 0.43, 95% CI: 0.22–0.84). Although in opposite directions in men and women, these results suggest a role for androgens and aromatase activity in colorectal carcinogenesis.

Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor beta (TGF- β) superfamily. The Müllerian ducts, present in the embryo, are precursors of the fallopian tubes, uterus, and upper vagina. In the fetus, AMH secretion by Sertoli cells of the testes halts Müllerian ducts from further development, whereas a lack of AMH promotes development of the female genital tracts. Postnatally, AMH secretion by granulosa cells of ovarian follicles is a sensitive marker of ovarian reserve. Levels are low in early life, increase during puberty and remain stable until about 25 years of age, and decline until undetectable in menopause, at which point the ovarian reserve is depleted.

The action of AMH binds its primary receptor (AMHR-II), which is present in organs of the reproductive tract, as well as in the breast. AMHR-II then phosphorylates the type I receptor, allowing mediation of downstream signaling. Through this mechanism, AMH activates growth inhibition pathways, and is hypothesized to protect against the development of tumors of the female reproductive tract, with extension to tumors of the breast. It has also been suggested to have a similar protective role against prostate cancer in males. Though animal and experimental studies support this hypothesis, the epidemiologic evidence does not support a protective effect of AMH on carcinogenesis. While the reason for discrepancy is unclear, concentrations of AMH well above normal physiologic levels were used in experimental studies.

Ovarian Cancer

Most ovarian surface epithelial (OSE) neoplasms arise from Müllerian epithelium or Müllerian-like ovarian surface mesothelium; thus, AMH may prevent ovarian cancer development. In animal and experimental studies in ovarian cancer cell lines AMH decreases proliferation, inhibits cell migration, and increases apoptosis. Despite this evidence, epidemiologic studies have consistently found no association between AMH levels and ovarian cancer risk.

Endometrial Cancer

In vitro, AMH inhibits growth in endometrial cancer cell lines, via apoptosis and cell cycle arrest, driven by regulation of proteins responsible for G1-to-S phase transition and cell cycle exit. However, the concentrations of AMH used in most experimental studies were higher than normal biological levels, and findings have not been reproduced in population studies.

In a large study of eight cohorts from the United States, Europe, and China ($n = 329$ cases), no association between AMH levels and endometrial cancer risk was observed (doubling of AMH RR = 1.07, 95% CI: 0.99–1.17).

Breast Cancer

Similar to experimental ovarian and endometrial cancer studies, supra-physiologic concentrations of AMH inhibit growth of breast cancer cells and increase apoptosis in vitro. However, the opposite effect is suggested by indirect epidemiologic evidence, as higher AMH levels are associated with later age at menopause, a known risk factor for breast cancer.

In prospective studies of the association between premenopausal AMH levels and breast cancer risk, positive associations have been observed between increasing AMH levels and incidence of breast cancer. A nearly 10-fold increased risk of breast cancer was observed in an initial epidemiologic study of AMH levels ($n = 309$ cases; 4th vs. 1st quartile RR = 9.8, 95% CI: 3.3–28.9). Two subsequent nested case–control studies ($n = 452$, 539 cases) confirmed the positive association, although the associations were not as strong (90th percentile vs. those with undetectable levels of AMH RR = 2.25, 95% CI: 1.26–4.02; 5th vs. 1st quintile RR = 2.20, 95% CI: 1.34–3.63, respectively). The latter study was conducted within NHSII and included women who were diagnosed with breast cancer prior to menopause, which indicates a biologic role for AMH in breast cancer etiology, and not simply an association that tracks with late menopause. Most recently, a pooled analysis of 2835 cases found a relative risk for breast cancer comparing the top versus bottom quartile of AMH of 1.60 (95% CI: 1.31–1.94).

Prostate Cancer

AMH is produced by the Sertoli cells of the testes in males, and following its initial role in sexual differentiation, AMH controls cell proliferation, differentiation, and apoptosis in normal tissues. Serum levels of AMH decline significantly following puberty and stabilize, remaining detectable throughout the lifespan. TGF-β has been shown to negatively regulate prostate growth through

induction of cellular apoptosis. As a member of the TGF-β growth factor family, AMH has been hypothesized to play a similar inhibitory role in the initial development of prostate cancer. In vitro evidence suggests, as with the cancers of the female reproductive tract, that AMH inhibits prostate cancer through similar mechanisms. However, no association was observed between AMH and prostate cancer risk (4th vs. 1st quartile RR = 1.15, 95% CI: 0.89–1.48) in the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial ($n = 1000$ cases).

Gonadotropins

The two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), regulate menstrual cycles in females. These hormones are synthesized by gonadotropic cells in the anterior pituitary gland and their secretion is regulated by gonadotropin-releasing hormone (GnRH). LH and FSH work synergistically to stimulate function within the gonads. LH is primarily responsible for increasing secretion of sex steroids in the ovarian theca cells, inducing ovulation, while FSH is essential for ovarian follicle growth prior to ovulation. Circulating levels of gonadotropins are controlled through a negative feedback loop, as sex steroids induced by LH stimulation inhibit GnRH and, in turn, FSH and LH levels.

The gonadotropin hypothesis posits that gonadotropins stimulate ovarian surface epithelium (OSE) to increase cell proliferation, as observed in vitro, which promotes tumor growth. In addition to proliferative effects, LH and FSH may promote tumorigenesis by increasing cell migration and suppressing apoptosis, through several different signaling mechanisms (e.g., via mitogen-activated protein kinases (MAPKs)).

Ovarian Cancer

Epidemiologic evidence indirectly supports the gonadotropin hypothesis on ovarian cancer risk, particularly given the protective effect of oral contraceptives, which suppress gonadotropin secretion. In addition, increased ovarian cancer risk among postmenopausal women provides support for this hypothesis, given that levels of FSH and LH increase in menopause in absence of ovarian steroid hormone feedback. Coupled with evidence that gonadotropin levels are higher in ovarian cancer patients, these findings support a potential role for FSH and LH in ovarian cancer development. However, among the few prospective studies of prediagnostic circulating gonadotropins on epithelial ovarian cancer (EOC), results have been inconclusive or contradictory to the gonadotropin hypothesis.

For example, while gonadotropin therapy for infertility, usually administered as a high dose of FSH, has been shown to increase risk of ovarian cancer, prospective studies of serum FSH levels have found null or contradictory results. In an analysis of three prospective cohorts, based in the United States, Sweden, and Italy ($n = 88$ postmenopausal cases), no association was observed between circulating FSH and epithelial ovarian cancer (3rd vs. 1st tertile RR = 0.85, 95% CI: 0.36–1.99). In a more recent but similarly small study in the United States and the United Kingdom (67 cases) an inverse association was observed between FSH levels and epithelial ovarian cancer risk (3rd vs. 1st tertile RR = 0.26, 95% CI: 0.10–0.70), that was consistent for both postmenopausal and premenopausal women. In either case, high circulating levels of FSH were not associated with increased ovarian cancer risk.

Null or suggestively inverse associations have also been observed for LH. A nested case–control study within the New York University Women's Health Study (NYUWHS) ($n = 58$ cases), observed an adjusted relative risk of 0.42 (95% CI: 0.09–2.09), comparing the 3rd vs. 1st tertile of LH.

Prolactin

Prolactin (PRL) is a neuroendocrine growth hormone secreted by the lactotroph cells of the anterior pituitary gland. Prolactin's role in breast development and lactation is well-known, though it acts on a variety of biologic pathways and in tissues aside from the mammary glands, including prostate, ovary, and liver tissue. Receptors are also expressed in adipocytes and immune system cells. Prolactin initiates multiple signaling pathways following binding to its receptor, a member of the cytokine receptor family. Signaling primarily occurs through the Janus protein kinase-2/signal transducer and activator of transcription-5 (JAK-2/STAT-5) pathway, which, as a growth promoting pathway, implicates prolactin in carcinogenesis. Supporting its protumorigenic action, prolactin has been shown to increase cell motility, cell proliferation, and tumor vascularization. These functions are particularly relevant in later-stage carcinomas.

Postmenopausal Breast Cancer

Animal and in vitro studies have shown prolactin overexpression accelerates or induces mammary tumor growth. Most epidemiologic studies examining the association between prolactin and breast cancer have correspondingly found increased breast cancer risk among women with higher circulating prolactin levels. However, there have been notable variations with respect to timing of measurement and menopausal status across studies.

In a study within NHS and NHSII ($n = 2468$ cases), a positive association was observed between prolactin levels measured among postmenopausal women and breast cancer incidence, though this was only apparent when prolactin was

measured <10 years prior to diagnosis ($n = 1445$ cases) (4th vs. 1st quartile RR = 1.20, 95% CI:1.03–1.04). The association was stronger among women with ER+ tumors (RR = 1.52, 95% CI: 1.19–1.93) and lymph node positive breast cancers (RR = 1.63, 95% CI: 1.08–2.44). No association was seen for prolactin measured >10 years prior to diagnosis, supporting the hypothesis that prolactin has an important role in later-stage carcinogenesis. A more recent study within EPIC ($n = 1738$ cases) found a similar positive association between prolactin and risk of postmenopausal breast cancer (4th vs. 1st quartile RR = 1.29, 95% CI: 1.05–1.58), although the association was only observed among women using hormone therapy (specifically, estrogen plus progestin).

Premenopausal Breast Cancer

A role for prolactin in premenopausal breast cancer is less clear than in postmenopausal breast cancer, with associations suggestively null overall. Although positive associations were observed in the NHS/NHSII with small case numbers, no association with premenopausal breast cancer was observed in larger subsequent analyses. In a recent study in EPIC ($n = 512$ cases), a suggested inverse association was reported (4th vs. 1st quartile RR = 0.70, 95% CI: 0.48–1.03). However, a positive association was observed in an analysis of in situ cases (3rd vs. 1st tertile RR = 1.49, 95% CI: 0.59–3.74). This finding contradicts the results among postmenopausal women suggesting a consistent positive association.

Prostate Cancer

Prolactin acts on the prostate indirectly by increasing testicular testosterone production through regulation of luteinizing hormone receptors, and directly influences prostate development and neoplasia by initiating signaling pathways after binding to prolactin receptors. As in breast cancer, prolactin may induce proliferation in prostate tumor cell lines and may also have an antiapoptotic effect. Animal studies have shown an influence of prolactin levels on prostate development and induced neoplasia in knockout mice, and prolactin is mitogenic in prostate epithelial cells. In addition, in human prostate tumors, prolactin is positively associated with high tumor grade and aggressive disease.

Despite biologic plausibility, epidemiologic evidence does not support a clear role for prolactin in prostate cancer development. For example, a large prospective study conducted within the Northern Sweden Health and Disease cohort ($n = 144$ cases), found no association between prediagnosis circulating prolactin levels and prostate cancer risk (4th vs. 1st quartile RR = 0.85, 95% CI: 0.49–1.47), agreeing with an earlier analysis combining results from two nested case–control studies, including a total of 320 prostate cancer cases.

Ovarian Cancer

Prolactin is essential for maintaining normal ovarian function, and also is produced in the ovaries, with its receptor expressed in normal ovarian tissues. Studies in ovarian cancer cell lines have shown that, under stressed conditions, addition of prolactin decreases apoptosis, and prolactin has also been shown to promote the growth of ovarian surface epithelial cells in animal models. A nested case–control study of three prospective cohorts in the United States, Sweden, and Italy ($n = 230$ cases) found a suggestive positive association between circulating prolactin and ovarian cancer risk (4th vs. 1st quartile RR = 1.56, 95% CI: 0.94–2.63), that was apparent only among overweight women (BMI ≥ 25 kg/m^2 RR = 3.10, 95% CI: 1.39–6.90).

Insulin/C-Peptide

Insulin, a peptide hormone produced and secreted by the pancreatic β-cells, is essential for maintaining glucose homeostasis, stimulating glucose uptake by insulin sensitive organs, including the liver, muscle, and adipose tissue, and reducing circulating glucose levels. The insulin receptor has two isoforms, α and β, which have distinct biologic functions given differential binding to insulin, and insulin-like growth factors IGF-I and IGF-II. Insulin receptors are ubiquitously expressed, and insulin may play an important role in linking cellular energy balance and cancer energetics.

Insulin is hypothesized to promote tumor development directly, by stimulating the insulin receptor, a member of the tyrosine kinase class, and promoting insulin signaling pathways in tumor cells, such as the MAPK pathway. It also may play an indirect role, increasing bioavailability of active growth factors (e.g., IGFs, described in the following section), and sex hormones, which promote carcinogenesis.

Type II diabetes is defined by insulin resistance, and cancer incidence is modestly increased among those with type II diabetes. Obesity, linked to energy balance and insulin resistance, is an established risk factor for a number of cancers; thus, insulin has been hypothesized as a biologic mechanism for obesity in cancer. Metformin, often used to treat type 2 diabetes, has been related to decreased cancer burden among patients with diabetes, and to reduced insulin-stimulated tumor growth in vivo. Insulin has also been shown to increase proliferation of certain cancer cell lines.

Insulin is initially linked to C-peptide during production as proinsulin; after secretion, insulin and C-peptide exist separately at equimolar concentrations in the blood. Unlike insulin, C-peptide is not metabolized in the liver, and thus measurement of C-peptide is often used to determine circulating insulin levels, as a more accurate representation of initially bioavailable hormone.

Several epidemiologic studies have examined circulating insulin or C-peptide levels in a variety of cancers, including breast, prostate, colorectal, pancreatic, and endometrial cancers.

Postmenopausal Breast Cancer

Indirect evidence for a role of insulin in breast cancer has accumulated, as postmenopausal overweight and obese subjects, and subjects with type II diabetes, have higher risk of breast cancer. Insulin receptor is expressed in breast tumor tissue, and insulin is hypothesized to affect risk by increasing proliferation of breast cancer cells directly or through indirect action described above.

A meta-analysis of prospective studies of circulating insulin or C-peptide and postmenopausal breast cancer risk included six studies of insulin ($n = 1239$ cases), and five studies of C-peptide ($n = 972$ cases). Overall, associations were near-null for C-peptide (summary relative risk (SRR) = 1.26, 95% CI: 0.80–1.96) and suggestively positive for insulin (SRR = 1.59, 95% CI: 0.92–2.74). In two studies that adjusted for BMI, a positive dose–response relationship between insulin levels and postmenopausal breast cancer risk was observed, while no dose–response relationship was apparent in the study that did not adjust for BMI.

Positive associations were observed in two analyses published since the meta-analysis. In the NHS I and II ($n = 796$ postmenopausal cases), C-peptide was associated with increased risk of invasive breast cancer (4th vs. 1st quartile RR = 1.5, 95% CI: 1.1–2.0), adjusting for BMI; the association was unchanged with estradiol adjustment. The association appeared stronger among women with ER− disease (RR = 2.0; 95% CI: 1.2–3.6). In the Cancer Prevention Study II (CPS-II) ($n = 302$ postmenopausal cases), a similarly large association between circulating C-peptide levels and breast cancer risk was observed (3rd vs. 1st tertile RR = 1.63, 95% CI: 1.08–2.45), with slight attenuation after adjustment for BMI. Overall, the current epidemiologic evidence points toward a positive association between circulating insulin and C-peptide and risk of breast cancer among postmenopausal women.

Premenopausal Breast Cancer

Interestingly, adiposity is associated with decreased risk of premenopausal breast cancer, making the investigation into insulin and breast cancer more complex. Several studies in the above-mentioned meta-analysis included premenopausal breast cancer cases; positive, inverse, and null associations between insulin or C-peptide levels and premenopausal breast cancer risk were noted by different studies. In the recent NHS/NHSII analysis ($n = 187$ premenopausal cases), a suggestively positive association was observed between C-peptide levels and premenopausal breast cancer (4th vs. 1st quartile RR = 1.4, 95% CI: 0.79–2.5).

Endometrial Cancer

Endometrial cancer is strongly associated with obesity, and has also been associated with type II diabetes and insulin resistance, even among nonobese women, indicating a potential role for insulin in its development.

In a large prospective study conducted within EPIC ($n = 286$ cases), serum C-peptide was associated with an increased risk of endometrial cancer (4th vs. 1st quartile RR = 2.13, 95% CI: 1.33–3.41), that was modestly attenuated with adjustment for BMI (RR = 1.56, 95% CI: 0.94–2.57). Results from several case–control studies agree with this prospective study.

Prostate Cancer

Insulin is thought to influence prostate cancer through cell proliferation and antiapoptotic functions, supported by in vitro studies in rat cell lines, where insulin has been shown to be mitogenic for both normal and adenocarcinoma prostate cells. While some prospective studies have found null results for overall prostate cancer risk based on C-peptide levels, others have observed differing associations by disease severity, with positive associations generally observed for aggressive disease. In the largest study to date, in the Health Professionals Follow-up Study (HPFS) ($n = 1314$ cases), no association was observed with C-peptide and risk overall prostate cancer (4th vs. 1st quartile RR = 1.05, 95% CI: 0.82–1.34); the null association remained when restricted to aggressive disease. Thus, circulating C-peptide levels do not appear directly associated with prostate cancer risk, and the association between obesity and prostate cancer (particularly aggressive disease) may be through a noninsulin-related pathway.

Colorectal Cancer

Obesity is an established risk factor for colorectal cancer, and insulin resistance has been posited as a potential mechanistic explanation for this association. Several epidemiologic studies have explored the association between circulating insulin or C-peptide and risk of colorectal adenoma, a precursor lesion. The current evidence indicates general agreement for a role of insulin in colorectal cancer development, though data are not entirely consistent.

A meta-analysis including 27 studies measuring either insulin or C-peptide, and risk of colorectal adenoma, found increased risk of adenoma with increasing insulin levels (highest vs. lowest quantile RR = 1.33, 95% CI: 1.12–1.58), and C-peptide levels (RR = 1.44, 95% CI: 1.13–1.83). Among studies adjusting for adiposity, the association was attenuated, though overall summary measures remained positive. In a more recent nested case–control study, in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) ($n = 273$ cases), no association was observed between serum C-peptide and colorectal adenoma (4th vs. 1st quartile RR = 0.83, 95% CI: 0.52–1.31).

Results from studies of insulin or C-peptide and risk of colorectal cancer have generally been positive. In the Physicians' Health Study ($n = 176$ cases), a strong positive association was observed between plasma C-peptide and colorectal cancer risk (5th vs. 1st quintile RR = 2.7, 95% CI: 1.2–6.2), including adjustment for BMI. A larger study within the EPIC cohort ($n = 1078$ cases) also found a positive association with C-peptide levels (5th vs. 1st quintile RR = 1.56, 95% CI: 1.16–2.09), which remained significant after adjustment for BMI and physical activity. In the WHI-OS ($n = 438$ cases), a positive association was observed between fasting insulin levels and colorectal cancer risk (4th vs. 1st quartile RR = 1.73, 95% CI:1.16–2.57), though the risk was attenuated after adjustment for waist circumference (4th vs. 1st quartile RR = 1.42, 95% CI: 0.91–2.23).

Pancreatic Cancer

In a pooled analysis of four US prospective studies ($n = 197$ cases), prediagnostic plasma C-peptide was positively associated with pancreatic cancer risk (4th vs. 1st quartile RR = 1.52, 95% CI: 0.87–2.64), which was not altered by BMI. Interestingly, the observed risk was attenuated when considering only fasting blood samples (RR = 1.21, 95% CI: 0.66–2.24). Insulin, measured in fasting samples, did not appear to be associated with risk in the same study (4th vs. 1st quartile RR = 1.08, 95% CI: 0.57–2.04).

Insulin-like Growth Factor

Insulin-like growth factors (IGFs, IGF-1 and IGF-2) are peptide hormones produced primarily in the liver that regulate proliferation and act as specific tissue growth factors. IGF-1 and IGF-2 are frequently expressed in neoplastic tissue, along with IGF-1 receptor, which is required for downstream signaling activity. In addition to their endocrine actions, IGFs also have autocrine and paracrine effects. IGF-1 has been shown to increase mitosis and inhibit apoptosis, indicating a procarcinogenic role. In vitro studies have observed positive dose–response relationships between neoplastic cell proliferation and IGF-1 concentration, and tumor progression in vivo has also been shown to depend on IGF-1 levels. In addition, IGFs, like insulin, are involved in signaling pathways that promote cell growth and migration in neoplastic tissue, including Akt and MAPK pathways. Drug candidates that target IGF-1R, either by influencing bioactivity or by blocking downstream signaling pathways, are being tested in clinical trials for response to chemotherapeutics. IGF-2R has not been targeted for such studies, as it exhibits properties of a tumor suppressor, and acts to block IGF-2 binding to IGF-1R, thereby reducing its activity.

Once produced, most IGFs associate with one of six IGF binding proteins (IGFBPs), while a small proportion circulate unbound. IGFBP-3 binds >75% of IGF-1 and IGF-2. In the bound state, IGFs are not available for IGF-I receptor activation. IGF bioactivity has thus been proposed to be a function of circulating levels of IGF, expression of IGF genes on tissues of interest, and protease action to cleave IGBP complexes to their functional form, though additional complexities also must be considered. For instance, most evidence supports an anticarcinogenic role for the binding proteins, particularly IGFBP-3 and IGFBP-1. However, certain binding proteins are also able to enhance the delivery of IGFs to certain tissues. Overexpression of both IGFBP-2 and IGFBP-5 have been associated with increased IGF action, and IGFBP-1, which is closely associated with insulin function, also increases IGF-1 induced DNA synthesis in its dephosphorylated state. Overall, an imbalance of IGFs and IGFBPs may indicate issues in cellular turnover and kinetics, characteristic of carcinogenesis.

Given the biologic plausibility that IGFs are important in carcinogenesis, these hormones, along with IGFBP-3 and IGFBP-1, have been investigated in several cancers to date, including breast, endometrial, ovarian, prostate, colorectal, pancreatic, and lung cancers. Serum measured IGFs and IGFBPs, used in epidemiologic studies, correlate well with tissue levels in murine models, supporting the use of blood-based measures as surrogates for tissue expression.

Postmenopausal Breast Cancer

In a recent pooled analysis combining data from 17 prospective studies in Europe, Australia, and the United States, 15 of which included women who were postmenopausal at blood collection ($n = 2853$ cases), IGF-1 was positively associated with breast cancer risk in postmenopausal women (5th vs. 1st quintile RR = 1.33, 95% CI: 1.14–1.55). Contrary to the idea that IGFBP-3 is inversely related to the bioactivity of IGF, IGFBP-3 levels were also positively associated with breast cancer risk (RR = 1.23, 95% CI: 1.04–1.45). Consistent with evidence from laboratory studies that estrogen and IGF signaling pathways interact in a synergistic manner, risk was only apparent among women with ER+ tumors (for 80% increase in IGF-1 RR = 1.38, 95% CI: 1.14–1.68), with no association noted among ER− tumors (RR = 0.80, 95% CI: 0.57–1.13). However, risk also increased consistently with increasing tertile of estrogen concentration, among women with either low, medium, or high levels of IGF-1, suggesting no clear interaction of effects, though these results were not confined to ER+ tumors.

Premenopausal Breast Cancer

IGF-1 has been associated with mammographic density in premenopausal women, a known risk factor for breast cancer, in a few recent studies. For instance, one study of 783 premenopausal healthy women found high IGF-1 and low IGFBP-3 to be independently correlated with high breast density. In the above-mentioned pooled analysis, 11 of the 17 studies included information on women who were premenopausal at blood collection ($n = 1937$ cases). The association between IGF-1 and breast cancer risk was

similarly positive (5th vs. 1st quintile RR = 1.21, 95% CI: 1.00–1.45). An analysis by age at diagnosis (< 50 years or ≥ 50 years) similarly observed a small, suggestive positive association between IGF-1 and breast cancer among younger women, though circulating IGFBP-3 levels were not significantly associated with breast cancer risk among women premenopausal at blood collection (RR = 1.00, 95% CI: 0.82–1.22).

Endometrial Cancer

A large nested case–control study within EPIC ($n = 286$ cases) examined prediagnostic concentrations of IGFBP-1 and IGFBP-2 and risk of endometrial cancer (the IGF hormones were not examined). No association between IGFBP-1 and endometrial cancer risk was observed (4th vs. 1st quartile RR = 0.76, 95% CI: 0.47–1.21), though there was an inverse association between IGFBP-2 and endometrial cancer risk (RR = 0.56, 95% CI: 0.35–0.90). This finding corresponds with a potential role for the IGF axis in the carcinogenesis of endometrial cancer; though, the null finding for IGFBP-1 suggests that the primary mode through which insulin resistance increases endometrial cancer risk is not through IGF signaling.

Prostate Cancer

A large pooled analysis of 17 prospective studies ($n = 7682$ cases) examined circulating IGF-1, IGF-2, IGFBP-2, and IGFBP-3 with prostate cancer risk (Fig. 4). IGF-1 was associated with an increased risk of prostate cancer (5th vs. 1st quintile RR = 1.29, 95% CI: 1.16–1.43), as was IGFBP-3 (RR = 1.25, 95% CI: 1.12–1.40). IGFBP-1 was inversely associated with risk (RR = 0.81, 95% CI: 0.68–0.96). After mutual adjustment for IGFs and IGFBPs, only IGF-1 remained associated with prostate cancer risk. While experimental evidence suggests that the activation of the IGF pathway is associated with progression of prostate cancer to lethal disease, the association between IGFs and prostate cancer risk did not appear to differ by stage or grade of disease in this pooled analysis.

Colorectal Cancer

Markers of hyperinsulinemia, such as obesity and central adiposity and type II diabetes have been associated with risk of colorectal cancer. Thus, insulin and corresponding IGF signaling pathways have been implicated in colorectal carcinogenesis. IGF-1 receptor is overexpressed in colon cancer cells, and overexpression of IGF-1 in a human CRC cell line resulted in a highly invasive tumor, further supporting a carcinogenic role. IGF-2 is also highly overexpressed in a subset of colorectal cancers. Moreover, CRC patients with low IGFBP-3 or high IGFBP-2 have worse prognosis, suggesting that these binding proteins negatively regulate IGF activity, or act independently, to influence tumor growth. Given the evidence for IGFs and IGFBPs in CRC progression, prospective epidemiologic studies have investigated this association.

A study within the Physicians' Health Study (PHS) examined IGF-1 and IGFBP-3 serum levels and colorectal cancer risk ($n = 193$ male cases). IGF-1 was positively associated with risk of colorectal cancer (5th vs. 1st quintile RR = 2.51, 95% CI: 1.15–5.46), and, correspondingly, IGFBP-3 was associated with decreased risk of colorectal cancer (RR = 0.28, 95% CI: 0.12–0.66). This association was attenuated among obese men, suggesting that obesity also contributes to colorectal cancer through non-IGF related pathways. These findings were supported by an investigation within the Northern Sweden Cohort. In contrast, no association was observed in the EPIC cohort ($n = 1121$ cases) between IGF-1 and CRC, or between IGFBP-3 (total and intact) and CRC. A meta-analysis combining these results with those of nine other prospective studies conducted between 1999 and 2008 ($n = 2862$ cases), found a small positive association between IGF-1 and CRC risk (per standard deviation increase RR = 1.07, 95% CI: 1.01–1.14).

Pancreatic Cancer

Experimental studies have shown the presence of IGF-1 and IGF-1R in pancreatic cell lines, and have demonstrated increased cellular proliferation and decreased apoptosis via signaling through IGF-1R. In the PLCO study ($n = 187$ cases) no statistically significant associations were observed for IGF-1, IGF-2, or IGFBP-3, although associations with IGF-1 (and the ratio of IGF-1/IGFBP-3, which would generally represent the proportion of bioavailable IGF-1) were suggestively positively associated with pancreatic cancer risk (e.g., IGF-1/IGFBP-3 4th vs. 1st quartile RR men = 1.39, 95% CI: 0.69–2.80; RR women = 1.47, 95% CI: 0.58–3.75). Another pooled study from the NHS, HPFS, PHS, and the WHI ($n = 212$ cases), observed no significant association between plasma IGF-1, IGF-2, IGFBP-3, or the IGF-1:IGFBP-3 M ratio and pancreatic cancer risk. Overall, the relatively limited data available to date suggests no substantial association of circulating IGFs and IGFBPs with pancreatic cancer risk.

Lung Cancer

IGF-1 exhibits mitogenic and antiapoptotic effects in lung cancer cell lines, while IGFBP-3 has been shown to inhibit IGF-1R signaling through interference with MAPK and Akt signaling pathways. A recent meta-analysis examining of circulating IGF-1 and IGFBP-3 and lung cancer risk included six nested case–control studies ($n = 1043$ cases). Neither IGF-1 nor IGFBP-3 were associated with increased lung cancer risk. One of the larger studies included found a positive association between IGF-1 and lung cancer risk, though this increased risk was only reported after controlling for IGFBP-3 levels. Overall, current evidence suggests no association between IGF-1 and IGFBP-3 and risk of lung cancer.

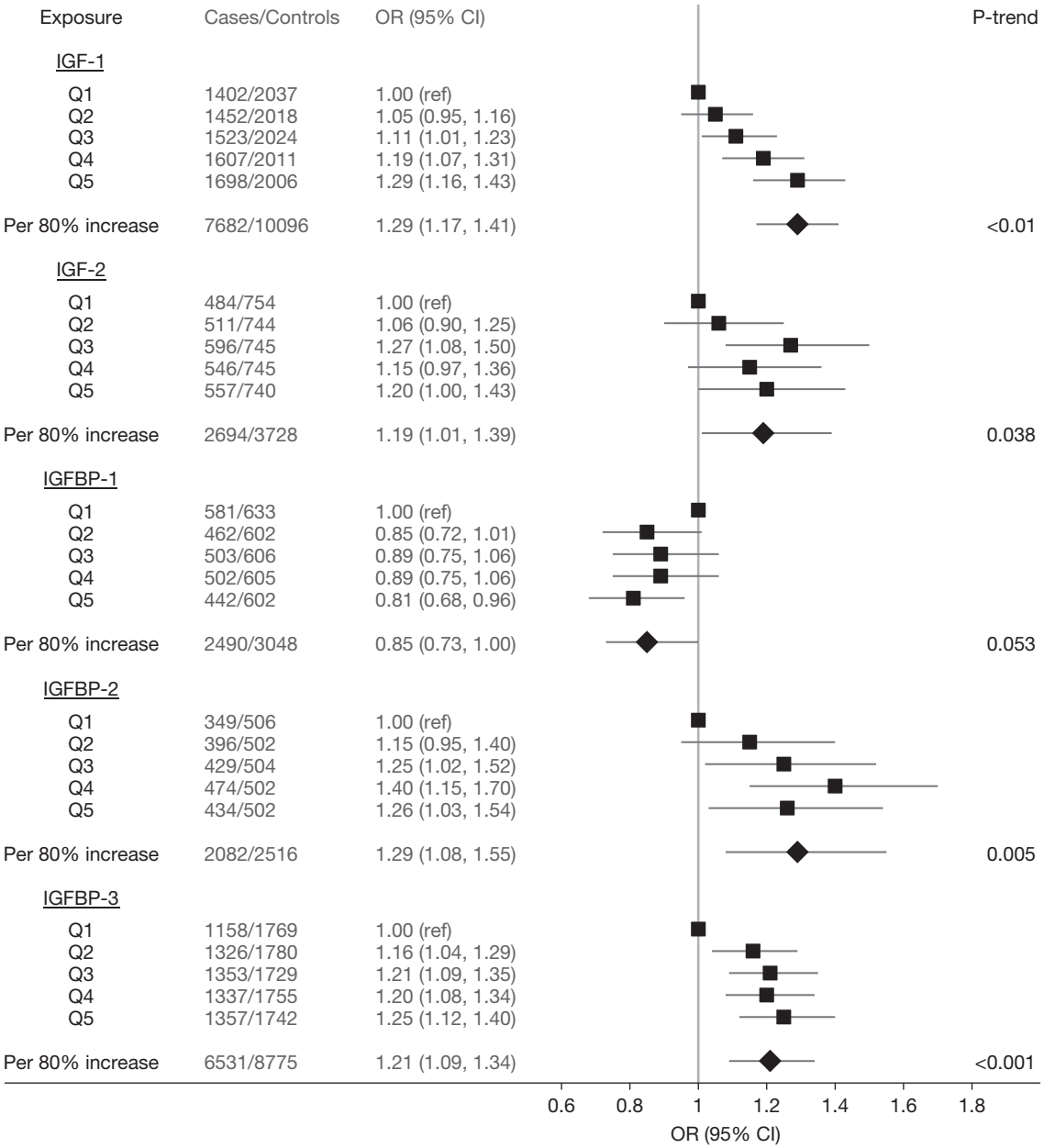

Fig. 4 IGF and IGFBP levels and prostate cancer risk. Odds ratios and 95% confidence intervals for prostate cancer associated with study-specific fifths of IGF and IGF binding protein concentrations. OR estimates from logistic regression conditioned on study-specific matching variables. Adapted from Fig. 1 in Travis, R. C., Appleby, P. N., Martin, R. M., et al. (2016). A meta-analysis of individual participant data reveals an association between circulating levels of IGF-I and prostate cancer risk. *Cancer research***76** (8), 2288–2300.

Adiponectin

Adiponectin is a peptide hormone secreted by adipocytes that acts in both a paracrine and endocrine manner. Adiponectin exists in two forms in circulation, full-length, and globular, both of which have antiinflammatory, antiangiogenic, and antidiabetic properties. Prior to secretion, high molecular weight (HMW) adiponectin is formed; as the dominant form in circulation, this is thought to be most physiologically relevant. Serum adiponectin is inversely associated with both obesity and type II diabetes, and low adiponectin is a sensitive marker for insulin resistance and hyperinsulinemia, independent of obesity.

Adiponectin may influence carcinogenesis via insulin-related pathways, or via a role in inflammation pathways and response. Its anticarcinogenic effect is also apparent at the cellular-level via activation of adenosine monophosphate-activated protein kinase (AMPK), which results in downstream inhibition of cellular proliferation and migration pathways of several key kinases, including phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT), mammalian target of rapamycin (mTOR), MAPK, glycogen synthase

kinase-3β (Wnt-GSK3β), and JAK/STAT. Overall, the activation of AMPK inhibits tumor growth and survival. Studies have examined the association between circulating adiponectin and breast, endometrial, prostate, colorectal, and pancreatic cancers.

Postmenopausal Breast Cancer

The inverse associations of adiponectin with adiposity and insulin resistance suggest its importance in postmenopausal breast cancer risk, as adiposity is an established risk factor in postmenopausal breast cancer. Experimental evidence also indicates a role for adiponectin in mammary carcinogenesis, with reduced breast cancer cell proliferation and inhibition of growth factor-induced cell migration.

One large prospective study nested within the NHS I and II ($n = 858$ cases) observed an inverse association between adiponectin and breast cancer risk (4th vs. 1st quartile RR = 0.73, 95% CI: 0.55–0.98). Results were similar after adjustment for IGF-I, C-peptide, IGFBP-1, and estradiol, indicating that this association is not simply a derivative of these connected biologic pathways. While this result was expected based on the biologic hypothesis, other epidemiologic studies have found no association between adiponectin and postmenopausal breast cancer risk.

Premenopausal Breast Cancer

In premenopausal women, adiposity is inversely associated with breast cancer risk, though the mechanisms underlying this association are unclear. In the Northern Sweden Health and Disease Cohort, a suggestively inverse association was observed among younger women < 55 years of age ($n = 227$ cases) (3rd vs. 1st tertile RR = 0.56, 95% CI: 0.28–1.11). In contrast, a positive association between adiponectin and premenopausal breast cancer risk was observed in the NHS/NHSII study ($n = 316$ cases) (4th vs. 1st quartile RR = 1.30, 95% CI: 0.80–2.10). This result corresponds with the evidence that obesity/adiposity is inversely related to breast cancer risk in this group.

Endometrial Cancer

Given the role of obesity and type II diabetes in endometrial cancer, adiponectin has been hypothesized to play a role in this disease. Two large prospective studies of adiponectin have shown conflicting results. A study conducted within EPIC ($n = 284$ cases), found an inverse association between adiponectin and endometrial cancer (4th vs. 1st quartile RR = 0.56, 95% CI: 0.36–0.86), which was independent of other obesity-related risk factors. However, a more recent study conducted within NHS ($n = 146$ cases) found no association between adiponectin and endometrial cancer risk (3rd vs. 1st tertile RR = 0.86, 95% CI: 0.53–1.39), which was true for both postmenopausal and premenopausal women.

Prostate Cancer

Both insulin resistance and obesity have been suggested as risk factors for prostate cancer incidence or progression, though the mechanisms underlying this relationship are unclear. Prospective epidemiologic studies do not indicate a role for adiponectin in the development of prostate cancer. Within the PHS ($n = 654$ cases) no overall association between serum adiponectin and prostate cancer risk was observed, though there was a lower risk of high-grade or lethal cancer with higher adiponectin levels (5th vs. 1st quintile RR = 0.25, 95% CI: 0.07–0.87).

Within a French nationwide cohort study (SUVIMAX), prostate cancer risk appeared to increase with increasing adiponectin (4th vs. 1st quartile RR = 1.34, 95% CI: 0.68–2.68), though this result was attenuated after adjustment for other inflammatory markers including high-sensitivity C-reactive protein (hs-CRP), which was strongly positively associated with prostate cancer risk, along with others (4th vs. 1st quartile RR = 1.18, 95% CI: 0.56–2.48).

Colorectal Cancer

In experimental studies, adiponectin inhibits colorectal cancer cell growth through activation of AMPK and consequent downregulation of the mTOR pathway. In addition, the strong link between BMI and colorectal cancer would indicate a potential role for adiponectin in colorectal cancer risk, though epidemiologic studies have not found a clear association. A prospective nested case–control study of Norwegian men ($n = 381$ cases) found no association between prediagnostic adiponectin and colorectal cancer risk (4th vs. 1st quartile RR = 0.8, 95% CI: 0.5–1.4). In another prospective study in EPIC ($n = 1206$ cases), HMW adiponectin was not associated with colorectal cancer risk (5th vs. 1st quintile RR = 1.05, 95% CI: 0.77–1.43), while non-HMW adiponectin was inversely associated with CRC risk (RR = 0.39, 95% CI: 0.26–0.60) with adjustment for BMI and waist circumference. This finding suggests the possibility that the proportion of non-HMW versus HMW adiponectin is important in CRC.

Pancreatic Cancer

Adiponectin's role in mediating insulin sensitivity makes it biologically plausible in pancreatic cancer. Receptors, AdipoR1 and AdipoR2 are expressed on the pancreatic beta cells and pancreatic tumor cells, and serum adiponectin is inversely correlated with

pancreatic tumor growth in mouse models. Plasma adiponectin has been consistently inversely associated with pancreatic cancer risk in prospective epidemiologic studies. A nested case–control study in Finland ($n = 311$ cases) found an inverse association between adiponectin and pancreatic cancer risk (5th vs. 1st quintile RR = 0.65, 95% CI: 0.39–1.07). A study within EPIC ($n = 452$ cases) similarly found higher circulating levels of adiponectin associated with a reduction in pancreatic cancer risk (4th vs. 1st quartile RR = 0.44, 95% CI: 0.23–0.82), though this was only apparent among never smokers. A study using pooled data from five US cohorts again reported an inverse association, with a risk reduction comparable to that seen in the Finnish cohort (5th vs. 1st quintile RR = 0.66, 95% CI: 0.44–0.97), but no modification in this association by smoking status was observed.

Melatonin

Melatonin is produced and secreted by the pineal gland, playing an integral role in the regulation of sleep and wake cycles, known as circadian rhythm. Melatonin production is controlled by a circadian signal from the suprachiasmatic nucleus (SCN), with function controlled through action on its receptors, MT1 and MT2. The SCN neuron firing declines later in the day, stimulating production of melatonin from serotonin conversion to tryptophan, with the majority of secretion taking place during the nighttime. Shift work that involves circadian disruption was identified as a probable human carcinogen in 2007 by the International Agency for Research on Cancer. Thus, melatonin is thought to play a role in carcinogenesis mainly through its regulation of circadian rhythm.

In addition to its pineal production, melatonin is also present in multiple extrapineal tissues such as the skin, reproductive tract, and gastrointestinal tract, where it exhibits antiinflammatory and antioxidant properties. Melatonin is a direct free radical scavenger, and has been shown to protect against ischemic neuronal damage. One pathway of interest for antioxidant activity involves reduction of cAMP and inhibition of the protein kinase A/cAMP pathway, resulting in modulation of antioxidant gene transcription. Melatonin also influences activity of MAPKs, which are involved in transcription and apoptosis, and acts as an angiogenesis inhibitor by targeting hypoxia induced-factor 1α and related genes. Thus, the antioxidant and proapoptotic functions of melatonin in tumor cells suggest it has an anticarcinogenic activity. Increasing evidence further suggests melatonin plays a protective role in carcinogenesis through regulation of the cell cycle and estradiol production.

Breast Cancer

In experimental studies, decreasing melatonin levels increases spontaneous tumor production, and restoration of melatonin prevents breast cancer development. Additionally, melatonin influences aromatase activity to reduce estrogen production and suppress tumor growth, and inhibits breast tumor growth and cell proliferation in vitro. Melatonin has been proposed as a biological mechanism explaining accumulating evidence that night-shift workers are at an increased risk of breast cancer. Epidemiologic evidence thus far indicates melatonin as a potential anticarcinogenic agent in postmenopausal, but not in premenopausal breast cancer.

Postmenopausal breast cancer

A recent meta-analysis of five prospective studies ($n = 1113$ cases), evaluating melatonin's primary urinary metabolite, 6-sulfatoxymelatonin (aMT6s), found an inverse association with postmenopausal breast cancer (4th vs. 1st quartile RR = 0.68, 95% CI: 0.49–0.92). Because melatonin has been associated with suppressed aromatase activity in breast tumors, which represents the primary mode to estrogen production in postmenopausal women, it is possible that melatonin decreases risk through this estrogen-mediated pathway.

Premenopausal breast cancer

In the meta-analysis mentioned above, aMT6s levels were not associated with risk of premenopausal breast cancer (RR = 1.05, 95% CI: 0.71–1.54). A more recent case–control study nested within the NHSII cohort, with approximately 75% premenopausal women ($n = 600$ cases), also found no association with aMT6s (4th vs. 1st quartile RR = 0.91, 95% CI: 0.64–1.28). The association, while remaining null, tended toward a decreased risk when diagnosis was less than 5 years from urine collection, consistent with an earlier analysis, and toward an increased risk when diagnosis was more than 5 years from urine collection, which may be indicative of differences in association by age or menopausal status at diagnosis. Thus, this warrants further study to better understand the issue of timing.

Ovarian Cancer

Melatonin is hypothesized to reduce ovarian cancer risk, based on both the broad argument that melatonin may influence carcinogenesis through circadian rhythm regulation, and biologic evidence suggesting that melatonin and its metabolites inhibit growth of ovarian cancer cell lines. Additionally, melatonin is synthesized in the ovary itself, and as such may influence ovarian function through modulation of luteinizing hormone and progesterone.

Urinary aMT6s levels within the NHS cohorts ($n = 152$ cases) were not associated with risk of ovarian cancer, consistent with studies indicating a null association between night-shift work and ovarian cancer.

Prostate Cancer

Previous epidemiologic studies have shown a positive association between sleep loss and proxies for circadian disruption including light at night and shift work, and prostate cancer, potentially supporting the circadian disruption hypothesis for melatonin action in prostate cancer. There is limited epidemiologic evidence for a role of melatonin in prostate cancer, though a case–cohort study within the Age, Gene/Environment Susceptibility (AGES)-Reykjavik cohort ($n = 111$ cases), found low aMT6s levels associated with increased risk for advanced disease (below vs. above median RR = 4.04, 95% CI: 1.26–12.98). Melatonin was associated with overall prostate cancer risk as well, though the result was not as strong (RR = 1.47, 95% CI: 0.94–2.30), and appeared to be driven by advanced prostate cancer risk.

Colorectal Cancer

Melatonin has been indicated in colorectal cancer, as night-shift work has been associated with increased risk of this cancer. Within the NHS, women who worked >15 years on rotating night shifts had a higher risk of CRC (RR = 1.35, 95% CI: 1.03–1.77) than those who did not work night shifts for this extended period of time. Recent work has also uncovered a role of melatonin in the inhibition of colorectal cancer cell proliferation, which may be suggestive of a potential risk mitigating effect of melatonin in CRC. However, to date there have been no epidemiologic studies of urinary melatonin and colorectal cancer risk.

See also: Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2. Ovarian Cancer: Diagnosis and Treatment.

Further Reading

Aleksandrova, K., Boeing, H., Jenab, M., et al., 2012. Total and high-molecular weight adiponectin and risk of colorectal cancer: The European Prospective Investigation into Cancer and Nutrition Study. Carcinogenesis 33 (6), 1211–1218.
Autier, P., Koechlin, A., Boniol, M., et al., 2013. Serum insulin and C-peptide concentration and breast cancer: A meta-analysis. Cancer Causes & Control 24 (5), 873–883.
Bernard, V., Young, J., Chanson, P., et al., 2015. New insights in prolactin: Pathological implications. Nature Reviews Endocrinology 11 (5), 265–275.
Bizzarri, M., Proietti, S., Cucina, A., et al., 2013. Molecular mechanisms of the pro-apoptotic actions of melatonin in cancer: A review. Expert Opinion on Therapeutic Targets 17 (12), 1483–1496.
Brinton, L.A., Trabert, B., Anderson, G.L., et al., 2016. Serum estrogens and estrogen metabolites and endometrial Cancer risk among postmenopausal women. Cancer Epidemiology, Biomarkers & Prevention 25 (7), 1081–1089.
Brown, S.B., Hankinson, S.E., Eliassen, A.H., et al., 2015. Urinary melatonin concentration and the risk of breast cancer in Nurses' Health Study II. American Journal of Epidemiology 181 (3), 155–162.
Choi, J.H., Wong, A.S., Huang, H.F., et al., 2007. Gonadotropins and ovarian cancer. Endocrine Reviews 28 (4), 440–461.
Cust, A.E., Allen, N.E., Rinaldi, S., et al., 2007. Serum levels of C-peptide, IGFBP-1 and IGFBP-2 and endometrial cancer risk; results from the European prospective investigation into cancer and nutrition. International Journal of Cancer 120 (12), 2656–2664.
Eliassen, A.H., Spiegelman, D., Xu, X., et al., 2012. Urinary estrogens and estrogen metabolites and subsequent risk of breast cancer among premenopausal women. Cancer Research 72 (3), 696–706.
Endogenous Hormones and Breast Cancer Collaborative Group, 2010. Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: Pooled individual data analysis of 17 prospective studies. The Lancet Oncology 11 (6), 530–542.
Fortner, R.T., Schock, H., Jung, S., et al., 2017. Anti-Mullerian hormone and endometrial cancer: A multi-cohort study. British Journal of Cancer 117 (9), 1412–1418.
Ge, W., Clendenen, T.V., Afanasyeva, Y., et al., 2018. Circulating anti-Müllerian hormone and breast cancer risk: A study in ten prospective cohorts. International Journal of Cancer. https://doi.org/10.1002/ijc.31249 [Epub ahead of print].
Giovannucci, E., 2001. Insulin, insulin-like growth factors and colon cancer: a review of the evidence. The Journal of Nutrition 131 (11 Suppl), 3109s–3120s.
Jamil, Z., Fatima, S.S., Ahmed, K., et al., 2016. Anti-Mullerian hormone: Above and beyond conventional ovarian reserve markers. Disease Markers 2016, 5,246,217.
Jenab, M., Riboli, E., Cleveland, R.J., et al., 2007. Serum C-peptide, IGFBP-1 and IGFBP-2 and risk of colon and rectal cancers in the European Prospective Investigation into Cancer and Nutrition. International Journal of Cancer 121 (2), 368–376.
Key, T., Appleby, P., Barnes, I., et al., 2002. Endogenous sex hormones and breast cancer in postmenopausal women: Reanalysis of nine prospective studies. Journal of the National Cancer Institute 94 (8), 606–616.
Key, T.J., Appleby, P.N., Reeves, G.K., et al., 2013. Sex hormones and risk of breast cancer in premenopausal women: A collaborative reanalysis of individual participant data from seven prospective studies. The Lancet Oncology 14 (10), 1009–1019.
Lauby-Secretan, B., Scoccianti, C., Loomis, D., et al., 2016. Body fatness and cancer—Viewpoint of the IARC working group. The New England Journal of Medicine 375 (8), 794–798.
Lihn, A.S., Pedersen, S.B., Richelsen, B., 2005. Adiponectin: Action, regulation and association to insulin sensitivity. Obesity Reviews 6 (1), 13–21.
Lin, J.H., Zhang, S.M., Rexrode, K.M., et al., 2013. Association between sex hormones and colorectal cancer risk in men and women. Clinical Gastroenterology and Hepatology 11 (4), 419-24.e1.
Moore, R.Y., 2007. Suprachiasmatic nucleus in sleep–wake regulation. Sleep Medicine 8 (Suppl. 3), 27–33.
Ose, J., Fortner, R.T., Rinaldi, S., et al., 2015. Endogenous androgens and risk of epithelial invasive ovarian cancer by tumor characteristics in the European Prospective Investigation into Cancer and Nutrition. International Journal of Cancer 136 (2), 399–410.
Pollak, M., 2012. The insulin and insulin-like growth factor receptor family in neoplasia: An update. Nature Reviews Cancer 12 (3), 159–169.
Rinaldi, S., Cleveland, R., Norat, T., et al., 2010. Serum levels of IGF-I, IGFBP-3 and colorectal cancer risk: Results from the EPIC cohort, plus a meta-analysis of prospective studies. International Journal of Cancer 126 (7), 1702–1715.
Roddam, A.W., Allen, N.E., Appleby, P., et al., 2008. Endogenous sex hormones and prostate cancer: A collaborative analysis of 18 prospective studies. Journal of the National Cancer Institute 100 (3), 170–183.
Trabert, B., Brinton, L.A., Anderson, G.L., et al., 2016. Circulating estrogens and postmenopausal ovarian cancer risk in the women's health initiative observational study. Cancer Epidemiology, Biomarkers & Prevention 25 (4), 648–656.
Tworoger, S.S., Eliassen, A.H., Kelesidis, T., et al., 2007. Plasma adiponectin concentrations and risk of incident breast cancer. The Journal of Clinical Endocrinology and Metabolism 92 (4), 1510–1516.

Tworoger, S.S., Eliassen, A.H., Zhang, X., et al., 2013. A 20-year prospective study of plasma prolactin as a risk marker of breast cancer development. Cancer Research 73 (15), 4810–4819.

Yager, J.D., 2000. Endogenous estrogens as carcinogens through metabolic activation. Journal of the National Cancer Institute Monographs 27, 67–73.

Zhang, X., Tworoger, S.S., Eliassen, A.H., et al., 2013. Postmenopausal plasma sex hormone levels and breast cancer risk over 20 years of follow-up. Breast Cancer Research and Treatment 137 (3), 883–892.

Induced Pluripotent Stem Cells and Yamanaka factors

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Glossary

ES cells Embryonic stem cells.
ICM Inner cell mas cells of the blastula.
iPS cells Induced pluripotent stem cells.
Pluripotency Means that the cell can give rise to any cell type in the body. The inner cell mass cells in the blastula and all cells before the blastula stage are considered as pluripotent. Pluripotency is defined as the ability of a cell to differentiate into any cell type of the three germ layers, namely endoderm, ectoderm and mesoderm.
Totipotency Means that one cell can give rise to any cell in the body, extra embryonic tissue and a new individual. The fertilized egg (also called the zygote, and up to the eight-cell stage in humans) is considered to be totipotent.
Zygote Fertlized egg.

Introduction

The challenge to delay aging in order to preserve youth and prolong life is counteracted by devastating diseases such as cancer that drastically shortens the average lifetime. It is tempting to speculate the enormous impact that scientific advancements could have when tweaking our longevity. All humans and other mammals start their life from a single cell the fertilized egg, called the zygote. This one cell replicates in a fast but regulated manner to give rise to a fully developed fetus in the mother's womb during gestation. All cells in our body have the genetic map of how one should look like and perfect regeneration of tissue, organs or limbs should be theoretically possible. Interestingly, a fetus in the mother's womb will not produce scar tissue if surgical procedure is done, but once born that perfect regeneration capacity is lost and scars are formed. One theory is that humans have lost their perfect regenerative capability in order to prevent cancer.

In 2012, Shinya Yamanaka and Sir John Gurdon were honored with the Nobel Prize in Physiology or Medicine for the discovery that mature cells can be reprogrammed to pluripotency. In other words, this meant that mature (differentiated/somatic) cells could be rejuvenated back to pluripotency using Yamanaka's protocol or even to totipotency using Gurdon's protocol. The definition of pluripotent cells is that they can give rise to all cell types in the body, whereas totipotent cells can become not only any cell type in the body but also gives rise to a new individual, the extra embryonic tissue including placenta and umbilical cord. In 2006, Yamanaka along with Takahasi made the landmark discovery of reprogramming differentiated cells into a pluripotent state. First, they succeeded in reprogramming mouse tail-tip fibroblasts into cells similar to embryonic stem (ES) cells. ES cells are in vitro cultured cells that are derived from the inner cell mass cells of the blastula, and are pluripotent. Yamanaka's method involved the retroviral mediated transduction of only four transcription factors to reprogram the differentiated cells into pluripotent cells, which were named induced pluripotent stem (iPS) cells. Their work started back in 2000, when they began analyzing mouse ES cells to identify genes responsible of their inherent characteristics, these are pluripotency and infinite self-renewal capacity. The outcome of these studies was the identification of several ES cell-associated transcripts, such as Nanog, signal transducer and activator of transcription 3 (STAT3), Krüppel-like factor 4 (Klf4), c-Myc, β-catenin, T-cell leukemia/lymphoma protein 1 (TCL1), growth factor receptor-bound protein 2 (GRB2), octamer binding protein 4 (Oct4) and sex determining region Y-box 2 (Sox2), among others. Thus, they elaborated a list of candidate-reprogramming factors that were tested in a system generated by a graduate student in the Yamanaka group. Briefly, this system was based on transducing different combinations of candidate genes into mouse embryonic fibroblasts, and then evaluating their resistance to geneticin. Using this system, they tested the 24 candidates (**Table 1**) and noticed that they got stem cell colonies similar to ES cell colonies, which was narrowed down to only four transcription factors. Recently, Shinya Yamanaka during an event in Gothenburg, Sweden (**Fig. 1**) revealed that it was Takahashi who cleverly excluded one factor at a time to quickly come up with only four factors, the so-called Yamanaka factors, Oct4, c-Myc, Klf4 and Sox2 (**Fig. 2**). A year after the discovery in mouse, human iPS cells were produced with the Yamanaka factors. Approximately at the same time, another group reported deriving human iPS cells with another combination of factors: Oct4, Sox2, Lin28 and Nanog. Currently, both sets of reprogramming factors are used globally.

The iPS cells share many characteristics with ES cells in terms of surface marker expression, morphology, proliferation and gene expression profiles. One of the advantages of iPS cells compared to ES cells is that these cells allow researchers to bypass ethical concerns related to the use of cells from embryos, thus offering amazing opportunities for regenerative medicine, drug discovery and disease modeling applications However, the main advantage of iPS cells is that one rejuvenates the patient cell into the blastula

Table 1 Yamanaka 24 candidate genes that generated ES like cells

Number	*Gene*	*Number*	*Gene*
1	Ecat1	13	Sall4
2	Dppa5	14	**Oct3/4 (Pou5f1)**
3	Fbxo15	15	**Sox2**
4	Nanog	16	Rex1 (Zfp42)
5	Eras	17	Utf1
6	Dnmt3l	18	Tcl1
7	Ecat8	19	Dppa3 (Stella)
8	Gdf3	20	**Klf4**
9	Sox15	21	β-catenin
10	Dppa4	22	**c-Myc**
11	Dppa2	23	Stat3
12	Fthl17	24	Grb2

Final four Yamanaka factors are marked in bold.

Fig. 1 Shinya Yamanaka's visit to Gothenburg in 2017, Nobel Prize Inspiration Initiative, sponsored by AstraZeneca, University of Gothenburg and Chalmers University of Technology.

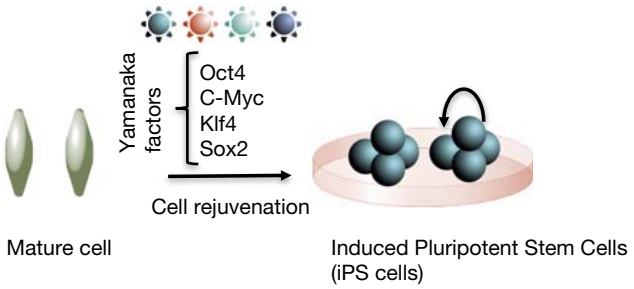

Fig. 2 Induced pluripotent stem cells (iPSCs) can be derived from mature (somatic) cells by expression of various transcription factors and were achieved in murine fibroblasts in 2006 by viral transduction of the Yamanaka factors Oct4, c-Myc, Sox2 and Klf4, and a year later in human fibroblasts. The method results in cellular rejuvenation back to blastula stage of development.

Table 2 Nomenclature of Oct4 and its identified pseudogenes

Gene symbol	*Gene description*
POU5F1	POU class 5 homeobox 1
POU5F1B	POU class 5 homeobox 1B
POU5F1P3	POU class 5 homeobox 1 pseudogene 3
POU5F1P4	POU class 5 homeobox 1 pseudogene 4
POU5F2	POU domain class 5, transcription factor 2

stage of development and can at least in theory attain an unlimited pool of pluripotent cells and these cells can be differentiated into the cell type of interest in any organ or tissue.

In addition to iPS cells, shairing similar properties with ES cells, they share some features with cancer stem cells, such as unlimited self-renewal, multi-lineage differentiation potential, maintenance of the stemness state and in vivo teratoma formation after implantation. Moreover, Yamanaka factors Oct4, Sox2, Klf4 and c-Myc have been identified in cancer cells. However, proteins encoded by the same gene can play very different roles in the cell (e.g., different pseudogenes or differently spliced versions) and the proteins can be modified to a different extent (e.g., glycosylation and phosphorylation, etc.). Therefore, the proteins that have been identified in cancer cells, although having the same name, do not necessarily have the same function in different contexts and further research is required to clarify their exact role. For example, the Oct4 protein is encoded by Pou5f1 and exists in at least five different versions and pseudogenes (Table 2).

Although the molecular mechanism underlying the reprogramming process is still unclear, it has been reported that "reprogramming" involves transcriptional induction that leads to the expression of pluripotency-related genes, therefore re-inducing the cell's capacity for unlimited cell growth. In 2008, Liu and colleagues performed a study to identify the global targets of endogenous Yamanaka factors in mouse ES cells. They found that these factors regulate developmental signaling networks of approximately a dozen signaling pathways to maintain the pluripotency of stem cells, including Notch, transforming growth factor β(TGFβ), Wingless/Wnt, and Hedgehog signaling pathways. Interestingly, they also found that the target genes of Oct4, Klf4, and Sox2 were linked to developmental processes, whereas the targets of c-Myc were mainly related to metabolic processes. Other studies have reported more pathways involved in the reprogramming process, including the overexpression of the Ha-Ras and hTERT oncogenes, inactivation of both the p53 and Rb tumor suppressor pathways by SV40 LT antigen, or the PTEN/PI3K/Akt pathway. Furthermore, it has been shown that the inactivation of p53 improves the efficiency of iPS cell generation. Taking into account that these pathways are considered as a hallmark of cancer and that Oct4, c-Myc, Klf4 and Sox2 are commonly overexpressed in some types of cancers, it seems obvious that cellular reprogramming and tumorigenesis share common features and mechanisms. Unraveling the mechanism of reprogramming could therefore help us to reveal new networks, potential biomarkers, develop cellular models for cancer progression and elucidate molecular mechanisms underlying the pathogenesis of human cancers.

In this article, we will describe each one of the four Yamanaka factors as well as discuss their role in the reprogramming process and relation to cancer (Fig. 3).

The Yamanaka Factor Pou5f1 (Oct4)

Oct4, also known as Pou5f1, Oct3, Otf3 or Oct3/4 (Oct3A, Oct3 B in human) is a member of the POU family of transcription factors and is expressed in the inner cell mass (ICM) of the blastocyst. Oct4 is involved in the initiation, maintenance and differentiation of pluripotent and germline cells during normal development. It is the first transcription factor known to be expressed during early mammalian embryogenesis and can be detected in the two-cell stage in human embryos and in the four-cell stage in mice. Oct4 has been proven to be important for implantation of the fetus in the uterus and was therefore not thought to have orthologues in species other than mammals, although orthologues in xenopus has been described. In mammals, the retention of totipotency has been correlated with the expression of Oct4. In the mouse, Oct4 is expressed from the fourth cell stage and in all of the early-cleavage nuclei of the morula and its expression becomes restricted to the ICM of the blastula. During gastrulation Oct4 is expressed solely in posterior epiblast cells lining the gut and those cells are thought to give rise to the primordial germ cells. These cells migrate using filopodia in the gut and end up where the gonads will be formed. Later, Oct4 expression is only seen in primordial germ cells and finally in oocytes but not in sperm or germ cells that reach the testis. However, Oct4 expression has been detected in primary testicular germ cell tumors and ovarian cancer; and this is associated with poor prognosis. In humans, opposed to mice, the expression of Oct4 has been detected in adult organs, for example Oct4 protein has been detected in numerous tissue/organ (Fig. 4A). Several reports argue that this detection of Oct4 in mature cells are false positive results due to pseudogene transcripts and DNA contamination. As mentioned earlier several versions of this gene exist (several splice variants and pseudogenes) and their different functions needs to be determined. The molecular mechanisms that regulate the first cell fate decisions in the human embryo are not well understood. The role of Oct4 during embryonic development in Oct4 −/− mice was studied and the conclusion was that the ICM formation was impaired. Recently Oct4 function during human embryogenesis was

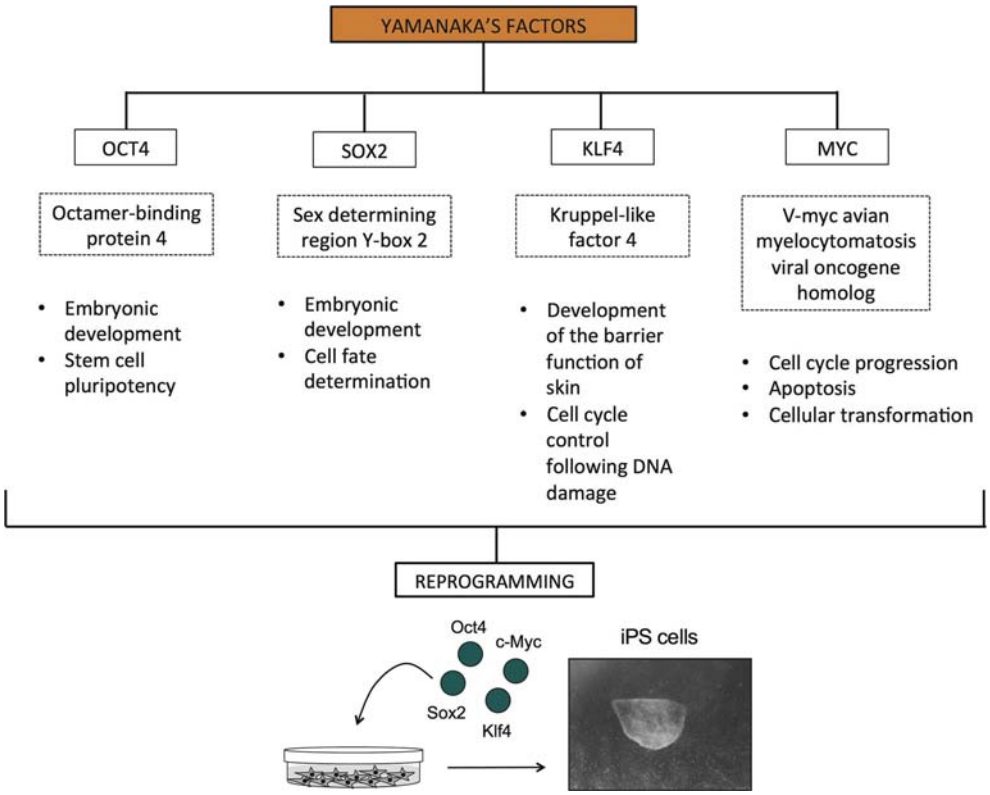

Fig. 3 Schematic picture representing the four Yamanaka factors, their main functions and the process of reprogramming.

investigated by genome editing, using CRISPR/Cas9-system to knock down its expression. Fogarty et al. specifically targeted the gene encoding Oct4 in diploid human zygotes and found that blastocyst development was compromised. Oct4 functions as a transcription factor and can form a heterodimer with Sox2 and function as a transcription activator or repressor. It induces the pluripotency gene Nanog. Oct4 needs to be at a certain concentration in order to maintain pluripotency, as little as a two-fold increase or decrease will cause pluripotent cells to differentiate. Therefore, accurate control of the Oct4 levels are crucial for maintaining pluripotency.

The Yamanaka Factor Myc (c-Myc)

Almost three decades back, the oncogene (v-Myc) from the myelocytomatosis virus (MC29) was traced back to a conserved cellular counterpart (c-Myc). In mammals, Myc has two other paralogs N-Myc (neuroblastoma derived homolog) and L-Myc (lung adenocarcinomas derived homolog). Myc codes for a basic-Helix-Loop Lelix (bHLHZ) domain containing the transcription factor of 439 amino acids and many of the conserved domains are shared with N-Myc and L-Myc. Like most members of the basic/helix-loop-helix/leucine zipper (bHLHZ) family, Myc is an obligate heterodimer. Myc heterodimerizes with Max (Myc-Associated Factor X; another bHLHZ protein) to bind specific DNA sequences (5′-CACGTG- 3′) called canonical *E*-boxes (enhancer boxes) and this interaction in turn activates transcription. On the other hand, binding of this heterodimers to non-canonical *E*-boxes is believed to result in transcriptional repression. Moreover, both N-Myc and L-Myc heterodimers are also known to bind the canonical and non-canonical *E*-boxes to interact with specific DNA sequences. Max is also known to form, self-homodimers to prevent phosphorylation of Max by Casein Kinase II (CKII). Yet, its binding affinity for DNA is extremely weak compared to the Myc-Max heterodimer. On the other hand, Max heterodimerizes with Mad to repress the same set of genes activated by Myc-Max. Furthermore, Myc also binds non- canonical *E*-boxes without Max and has probable Max-independent regulation.

The Myc protein has a half-life of less than ~30 min under normal physiological condition. Myc is typically degraded by tagging the protein for ubiquitin-mediated degradation. The hotspot residues (Serine 62 and Threonine 58) in Myc homology box I (MBI) is the target for ubiquitin ligase Fbw7 and is subsequently degraded by proteasome-mediated pathways (Fig. 5). Not surprising that the T58 residue is one of the most frequently found mutations in Myc. Myc homology box II (MBII) is the most extensively studied Myc domain because of its indispensable role in transcriptional activation, owing to its interaction with a number of histone acetyl transferase and other co-factors. Furthermore, the TAD domain is known to interact with the positive transcription elongation complex (P-TEFB).

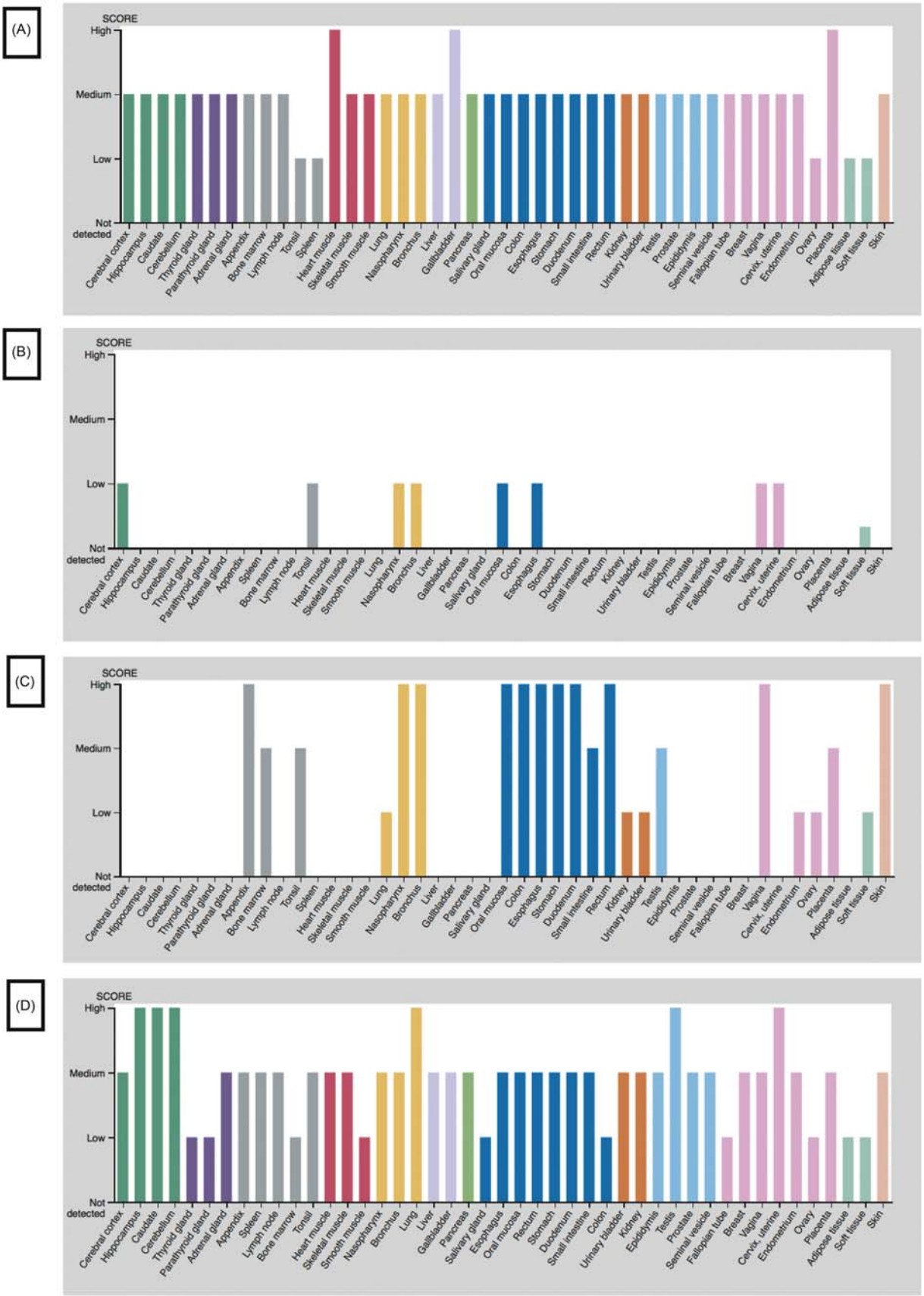

Fig. 4 Bar graph representing the protein levels in the different organs of the body of (A) OCT4 factor, (B) Sox2, (C) KLF4 and (D) MYC. Image obtained and modified from https://www.proteinatlas.org.

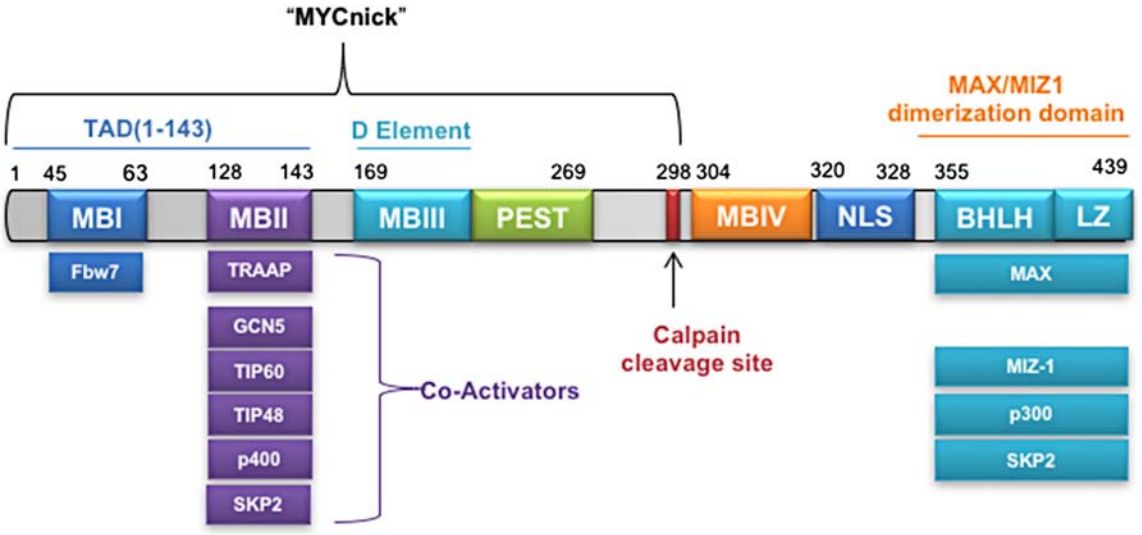

Fig. 5 Myc domains along with the associated factors. Myc contains two Myc boxes (MBI and MBII) in the N-terminal and together this is called a transcriptional activator domain (TAD). The PEST (Proline, Glutamic Acid, Threonine and Proline) domain; two other Myc boxes (MBIII and MBIV) and the nuclear localization signal then follow it. The C-terminal domain of Myc contains the bHLHZ domain. Figure adapted from PhD thesis of Joydeep Bhadury.

Most cells always maintain a delicate equilibrium of Myc levels under normal physiological condition. When deregulated, its level dictates if the cell opts for induction apoptosis or cellular transformation; a characteristic very explicit to Myc. Recently, how varying levels of Myc molecules regulate various Myc dependent functions has been elucidated. Myc regulates a wide range of target genes transcription governed by RNA Pol II by regulating their transcriptional elongation. Moreover, Myc also directly interacts with RNA Pol I and III to stimulate rRNA and tRNA synthesis. Myc not only induces the transcription of target genes but is also known to repress many genes. Despite having an enormous number of target genes, Myc still manages to have its specificity for either activating or repressing genes. YC regulates a wide range of target genes transcription governed by RNA Pol II by regulating their transcriptional elongation. Moreover, MYC also directly interacts with RNA Pol I and III to stimulate rRNA and tRNA synthesis. MYC not only induces the transcription of target genes but is also known to repress many genes. Despite having an enormous number of target genes, MYC still manages to have its specificity for either activating or repressing genes.

Myc is indispensable in embryonic development and only Myc heterozygous mice are born, albeit with not completely normal phenotypes. On the other hand, Myc null rat fibroblasts remain viable in spite of having smaller cell size and slower replication cycles; though the precise molecular mechanism owing to their propagation without Myc remains to be defined. Myc remains to be targeted directly by pharmacological means to date. This is primarily due to its intrinsically disordered protein nature (IDP) and IDPs are in general extremely difficult targets for therapeutic interventions. Indirect targeting of Myc by interfering with the Myc-Max heterodimer is an attractive therapeutic strategy. Among many compounds, 10058-F4 was extensively tested, but could not be used clinically because of its poor pharmacokinetics and bioavailability. Another approach is to use a dominant negative dimerization variant of Myc called "Omomyc", which is known to inhibit Myc function both in vitro and in vivo.

With regard to iPS cells, Myc is dispensable, albeit with significant reduction in the generation of iPS cell colonies. Yamanaka and colleagues had previously shown that either L-Myc or transformation deficient (TD) Myc can be used for iPS cell generation, but is not as efficient as c-Myc. Michael Cole and colleagues first reported the TD-Myc variant. By specifically mutating Myc at W136 to W136E, they showed that this inhibits the binding of Tip49a, a protein known for its role in ATP dependent remodeling of various targets including histones and has numerous other functions. This point mutation only leads to c-Myc losing its transformation capabilities, without significantly effecting its roles in maintaining cell cycle, apoptosis and other functions. Surprisingly, Myc despite being embryonically lethal, seems to be dispensable in maintaining ES cells or the naïve state of pluripotency. Okuda and colleagues have shown that ES cells can self-renew and maintain pluripotency without Max, the obligate heterodimer of Myc. In line with this, Myc transcript level are significantly reduced when ES cells are maintained in two inhibitors (MEK and GSK3-beta inhibitor), the so called 2i condition, a culture condition globally used to derive and maintain ES cells in their naïve or ground state. Finally, Andreas Trumpp and colleagues have recently shown that by transiently inhibiting Myc in mouse embryos, they could induce a diapause-like state. These embryos develop normally when transferred to pseudo-pregnant sows, thus showing a dispensable role of Myc in maintaining pluripotency, at least in the pre-implantation stages. On the other hand, the important role of Myc in mediating cell competition and shaping the developing epiblast during embryogenesis may not be overlooked. Recently, Miguel Torres and colleagues have shown that the ES cells that maintain high levels of Myc stay in naïve state, as compared to Myc low cells, which are found in the primed or epiblast stem cells states. These contradicting roles of Myc being dispensable in ES cells, as compared to the historically observed embryonic lethal phenotypes or its important role in cell competition, to shape the developing post implantation embryo, opens numerous questions. Cell competition is a conventional view of development which

involves elimination of neighboring cells to build an organism. How is Myc dispensable in maintenance of ES cells but still remains a critical factor for cell competition mediated processes of a developing post implantation embryo? Are other members of the Myc family replacing c-Myc during these processes? How are the Myc target genes regulated in absence of detachable Myc protein in ES cells? However, a possible explanation could be as ES cells are retrieved from the ICM cells of the blastula before the formation of epiblast, the embryonic lethal stage when Myc is depleted, post implantation and the cell competition stages. This supports the fact that Myc may be dispensable for reprogramming and in ES cells maintance. (Note: The part of this review partaining to the description of Myc has been adpated and modified with consent from the PhD thesis of Joydeep Bhadury (a co-author in this article). Using genetics to identify epigenetic and signal transduction targets in cancer. http://hdl.handle.net/2077/42347. ISBN: 978-91-628-9850-2 (PRINT); 978-91-628-9851-9 (PDF).)

The Yamanaka Factor Klf4

Krüppel-like factor 4 (Klf4) is a zinc finger-containing transcription factor that belongs to the Krüppel-like factor (Klf) family, and is expressed in various human tissues (**Fig. 4**C). Members of the Klf family possess characteristic carboxyterminal zinc finger domains that allow them to bind specific sites in promoter and enhancer regions of the genes they regulate. These zinc finger motifs at the carboxyterminal end of the proteins are highly conserved from flies to humans.

The Klf family is composed of 17 members, which play crucial roles in different body processes including differentiation, development and programmed cell death. Thus, several human diseases such as cardiovascular disease, metabolic disorders and cancers have been associated with the alteration of the function of these proteins. Specifically, Klf4 is thought to be involved in the normal development of the skin barrier function and consequently, it is involved in the pathogenesis of squamous cell carcinoma. Apart from playing a key role in normal development of the skin barrier, it is also involved in apoptosis, epithelial-to-mesenchymal transition, epithelial differentiation and cell cycle regulation. However, contradictory data exists regarding the regulation of the cell cycle by Klf4. For example, it seems to mediate the G1-to-S transition following DNA damage, by mediating the tumor suppressor gene p53. Also, Klf4 is able to induce cell cycle arrest by mediating p21, thus acting as a tumor suppressor. In fact, a study conducted by Dang and colleagues in 2003 revealed that induced Klf4 expression in cells that do not express endogenous Klf4, resulted in reduced colony formation, cell migration, invasion, and in vivo tumorigenicity. On the contrary, Klf4 can also contribute to tumor progression as it inhibits apoptosis. Furthermore, it has been seen that both Klf4 mRNA and protein are present in a high proportion in breast tumor samples as well as in gastrointestinal tumors. It seems that Klf4 could play different roles – as an oncogene or tumor suppressor - depending on the microenvironment in which it is functioning.

The fact that Klf4 is necessary for the efficient generation of iPS cells could be explained by its role in the maintenance of ES cells pluripotency. Klf4 has been observed to be upregulated by the leukemia inhibition factor (LIF), which maintains pluripotency in mouse ES cells. Additionally, Klf4 enables the maintenance of the self-renewal capacity in iPS cells by its cooperation with c-Myc. In iPS cells, there is a balance between these two factors, in which Klf4 suppress apoptosis induced by c-Myc, and c-Myc neutralizes the cytostatic effect of Klf4, thus maintaining the immortality of iPS cells. Apart from c-Myc, this factor can also cooperate with another member of the Klf family, such as Klf2 or Klf5 in the self-renewal of ES cells, and it is thought to play a crucial role in the ICM development at the blastocyst stage.

Regarding its relationship with the other Yamanaka factors, Klf4 co-occupies a substantial proportion of its downstream genes with Oct4 and Sox2. In fact, when Liu et al. (2008) studied the developmental signaling network in mouse ES cells, they found that Klf4 functioned to enhance Oct4 and Sox2 core factors for developmental regulation. On the other hand, Klf4 was identified as an upstream regulator of a large, feed-forward transcription factor loop that contains Oct4, Sox2, and c-Myc as well as other common downstream factors such as Nanog.

Despite of the fact that the exact mechanism of reprogramming is still poorly understood, new biological and pathobiological roles for Klf factors are continually being discovered. The critical role of Klf4 in important processes such as development, cell cycle, reprogramming or cancer, makes it necessary to study this protein and its pathways further.

The Yamanaka Factor Sox2

Sox2 (sex-determining region Y-box 2) is one of 20 members of the Sox family of transcription factors. Like Oct4, c-Myc and Klf4, it is expressed in embryonic and adult stem cells, where it functions as a master regulator of pluripotency and self-renewal.

During early embryogenesis, Sox2 levels are carefully controlled to inhibit mesendodermal differentiation and to promote differentiation into the neural ectoderm germ layer. Knock-down and knock-out, result in neural deformations and lethality of the developing embryo shortly after the epiblast stage, respectively. Subsequently, Sox2 remains constitutively elevated during neural tube formation and in central nervous system (CNS) progenitor cells until those are fully differentiated. Next to this, Sox2 dictates the branching pattern of the developing bronchial tree as well as differentiation of the airway epithelium. Up- and down-regulation studies show that Sox2 must be tightly regulated at all stages of development. Even a two-fold increase was reported to induce differentiation in mouse ES cells.

In adulthood, Sox2 regulates stemness and homeostasis of basal cells in the tracheal epithelium and the gastric tract; and also, neural stem cells (NSCs) maintain high levels of Sox2. These cells can produce either more Sox2-positive stem cells or differentiated

neural precursors. The high endogenous expression of Sox2 as well as c-Myc by NSCs allows them to be reprogrammed towards pluripotency by the mere addition of Oct4. This decreases the risks associated with the introduction of multiple exogenous reprogramming factors. For most other cell types, at least Sox2 and Oct4 are required to induce pluripotency. However, when reprogramming fibroblasts using small molecule, only Oct4 is required. Sox2 is regulated by LIF (leukemia inhibitory factor) signaling via the Jak/Stat3 (Janus-kinase/signal transducer and activator transcription) pathway activating Klf4 which further activates Sox2. It regulates itself and other pluripotency factors, namely Oct4 and Nanog, as well as developmental genes like FGF4 and is part of a complex network of interacting factors on multiple levels. All members of the Sox family contain the HMG box DNA binding domain (~80 amino acids), named after the highly similar DNA binding domains of High Mobility Group DNA binding proteins. With this domain, Sox2 binds to an eight base-pair DNA motive in the major groove, present in many developmental genes. Yet, Sox2 not only regulates the transcription of developmental genes but also its own by means of positive and negative feedback loops. Additionally, Sox2 has been found to collaborate with Oct4 while possibly also regulating its transcription. Together, Sox2 and Oct4 bind to non-palindromic sequences, the HMG/POU cassette, activating a subset of pluripotency and developmental factors. However, it was found that the HMG/POU cassette of many genes can also be bound by other families of the Sox family, namely Sox4, Sox11 and Sox15, indicating redundancy of Sox2 function. Yet, Sox2 is essential over other Sox protein family members to maintain a pluripotent state. Interestingly, the HMG/POU cassette is also present in the promoter of Sox2 and Oct4 and in at least one of three known Sox2 enhancers, namely SRR2. This implies their ability to regulate themselves and each other, as noted before, and explains why minor changes in Sox2 expression can have such dramatic effects on cell physiology. Together Sox2 and Oct4 might further work as co-activators or co-suppressors, enabling cells to rapidly differentiate during development.

Next to its vital role during nearly all stages of mammalian development, Sox2 has recently been detected in a growing number of cancers. These discoveries have shifted the focus of studies on Sox2 in development towards its involvement with tumor initiation and growth. A PubMed search of "Sox2 in Cancer" yields 2131 results and accumulating evidence of Sox2 upregulation in at least 25 different types of cancers confirms its state as a potent oncogene. Sox2 upregulation relative to healthy tissue is found in many solid tumors but remains low or absent in the surrounding tissue, tightly tying the gene to the disease. Further, enhanced migration and anchorage-independent growth of Sox2 over-expressing cells has been observed in pancreatic cancer, lung and esophageal cell lines and human embryo teratocarcinoma cell lines which links it to metastasis formation. But also in ovarian, skin, esophageal, lung, prostate, breast, and glioblastoma cancers, Sox2 upregulation is found in a number of patient samples. Notably, Sox2 expression is not necessarily uniformly upregulated throughout the tumor tissue but a cancer stem cell (CSCs) subpopulation primarily expresses the gene. Ectopic upregulation of Sox2 in epithelial and mesenchymal breast cancer was shown to enhance cell proliferation and invasion while knocking it down had mitigating effects. The occurrence of Sox2 upregulation in a great number of cancer types has tempted researches to establish a relation between Sox2 levels or Sox2-positive cell count and a possible clinical outcome. Together, these studies motivate the search for possible relations between the percentage of Sox2-positive cells and tumor malignancy in order to ultimately improve clinical diagnoses and patient survival.

However, a number of studies using knock-down and knock-out of Sox2 have brought up contradicting results. For instance, in head and neck squamous cell carcinoma, the knock-down of Sox2 has been related to a five-fold increased recurrence of cancer. The same effect has been observed in ovarian cancer by Wen et al., who found that cancer spheroid formation, proliferation, migration and tumorigenicity decreases under Sox2 knock-down. Although these studies constitute only a small part of the current research on Sox2, they contradict the theory of Sox2-positive tumor initiating CSCs and indicate that Sox2 is not necessarily involved in all types of cancer.

Concluding Remarks

In summary, the invention of iPS cells by Yamanaka and colleagues have led the opening of an entirely new era of research. To date the precise genetic, molecular and epigenetic mechanisms leading to iPS cell generation remains to be fully elucidated. Numerous key discoveries have been made using iPS cells, since its initial publication in 2006, leading to the first clinical trial to treat macular degenerative disease that was first initiated in 2014 and is still ongoing. One of the drawbacks with the iPS cell technology is the random integration of DNA into the genome. This has been an impediment of the iPS cells method for clinical use, and the search for ways to induce pluripotency without causing genetic change has become the focus of intense research. The criteria for the selection of a good manufacturing practice (GMP) grade iPS cell line would be a cell line without any exogenous genomic modifications. For example, iPS cell lines generated by the messenger RNA (mRNA) technology or by using Sendai virus mediated transduction, are known to result in generation of foot-print free iPS cells.

Recently, researchers studied the role of knocking down Oct4 in human embryos and the result is similar but not identical to what has been previously found in mice, which was that the inner cell mass (ICM) cells were absent. Under normal conditions, the ICM cells give rise to the individual. This data supports Oct4 as a key player for the development process and further support earlier findings, that out of the four Yamanaka factors, Oct4 has a primary role in reprograming and as being a totipotent and pluripotent marker. Although, it is important to note that iPS cells generated from murine cells closely resemble the ICM or the naïve state, whereas iPS cell generate using human cells are closer to the epiblast stem cells or primed state. The exact reasons and the factors regulating this difference between the human and murine cells is not fully understood, but several protocols have been developed to convert the human primed state to naïve like state or directly derive naïve state like human ES cells. An imbalance in cellular signaling networks, which are necessary during normal embryonic development, can either result in cancer or birth defects.

However, cancer and birth defects are each other's counterpart since molecules identified to cause birth defects, for example, retinoic acid or thalidomide can be used as cancer drugs. Therefore, in theory all drugs that disrupt normal development are promising candidates for hindering the development of cancer. Scientists hypothesize that the development of certain cancers and embryonic development are closely related events and have particularly been proven for teratocarcinomas. Indeed, the opportunity with the iPS cell technology to rejuvenate cells from patients back to the pluripotent state of development opens up an avenue of experimental approaches. One example is the generation of organoids from iPS cells, to recapitulate development in the test tube, to increase our understanding of human cell biology, genomics and epigenetics that potentially cause a specific devastating disease such as cancer. An additional example is that we have recently made cartilage by 3D bioprinting iPS cells and anticipate this as a future treatment option to repair damaged cartilage in joints. In theory, the strategy of 3D bioprinting iPS cells, followed by differentiation into the desired cell type or tissue, can potentially be used as "tissue patches" that can be stitched into any injured organ. 3D bioprinted tissue-replicas generated from iPS cells are juvenile and if such tissue is proven safe it could be like a perpetual machine and be industrially mass produced.

Further Reading

Amati, B., Brooks, M.W., Levy, N., Littlewood, T.D., Evan, G.I., Land, H., 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell 72 (2), 233–245.

Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fatyol, K., et al., 2005. c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nature Cell Biology 7 (3), 303–310. https://doi.org/10.1038/ncb1225.

Arnold, K., Sarkar, A., Yram, M.A., Polo, J.M., Bronson, R., Sengupta, S., et al., 2011. Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell 9 (4), 317–329. https://doi.org/10.1016/j.stem.2011.09.001.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., Lovell-Badge, R., 2003. Multipotent cell lineages in early mouse development depend on Sox2 function. Genes & Development 17 (1), 126–140. https://doi.org/10.1101/gad.224503.

Bahram, F., von der Lehr, N., Cetinkaya, C., Larsson, L.G., 2000. c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. Blood 95 (6), 2104–2110.

Bass, A.J., Watanabe, H., Mermel, C.H., Yu, S., Perner, S., Verhaak, R.G., et al., 2009. Sox2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. Nature Genetics 41 (11), 1238–1242. https://doi.org/10.1038/ng.465.

Berberich, S.J., Cole, M.D., 1992. Casein kinase II inhibits the DNA-binding activity of Max homodimers but not Myc/Max heterodimers. Genes & Development 6 (2), 166–176.

Berezovsky, A.D., Poisson, L.M., Cherba, D., Webb, C.P., Transou, A.D., Lemke, N.W., et al., 2014. Sox2 promotes malignancy in glioblastoma by regulating plasticity and astrocytic differentiation. Neoplasia 16 (3), 193–206 e19–25. https://doi.org/10.1016/j.neo.2014.03.006.

Berg, T., Cohen, S.B., Desharnais, J., Sonderegger, C., Maslyar, D.J., Goldberg, J., et al., 2002. Small-molecule antagonists of Myc/Max dimerization inhibit Myc-induced transformation of chicken embryo fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 99 (6), 3830–3835. https://doi.org/10.1073/pnas.062036999.

Bhadury, J., Nilsson, L.M., Muralidharan, S.V., Green, L.C., Li, Z., Gesner, E.M., et al., 2014. BET and HDAC inhibitors induce similar genes and biological effects and synergize to kill in Myc-induced murine lymphoma. Proceedings of the National Academy of Sciences of the United States of America 111 (26), E2721–E2730. https://doi.org/10.1073/pnas.1406722111.

Blackwell, T.K., Huang, J., Ma, A., Kretzner, L., Alt, F.W., Eisenman, R.N., Weintraub, H., 1993. Binding of Myc proteins to canonical and noncanonical DNA sequences. Molecular and Cellular Biology 13 (9), 5216–5224.

Borestrom, C., Simonsson, S., Enochson, L., Bigdeli, N., Brantsing, C., Ellerstrom, C., et al., 2014. Footprint-free human induced pluripotent stem cells from articular cartilage with redifferentiation capacity: A first step toward a clinical-grade cell source. Stem Cells Translational Medicine 3 (4), 433–447. https://doi.org/10.5966/sctm.2013-0138.

Boumahdi, S., Driessens, G., Lapouge, G., Rorive, S., Nassar, D., Le Mercier, M., et al., 2014. Sox2 controls tumor initiation and cancer stem-cell functions in squamous-cell carcinoma. Nature 511 (7508), 246–250. https://doi.org/10.1038/nature13305.

Bres, V., Yoh, S.M., Jones, K.A., 2008. The multi-tasking P-TEFb complex. Current Opinion in Cell Biology 20 (3), 334–340. https://doi.org/10.1016/j.ceb.2008.04.008.

Brough, D.E., Hofmann, T.J., Ellwood, K.B., Townley, R.A., Cole, M.D., 1995. An essential domain of the c-Myc protein interacts with a nuclear factor that is also required for E1A-mediated transformation. Molecular and Cellular Biology 15 (3), 1536–1544.

Byrne, J.A., Simonsson, S., Western, P.S., Gurdon, J.B., 2003. Nuclei of adult mammalian somatic cells are directly reprogrammed to Oct-4 stem cell gene expression by amphibian oocytes. Current Biology 13 (14), 1206–1213.

Chen, X., Whitney, E.M., Gao, S.Y., Yang, V.W., 2003. Transcriptional profiling of Krüppel-like factor 4 reveals a function in cell cycle regulation and epithelial differentiation. Journal of Molecular Biology 326, 665–677.

Cheng, L., Thomas, A., Roth, L.M., Zheng, W., Michael, H., Karim, F.W., 2004. OCT4: A novel biomarker for dysgerminoma of the ovary. The American Journal of Surgical Pathology 28 (10), 1341–1346.

Claveria, C., Giovinazzo, G., Sierra, R., Torres, M., 2013. Myc-driven endogenous cell competition in the early mammalian embryo. Nature 500 (7460), 39–44. https://doi.org/10.1038/nature12389.

Dai, X., Lau, P., Lau, A.W., Liu, Y., Inuzuka, H., 2014. Acetylation-dependent regulation of essential iPS-inducing factors: A regulatory crossroad for pluripotency and tumorigenesis. Cancer Medicine 3 (5), 1211–1224. https://doi.org/10.1002/cam4.298.

Dang, D.T., Chen, X., Feng, J., Torbenson, M., Dang, L.H., Yang, V.W., 2003. Overexpression of Kruppel-like factor 4 in the human colon cancer cell line RKO leads to reduced tumorigenecity. Oncogene 22, 3424–3430.

Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R., Bradley, A., 1993. A null c-Myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes & Development 7 (4), 671–682.

Diaz-Diaz, C., Fernandez de Manuel, L., Jimenez-Carretero, D., Montoya, M.C., Claveria, C., Torres, M., 2017. Pluripotency surveillance by Myc-driven competitive elimination of differentiating cells. Developmental Cell 42 (6), 585–599 e584. https://doi.org/10.1016/j.devcel.2017.08.011.

Dong, C., Wilhelm, D., Koopman, P., 2004. Sox genes and cancer. Cytogenetic and Genome Research 105 (2–4), 442–447. https://doi.org/10.1159/000078217.

Drakulic, D., Vicentic, J.M., Schwirtlich, M., Tosic, J., Krstic, A., Klajn, A., Stevanovic, M., 2015. The overexpression of Sox2 affects the migration of human teratocarcinoma cell line NT2/D1. Anais da Academia Brasileira de Ciências 87 (1), 389–404. https://doi.org/10.1590/0001-3765201520140352.

Ema, M., Mori, D., Niwa, H., Hasegawa, Y., Yamanaka, Y., Hitoshi, S., et al., 2008. Kruppel-like factor 5 is essential for blastocyst development and the normal self-renewal of mouse ESCs. Cell Stem Cell 3 (5), 555–567. https://doi.org/10.1016/j.stem.2008.09.003.

Fogarty, N.M.E., McCarthy, A., Snijders, K.E., Powell, B.E., Kubikova, N., Blakeley, P., et al., 2017. Genome editing reveals a role for OCT4 in human embryogenesis. Nature. https://doi.org/10.1038/nature24033.

Foster, K.W., Frost, A.R., McKie-Bell, P., Lin, C.-Y., Engler, J.A., Grizzle, W.E., Ruppert, J.M., 2000. Increase of GKLF messenger RNA and protein expression during progression of breast cancer. Cancer Research 60, 6488–6495.

Gargano, B., Amente, S., Majello, B., Lania, L., 2007. P-TEFb is a crucial co-factor for Myc transactivation. Cell Cycle 6 (16), 2031–2037.
Ghaleb, A.M., Katz, J.P., Kaestner, K.H., Du, J.X., Yang, V.W., 2007. Kruppel-like factor 4 exhibits antiapoptotic activity following gamma-radiation-induced DNA damage. Oncogene 26 (16), 2365–2373.
Gomez-Roman, N., Grandori, C., Eisenman, R.N., White, R.J., 2003. Direct activation of RNA polymerase III transcription by c-Myc. Nature 421 (6920), 290–294. https://doi.org/10.1038/nature01327.
Gontan, C., de Munck, A., Vermeij, M., Grosveld, F., Tibboel, D., Rottier, R., 2008. Sox2 is important for two crucial processes in lung development: Branching morphogenesis and epithelial cell differentiation. Developmental Biology 317 (1), 296–309. https://doi.org/10.1016/j.ydbio.2008.02.035.
Graham, V., Khudyakov, J., Ellis, P., Pevny, L., 2003. Sox2 functions to maintain neural progenitor identity. Neuron 39 (5), 749–765.
Gurdon, J.B., 1962. Adult frogs derived from the nuclei of single somatic cells. Developmental Biology 4, 256–273.
Hahn, W.C., Counter, C.M., Lundburg, A.S., Beijersbergen, R.L., Brooks, M.W., Weinberg, R.A., 1999. Creation of human tumor cells with defined genetic elements. Nature 400, 464–468.
Hann, S.R., Eisenman, R.N., 1984. Proteins encoded by the human c-Myc oncogene: Differential expression in neoplastic cells. Molecular and Cellular Biology 4 (11), 2486–2497.
Hansis, C., Grifo, J.A., Krey, L.C., 2000. Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. Molecular Human Reproduction 6 (11), 999–1004.
Herkert, B., Eilers, M., 2010. Transcriptional repression: The dark side of Myc. Genes & Cancer 1 (6), 580–586. https://doi.org/10.1177/1947601910379012.
Hishida, T., Nozaki, Y., Nakachi, Y., Mizuno, Y., Okazaki, Y., Ema, M., et al., 2011. Indefinite self-renewal of ESCs through Myc/Max transcriptional complex-independent mechanisms. Cell Stem Cell 9 (1), 37–49. https://doi.org/10.1016/j.stem.2011.04.020.
Hopewell, R., Ziff, E.B., 1995. The nerve growth factor-responsive PC12 cell line does not express the Myc dimerization partner Max. Molecular and Cellular Biology 15 (7), 3470–3478.
Hussenet, T., Dali, S., Exinger, J., Monga, B., Jost, B., Dembele, D., et al., 2010. Sox2 is an oncogene activated by recurrent 3q26.3 amplifications in human lung squamous cell carcinomas. PLoS One 5 (1), e8960. https://doi.org/10.1371/journal.pone.0008960.
James, L., Eisenman, R.N., 2002. Myc and Mad bHLHZ domains possess identical DNA-binding specificities but only partially overlapping functions in vivo. Proceedings of the National Academy of Sciences of the United States of America 99 (16), 10429–10434. https://doi.org/10.1073/pnas.162369299.
Jha, S., Dutta, A., 2009. RVB1/RVB2: Running rings around molecular biology. Molecular Cell 34 (5), 521–533. https://doi.org/10.1016/j.molcel.2009.05.016.
Jones, T.D., Ulbright, T.M., Eble, J.N., Baldridge, L.A., Cheng, L., 2004. OCT4 staining in testicular tumors: A sensitive and specific marker for seminoma and embryonal carcinoma. The American Journal of Surgical Pathology 28 (7), 935–940.
Jones, T.D., Wang, M., Sung, M.T., Zhang, S., Ulbright, T.M., Eble, J.N., et al., 2006. Clonal origin of metastatic testicular teratomas. Clinical Cancer Research 12 (18), 5377–5383. https://doi.org/10.1158/1078-0432.CCR-06-0444.
Kanazawa, S., Soucek, L., Evan, G., Okamoto, T., Peterlin, B.M., 2003. c-Myc recruits P-TEFb for transcription, cellular proliferation and apoptosis. Oncogene 22 (36), 5707–5711. https://doi.org/10.1038/sj.onc.1206800.
Kim, J., Zaret, K.S., 2015. Reprogramming of human cancer cells to pluripotency for models of cancer progression. The EMBO Journal 34 (6), 739–747. https://doi.org/10.15252/embj.201490736.
Kim, J., Chu, J., Shen, X., Wang, J., Orkin, S.H., 2008. An extended transcriptional network for pluripotency of embryonic stem cells. Cell (6), 1049–1061.
Kopp, J.L., Ormsbee, B.D., Desler, M., Rizzino, A., 2008. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells 26 (4), 903–911. https://doi.org/10.1634/stemcells.2007-0951.
Lee, S.H., Oh, S.Y., Do, S.I., Lee, H.J., Kang, H.J., Rho, Y.S., et al., 2014. Sox2 regulates self-renewal and tumorigenicity of stem-like cells of head and neck squamous cell carcinoma. British Journal of Cancer 111 (11), 2122–2130. https://doi.org/10.1038/bjc.2014.528.
Leis, O., Eguiara, A., Lopez-Arribillaga, E., Alberdi, M.J., Hernandez-Garcia, S., Elorriaga, K., et al., 2012. Sox2 expression in breast tumors and activation in breast cancer stem cells. Oncogene 31 (11), 1354–1365. https://doi.org/10.1038/onc.2011.338.
Li, Y., McClintick, J., Zhong, L., Edenberg, H.J., Yoder, M.C., Chan, R.J., 2005. Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. Blood 105, 635–637.
Liu, X., Huang, J., Chen, T., Wang, Y., Xin, S., Li, J., et al., 2008. Yamanaka factors critically regulate the developmental signaling network in mouse embryonic stem cells. Cell Research 18 (12), 1177–1189. https://doi.org/10.1038/cr.2008.309.
Lorenzin, F., Benary, U., Baluapuri, A., Walz, S., Jung, L.A., von Eyss, B., et al., 2016. Different promoter affinities account for specificity in Myc-dependent gene regulation. eLife 5. https://doi.org/10.7554/eLife.15161.
Lu, Y., Futtner, C., Rock, J.R., Xu, X., Whitworth, W., Hogan, B.L.M., Onaitis, M.W., 2010. Evidence that Sox2 overexpression is oncogenic in the lung. PLoS One 5 (6), e11022. https://doi.org/10.1371/journal.pone.0011022.
Ma, A., Moroy, T., Collum, R., Weintraub, H., Alt, F.W., Blackwell, T.K., 1993. DNA binding by N- and L-Myc proteins. Oncogene 8 (4), 1093–1098.
Marks, H., Kalkan, T., Menafra, R., Denissov, S., Jones, K., Hofemeister, H., et al., 2012. The transcriptional and epigenomic foundations of ground state pluripotency. Cell 149 (3), 590–604. https://doi.org/10.1016/j.cell.2012.03.026.
Mateyak, M.K., Obaya, A.J., Adachi, S., Sedivy, J.M., 1997. Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth & Differentiation 8 (10), 1039–1048.
McConnell, B.B., Yang, V.W., 2010. Mammalian Kruppel-like factors in health and diseases. Physiological Reviews 90 (4), 1337–1381. https://doi.org/10.1152/physrev.00058.2009.
McKeown, M.R., Bradner, J.E., 2014. Therapeutic strategies to inhibit Myc. Cold Spring Harbor Perspectives in Medicine 4 (10). https://doi.org/10.1101/cshperspect.a014266.
McMahon, S.B., Van Buskirk, H.A., Dugan, K.A., Copeland, T.D., Cole, M.D., 1998. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 94 (3), 363–374.
Müller, M., Liebau, H.P.C.S., Weidgang, C., Seufferlein, T., Kleger, A., Perkhofer, L., 2016. The role of pluripotency factors to drive stemness in gastrointestinal cancer. Stem Cell Research 16, 349–357. https://doi.org/10.1016/j.scr.2016.02.005.
Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., et al., 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nature Biotechnology 26 (1), 101–106. https://doi.org/10.1038/nbt1374.
Nakagawa, M., Takizawa, N., Narita, M., Ichisaka, T., Yamanaka, S., 2010. Promotion of direct reprogramming by transformation-deficient Myc. Proceedings of the National Academy of Sciences of the United States of America 107 (32), 14152–14157. https://doi.org/10.1073/pnas.1009374107.
Narsinh, K.H., Plews, J., Wu, J.C., 2011. Comparison of human induced pluripotent and embryonic stem cells: Fraternal or identical twins? Molecular Therapy 19, 635–638.
Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., et al., 1985. L-Myc, a new Myc-related gene amplified and expressed in human small cell lung cancer. Nature 318 (6041), 69–73.
Nguyen, D., Hagg, D.A., Forsman, A., Ekholm, J., Nimkingratana, P., Brantsing, C., et al., 2017. Cartilage tissue engineering by the 3D bioprinting of iPS cells in a Nanocellulose/alginate bioink. Scientific Reports 7, 658.
Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., et al., 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95 (3), 379–391.
Nilsson, J.A., Cleveland, J.L., 2003. Myc pathways provoking cell suicide and cancer. Oncogene 22 (56), 9007–9021. https://doi.org/10.1038/sj.onc.1207261.
Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M., Hamada, H., 1990. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 60 (3), 461–472.

Patel, M., Yang, S., 2010. Advances in reprogramming somatic cells to induced pluripotent stem cells. Stem Cell Reviews 6 (3), 367–380. https://doi.org/10.1007/s12015-010-9123-8.
Ramos-Mejía, V., Fraga, M.F., Menendez, P., 2012. iPS cells from cancer cells: Challenges and opportunities. Trends in Molecular Medicine 18 (5), 245–247.
Rizzino, A., 2009. Sox2 and Oct-3/4: A versatile pair of master regulators that orchestrate the self-renewal and pluripotency of embryonic stem cells. Wiley Interdisciplinary Reviews. Systems Biology and Medicine 1 (2), 228–236. https://doi.org/10.1002/wsbm.12.
Rony, I.K., Baten, A., Bloomfield, J.A., Islam, M.E., Billah, M.M., Islam, K.D., 2015. Inducing pluripotency in vitro: Recent advances and highlights in induced pluripotent stem cells generation and pluripotency reprogramming. Cell Proliferation 48, 140–156.145.
Rosner, M.H., Vigano, M.A., Ozato, K., Timmons, P.M., Poirier, F., Rigby, P.W.J., Staudt, L.M., 1990. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 345 (6277), 686–692. https://doi.org/10.1038/345686a0.
Roussel, M., Saule, S., Lagrou, C., Rommens, C., Beug, H., Graf, T., Stehelin, D., 1979. Three new types of viral oncogene of cellular origin specific for hematopoietic cell transformation. Nature 281 (5731), 452–455.
Rowland, B.D., Peeper, D.S., 2006. KLF4, p21 and context-dependent opposing forces in cancer. Nature Reviews. Cancer 6, 11–23.
Russo, M.V., Esposito, S., Tupone, M.G., Manzoli, L., Airoldi, I., Pompa, P., et al., 2016. Sox2 boosts major tumor progression genes in prostate cancer and is a functional biomarker of lymph node metastasis. Oncotarget 7 (11), 12372–12385. https://doi.org/10.18632/oncotarget.6029.
Sabo, A., Amati, B., 2014. Genome recognition by Myc. Cold Spring Harbor Perspectives in Medicine 4 (2). https://doi.org/10.1101/cshperspect.a014191.
Sabo, A., Kress, T.R., Pelizzola, M., de Pretis, S., Gorski, M.M., Tesi, A., et al., 2014. Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis. Nature 511 (7510), 488–492. https://doi.org/10.1038/nature13537.
Savino, M., Annibali, D., Carucci, N., Favuzzi, E., Cole, M.D., Evan, G.I., et al., 2011. The action mechanism of the Myc inhibitor termed Omomyc may give clues on how to target Myc for cancer therapy. PLoS One 6 (7), e22284. https://doi.org/10.1371/journal.pone.0022284.
Sayed, N., Chun, L.J.C.W., 2016. Translation of human-induced pluripotent stem cells from clinical trial in a dish to precision medicine. Journal of the American College of Cardiology 67, 2161–2176.
Scholer, H.R., Dressler, G.R., Balling, R., Rohdewohld, H., Gruss, P., 1990. Oct-4: A germline-specific transcription factor mapping to the mouse t-complex. The EMBO Journal 9 (7), 2185–2195.
Scholer, H.R., Ruppert, S., Suzuki, N., Chowdhury, K., Gruss, P., 1990. New type of POU domain in germ line-specific protein Oct-4. Nature 344 (6265), 435–439. https://doi.org/10.1038/344435a0.
Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., et al., 1983. Amplified DNA with limited homology to Myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. Nature 305 (5931), 245–248.
Scognamiglio, R., Cabezas-Wallscheid, N., Thier, M.C., Altamura, S., Reyes, A., Prendergast, A.M., et al., 2016. Myc depletion induces a pluripotent dormant state mimicking diapause. Cell 164 (4), 668–680. https://doi.org/10.1016/j.cell.2015.12.033.
Sheiness, D., Bishop, J.M., 1979. DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. Journal of Virology 31 (2), 514–521.
Sheiness, D.K., Hughes, S.H., Varmus, H.E., Stubblefield, E., Bishop, J.M., 1980. The vertebrate homolog of the putative transforming gene of avian myelocytomatosis virus: Characteristics of the DNA locus and its RNA transcript. Virology 105 (2), 415–424.
Simonsson, S., Gurdon, J., 2004. DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nature Cell Biology 6 (10), 984–990. https://doi.org/10.1038/ncb1176.
Simonsson, S., Svensson, F., Odén, A., Simonsson, T., 2014. The embryonic stem cell protein Oct4 may be a prognostic variable for death from ovarian cancer. Trends in Cancer Research 10, 79–84.
Singh, V.K., Kalsan, M., Kumar, N., Saini, A., Chandra, R., 2015. Induced pluripotent stem cells: Applications in regenerative medicine, disease modeling, and drug discovery. Frontiers in Cell and Development Biology 3 (2).
Smith, A.G., 2001. Embryo-derived stem cells: Of mice and men. Annual Review of Cell and Developmental Biology 17, 435–462. https://doi.org/10.1146/annurev.cellbio.17.1.435.
Soucek, L., Evan, G.I., 2010. The ups and downs of Myc biology. Current Opinion in Genetics & Development 20 (1), 91–95. https://doi.org/10.1016/j.gde.2009.11.001.
Soucek, L., Whitfield, J., Martins, C.P., Finch, A.J., Murphy, D.J., Sodir, N.M., et al., 2008. Modeling Myc inhibition as a cancer therapy. Nature 455 (7213), 679–683. https://doi.org/10.1038/nature07260.
Soucek, L., Whitfield, J.R., Sodir, N.M., Masso-Valles, D., Serrano, E., Karnezis, A.N., et al., 2013. Inhibition of Myc family proteins eradicates KRas-driven lung cancer in mice. Genes & Development 27 (5), 504–513. https://doi.org/10.1101/gad.205542.112.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861–872. https://doi.org/10.1016/j.cell.2007.11.019.
Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126 (4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024.
Takahashi, K., Yamanaka, S., 2016. A decade of transcription factor-mediated reprogramming to pluripotency. Nature Reviews. Molecular Cell Biology 17 (3), 183–193. https://doi.org/10.1038/nrm.2016.8.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell (5), 861–872.
Tokuzawa, Y., Kaiho, E., Maruyama, M., Takahashi, K., Mitsui, K., Maeda, M., Niwa, H., Yamanaka, S., 2003. Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Molecular and Cellular Biology 23 (8), 2699–2708.
Tomioka, M., Nishimoto, M., Miyagi, S., Katayanagi, T., Fukui, N., Niwa, H., et al., 2002. Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. Nucleic Acids Research 30 (14), 3202–3213.
Wang, Q., He, W., Lu, C., Wang, Z., Wang, J., Giercksky, K.E., et al., 2009. Oct3/4 and Sox2 are significantly associated with an unfavorable clinical outcome in human esophageal squamous cell carcinoma. Anticancer Research 29 (4), 1233–1241.
Wang, Y., Zhou, J., Wang, Z., Wang, P., Li, S., 2017. Upregulation of Sox2 activated LncRNA PVT1 expression promotes breast cancer cell growth and invasion. Biochemical and Biophysical Research Communications. https://doi.org/10.1016/j.bbrc.2017.09.005.
Welcker, M., Orian, A., Grim, J.A., Eisenman, R.N., Clurman, B.E., 2004. A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. Current Biology 14 (20), 1852–1857. https://doi.org/10.1016/j.cub.2004.09.083.
Welcker, M., Orian, A., Jin, J., Grim, J.E., Harper, J.W., Eisenman, R.N., Clurman, B.E., 2004. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proceedings of the National Academy of Sciences of the United States of America 101 (24), 9085–9090. https://doi.org/10.1073/pnas.0402770101.
Wen, Y., Hou, Y., Huang, Z., Cai, J., Wang, Z., 2017. Sox2 is required to maintain cancer stem cells in ovarian cancer. Cancer Science 108 (4), 719–731. https://doi.org/10.1111/cas.13186.
Wood, M.A., McMahon, S.B., Cole, M.D., 2000. An ATPase/helicase complex is an essential cofactor for oncogenic transformation by c-Myc. Molecular Cell 5 (2), 321–330.
Wuebben, E.L., Rizzino, A., 2017. The dark side of Sox2: Cancer—A comprehensive overview. Oncotarget 8 (27), 44917–44943. https://doi.org/10.18632/oncotarget.16570.
Wuebben, E.L., Wilder, P.J., Cox, J.L., Grunkemeyer, J.A., Caffrey, T., Hollingsworth, M.A., Rizzino, A., 2016. Sox2 functions as a molecular rheostat to control the growth, tumorigenicity and drug responses of pancreatic ductal adenocarcinoma cells. Oncotarget 7 (23), 34890–34906. https://doi.org/10.18632/oncotarget.8994.

Yamanaka, S., Takahashi, K., 2006. Induction of pluripotent stem cells from mouse fibroblast cultures. Tanpakushitsu Kakusan Koso 51 (15), 2346–2351.
Yin, X., Giap, C., Lazo, J.S., Prochownik, E.V., 2003. Low molecular weight inhibitors of Myc–Max interaction and function. Oncogene 22 (40), 6151–6159. https://doi.org/10.1038/sj.onc.1206641.
Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., et al., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318 (5858), 1917–1920. https://doi.org/10.1126/science.1151526.
Zhang, S., Cui, W., 2014. Sox2, a key factor in the regulation of pluripotency and neural differentiation. World Journal of Stem Cells 6 (3), 305–311. https://doi.org/10.4252/wjsc.v6.i3.305.
Zhao, W., Jin, X., Cong, Y., Liu, Y., Fu, J., 2013. Degradable natural polymer hydrogels for articular cartilage tissue engineering. Journal of Chemical Technology and Biotechnology 88, 327–339.
Zhu, S., Li, W., Zhou, H., Wei, W., Ambasudhan, R., Lin, T., et al., 2010. Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7 (6), 651–655. https://doi.org/10.1016/j.stem.2010.11.015.

Inhibitors of Lactate Transport: A Promising Approach in Cancer Drug Discovery

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Glossary

α-CHC *alpha* cyano-4-hydroxycinnamic acid, a tool compound MCT inhibitor.
ALL Acute lymphoblastic leukemia.
ASCT2 The glutamine transporter SLC1A5.
CD147 Cluster of differentiation 147, chaperone protein for MCTs.
CLL Chronic lymphocytic leukemia.
CML Chronic myeloid leukemia.
DIDS 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid.
GADP Glyceraldehyde 3-phosphate.
GADPH Glyceraldehyde 3-phosphate dehydrogenase.
GLS1 Glutaminase.
HIF1-α Hypoxia-inducible factor 1-*alpha*, a transcription factor.
LAT1 Large neutral amino acid transporter.
LDH Lactate dehydrogenase, family of enzymes that interconvert lactate and pyruvate.
MCF7 The cancer cell line Michigan Cancer Foundation-7.
MCT Monocarboxylate transporter.
MCT1 (aka SLC16A1) Isoform 1 in the MCT superfamily.
MCT2 (aka SLC16A7) Isoform 2 in the MCT superfamily.
MCT3 (aka SLC16A8) Isoform 3 in the MCT superfamily.
MCT4 (aka SLC16A3) Isoform 4 in the MCT superfamily.
NAD Nicotinamide adenine dinucleotide.
NHL Non-Hodgkin's lymphoma.
pCMBS *para*-chloromercuribenzene sulfonate, a tool compound MCT inhibitor.
PET Positron emission tomography.
PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase.
SLC1A5 A glutamine transporter ASCT2.

Introduction

Altered or deregulated energy metabolism has been widely recognized as a defining characteristic of cancer cells ever since the pioneering work in the 1920s through the 1950s by the German biochemist Otto Warburg. The cancer cell metabolic phenotype arises largely by a preferential dependence upon glycolysis rather than oxidative phosphorylation for energy production, regardless of the availability of oxygen (a reliance termed "the Warburg effect"). The defining components of the Warburg effect are increased glucose consumption, decreased oxidative phosphorylation, and greatly increased lactate production by tumor cells. Here we focus on the roles of lactate in cancer, specifically its transport and utilization.

Why tumor cells rely on glycolysis (i.e., what is the evolutionary advantage gained) has long been a subject of great discussion and debate. Another controversial issue is whether the reliance upon glycolysis is not merely a defining characteristic but rather provides a causal relationship for cancer progression. Recently it has become widely accepted that the glycolytic phenotype does indeed drive both tumorigenesis and cancer progression. Since glycolysis converts glucose into pyruvate and ultimately produces lactate, several biochemical pathways cooperatively maintain the cancer cell metabolic phenotype, including: (1) those that permit increased uptake of nutrient inputs, such as glucose, glutamine, and other amino acids, (2) those that make efficient use of the metabolic intermediates of glycolysis for conducting essential cellular processes, or (3) those that permit lactate utilization and removal. Conversely, targeting glycolysis at various checkpoints are attractive general strategies for cancer treatment. Blocking the influx of nutrients that drive anabolic growth, inhibiting glycolysis pathway enzymes (e.g., lactate dehydrogenase, isocitrate dehydrogenase), and impeding lactate active transport and/or utilization are all areas of very active interest for the development of anticancer therapeutic agents.

High-value biological targets, whether specific proteins or entire pathways, are those that are differentially activated in cancer cells versus normal cells. Targeting cell division processes, for example, historically drove the development of traditional chemotherapy drugs and drug cocktails, relying on a rapid rate of cell growth to achieve a useful therapeutic index. More recently the trend

had been to target proteins and pathways that are more highly specific to cancer cells, defining at the molecular level the characteristics that constitute a unique biochemical fingerprint reflecting one or more of the hallmarks of cancer. Such high-value targets offer the promise of avoiding mechanism-based side effects that limit the usefulness of traditional chemotherapy treatment regimens.

Advances in genomic and metabolomic technologies have helped identify various key proteins and regulatory transcription factors that maintain the glycolytic phenotype and that confer selective advantages to cancer cells, including an uninterrupted growth potential and a high capacity for the evolution of drug resistance. This marks them as potential high-value cancer targets. Protein kinase B (aka AKT kinase), as an example, drives increased glucose uptake. Hypoxia-inducible factor 1-alpha (HIF1α) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) are proteins that drive the metabolic reprogramming of cancer cells to rely upon glycolysis. The powerful and oft-mutated oncogene RAS has multiple essential roles supporting the glycolytic phenotype. The transcription factors perhaps most relevant to the maintenance of the glycolytic phenotype are the MYC oncoproteins, which are known to directly activate the expression of two isoforms of lactate transporters, which are monocarboxylate transporters known as MCT1 (aka SLC16A1) and MCT4 (aka SLC16A3). MYC also drives the expression of several metabolic genes, including proteins that are involved in glutamine catabolism (e.g., the glutamine transporter SLC1A5 [aka ASCT2] and glutaminase GLS1) and proteins that are responsible for glucose transport. MYC also regulates the biosynthesis of lipids, purines, and pyrimidines. MYC overexpression is sufficient to promote tumorigenesis in mouse models. In humans, high MYC expression levels correlate with aggressive tumor growth and with poor treatment prognosis. Direct inhibition of MYC function by small molecules or by antibodies has often extensively investigated, but to date no MYC inhibitors have translated to clinical use. This is perhaps due to the considerable number of proteins that are unrelated to tumor growth that are also regulated by MYC. Direct inhibition of such an essential transcription factor seems to be an approach that would offer serious complications with respect to therapeutic index. Alternatively, a more attractive means of targeting MYC would be to identify the specific MYC targets that are both necessary and sufficient for driving tumor growth, such as the monocarboxylate transporters (MCTs).

Because cancer cells convert most available glucose into pyruvate and then to lactate, the issue of dealing with the high rate of lactate production, termed the lactate dilemma, is critical for cancer cell survival. An intracellular build-up of lactate would result in cytoplasmic and nuclear pH lowering, adversely affecting a multitude of pH-dependent processes that are involved in cellular homeostasis and cell division. Thus, the active efflux of lactate from intracellular stores to the extracellular matrix is essential for cancer cell survival. Measuring extracellular lactate levels, or as a surrogate, measuring the pH of the tumor's exterior microenvironment, are means to ascertain the glycolytic state of the tumor cells. A lowered extracellular pH, even as low as 6.0, is reflective of increased lactate export from malignant cells by monocarboxylate transporters. While lactate can be considered to be the waste product of energy production via glycolysis, lactate serves other functions that apparently aid tumor survival. The lowered extracellular pH, for example, facilitates the loss of cellular adhesion, thereby aiding tumor migration. The decreased pH also facilitates tumor invasiveness. More recently it has become clear that certain tumor types shift from glycolysis to oxidative phosphorylation, opportunistically using excess lactate generated from glycolysis as a carbon source for energy production through the TCA cycle. For example, when human non small cell lung cancer (NSCLC) patients were infused with ^{13}C-lactate, extensive labeling of TCA cycle metabolites was noted. Surprisingly, lactate's contribution to the TCA cycle is greater than that of glucose in these tumors.

Lactate homeostasis is critical for maintaining the malignant state, either blocking lactate production or inhibiting lactate transport can rapidly compromise the viability of established tumors, stunting growth, preventing metastases, and even eradicating established tumors. As discussed above, in principle all of the glycolytic pathway enzymes are viable targets to impede glycolysis and hence to interrupt lactate production. Many of these targets have been studied and their detailed discussion is beyond the scope of this synopsis, though certain high-value targets are discussed below. The main focus here is on the possibility of controlling the fate of lactate after its production, however. In particular, the strategy of interrupting lactate transport is shown to have significant promise. Such an approach both alters pH homeostasis and precludes the possibility of lactate utilization in the TCA cycle after it is exported. We discuss the therapeutic opportunities, the progress made, and the future translational potential for lactate transport inhibition.

Growth arrest following lactate transport inhibition arises from drastically reducing the internal rather than the external pH of affected cells. Lactate transport inhibitors should also be chemopreventative, since the glycolytic phenotype is manifest even in premalignant cells, as demonstrated for EμMyc premalignant B cells and for many lymphoma cell lines, which show increased expression levels of MCTs prior to their transformation. The transformed cells, in turn, express even higher levels of MCTs. Inhibitors of MCT1 and/or MCT4 should have efficacy as single agents in cells highly expressing that specific transporter. Perhaps more importantly, at least in a clinical setting, MCT inhibitors should augment the efficacy of many other agents, including those that target the glycolytic pathway by other mechanisms, and moreover they should have limited effects on nontransformed cells, which rarely rely upon glycolysis for energy production.

Related Targets in the Glycolytic Pathway

Because lactate is produced from pyruvate as the end product of glycolysis, blocking glucose transporters or blocking any step of glycolysis by inhibiting any of the glycolytic enzymes can be considered de facto means of disrupting lactate homeostasis. Glycolysis enzymes upstream of pyruvate production include hexokinase, phosphoglucose isomerase, phosphofructokinase, fructose 1,6-biphosphatase, aldolase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate mutase, enolase, pyruvate kinase, and the lactate dehydrogenase (LDH), which is a family of enzymes that interconvert pyruvate and lactate, requiring a nicotinamide

cofactor. This step in glycolysis is critical for the recycling of nicotinamide for the continuation of glycolysis, as NAD+ is required in the conversion of glyceraldehyde 3-phosphate (GADP) to D-1,3-bisphosphoglycerate by glyceraldehyde phosphate dehydrogenase (GAPDH). Lactate dehydrogenase plays a key direct role in lactate homeostasis and the reader is directed to reviews describing anticancer efforts relying on selective (ideally) inhibition of the LDH5 isoform. LDH5 has proven to be a difficult target due to the polar and charged nature of its native substrates, pyruvate and lactate. Compounds binding at or near the substrate binding pocket tend to be very polar in nature and thus tend to lack favorable drug-like properties. LDH and many glycolytic pathway enzymes exist in multiple isoforms, some specifically upregulated in various cancers and that thus have been actively targeted in anticancer therapeutics development efforts. The discussion of targeting glycolysis enzymes and LDH is beyond the scope of this review, which rather focuses specifically upon lactate transport.

MCT1 and MCT4: CD147-Associated Proteins That Transport Lactate in Cancer Cells

The SLC16A family of proteins, termed the monocarboxylate transporters (MCTs), are 12-membrane pass cell surface proteins. While 14 MCT isoforms are known, only the MCT1, MCT2, MCT3, and MCT4 isoforms transport lactate. In humans, this transport across plasma membranes is bidirectional and is a proton-linked exchange. Expression profiling studies show that many aggressive tumor types, particularly those linked with activation of the oncogenic transcription factor MYC but also those with elevated expression levels of RAS, PI3K, and HIF-1α, express markedly elevated levels of the MCT1 isoform, the MCT4 isoform, or both.

Fig. 1 shows a modeled structure of the open conformation of the MCT1 transporter (blue and red) shown associated with the type I integral membrane receptor "cluster of differentiation 147" (aka CD147), shown in green. CD147 is a highly glycosylated chaperone protein that is also widely known as basigin or as the extracellular matrix metalloproteinase inducer (EMMPRIN). CD147 is required for proper localization of MCTs in the membrane. In this depiction, MCT1 is bound by the small molecule DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate), which is a strong electrophile and covalently binds to a lysine residue of MCT1.

Directly targeting the chaperone, CD147, rather than MCT1 itself with small molecule inhibitors or with antibodies is a potentially viable strategy for disrupting lactate homeostasis and therefore is an area of high interest for therapeutics development. Agents that target CD147 would compromise the activity of both MCT1 and MCT4 transporters. This would have the advantage of decreasing the viability of: (A) tumors that express both MCT1 and MCT4; (B) tumors that normally express MCT1 but that, under evolutionary pressure, are capable of upregulating MCT4 expression, and (C) tumors that normally express MCT4 but that are capable of upregulating MCT1 expression. The most significant disadvantage of targeting CD147 is perhaps that this protein has

Fig. 1 Calculated molecular structure of the lactate transporter MCT1. From Halestrap and Meredith (2004). *European Journal of Physiology*, **447**, 619–628.

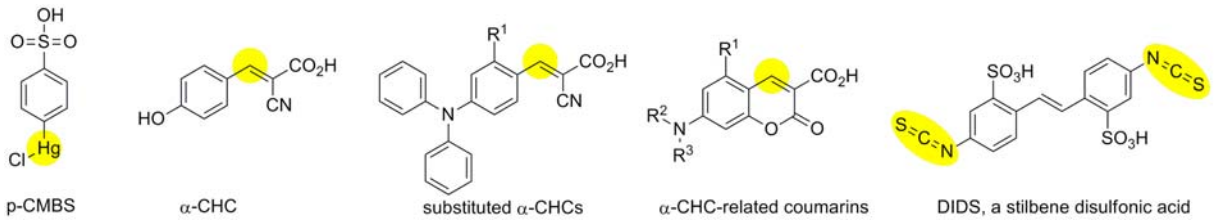

Fig. 2 Purported MCT inhibitors that are cysteine-reactive and that may target CD147 (reactive sites are highlighted in yellow).

a wide variety of biological functions beyond serving as a chaperone for MCTS, functions that are important to nonmalignant cells. As an example, CD147 forms complexes with various matrix metalloproteases. Thus, the blockade of CD147 function has the potential to elicit many dose-limiting side effects, giving a diminished therapeutic index. Perhaps for this reason, a more popular approach has been to directly target MCTs rather than CD147.

Many compounds that have been reported to be MCT inhibitors, especially those that simultaneously inhibit multiple MCTs, apparently interact with CD147 rather than with the associated MCT. This effect was first demonstrated with the organomercurial compound *para*-chloromercuribenzene sulfonate (pCMBS) (see Fig. 2). This compound is highly electrophilic: the mercury atom (highlighted in yellow) binds to sulfhydryl groups on accessible cysteine side chains in proteins. CD147 has several cysteine residues and in a genetic mutation study it was shown that these cysteines, and not those on MCTS, are responsible for the sensitivity of CD147/MCT complexes to pCMBS. Other electrophilic species that are thought to be MCT inhibitors are also cysteine-reactive, typically as Michael acceptors, such as alpha cyano-4-hydroxycinnamic acid (α-CHC). This compound, and related species, can also covalently bind accessible cysteine residues, including those in CD147 (the reactive center is highlighted in yellow in Fig. 2).

Many biological studies have used reactive compounds, especially α-CHC, as tools for MCT inhibition, with implicit assumptions: (1) that the compound has high target specificity; and (2) that any resulting phenotype that results from administering the compound in vitro or even in vivo is driven specifically by MCT inhibition. Care should be taken to establish whether off-target effects could also be important, however, with such broadly reactive species. α-CHC, for example, is also a very potent inhibitor of the mitochondrial pyruvate carrier. Any purported MCT inhibitor that is electrophilic toward cysteine residues, whether reversible or irreversible, might target CD147 rather than the MCTs themselves. Such a compound may have limitations in therapeutic index because of CD147's many roles, as discussed above. It also may target many other cysteine-reactive members of the proteome at large, providing further concerns from a target selectivity and side effect perspective.

This class of electrophilic compounds (i.e., Michael acceptors, like α-CHC) includes potent purported dual MCT1/MCT4 inhibitors that are essentially more highly substituted analogs of α-CHC, as well as closely related coumarin analogs (Fig. 2). Certain compounds among these coumarin inhibitors have properties that appear to be unique among the reactive inhibitors, however. They impede lactate efflux but not lactate influx, even though the MCTs are bidirectional transporters. Thus, if they act by modifying cysteine residues of CD147, the modified complex oddly retains its lactate import function while losing its lactate export capabilities. Certain coumarins also impede MCT1 function to as greater extent than MCT4, suggesting that they either bind to MCT1 directly or that they differentially alter the function of MCT1/CD147 complexes relative to MCT4/CD147 complexes.

Another class of MCT inhibitors are the distilbene sulfonates, which bear two highly reactive isothiocyanate groups. This class of inhibitors is also capable of covalently binding accessible cysteine residues on CD147. However it is also known to be reactive toward lysine side chains, if they are proximally held through other protein–ligand interactions. The distilbene sulfonate DIDS (shown bound to MCT1 in Fig. 1) forms a covalent interaction with a specific lysine residue in MCT1. Unfortunately the high chemical reactivity of isothiocyanates precludes their use as drugs to target MCT1, since a variety of other protein targets would certainly also be covalently modified, conferring unacceptable side effects.

As discussed above, given CD147's many functions, reactive species binding to CD147 may have significant drawbacks. The argument for disfavoring reactive inhibitors in this instance in no way excludes cysteine-reactive species from being useful tool compounds or even drugs; indeed, many recent clinical kinase inhibitors include in their structures appended acrylamide groups that specifically target nearby cysteine residues, for example. Care must always be taken, however, to identify what members of the proteome are targeted by such molecules and, thereby, what therapeutic consequences may result that are unrelated to affecting the intended target. Thankfully recent advantages in proteomics has made target profiling of covalent modifiers trend toward becoming a routine process.

Selective MCT1 Inhibition in Cancer Therapy

For the target selectivity/therapeutic index reasons cited above, directly targeting MCT1, MCT4, or both, preferably with species that are generally unreactive toward other proteins, would seem to be preferable to targeting CD147. Of these modalities, selective MCT1 inhibition has been most extensively reported. Early reports of weak MCT1 inhibitors include the Fig. 2 inhibitors such as pCMBS and α-CHC, but also includes species that are not covalently reactive, including dietary flavonoids and related polyphenols such as phloretin. Such polyphenols have often proved difficult to optimize with regard to their potency and target specificity.

Much more potent MCT1 inhibitors that are also better-suited for therapeutic translation are the structurally related compounds 1–3 (Fig. 3), the pyrrolopyridazinones, fused uracil pyrroles, and fused uracil thiophenes from AstraZeneca. These compounds inhibit MCT1 but not MCT4. They also inhibit MCT2, though the inhibition of the MCT2 isoform should have a negligible effect upon antitumor efficacy, given that the MCT2 isoform has a lesser role than does MCT1 (and MCT4 as well) in human cancers, a finding based upon tumor expression studies for the MCT isoforms. It is apparently very difficult to separate inhibition of MCT1 from MCT2, because these two isoforms have very high sequence homology.

The initial AstraZeneca compounds 1–3 were not ideal for therapeutic translation due to their poor pharmacokinetic (PK) properties. Nevertheless, the probe compound 1 and related analogs have been shown to rapidly block tumor cell growth, leading to cell death ex vivo and to tumor regression in vivo, in the case of both Raji Burkitt lymphoma cells and in the case of estrogen receptor-positive MCF7 breast cancer cells, both of which highly express MCT1 and express very low or undetectable levels of MCT4. Knockdown of MCT1 mRNA and protein in MCF7 breast cancer cells using MCT1-selective siRNA was also shown to impair proliferation, indicating that the antiproliferative activity of the probe compound 1 is due to its on-target effects at MCT1.

The fused uracil thiophene series of probe compounds, related to structure 3, was amenable to further optimization in regions of the molecule where there were specific metabolic and physical property concerns, such as the metabolically labile thioether side chain and the highly lipophilic and extensively metabolized naphthyl group. Changes in these two specific regions of probe compound 3 led to the clinical compound AR-C155858. The changes that apparently improved PK properties are shown in green and in yellow in Fig. 3. Further minor alterations in structure in three areas (see pink shading, Fig. 3) then gave the clinical compound AZD3965, which replaced the previous candidate in 2013. Cancer Research UK has sponsored the clinical studies, which first have been focused on the treatment of advanced solid tumors that express MCT1. Communications from Cancer Research UK have indicated the potential for additional clinical studies versus MCT1-expressing lymphomas and also versus small cell lung cancer (SCLC).

Though the full clinical findings are yet unpublished, some reports of AZD3965's effects, both preclinically and clinically, have appeared. As mentioned, MCT1-expressing tumors are targeted for treatment. In animal models, significant levels of MCT4 expression is associated with the emergence of treatment resistance. The use of MCT4 RNA interference agents in such resistant tumor cell lines restores sensitivity to AZD3965. Not reported, but generally anticipated, is that efficacy may also correlate with the extent of MYC activation, the presence of p53 mutations, and the extent of hypoxia in the treated tumors. In agreement with PK modeling, BID oral dosing of 30 mg of AZD3965 gives 24 h coverage in humans. In animal models, xenografts in mice bearing H526 tumors were treated with 100 mg/kg AZD3965 BID, a relatively high dose, for 7 days, alone or in combination with radiation. Both treatment regimens gave a statistically significant reduction in tumor size, with a more substantial reduction arising from the combination therapy. The compound was generally well-tolerated with respect to maintenance of animal body weight, also without overt effects on animal behavior.

Elevated levels of MCT1 with low levels of MCT4 have been detected in certain neuroblastomas, gliomas, and in certain specific breast, colorectal, gastric, and cervical cancer cell lines. As mentioned above, a potential future clinical application for AZD3965 is targeting MCT1-expressing lymphomas. In general, many hematological malignancies should be sensitive to MCT1 inhibitors that have no anti-MCT4 activity. Table 1, derived from patent data, summarizes the demonstrated or expected efficacy for an MCT1-specific inhibitor versus several clinically relevant hematological cell lines.

It is somewhat unclear what potential toxicities might arise from MCT1 inhibition, including from the clinical use of AZ3965. In an immunohistochemistry study in wild-type mice, MCT1 was found to be expressed in several tissues, including the retina, kidney, heart, stomach, duodenum, and liver. Many of these tissues also express MCT4, often to a greater extent, which might preserve normal function in these tissues should MCT1 be specifically inhibited. MCTs are also highly expressed in red blood cells.

Undesired retinal effects, including abnormal photoreceptor cell function and degeneration, is perhaps the major clinical concern for MCT1 inhibitors, though MCT3 was found to be the most highly expressed MCT in the retinal pigment epithelium and could perhaps compensate for MCT1 inhibition. MCT3 knockout animals display reversibly impaired retinal function. In CD147 knockout mice (all MCTs are effected) retinal tissue sections, especially Müller and photoreceptor cells, had lowered

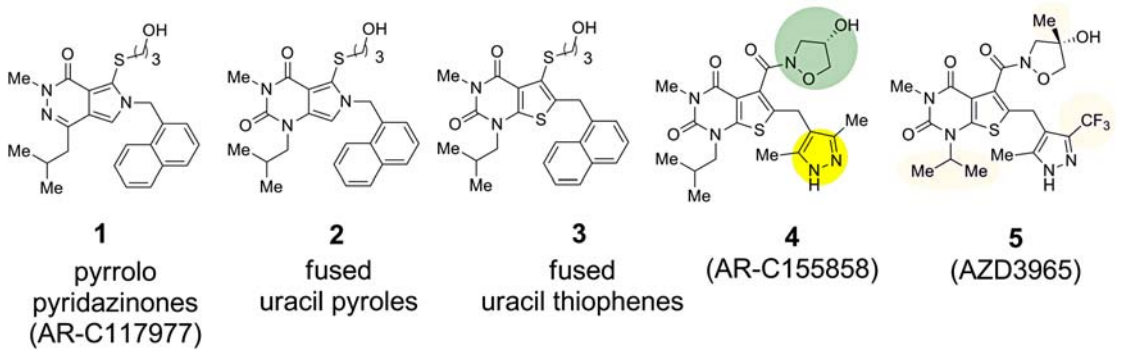

Fig. 3 Potent MCT1/MCT2 inhibitors from AstraZeneca and Cancer Research UK.

Table 1 MCT1 inhibitor-sensitive hematological malignancies[a]

Cell line	*Description*	*MCT1, MCT4 protein levels*
Cell lines highly sensitive to MCT1 inhibitors ex vivo, with $EC_{50} < 10$ nM		
SC-1	B cell non-Hodgkin's lymphoma (NHL)	MCT1 high, MCT4 not detectable
OCI-LY-19	B cell non-Hodgkin's lymphoma (NHL)	MCT1 high, MCT4 not detectable
WSUDLCL-2	B cell non-Hodgkin's lymphoma (NHL)	MCT1 high, MCT4 low
DOHH-2	B cell non-Hodgkin's lymphoma (NHL)	MCT1 medium, MCT4 not detectable
Raji	Burkitt's lymphoma	MCT1 high, MCT4 not detectable
Ramos	Burkitt's lymphoma	MCT1 medium, MCT4 not detectable
Jeko-1	Burkitt's lymphoma	MCT1 medium, MCT4 low
Namalwa	Burkitt's lymphoma	MCT1 high, MCT4 low
K562	CML	MCT1 high, MCT4 not detectable
BV-173	CML	MCT1 medium, MCT4 not detectable
Karpas-231	B cell ALL	MCT1 high, MCT4 low
EHEB	B cell CLL	MCT1 medium, MCT4 not detectable
Cell lines expected to be sensitive, based upon MCT1/4 expression levels		
SUP-B15	B cell precursor ALL	MCT1 medium, MCT4 not detectable
REH	ALL (non-T, non-B)	MCT1 medium, MCT4 not detectable
MEC-2	B cell CLL	MCT1 medium, MCT4 not detectable
HT	B cell NHL	MCT1 high, MCT4 low
SU-DHL-5	B cell NHL	MCT1 high, MCT4 not detectable
SU-DHL-4	B cell NHL	MCT1 high, MCT4 not detectable
Karpas-422	B cell NHL	MCT1 high, MCT4 not detectable
WSU-NHL	B cell NHL	MCT1 high, MCT4 not detectable
KG-1a	AML	MCT1 high, MCT4 low
MOLM-16	AML	MCT1 medium, MCT4 not detectable
OCI-M1	AML & CML	MCT1 high, MCT4 not detectable
KU812	CML	MCT1 high, MCT4 low
Daudi	Burkitt's lymphoma	MCT1 high, MCT4 not detectable
ESKOL	Hairy cell leukemia	MCT1 high, MCT4 low
Jurkat	T-cell leukemia	MCT1 high, MCT4 not detectable
KIT 225	T-cell leukemia	MCT1 high, MCT4 not detectable

[a]All data from Critchlow et al. WO 2010089580 A1 using AZD3965.

MCT1 expression based upon an immunohistochemical study using MCT1 antibodies. Areas to carefully monitor in the clinic thus certainly include retinal function, and if effects are seen, the reversibility of any retinal changes after the cessation of drug treatment.

To lessen the potential for retinal toxicity, or even for other tissue-specific toxicity issues that might arise, it is wise to closely assess the tissue distribution of candidate MCT1 inhibitors. For example, one would choose to advance compounds that have limited retinal exposure relative to the systemic drug levels, and especially relative to intertumoral drug levels. Certain MCT1 inhibitors, including the original AstraZeneca pyrrole pyridazinone probe compound 1 (**Fig. 3**), have been observed to rapidly accumulate in tumors to high levels, a property that could raise efficacy, mitigate potential side effects in normal tissues, and prolong the compound's duration of action, due to low liver exposure and concomitant lower-than-expected metabolism and systemic clearance.

As mentioned above, MCT1 is expressed in the liver, where its primary function appears to be the import of lactate for conversion to glucose (gluconeogenesis) via the Cori cycle. Thus, systemic MCT1 inhibition might be expected to elicit a modest reduction in blood glucose levels. In fact, this effect might well be amenable to therapeutic translation, where MCT1 inhibition could be used for modest glucose lowering in diabetic and/or obese individuals. For long-term use in therapy for diabetes or obesity, however, safety issues must be even more carefully considered. In any event, even in cancer treatment the monitoring of liver function would be advised in clinical trials of MCT1 inhibitors. The kidney, heart, and red muscles are also sites for gluconeogenesis and thus the function of these organs should also be monitored.

As discussed earlier, certain tumors (NSCLC in particular) also use lactate as fuel for entry into the TCA cycle, expanding its metabolic functions in cancer. MCT1, MCT2 and MCT4 are expressed in mitochondria in addition to the plasma membrane. It is unclear if MCT isoform distribution in mitochondria tracks with plasma membrane distribution, but this seems most likely. Thus tumor cells that rely primarily on MCT1, for example, for lactate export also would primarily use MCT1 for lactate flux into the mitochondria. The inhibition of this isoform would have two separate but synergistic effects, upon lactate efflux (driving adhesion and invasiveness, e.g.) and impeding energy production through lactate utilization in the TCA cycle. Similar synergies would exist for inhibitors of other MCT isoforms in tumor types reliant on that isoform for lactate flux.

Immune system responses should also be monitored in the clinical setting. The AstraZeneca probe compound 1 was, in fact, initially identified in a cell-based phenotypic screen for immunosuppressants, with MCT1 inhibition identified as its mechanism of action only a decade later. The AstraZeneca group showed that the inhibition of MCT1 during T lymphocyte activation resulted

in the inhibition of an extremely rapid phase of T-cell division that is believed to be essential for an effective immune response. MCT1 activity, however, is not required for most normal physiological functions of lymphocytes, thus it is unclear if immunosuppression would be a significant concern, especially in an acute treatment regimens that are characteristic of cancer therapy.

Although l-lactate is most often the substrate of MCT1–MCT4, these isoforms also transport other monocarboxylates, including the ketone bodies acetoacetate and β-hydroxybutyrate, as well as branched-chain keto acids. In the colon, MCT1 also facilitates the uptake of short-chain fatty acids. Biological processes dependent upon these species may thus be affected by MCT inhibition. MCTs have also been implicated in the transport of some carboxylate-containing drugs, such as salicylate, valproic acid, atorvastatin, γ-aminobutyric acid (GABA), and nateglinide. Thus MCT inhibitors should be assessed for drug–drug interactions, in particular for their ability to alter exposure of co-administered monocarboxylate drugs.

The emergence of therapeutic resistance is a general concern in the design of antitumor agents, and MCT inhibitors, including MCT1-specific inhibitors, are no exception. Resistance might emerge by at least three different mechanisms: (a) the ectopic induction of the MCT4 isoform, which can then replace MCT1's role in wild-type tumor cells in maintaining lactate homeostasis, (b) mutations to the MCT1 transporter that preserve lactate transport efficiency but that lessen the binding energy of the interactions of the transporter with the inhibitor, and (c) a shift from the reliance upon glycolysis to a reliance on oxidative phosphorylation for energy production, though this can in principle be impeded by inhibition of lactate transport into the mitochondria.

Resistance gained by MCT4 induction would likely be only a minor concern for tumor types that normally express very little MCT4. Tumor genotyping, in particular to ascertain the MCT1/MCT1 expression ratio, is likely to be essential for the successful identification of patient populations that are most likely to respond favorably to MCT1 inhibitors.

The second scenario, mutation to create a functional MCT that doesn't bind to the inhibitor, is perhaps the most problematic. Little is known about what mutations, if any, permit lactate transport while disfavoring the binding of inhibitors. Moreover, the emergence of resistance requires longer-term studies in animals, and preferably in humans. Presumably phase III clinical data on the most advanced MCT1 inhibitors will define the susceptibility for resistance emerging through point mutations in the transporter.

Regarding the third scenario, there is now substantial evidence to suggest that the efficacy of MCT inhibitors can be increased by the co-administration of an inhibitor of mitochondrial complex I, a drug class which includes the biguanides, one of which is the widely used diabetes drug metformin. The added efficacy may be the outcome of thwarting the emergence of resistance that could otherwise arise from a metabolic shift to oxidative phosphorylation. By a yet-unknown mechanism, biguanides seem to disfavor this metabolic shift by tumor cells. MCT1 inhibitor/metformin co-therapy can thus be superior to MCT1 inhibitor therapy alone, with lower doses of the inhibitor needed and with at least short-term resistance averted.

It should also be noted that, because MCTs are bidirectional transporters, antitumor effects may in principle arise via disruption of either lactate export or import. Though some reports attribute effects of MCT1 inhibition to the blockade of lactate import, this seems to be the exception rather than the rule, applying to oxygen-rich tumors relying on oxidative phosphorylation rather than glycolysis for energy production. MCT1-expressing tumor cells treated with MCT1 inhibitors had a decreased level of extracellular acidification, reflective of the blockade of lactate export. The most critical role of MCT1 and MCT4 in tumor cells thus appears to be the export of lactate that has been produced by glycolysis. It is important to keep in mind, however, that nontumor cells that require either the export or import of lactate can be affected.

Proposed Binding Mode(s) of AZ3965 to MCT1

No X-ray structures of MCTs have yet been obtained. Such structures would likely spur structure-based design efforts. Models of how DIDS binds to MCT1 and how AR-C155858 binds to MCT1 have been proposed by Halestrap et al., in both cases based upon extensive site-directed mutagenesis studies, and in the case of AR-C155858 binding, based upon the wealth of reported SAR information from AstraZeneca. The current clinical compound AZ3965 is expected to share the same binding characteristics of AR-C155858, as they are closely related in structure. Interestingly, Halestrap et al. concluded that one binding pose alone for AR-C155858 does not adequately fit the SAR data, and so they have proposed a dynamic model wherein the ligand transiently binds on the cytoplasmic side of MCT1 and then relocates to another, more deeply embedded binding pocket. In the first binding site, the key interactions are with Asn147, Ser264, and Arg306, while in the deeper site the key interactions are with Arg306, Asp302, Phe360, Leu274, Ser278, and Lys38. In aggregate, there are several essential pharmacophore features of this ligand class, as shown in Fig. 4 for AZ3965. The secondary alcohol of the ligand (in yellow) is an H-bond donor to Asp302 in both conformations, and also is an H-bond acceptor to other polar residues in one or both conformations. The A-ring carbonyl group (green) is a hydrogen bond acceptor, and this interaction appears to be the only key interaction involving the core [6,5] ring system of the scaffold. The isopropyl group (blue) fills a hydrophobic pocket in MCT1 that is comprised of lipophilic side chains. Finally, the pyrazole group (pink) makes both hydrophobic and polar interactions with neighboring residues. Presumably the hydrophobic interactions in this region are more important, given that original AstraZeneca pyrrolo pyridazinone inhibitor AR-C117977 (compound 1, Fig. 3) has a highly lipophilic naphthalene ring in this region, but it tightly binds to MCT1. The introduction of polarity in this region likely serves to lower the ligand's lipophilicity, thus conferring improved drug-like properties.

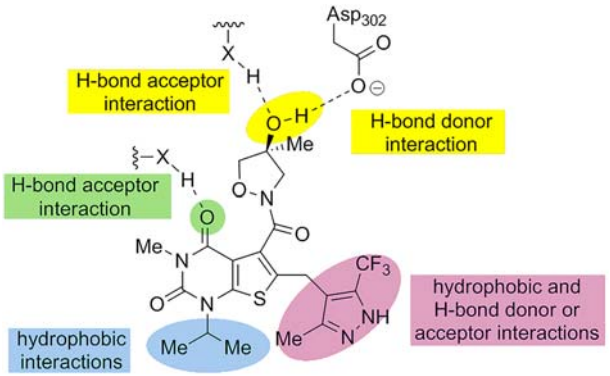

Fig. 4 Pharmacophore model for AZ3965 binding to the lactate transporter MCT1.

Scaffold-Hopping Efforts

The model in Fig. 4 suggests that the core scaffold of AZ3965 (the central 6-5 ring system) makes only one direct interaction with MCT1. The scaffold serves largely as a template for appropriately positioning a hydroxyl-containing group (yellow), a small hydrophobic group (blue) and a heteroaromatic group (pink). This explains the structural diversity allowed in the core scaffolds of the AstraZeneca tool compounds 1–3 (see Fig. 3) and it further suggests that additional scaffold-hopping efforts could identify compounds with groups that can mimic the key binding interactions that are proposed for AZ3965 but that may confer altered physical and/or DMPK properties. This hypothesis is supported by the identification of pteridine dione and pteridine trione MCT1 inhibitors 6–10 (Fig. 5), which have a [6,6] core ring system yet preserve key interactions regarded as essential in the binding model. Compounds in this structural series, such as compound 10, display low nanomolar potency in MTT assays for blocking the growth of the MCT1-dependent Raji Burkitt lymphoma cell line.

Given the activity seen in the pteridine-derived series, one might expect that other [6,6] and [6,5] ring systems might also be well-suited as core scaffolds for MCT1 inhibition. Indeed, in the recent patent literature both chromenones (e.g., compound 11, Fig. 6) and biaryl compounds (e.g., compound 12) have been reported to be potent MCT1 inhibitors. Both of these series, and the pterdinones as well, presumably fit the pharmacophore model shown earlier (Fig. 4) for MCT1 inhibition by AZ3965. While chromenones such as compound 11 share the core structure of dietary flavonoids, this common feature is likely coincidental. Biaryl compounds such as structure 12 have the essential hydroxyl group positioned to bind to MCT1 through a phenyl ring spacer rather than though an amide group, as in AZ3965 (Fig. 4).

Selective MCT4 Inhibition in Cancer Therapy

Much less information is available on the design of potent and selective inhibitors of the MCT4 isoform. Lipophilic statins (but not more hydrophilic statins) have been found to be MCT4-specific inhibitors, though with very low potency (IC_{50} values in the 30–100 μM range, with fluvastatin having the highest affinity for MCT4 among the statins). Such low affinity for relatively large structures offers little promise for optimization to inhibitors with potency at low nanomolar levels.

In 2014 Vettore Bioscience LLP received a Small Business Innovation Research Grant from the NIH to develop MCT4 inhibitors, with an aim of optimizing from a then-undisclosed starting point. A subsequent patent application disclosed an aryl pyrazole lead

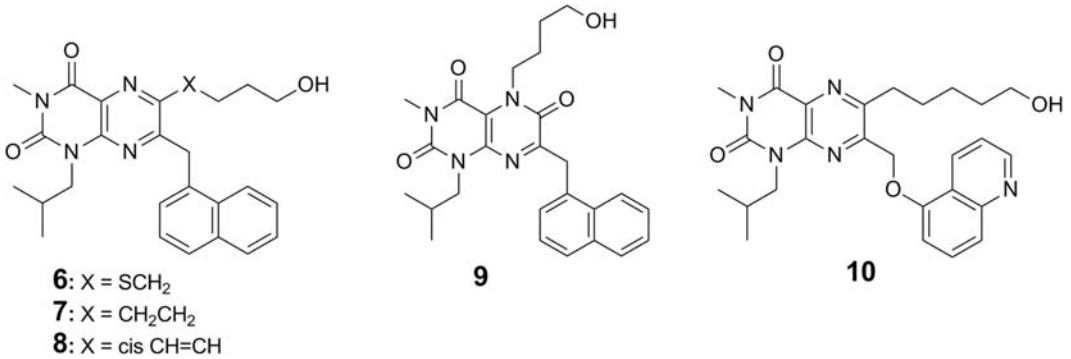

Fig. 5 Pteridine dione/trione MCT1 inhibitors.

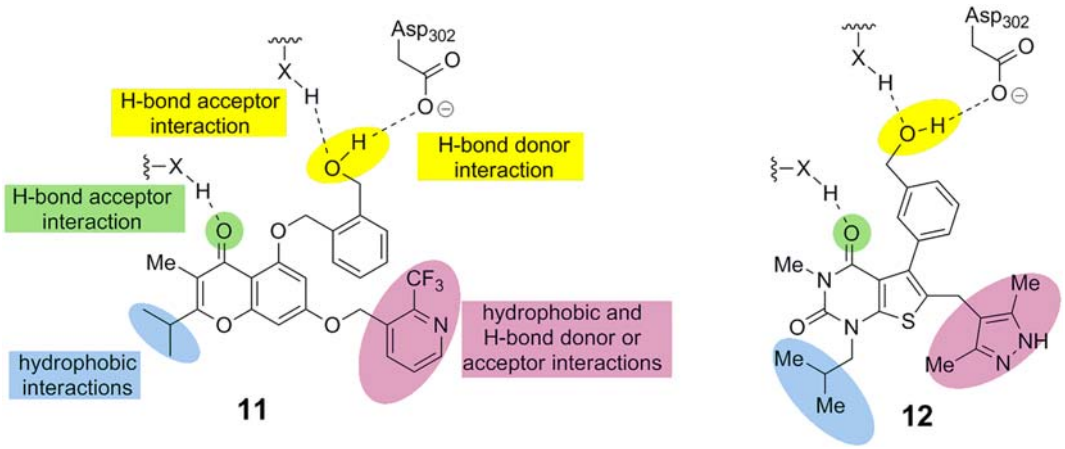

Fig. 6 Chromenone and biaryl MCT1 inhibitors.

series of potently active MCT4-specific inhibitors (Fig. 7). Compound 13 is one of the more potent and highly liver enzyme-stable analogs in the series. In this 2-aryl-*N*-benzyl pyrazole series, the highlighted groups are essential for activity, with some structural variation allowed, as indicated. No binding mode for these compounds to MCT4 had been proposed. Further, no data has yet emerged on the antitumor effects of these compounds in vivo or the side effects of MCT4-specific inhibition, but they should serve as useful tool compounds for making such determinations in animal models.

One might expect a rational design approach would be feasible for designing MCT4 inhibitors, if one might adapt the MCT1 pharmacophore model (Fig. 4) and consider the differences in amino acid sequence for MCT4 versus MCT1, altering the ligand to maximize desired interactions with differing MCT4 residues. To date, however, no MCT4-specific ligands are known to have been identified by such an approach. High-throughput screening presumably led to the Vettore lead MCT4 inhibitors, thus screening other collections might also be expected to give a suitable starting point for selective MCT4 inhibition, perhaps by cell-based screening for reduction in viability of an MCT4-expressing cell line followed by assaying any active compounds for their ability to disrupt the transport of ^{14}C-lactate. This approach could also reveal target binding sites that may be present in MCT4 but absent in MCT1. To date no screening campaigns for identifying MCT4 inhibitors have been reported, however.

Given that small molecule MCT4-specific inhibitors have only recently been disclosed, little empirical evidence has emerged regarding what tumor types they are capable of effectively targeting, though there is enough mounting evidence from other studies that we can anticipate what tumor types would be high impacted. MCT4 silencing by RNA interference was shown to drive clear cell renal cell carcinoma (ccRCC), the most common pathological subtype of kidney cancer, to cell cycle arrest and apoptosis. Other studies have implicated MCT4 as a key mediator of tumorigenicity in various pancreatic cancers, breast cancers (in particular, triple-negative breast cancer), oral squamous cell carcinoma, hepatocellular carcinoma, certain forms of colorectal cancer, and specific forms of prostate cancer. The ability of tumor cell line to express MCT1 would be expected to compromise its sensitivity to MCT4-specific inhibitors, so an analysis of relative expression levels, MCT1 versus MCT4, in tumors is likely to be important to maximizing the potential for therapeutic success.

There are significant mechanism-based safety concerns for systemic MCT4 inhibition, again not based upon reported studies with MCT4 inhibitors but rather based upon proteomics analysis. MCT4 is the major monocarboxylate transporter isoform found in white skeletal muscle and it is essential for muscle homeostasis. Medium levels of MCT4 are also found in all smooth muscles and

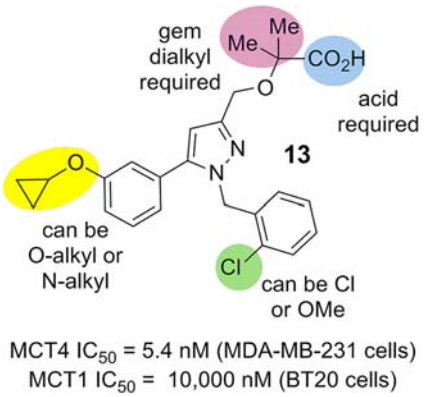

Fig. 7 Vettore's MCT4 inhibitor series.

in the heart. The common phenomenon wherein athletes experience muscle cramps after prolonged periods of heavy exercise is thought to arise from excessive lactate production that overwhelms the capacity of available MCT4s to export lactate. Thus, smooth muscle pain in general is an area of concern in the use of MCT4 inhibitors for treating cancer. Interestingly, muscle pain has been found to be more pronounced in humans taking statins that exercise heavily. As cited above, lipophilic statins are but weak MCT4 inhibitors, thus they likely do not act by direct inhibition. Rather it may arise from the downregulation of MCT4 expression, which has been observed for statins. Statin users might be poor subjects for anti-MCT4 antitumor therapy. There are also elevated levels of MCT4 expressed in the human placenta, in many sections of the GI tract, spleen, testes, and thyroid. Many of these tissues also have moderate or high expression of the MCT1 isoform, which could compensate for MCT4-specific inhibition and thereby prevent unwanted effects from arising. Complicating the analysis further, in a mouse study the expression levels of MCTs was found to differ depending upon whether the animals studied were in a feeding or fasting state, thus clinical studies on MCT4 inhibitors should consider this factor as well.

Dual Inhibition of MCT1 and MCT4 in Cancer Therapy

A few small molecules have been proposed to function as dual inhibitors of MCT1 and MCT4. Many of these species, however, are cysteine-reactive electrophiles (see **Fig. 2**) and thus they may inhibit the function of multiple MCTs by binding to their common CD147 chaperone. Several dietary flavonoids (e.g., quercetin and luteolin) disrupt function of several MCTs, albeit only at high micromolar concentrations. Because these compounds lack a cysteine-reactive warhead, perhaps they indeed do form direct interactions with the MCTs rather than with CD147. Unfortunately, the dietary flavonoids suffer from poor drug-likeness, as multiple metabolically labile phenol groups that are present are also essential for their modest activity. Thus, the series would seem to be ill-suited for substantial potency optimization to the point that physiological effects could be expected after following a reasonable dosing regimen. Dietary flavonoids are also electron transfer agents, with their antioxidant potential contributing to several pharmacological effects that are entirely unrelated to MCT binding.

SAR studies of potent MCT1 inhibitors in multiple scaffolds (**Figs. 3, 5 and 6**) identified no compounds in any series that also potently inhibit MCT4. Conversely, an SAR study of potent MCT 4 inhibitors (**Fig. 7**) also identified no compounds that were also potently active at MCT1. It may be generally true that the significant sequence differences between these two isoforms in their binding pockets will make direct dual inhibition of both MCT1 and MCT4 an elusive goal. Inhibitors of the interaction of CD147 and MCTs could achieve the same result, however. It might also be possible to rationally design dual MCT1/MCT4 inhibitors based upon the binding model for the AstraZeneca MCT1 inhibitors, considering differences in amino acid sequence between the MCT1 and MCT4 transporters and targeting only the common aspects of their respective binding pockets. To date, however, no highly potent dual inhibitors that clearly bind the MCTs directly have been reported.

The dynamic, two-binding site model for interactions of the AstraZeneca MCT1 inhibitors with MCT1 (see **Fig. 4** and associated discussion) may explain why MCT4 cannot be similarly targeted, as perhaps one or both of MCT1's ligand binding sites is much altered or absent in MCT4. A high-throughput screening approach, wherein even alternate binding sites might be occupied, might hold more promise for identifying dual MCT1/MCT4 inhibitors. If tight binding to both isoforms by a single molecule proves not to be feasible, the co-administration of a cocktail of two separate species, an MCT1 inhibitor and an MCT4 inhibitor, might be the most straightforward way to simultaneously block the function of both transporters.

Dual MCT1/MCT4 inhibition could have both significant advantages and disadvantages relative to selectively targeting either MCT1 or MCT4. Because several tumor types express both MCT1 and MCT4, selectively blocking only one transporter is unlikely to impede their growth, as the uninhibited transporter can function to maintain lactate homeostasis. Dual inhibitors should have the advantage of being much more effective versus such cell lines. Dual inhibition may also be advantageous for tumor cell types that, while expressing much more of one of these transporters (MCT1 or MCT4), is also capable of upregulating the other relevant transporter and thereby conferring resistance to any agent targeting only the more highly expressed transporter. In general, dual MCT1/MCT4 inhibitors (or a cocktail containing both an MCT1 inhibitor and an MCT4 inhibitor) are expected be cytotoxic toward wider range of patient-derived tumor cell lines than would either an MCT1 inhibitor or an MCT4 inhibitor.

The drawback to a dual MCT1/MCT4 inhibitor approach would be that side effect risks could also be augmented, given that normal cells that are dependent on *either* MCT1 or MCT4 for either lactate import or export at any stage in their development would be sensitive to dual inhibition. Dual MCT1/MCT4 inhibitors for cancer therapy would be used acutely, however, and perhaps even an extensive side effect profile could be tolerated or managed. The concerns regarding a wider spectrum of potential side effects would also apply to targeting CD147, either by antibody or by small molecule approaches, and perhaps more so, given that CD147 performs other essential physiological functions besides serving as an MCT chaperone.

Additive or Synergistic Effects With Other Drugs

As described earlier, in animal models the administration of mitochondrial complex I inhibitor biguanides, such as the widely used diabetes drug metformin or the related compound phenformin, augments the efficacy of MCT1 inhibitors. In animals receiving AZ3965, radiation therapy was shown to provide a synergistic benefit. Inhibitors of other biological targets that also facilitate glycolysis should also act additively, if not synergistically, with MCT inhibitors. One example is PI3K inhibitors, which cause a significant

decrease in glycolysis by decreasing aldolase activity. MCT inhibitors are likely to also act additively or perhaps synergistically with a variety of other anticancer drugs that act by fully orthogonal mechanisms, such as antiangiogenic compounds, immunologic therapies, and cell cycle disruptors. Now that tool compounds exist for both selective MCT1 and MCT4 inhibition, studies of the potential of such polypharmacological approaches will undoubtedly be thoroughly investigated.

Selective Tissue Targeting

MCT inhibitors can clearly thwart the growth of cell types that rely on the specific MCT isoform being inhibited. Much of the concerns with using MCT inhibitors relate to possible off-target effects at normal cells that at some stage are lactate-producing and that express MCTs. As mentioned earlier, one might fortuitously find small molecules that distribute well to tumors or to the target organ bearing the tumor. A more reliable approach may be to use a mechanism that will very specifically deliver the drug where it is required, that is, in the vicinity of the tumor that is being targeted. Such approaches often provide three important advantages: lower systemic toxicity due to low systemic exposure, prolonged duration of action for the same reason, and improved potency at the site of action due to greatly elevated local exposure.

The emergence of antibody therapies and, more recently, antibody–drug conjugates, has made it clear that tumors can be targeted with high specificity and that small molecule payloads that are highly cytotoxic can also be delivered to tumors, such that the toxic payload has negligible effects on normal cells and devastatingly lethal effects on the tumor. An MCT inhibitor may be a candidate for antibody delivery, although certain conditions much be met. The antibody-MCT inhibitor conjugate must be recognized by the tumor cell, must then be internalized, and then the MCT inhibitor must be released so that it reaches its binding site, near the cytosolic surface of the membrane-spanning transporter. The released MCT inhibitor should then remain in the cell and be neither effluxed nor lost to the cell by diffusion. Thus, while there are significant technological obstacles to overcome, the antibody delivery approach is attractive and warrants further study. To date there have been no reports of antibody-MCT inhibitor conjugates used either in vitro or in vivo.

Antibodies are not the only option for effective tumor targeting. Proteomics studies have identified many active transport systems that are very highly upregulated in numerous tumor types. For example, a folate-tagged small molecule can be delivered to tumors having upregulated folate transporters, meeting the tumor's increased appetite for folate. Tumor cells also aggressively compete for a variety of other nutrients that support the glycolytic phenotype, including glucose, glutamine, and other amino acids. An MCT inhibitor that is a substrate for a glucose transporter, a glutamine transporter (in particular, ASCT2, which is broadly upregulated in tumors), or the transporter for large neutral amino acids (LAT1, which is also highly expressed in tumors) will be internalized by tumor cells through active transport and then can block the function of the MCT.

The strategy of successfully exploiting the upregulation of nutrient transporters has gained substantial validation in the tumor imaging field. The general strategy used is that an imaging group is tethered to, or is part of, a tumor-targeting group. As an example, in ^{18}F-Fluorodeoxyglucose positron emission tomography (PET), tumors are imaged in preference to the background of nonmalignant cells. The extent of imaging contrast arises from the tumor's ability to take up fluorodeoxyglucose (compound 14, **Fig. 8**, also called fludeoxyglucose) via glucose transporters, which are highly upregulated in tumor cells. Similarly, ^{18}F-FBPA (compound 15) is a phenylalanine analog that is taken up preferentially by tumors via LAT1, a transporter for large neutral amino acids (e.g., Phe, Tyr, Leu, Trp, etc.) that is also widely upregulated in tumors. As a third example, gadolinium-containing MRI contrast agents, such as compound 16, have one or more appended glutamine residues. This appended amino acid allows the entire complex to be taken up preferentially by tumors via ASCT2 (aka SLC1A5), a MYC-driven tumor-upregulated transporter for glutamine, a nutrient that is essential for tumor growth.

The sample principles can be applied to the targeted delivery of a therapeutic agent rather than an imaging agent. The LAT1-directed and ASCT2-directed tumor delivery strategies used for imaging agents 15 and 16 have been applied to the targeted delivery of MCT1 inhibitors. Briefly, SAR studies with the AstraZeneca tool compound 1 (**Fig. 3**) revealed that the naphthalene region was open to substitution by even large functional groups without a significant loss of MCT1 inhibitory activity. Thus, a linker group could be attached to the naphthyl ring (or to an isosteric quinoline ring) to permit the attachment of a phenylalanine residue or a glutamine residue, appended as shown in compounds 17 and 18, respectively. The Phe-MCT1 inhibitor and Gln-MCT1 inhibitor conjugates 17 and 18 were designed to be transported by LAT1 and ASCT2, respectively. Since these two amino acid transporters are widely upregulated in tumors (they have even been dubbed "partners in crime" with regard to sustaining tumor growth), the conjugates 17 and 18 are tumor-targeted, displaying higher efficacy versus Raji Burkitt lymphoma cells than compounds lacking a tethered amino acid. Moreover, tumor intracellular drug levels are much higher for the amino acid conjugated compounds, and also for a prolonged duration. No in vivo results using such compounds have yet appeared, but it will be interesting to see if these or other tumor-targeting strategies can be used to widen the therapeutic index of MCT inhibitors.

Prospective Vision

The reliance of cancer cells on glycolysis to meet their energy needs has spurred many efforts to exploit this dependence for cancer therapeutics development. The major impediments to impeding glycolysis with small molecules have been overcoming several potentially toxic side effects and thwarting the emergence of treatment resistance. At the forefront of cancer metabolism mechanistic

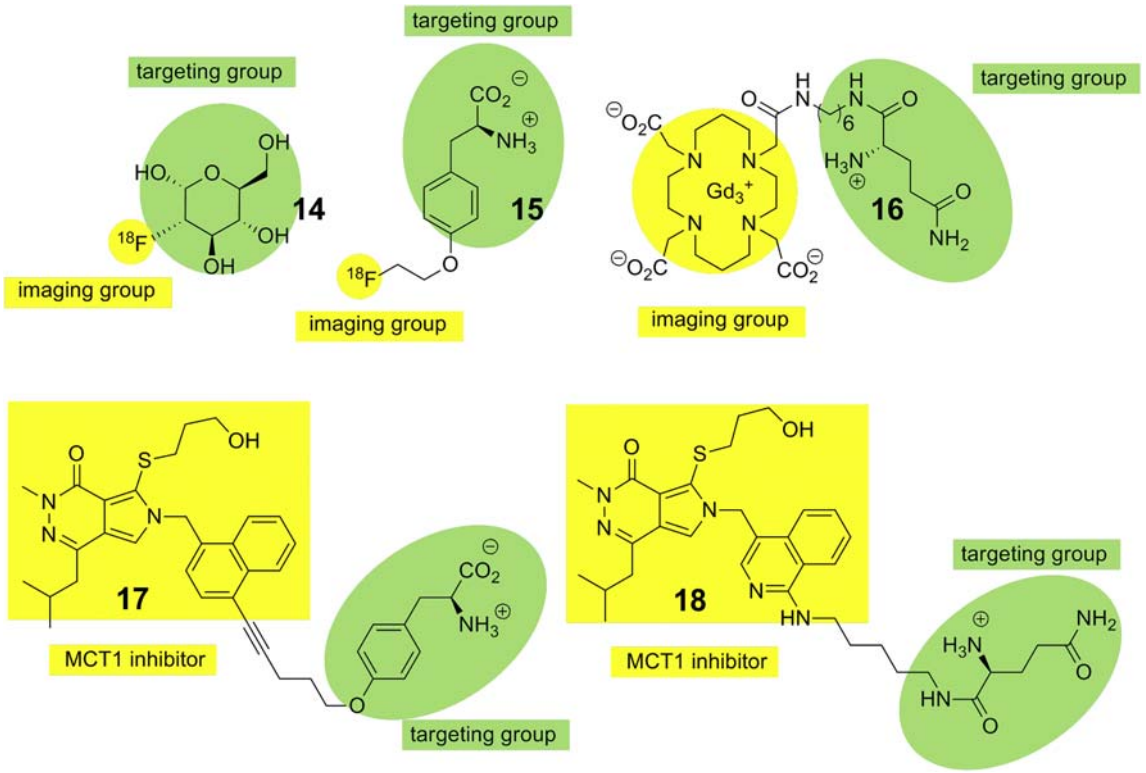

Fig. 8 Tumor upregulation of amino acid transporters can be exploited for the targeted delivery of imaging agents and toxic payloads, such as an MCT1 inhibitor.

targets are the lactate transporters most highly expressed in tumors, MCT1 and MCT4. In recent years potent and drug-like MCT1 inhibitors have emerged and have even reached the clinic. Promising MCT4 inhibitors, while slower to emerge, are now available as tool compounds and possible forerunners of anticancer drugs. We now have the molecular tools necessary allowing researchers to test the idea that MCT inhibition will broadly impede lactate flux in tumors, affecting both the export of lactate and also its utilization, in at least some cases, as an energy source. Controlling lactate flux may be a powerful approach to thwart the growth of many clinically important and otherwise poorly treated tumor types, to evaluate possible treatment synergies, and to address concerns related to the therapeutic index of potent antitumor agents.

Further Reading

Birsoy, K., Sabationi, D.M., Possemato, R., 2012. Targeting cancer metabolism: A bedside lesson. Nature Medicine 18 (7), 1022–1023.
Chesney, J., Telang, S., 2013. Regulation of glycolytic and mitochondrial metabolism by ras. Current Pharmaceutical Biotechnology 14 (3), 251–260.
Curtis, N.J., Mooney, L., Hopcroft, L., Michopoulos, F., Whalley, N., et al., 2017. Pre-clinical pharmacology of AZD3965, a selective inhibitor of MCT1: DLBCL, NHL and Burkitt's lymphoma anti-tumor activity. Oncotarget. https://doi.org/10.18632/oncotarget.18215 (Epub ahead of print).
Doherty, J.R., Cleveland, J.L., 2013. Targeting lactate metabolism for cancer therapeutics. The Journal of Clinical Investigation 123 (9), 3685–3692.
Doherty, J.R., Yang, C., Scott, K., Cameron, M., Fallahi, M., et al., 2014. Blocking lactate export by inhibiting the Myc target MCT1 disables glycolysis and glutathione synthesis. Cancer Research 74, 908–920.
Doyen, J., Trastour, C., Ettore, F., Peyrottes, I., Toussant, N., et al., 2014. Expression of the hypoxia-inducible monocarboxylate transporter MCT4 is increased in triple negative breast cancer and correlates independently with clinical outcome. Biochemical and Biophysical Research Communications 451 (1), 54–61.
Enereson, B.E., Drewes, L.R., 2003. Molecular features, regulation, and function of monocarboxylate transporters: Implications for drug delivery. Journal of Pharmaceutical Sciences 92 (8), 1531–1544.
Faubert, B., Li, K.Y., Cai, L., Young, J.D., Kemstine, K., DeBernardinis, R.J., 2017. Lactate metabolism in human lung tumors. Cell 171, 358–371.
Galluzzi, L., Kepp, O., Vander Heiden, M.G., Kroemer, G., 2013. Metabolic targets for cancer therapy. Nature Reviews. Drug Discovery 12, 829–846.
Ganapathy-Kanniappan, S., Geschwind, J.-F.H., 2013. Tumor glycolysis as a target for cancer therapy: Progress and prospects. Molecular Cancer 12, 152.
Gilmour, R., Velasco, S., 2017. Back to the future: Therapeutic targeting of cancer cell metabolism. SLAS Discovery 22 (4), 333–337.
Halestrap, A.P., Meredith, D., 2004. The SLC16 gene family—From monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. European Journal of Physiology 447, 619–628.
Halestrap, A.P., Price, N.T., 1999. The proton-linked monocarboxylate transporter (MCT) family: Structure, function and regulation. The Biochemical Journal 343 (2), 281–299.
Hsu, P.P., Sabatini, D.M., 2008. Cancer cell metabolism: Warburg and beyond. Cell 134, 703–707.
Kroemer, G., Pouyssegur, J., 2008. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 13, 472–482.
Mathupala, S.P., Colen, C.B., Parajuli, P., Sloan, A.E., 2007. Lactate and malignant tumors: A therapeutic target at the end stage of glycolysis. Journal of Bioenergetics and Biomembranes 39, 73–77.

Meredith, D., Christian, H.C., 2009. The SLC16 monocarboxylate transporter family. Xenobiotica 38, 1072–1106.
Morais-Santos, F., Granja, S., Miranda-Goncalves, V., Moreira, A.H., Queiros, S., et al., 2015. Targeting lactate transport suppresses in vivo breast tumour growth. Oncotarget 6 (22), 19177–19189.
Nancolas, B., Sessions, R.B., Halestrap, A.P., 2015. Identification of key binding site residues of MCT1 for AR-C155858 reveals the molecular basis of its isoform selectivity. The Biochemical Journal 466, 177–188.
Semenza, G.L., 2008. Tumor metabolism: Cancer cells give and take lactate. The Journal of Clinical Investigation 118 (12), 3835–3837.
Spanier, J.A., Drewes, L.R., 2006. Monocarboxylate transporters. In: You, G., Morris, M.E. (Eds.), Drug transporters: Molecular characterization and role in drug disposition. Wiley-Interscience, Hoboken, N.J., pp. 147–170
Voss, D.M., Spina, R., Carter, D.L., Bar, E.E., Lim, K.S., Jeffery, C.J., 2017. Disruption of the monocarboxylate transporter-4-basigin interaction inhibits the hypoxic response, proliferation, and tumor progression. Scientific Reports 7 (1), 4292.
Wilson, M.C., Meridith, D., Bunnun, C., Sessions, R.B., Halestrap, A.P., 2009. Studies on the DIDS binding site of monocarboxylate transporter 1 suggest a homology model of the open conformation and a plausible translocation cycle. The Journal of Biological Chemistry 284, 20011–20021.

Integrative Molecular Tumor Classification: A Pathologist's View

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Abbreviations

AFIP Armed Forces Institutes of Pathology
CAP College of American Pathologists
CMS Consensus molecular classification
ICD International Classification of Diseases
ICD-O International Classification of Diseases for Oncology
ICGC International Cancer Genome Consortium
NGS Next generation sequencing
SNOP Systematized Nomenclature Of Pathology
TCGA The Cancer Genome Atlas
UICC Union for International Cancer Control
WHO World Health Organization

Introduction

Understanding the biology of cancer and using this understanding to improve (early) diagnosis, treatment and follow-up of cancer patients is very much a multidisciplinary effort. Clinicians, clinician-scientists and basic scientists work together in what has become known as translational research: results of laboratory studies are translated into new approaches towards diagnosis and treatment and observations in the clinics generate questions for which laboratory studies are necessary. This is exemplified in the well-known metaphor "bench to bedside and back." Pathology is well positioned in this field, as it is considered "the science behind the cure": understanding cancer will eventually lead to earlier and better diagnosis underpinning therapeutic approaches targeting the molecular mechanisms responsible for the development of individual cancers.

Another reason why pathology plays a central role is the availability of biospecimens which are used for detailed molecular mapping of cancer. These biospecimens include tissue obtained through biopsy, cells from bodily fluids, tissue samples from surgical specimens and of late also tumor derived nucleic acids in circulating blood. Such specimens are collected in pathology departments in "biobanks," which have become a cornerstone in cancer research. Detailed questions regarding molecular mechanisms often require in vitro or in vivo (animal) models, which have been refined to closely mimic cancer in man. But invariably, application of new understanding, in terms of biomarkers for detailed therapy-oriented classification or development of new (targeted) therapeutic approaches, will pass through a phase of research on patients or patient samples.

History of Cancer Classification

It is hard to imagine, but as little as 100 years ago there was no standard classification or nomenclature (system of names) for diseases. Case records and causes of death were often recorded using narratives of symptoms. It was only in the second half of the 19th century that attempts were made to get to more systematic classifications of disease. Important groundwork was done not by pathologists or clinicians but by early medical statisticians, notably William Farr (1807–83) and the Frenchman Jacques Bertillon (1851–1922). The latter was instrumental in stimulating the French government to support the first International Conference for the revision of the International Classification of Causes of Death in 1900, which is the predecessor of the WHO International Statistical Classification of Diseases.

Tumor classifications came even later. In 1924 the Health section of the League of Nations decided that disease classifications needed to be revised every 10 years. When the WHO was created in 1947, this responsibility was bestowed upon this organization. In parallel, the Committee on Pathology of the National Academy of Sciences (USA) and the National Research Council of the USA took the initiative to develop the Atlas of Tumor Pathology published by the Armed Forces Institutes of Pathology (AFIP), which rose to fame as the AFIP fascicles. The American Cancer Society (ACS) in 1951 published the Manual of Tumor Nomenclature and Coding (MOTNAC), which consisted of a two-digit code for morphology with a third digit indicating the behavior of the neoplasm. In the 1960s the College of American Pathologists (CAP) decided to develop a classification for all pathologic entities, which evolved into the Systematized Nomenclature Of Pathology (SNOP). Meanwhile, the Union for International Cancer Control (UICC) published in 1952 the "Uniform Technique for a Clinical Classification by the TNM System," of which recently the 8th edition has been published. As of 1956 the International Classification of Disease (ICD) saw the light of day and now its 11th

revision is underway. In 1976 the 1st edition of the International Classification of Diseases for Oncology (ICD-O) was published and presently in use is its 3rd edition dating from 2013.

The role of the WHO in tumor classification started in 1956 when the WHO executive board in 1957 requested the Director-General to explore the possibility that WHO might organize centres in various parts of the world to collect human tissues and their histological classification. The main purpose of such centres would be to develop histological definitions of cancer types and to facilitate the wide adoption of a uniform nomenclature. The first such centre was created in 1958 in Oslo with as task to collect a case set that would underpin a WHO classification of lung tumors and the tangible result came in 1965 with the publication of the first volume of the first edition of the WHO classification of lung cancer. Publication of the 24 volumes of the first edition took until 1980. The second edition was published between 1988 and 2002 in 26 volumes. The third edition was published in 10 volumes between 2004 and 2007 and the publication of the fourth edition in 11 volumes will be completed with the last volume on Tumors of the Eye and Orbita in 2018.

What this brief review of the definition, scientific basis, development and history of classification tells us is that tumor classifications are in constant motion. Understanding of the biology of cancer has improved spectacularly, methods for (early) detection and specific diagnosis become more and more sophisticated, treatment options evolve rapidly and all these need to be taken into consideration in adapting existing classifications to the requirements of the users: patients, their physicians, pathologists, registrars, health care policy makers and (inter)national bodies such as the WHO.

Tumor Classification Now: How and Why?

Disease classification is the process in which signs and symptoms of disease are recognized, differentiated, understood and translated into a universally accepted diagnostic term or disease name, which guides treatment decisions clinicians take, and can be transformed into a universal medical code. These diagnoses and codes are used by health care providers, public health officials, health insurance companies, epidemiologists, registrars etc. among others to decide on therapeutic actions, analyze disease statistics and justify reimbursement.

It is important to emphasize that disease classifications do not develop through a process of deductive but rather through inductive reasoning. In deductive reasoning the starting point is a scientifically verified theoretical framework, within which the consequences of for example, an intervention can be predicted and subsequently experimentally verified. This is the classical model of hypothesis driven experimental studies. Inductive reasoning, on the contrary, starts with a set of observations, which form the basis for discerning a pattern that can be translated into a generalization, a theory or in the context of cancer classification a proposal for a new class or subtype of disease. The result of inductive reasoning can be a new hypothesis, which can subsequently be tested in an experimental approach, using deductive reasoning to establish a logical experimental design.

> "A recent example of this is the birth of the sessile serrated adenoma of the colon. In a well-known context, that of hyperplastic polyps of the colon, pathologists started to recognize lesions that look a little bit different, as they show more architectural disturbance with aberrant crypt shapes and intracrypt differentiation patterns. A set of characteristics was gradually identified, allowing a histological definition of a purported new class of disease. Subsequent clinical-pathological studies linked this new category with an increased risk for the development of colon cancer and molecular studies showed that they are characterized by aberrant gene promoter methylation patterns and *BRAF* mutation. The term sessile serrated adenoma of polyp was finally canonized in the WHO classification of human tumors and earned its own new ICD-O code."

The interplay between inductive and deductive reasoning characterizes translational research: "from bedside to bench and back." The implication of the inductive nature of the evolution of disease classification is the absence of an absolute scientifically verified theoretical framework, like the periodic table. Consequently, new observations might require an established classification framework to be modified. A striking example of the need for adaptation/modification is the emergence of the wealth of "omics" data on cancer, which seriously interrogates the relevance of the anatomo-morphological basis of the current approach to the classification of cancer.

A key characteristic of a scientifically robust classification is that class properties are shared among the members of a class, and new members of a class will share the properties of existing members. Identification of a condition as member of a specific class is of great practical importance, as this instantly conveys important information regarding biological behaviour, possible treatment options and potential outcome. The ideal tumor classification organizes tumors in hierarchical classes, with as basic principle that all members of a class share the same essential characteristics. As we will see, current classifications do not (completely) abide by this rule. Many tumor types occur in several organs, for example adenocarcinomas occurr in practically all parenchymatous organs. This leads to a certain level of redundancy and confusion, as the biological behaviour of an adenocarcinoma of the lung is quite different from that of an adenocarcinoma of the colon. Site can be a more important determinant of behaviour than type. Tumors of the same type at a single site may have different molecular (genetic) characteristics, going along with significant differences in biological behaviour. As a result, the current WHO classification (as all other tumor classifications) has more the character of a standard nomenclature than that of a coherent classification. A list of all entities in the WHO classification does provide a complete taxonomy of cancer, with which then the ICD-O codes can be associated. Using this classification as a tool, pathologists *identify*: they assign a standard diagnostic term from the WHO classification to a tumor using identifying characteristics. In simple

terms "they make a diagnosis." Pathologists also *discriminate*: they recognize features that define sub-types which differ in behaviour. As an example, a class or type would be "adenocarcinoma of the colon" and members of this type can be further subdivided according to anatomical criteria into TNM based stage groups or according to histological characteristics into different morphological subtypes or grades ("low grade" or "high grade").

The current classifications group human tumors according to the following hierarchical characteristics:

- Site
- Tumor category
- Tumor family
- Tumor type
- Tumor sub-type
- Tumor grade

Tumor *site* is usually an organ (e.g., lung, breast, prostate) or system (e.g., hematopoietic system, lymphatic system). Tumor *category* refers to major distinct cancer types, for which over time direction of differentiation homologous to developmental biological concepts were chosen, for example carcinoma (epithelial differentiation), sarcoma (mesenchymal differentiation), lymphoma and leukemia (lympho- and hematopoietic differentiation), and germ cell tumors (germ cell differentiation).

Within these categories, tumor *type* refers to distinct cancer types showing specific patterns of differentiation. For carcinomas typical examples are squamous cell carcinoma, adenocarcinoma or neuroendocrine carcinoma. For sarcomas typical examples are fibrosarcoma, osteosarcoma, chondrosarcoma or angiosarcoma. Tumor types over time segregated into tumor *subtypes*. As an example, adenocarcinoma of the colon has as subtypes cribriform comedo-type adenocarcinoma, medullary carcinoma, micropapillary carcinoma, mucinous adenocarcinoma, serrated adenocarcinoma and signet ring cell carcinoma. The evolution of subtypes was mostly driven by morphological observations, even though their relevance is to a large extent determined by association with differences in clinical behavior and more recently differences in molecular characteristics. The sessile serrated adenoma of the colon, as elaborated above, is an example of this evolutionary pattern of tumor classification.

Tumor *grade* is the final step. Grade refers to the histological similarity of cancer tissue to the tissue type in which the cancer arose. A well differentiated, low-grade adenocarcinoma of the colon shows a histological pattern close to that of normal colon mucosa, while a poorly differentiated high-grade colon cancer has lost many morphological differentiation characteristics. The importance of grading is the association with tumor behavior: high-grade cancers tend to be more aggressive with poorer prognosis than low-grade cancers.

The problem with this approach, used in the World Health Organization classification which list the tumors that occur at different body sites, is that in every organ cell types exist which are organ specific but also cell types not specific for that organ. The colon, for instance, contains a mucosal surface with an epithelial covering which is relatively specific for that organ. Tumors originating in the mucosa are also relatively specific for the colon. However, connective tissue, smooth muscle, fatty tissue, nerves and lymphoid tissue are also components of the bowel wall and give rise to tumors. The list of tumors in the colon therefore does contain, in addition to adenocarcinomas, also fibromas, leiomyomas, gastrointestinal stromal tumors (derived from the interstitial cells of Cajal, the pacemakers of intestinal peristaltic movement), lipomas, neurofibromas, ganglioneuromas, and lymphomas. These all occur in several other sites and henceforth will be mentioned there, which incurs significant redundancy. A comprehensive approach to tumor classification which would avoid such redundancy would be of interest but attempts to this end have not been undertaken.

Tumor Classification in the Post-Genomic Era

In this complex of "moving targets," molecular (genetic) analysis has introduced a whole new layer of complexity. Conceptually, a scientifically validated theoretical framework as a basis for classification should allow new data to be assimilated smoothly within the existing classification structure. New information regarding class members accumulates and/or new class members are identified, and both can be comfortably accommodated within the existing classification: class definitions do not have to be changed. It is now evident that molecular data only partially fit within existing tumor classification approaches and there are several examples of molecular data which have upset them. It is therefore imperative that new approaches to tumor classification are considered.

What would a "second generation" tumor classification accommodating this complex molecular knowledge look like? The following list summarizes some essential features of an ideal tumor classification.

- The classification should be based on biological principles, using features from developmental biology, molecular cell biology and genetics, to segregate tumors into distinct classes, preferably based upon a scientifically valid conceptual framework.
- The classification should be informative in providing basic biological and clinically relevant information regarding the essential characteristics of each class member.
- The classification should be comprehensive (e.g., every human tumor can be easily accommodated within the classification) and based upon consensus: "everyone should speak the same language."
- New subdivisions should be readily accommodated in the classification, allowing for example easy assimilation of the wealth of "omics" data.

- The classification should allow migration of a subdivision to a different class. With the continuous emergence of new molecular data, subtypes might have to be united or repositioned into a different class.
- The classification should allow assimilation of results of basic research with needs of clinicians.
- The classification should be compatible with earlier classifications to allow easy translation of earlier classification results (e.g., pathological diagnoses) into the new classification.

The question arises weather the wealth of molecular genetic data, presently available for almost all human tumor types following the TCGA and ICGC projects, provides a biologically valid basis for a new approach towards tumor classification. If so, the new classification should manage the complexity of voluminous and enormously complex molecular tumor data, allowing elucidation of relationships between different data elements by reclassifying tumors under redefined group hierarchies. Much of the presently available molecular (genetic) data on human tumors represent discriminant analysis within a class, but not necessarily an attempt to reclassify based upon molecular characteristics across the boundaries of existing tumor classifications. Typically, in a gene expression profiling (or genomics) study unsupervised clustering of expression (or genome aberration) data separates the tumors into groups with (partially) shared patterns of gene expression or genome aberrations. To be significant, such new groups will also share specific biologic and/or clinical features (e.g., high propensity for metastasis, response to chemo- or targeted therapy, better prognosis). But this merely represents new intraclass variability, and rarely justifies the creation of a new tumor type. An example is the revised 4th edition of the WHO classification of tumors of the nervous system, "which breaks with the century-old principle of diagnosis based entirely on microscopy by incorporating molecular parameters into the classification of CNS tumor entities" as David Louis states in the introduction. Glioblastomas are now subdivided in *IDH1* wild type and *IDH1* mutated, with differences in biological properties and therapy options, but they are both still considered as belonging to the glioblastoma class.

Some molecular cancer biologists like to convey the impression that we are on the verge of a totally new approach towards tumor classification, based first and foremost on molecular (genetic) information and principles. The question is whether this is indeed the way to go: abandoning presently used principles of tumor classification by reclassifying based upon the presumed primacy of molecular (genetic) data.

Can Human Cancer Be Classified Based Uniquely Upon Molecular (Genetic) Characteristics?

Conceivably, a completely new classification approach might eventually be developed using molecular (genetic) characteristics as dominant ordering principle. Such an effort, however, would face at least four major obstacles.

The Complexity of the "Omics Data"

Molecular (genetic) analysis of cancer has been around for decades. Immunohistochemical studies on gene expression at protein level by immunohistochemistry have become mainstream in diagnostic pathology and for many cancer types this is presently an essential step to arrive at proper classification. Early ground-breaking studies on chromosomal abnormalities in cancer contributed essential new insight, of which the discovery of the Philadelphia chromosome in chronic myeloid leukemia (CML) is the classical example. The recognition of the t9;22 translocation led to the discovery of the *BCR-ABL* fusion gene and subsequently to one of the first success stories of targeted therapy, the tyrosine-kinase inhibitor Imatinib turning CML from a lethal into a manageable chronic disease. Things changed with the arrival of the "omics" era. Cancer is now characterized at exome, whole genome, methylome, transcriptome and proteome level and the end of this revolution is not in sight with exploration of the metabolome and kinome, to name just two. Different "omics" are functionally interrelated and one of the tasks of bioinformatics will be to make sense of this mountain of confusing information. Basing a classification of cancer on one of these "omics" levels does not seem the right way to go. Integration of these levels in experimental biological research has led to a new emerging scientific discipline, that of systems biology. Conceivably, functional integration of these levels of molecular detail will be required to get to more profound understanding as to what exactly determines the behaviour of a tumor. We might therefore see the emergence of "systems oncology." Until then, it is unlikely that the anatomical/histomorphological basis of tumor classification will be replaced by a molecular basis.

The Context Dependency of the Significance of Molecular Characteristics

A further complication is the context dependency of (some?) molecular genetic traits. The same molecular (genetic) aberration may have different functional implications, depending on the tumor type. It is rather likely that integration of the different strata of molecular information, as suggested in the previous paragraph, will eventually explain why. But for the time being this functional inconsistence in the effects of for example, a gene mutation complicates the use of such aberrations as starting point for tumor classification. An example of this problem are the mutations in *BRAF* in a variety of neoplastic lesions: non-Hodgkin lymphoma, colorectal cancer, malignant melanoma, papillary thyroid carcinoma, non-small-cell lung carcinoma, adenocarcinoma of the lung, brain tumors including glioblastoma and pilocytic astrocytomas. Strikingly, sessile serrated adenomas of the colon often harbor the characteristic V600E *BRAF* mutation, even though most of these lesions will spontaneously regress. In contrast, once a colon carcinoma has developed, the same mutation confers aggressive properties to the tumor, which is reflected in poor prognosis. Depending on

the site or histological characteristics of a *BRAF* mutated tumor, it might or might not respond to the BRAF inhibiting molecule vemurafenib. It is clearly not only the mutation as such but also the functionally integrated molecular biological context that determines the influence of an isolated parameter on the behaviour of a cancer. This is a further impediment in using molecular characteristics as guiding principle in tumor classification.

The Need for Complementarity With Existing Tumor Classifications

Any new tumor classification, no matter how biologically relevant, will need to have a clear relationship with existing classification(s). Continuity in cancer registration for epidemiological purposes is essential. Clinicians will have to be able to continue to apply knowledge based upon previous classification systems in giving care to cancer patients. In addition, there is a conceptual dimension: even though a classification might become obsolete, the knowledge gained through its use does not necessarily loose its relevance.

The Spatial and Temporal Heterogeneity of Tumors

Tumors are heterogeneous. To begin with, all tumors are composed of neoplastic cells surrounded by tumor stroma, the generic name for what tumor biologists tend to call the tumor micro-environment. Stromal components are provided by the host, constitute an essential element of the tumor and co-determine tumor behavior. Furthermore, it is not unusual for cancer cells in a tumor to vary in architectural arrangement, size and shape. There may be well differentiated areas, resembling the tissue of origin; other areas may be undifferentiated, having lost identifying morphological characteristics. In some tumors heterogeneous differentiation patterns occur: some lung cancers are partly adenocarcinoma and partly squamous cell carcinoma. Immunohistochemical staining patterns for marker proteins are often heterogeneous. Areas may be strongly positive and others completely negative; sometimes the immunoreactive cells are scattered throughout the tumor tissue. Molecular genetic analyses have shown that tumors are more heterogeneous than earlier assumed and with the advent of next generation sequencing (NGS) we now must face the reality that at all levels all tumor are heterogeneous, with significant implications for diagnosis and treatment.

This heterogeneity is not only spatial but also temporal. Different tumors have different levels of chromosomal instability or genome hypermutability, and both factors continuously introduce new genomic traits over time. Intrinsic "selective sweeps" or external selection, for example chemotherapy affecting responsive tumor cell clones but not those non-responsive, will determine which tumor cell clones emerge over time as dominant clones largely responsible for prognosis.

Does present day practice in molecular analysis reflect tumor heterogeneity?

This is unfortunately not the case. A small endoscopic biopsy of a carcinoma of the colon does not necessarily represent all the clones present in the primary tumor. DNA isolated from the sample is then unlikely to be representative of all different tumor clones. This has immediate practical consequences, as the single maybe 1 cm^2 but 5 μm thin tissue section sample of a colon cancer submitted for determination of *KRAS* mutation status represents an extremely small fraction of all tumor DNA. Small emerging subclones are unlikely to be represented in a small biopsy. This is one of the explanations for the development of secondary resistance to targeted treatment: a small *KRAS* mutated subclone may remain undetected in the analyzed sample but may emerge in a treatment resistant recurrent lesion.

Does molecular heterogeneity affect patient treatment?

To what extent molecular heterogeneity affects patient treatment is not entirely clear yet. We do know that molecular heterogeneity can be found in all tumors but with strikingly different levels between different tumor types or even between different cases of the same tumor. A landmark paper by the Swanton group reported a disturbingly high level of intratumor heterogeneity in renal cell carcinoma but this is not the case in all cancer types. Most colon cancers have a relatively stable genome and driver mutations in the primary tumor also drive (liver) metastases. A notorious exception in colon cancer are mismatch repair and POLE deficient cases, which have a hypermutating genotype. However, most of these mutations are (silent) passengers and do not drive the metastatic lesion. We need more insight in the patterns of molecular evolution/phylogeny of different tumor types. This will allow better understanding which genome alterations are in the trunk of the (phylogenetic) tree, which are in the branches at which level and what drives the development of new branches.

The wealth of molecular data in The Cancer Genome Atlas (TCGA) or the International Cancer Genome Consortium (ICGC) databases only partly allows to address some of these issues. As a rule, in their studies single decentrally collected tumor tissue samples are analyzed, with relatively scanty follow-up data. Despite current NGS deep sequencing and sophisticated bioinformatics analysis, it will require study of multiple samples on large numbers of primary tumors, long term follow-up of the patients with multiple (liquid or tissue) biopsies including from metastatic sites, along with detailed documentation of clinical evolution and treatment response, to answer the most burning and clinically relevant questions.

A New Classification or a New Layer of Complexity in Existing Classifications?

What emerges from the arguments elaborated in the preceding paragraphs is that the present state of knowledge does not justify the creation of a new classification based primarily upon molecular knowledge. A recent TCGA publication on soft tissue sarcomas

provides a striking example of how "omics" data confirms the solid biological basis of existing classifications. Unsupervised clustering of sarcomas based upon genome aberrations nicely put synovial sarcomas and leiomyomas in distinct clusters, confirming the strength of the morphological approach. An interesting finding in this paper is also that nuclear pleomorphism could at least in part be explained by high genome variability. Automated computational analysis of digital pathology images allowed calculating a nuclear pleomorphism score for each case and increased nuclear pleomorphism was found to correlate significantly with genomic complexity. The earlier cited subclassification of glioblastomas according to *IDH1* mutation status is another example: the class remains glioblastoma, but the subtype is based upon molecular characteristics. What emerges as general conclusion is that class definitions in the WHO classification will remain, but that subtypes will be increasingly defined by molecular parameters. Another example is the development of the consensus molecular classification (CMS) of colorectal cancer. Morphologically defined subtypes have had limited clinical impact, apart from the association of carcinomas in the right colon and of medullary or mucinous subtype and mismatch repair deficiency. In contrast, the molecularly defined CMS subtypes differ significantly in behaviour and in the presence of actionable targets.

In clinical perspective extraction of clinically relevant findings from huge amounts of "omics" data is an important issue. This is manageable for pathologists with profound understanding of molecular data interpretation when it comes to testing for a limited set of known cancer associated genome aberrations. However, for exome, RNA and most obviously for whole genome sequencing data expert bioinformatics support is essential. This requires on the one hand pathologists and clinicians sufficiently comfortable with molecular data to communicate effectively with bioinformaticians, and on the other bioinformaticians with good operational knowledge of cancer genomics and its application in a clinical context. Onco(patho)logists need to train bioinformaticians in the pathobiology of cancer and need to adapt curriculum content of their postgraduate education programs to the molecular world. Digital microscopy will gain in importance, in view of its potential in terms of its more objective character, notably for quantitative parameters, and for decision support to be provided by artificial intelligence.

A final important issue is the need for data sharing. TCGA and ICGC data are based upon significant case cohorts, but to document in detail characteristics of emerging subtypes, some of which will be quite rare, it will be important to dispose of very large case collections. While the wealth of data from diagnostic molecular analyses of human tumors will first and foremost serve individual patients, it would be wasteful not to use these data to increase our understanding of the molecular pathobiology of cancer. An obvious way to do this is large scale data sharing. Cancer care centers might form consortia with translational research units, conceivably with participation of industry partners and obviously supervised by a legal and ethics framework to guarantee patient safety and privacy protection. Molecular data are only useful when accompanied by clinical and pathological data and within such consortia optimal data sets can be developed to complement molecular data. To gain support for this approach, onco(patho)logists need to participate actively in campaigns to inform the public at large and intensify productive interactions with patient advocacy organizations.

Conclusions

The cancer research and care providing community is gradually introducing molecular characteristics into cancer classification. This will have significant implications for but will not completely upset present approaches.

1. The current WHO approach to cancer classification will remain a solid basis for the years to come. The definition of main tumor classes will remain histomorphologic. As David Huntsman and Mark Ladanyi put it in the concluding phrase of their introduction to the 2018 Annual Review Issue of the Journal of Pathology: "Thus, oncological pathology's oldest technology remains meaningfully connected with and continues to inform genomic pathology."
2. New cancer subtypes will continue to emerge, of which the definition will be largely or entirely "omics" oriented.
3. Digital microscopy will rapidly complement and gradually replace conventional microscopy. This will provide a higher level of objectivity to cancer diagnostics, notably when it comes to quantitative parameters. Artificial intelligence through machine deep learning might provide decisional support in the future.
4. Pathologists will need to adapt postgraduate training programs to this shift in emphasis in cancer classification. In the 20th century the endpoint of cancer classification was the information a histological section offered. In the 21st century the histological section represents its first phase, providing a solid basis upon which "genomic pathology" will be grafted, and this should be reflected in the curricula. This holds true also for postgraduate education in other oncology oriented clinical disciplines.
5. Bioinformaticians contribute essential competencies to molecular based classification. A new class of "in silico" oncobiologists with profound understanding of cancer biology and its clinical implications needs to be educated to face increasing demand for this type of support.
6. The likelihood that many new "omics" defined subtypes will emerge goes along with decreasing case numbers per subtype. To nonetheless arrive at case collections allowing solid conclusions as to the relationship between molecular characteristics and clinical behaviour including treatment response, data sharing will become imperative. This needs to be supervised by a legal and ethics framework to guarantee patient safety and privacy protection.

See also: Hormones and Cancer.

Further Reading

Alizadeh, A.A., Aranda, V., Bardelli, A., Blanpain, C., Bock, C., Borowski, C., Caldas, C., Califano, A., Doherty, M., Elsner, M., Esteller, M., Fitzgerald, R., Korbel, J.O., Lichter, P., Mason, C.E., Navin, N., Pe'er, D., Polyak, K., Roberts, C.W., Siu, L., Snyder, A., Stower, H., Swanton, C., Verhaak, R.G., Zenklusen, J.C., Zuber, J., Zucman-Rossi, J., 2015. Towards understanding and exploiting tumor heterogeneity. Nature Medicine 21, 846–853.

Bailey, M.H., Tokheim, C., Porta-Pardo, E., Sengupta, S., Bertrand, D., Weerasinghe, A., Colaprico, A., Wendl, M.C., Kim, J., Reardon, B., Ng, P.K., Jeong, K.J., Cao, S., Wang, Z., Gao, J., Gao, Q., Wang, F., Liu, E.M., Mularoni, L., Rubio-Perez, C., Nagarajan, N., Cortés-Ciriano, I., Zhou, D.C., Liang, W.W., Hess, J.M., Yellapantula, V.D., Tamborero, D., Gonzalez-Perez, A., Suphavilai, C., Ko, J.Y., Khurana, E., Park, P.J., Van Allen, E.M., Liang, H., MC3 Working Group; Cancer Genome Atlas Research Network, Lawrence, M.S., Godzik, A., Lopez-Bigas, N., Stuart, J., Wheeler, D., Getz, G., Chen, K., Lazar, A.J., Mills, G.B., Karchin, R., Ding, L., 2018. Comprehensive characterization of Cancer driver genes and mutations. Cell 173, 371–385.e18.

Berman, J.J., 2004. Tumor classification: Molecular analysis meets Aristotle. BMC Cancer 4, 10.

Boesch, M., Sopper, S., Zeimet, A.G., Reimer, D., Gastl, G., Ludewig, B., Wolf, D., 1866. Cancer stem cells: Rationale for targeting the stem cell niche. Biochimica et Biophysica Acta 2016, 276–289.

Gerlinger, M., Rowan, A.J., Horswell, S., Larkin, J., Endesfelder, D., Gronroos, E., Martinez, P., Matthews, N., Stewart, A., Tarpey, P., Varela, I., Phillimore, B., Begum, S., McDonald, N.Q., Butler, A., Jones, D., Raine, K., Latimer, C., Santos, C.R., Nohadani, M., Eklund, A.C., Spencer-Dene, B., Clark, G., Pickering, L., Stamp, G., Gore, M., Szallasi, Z., Downward, J., Futreal, P.A., Swanton, C., 2012. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. The New England Journal of Medicine 366, 883–892.

Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D., Lander, E.S., 1999. Molecular classification of cancer: Class discovery and class prediction by gene expression monitoring. Science 286, 531–537.

Greaves, M., Maley, C.C., 2012. Clonal evolution in cancer. Nature 481, 306–313.

Guinney, J., Dienstmann, R., Wang, X., de Reyniès, A., Schlicker, A., Soneson, C., Marisa, L., Roepman, P., Nyamundanda, G., Angelino, P., Bot, B.M., Morris, J.S., Simon, I.M., Gerster, S., Fessler, E., De Sousa E Melo, F., Missiaglia, E., Ramay, H., Barras, D., Homicsko, K., Maru, D., Manyam, G.C., Broom, B., Boige, V., Perez-Villamil, B., Laderas, T., Salazar, R., Gray, J.W., Hanahan, D., Tabernero, J., Bernards, R., Friend, S.H., Laurent-Puig, P., Medema, J.P., Sadanandam, A., Wessels, L., Delorenzi, M., Kopetz, S., Vermeulen, L., Tejpar, S., 2015. The consensus molecular subtypes of colorectal cancer. Nature Medicine 21, 1350–1356.

Hamilton, P.W., Wang, Y., Boyd, C., James, J.A., Loughrey, M.B., Hougton, J.P., Boyle, D.P., Kelly, P., Maxwell, P., McCleary, D., Diamond, J., McArt, D.G., Tunstall, J., Bankhead, P., Salto-Tellez, M., 2015. Automated tumor analysis for molecular profiling in lung cancer. Oncotarget 6, 27938–27952.

Huntsman, D.G., Ladanyi, M., 2018. The molecular pathology of cancer: From pan-genomics to post-genomics. The Journal of Pathology 244, 509–511.

Jamal-Hanjani, M., Quezada, S.A., Larkin, J., Swanton, C., 2015. Translational implications of tumor heterogeneity. Clinical Cancer Research 21, 1258–1266.

Louis, D.N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W.K., Ohgaki, H., Wiestler, O.D., Kleihues, P., Ellison, D.W., 2016. The 2016 World Health Organization classification of tumors of the central nervous system: A summary. Acta Neuropathologica 131, 803–820.

Maley, C.C., Aktipis, A., Graham, T.A., Sottoriva, A., Boddy, A.M., Janiszewska, M., Silva, A.S., Gerlinger, M., Yuan, Y., Pienta, K.J., Anderson, K.S., Gatenby, R., Swanton, C., Posada, D., Wu, C.I., Schiffman, J.D., Hwang, E.S., Polyak, K., Anderson, A.R.A., Brown, J.S., Greaves, M., Shibata, D., 2017. Classifying the evolutionary and ecological features of neoplasms. Nature Reviews. Cancer 17, 605–619.

Song, Q., Merajver, S.D., Li, J.Z., 2015. Cancer classification in the genomic era: Five contemporary problems. Human Genomics 9, 27.

Sottoriva, A., Kang, H., Ma, Z., Graham, T.A., Salomon, M.P., Zhao, J., Marjoram, P., Siegmund, K., Press, M.F., Shibata, D., Curtis, C., 2015. A big bang model of human colorectal tumor growth. Nature Genetics 47, 209–216.

The Cancer Genome Atlas Research Network, 2017. Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. Cell 171, 950–965.

Interferons: Cellular and Molecular Biology of Their Actions☆

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Introduction

Vertebrates mount two major types of responses to thwart the threat from invading pathogens and progression of neoplastic cell growth: (1) innate (non-specific) immunity and (2) acquired (specific) immunity. The former, an immediate response, sets the stage for the latter, which takes longer time (few weeks) to develop. Cytokines play a central role in both these processes. Interferons (IFNs) are primary responders and drivers of innate immunity, which later continue to collaborate with other cytokines in developing acquired immunity. The name "Interferon" was coined about 59 years back by Isaacs and Lindenmann on the basis of their earliest observation that these cytokines, secreted by virus-infected cells, interfered with virus replication in the neighboring cells. Since their original description, studies on these cytokines provided remarkable insights into anti-pathogen immunity, non-self and self-recognition, cancer biology and signal transduction, gene regulation and many other fundamental processes involved in cellular physiology including RNA stability and translational control of gene expression. As a result IFNs occupy a central place in mammalian biology and continue to provide trailblazing insights. This chapter will provide a bird's eye view of a current understanding of this field.

IFNs—A Family of Cytokines

Although presumed to be a single protein at the time of their discovery, it is now clear that IFNs are a collection of cytokines (α, β, γ, δ, k, ε, λ, τ, ω, ζ). In mammals, there are 13 subtypes of IFN-α, 4 subtypes of IFN-λ and single forms of IFN-β, IFN-γ, IFN-ε, IFN-κ, IFN-δ, IFN-τ, IFN-ω, and IFN-ζ. IFN-κ and IFN-ε are exclusively expressed in the skin keratinocytes and the female secondary reproductive organs, respectively. IFN-δ and IFN-τ are only found in pigs and ruminants, respectively. IFN-τ is expressed only in the trophoblasts. Similarly, one other IFN, Limitin, which downsizes B-cell populations via apoptosis after the initial antigen stimulus, is only found in mice, for which no known orthologues are present in other species. There are 4 IFN-λ subtypes viz., -λ1,-λ2, -λ3 and -λ4 in humans. IFN-λ1, -λ2 and -λ3 were previously known as IL-29, IL-28A and IL-28B, respectively. After realizing their functional equivalence to other IFNs in terms of signal transduction and gene-induction profiles, they were regrouped and named IFN-λ. In the laboratory mouse strains, IFN-λ1 is a pseudogene, whereas all 4 genes are expressed in humans. Some of these IFNs express in a tissue-dependent manner and exhibit as high as 90–100% (e.g., among IFN-α subtypes) and as little as 20% (e.g., IFN-α and IFN-λ) sequence similarity with other members of their family. Despite their significant sequence divergences, most of these IFNs are capable of inducing an anti-viral state in target cells, which unifies them functionally. In humans, the 13 IFN-α, and one of each IFN-β, IFN-ω, IFN-k and IFN-ε genes all are located in a gene cluster on chromosome 9. It is still unclear why 13 different IFN-α subtypes are produced. IFNs are also grouped on the basis of their receptor usage: type I (IFN-α, IFN-β, IFN-ε, IFN-k, IFN-δ, IFN-τ, IFN-ω, and IFN-ζ); type II (IFN-γ) and type III (IFN-λs). All type I IFN genes have no introns, consistent with their activity as first messengers after an active infection, or danger response. It is likely that the lack of introns allows a rapid processing of mRNAs for translation to prevent infections. The IFN-γ gene has 3 introns while IFN-λ genes have 4 introns. All IFNs are produced as 16–18 kDa secretory proteins.

Inducers of IFN Genes

Most IFNs are induced in response to a pathogen or danger signal(s). These signals include viral and cellular antigens, viral genomic nucleic acids or their replication intermediates, bacterial cell wall products such as LPS, lipids, proteins like flagellin, nucleic acids, radiation damage, DNA-damaging drugs like the anthracycline antibiotics, parasite DNAs and their cell constituents, non-DNA-damaging drugs like Imiquimod, and 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) and modified cellular nucleic acids (**Table 1**). Most potent among these are the viral nucleic acids that is, single-stranded (ss) and double-stranded (ds) RNAs, RNA-DNA hybrids (retroviruses), endogenous retroviral nucleic acids, retro-transposon elements, and viral DNAs, along with synthetic nucleic acids like poly I:C and poly dI:dC. The signaling pathways that induce IFN-β have been well worked out using several experimental models (see below). The most commonly shared transducers of these signals are: Tumor-Necrosis Factor (TNF) Receptor-Associated Factor (TRAF) family member-Associated NF-kB activator (TANK)-Binding Kinase 1 (TBK1) and IFN-gene Regulatory Factor 3 (IRF3). Finally, basal IFN-β production under physiological conditions is induced by the commensal microbial flora.

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Table 1 The ligands and effectors of PRRs

PRR	*Ligand*	*Source of Ligand*	*Effector(s) induced*
TLRs			
TLR1	Multiple triacyl lipopeptides	Bacteria	Cytokines
TLR2	Peptidoglycans, Triacyl lipopeptides, Hemagglutinin, Porins	Bacteria, viruses, parasites, self	Cytokines
TLR3	dsRNA, ssRNA, Poly A:U, Poly I:C	Viruses	Cytokines, type I IFNs
TLR4	LPS, Mannan, Envelope proteins	Bacteria, viruses, self	Cytokines, type I IFNs
TLR5	Flagellin	Bacteria	Cytokines
TLR6	Multiple diacyl lipopeptides	Bacteria, viruses	Cytokines
TLR7	ssRNA, Imidazoquinoline	Viruses, bacteria, self	Cytokines, type I IFNs
TLR8	ssRNA, Small synthetic compounds	Viruses, bacteria, self	Cytokines, type I IFNs
TLR9	CpG-DNA, dsDNA	Viruses, bacteria, parasites, self	Cytokines, type I IFNs
TLR10	Bacterial lipopeptides	Bacteria	Cytokines
NLRs			
NOD1	iE-DAP(PGN)	Bacteria	Cytokines
NOD2	MDP(PGN)	Bacteria	Cytokines, type I IFNs
RLRs			
RIG-I	Short dsRNA	RNA viruses, DNA viruses	Type I IFNs, cytokines
MDA5	Long dsRNA	RNA viruses	Type I IFNs, cytokines
LGP2	Unknown	RNA viruses	Reduced IFN-response
CDSs			
AIM2	dsDNA	DNA viruses	IFNs, cytokines
DNA-PK	dsDNA	DNA viruses	IFNs, cytokines
cGAS	dsDNA	DNA viruses	IFNs, cytokines
DHX36	dsDNA	DNA viruses	IFNs, cytokines
STING	dsDNA	DNA viruses	IFNs, cytokines
DHX9	dsDNA	DNA viruses	IFNs, cytokines
DAI	dsDNA	DNA viruses	IFNs, cytokines
IFI16	dsDNA	DNA viruses	IFNs, cytokines
LRRF1P1	dsDNA	DNA viruses	IFNs, cytokines

Abbreviations: AIM2, Absent in melanoma 2; CDS, Cytosolic DNA sensors; cGAS, cyclic-GAMP synthase; CpG DNA: DNA containing unmethylated Cytosine in CG repeates DAI, DNA - dependent activator of IRFs; DHX, DEAH box helicase; DNA-PK, DNA dependent protein kinase; dsDNA, double stranded DNA; dsRNA, double-stranded RNA; iE-DAP,gamma-D-glutamyl-meso-diaminopimelic acid; IFI16, Interferon gama inducible protein 16; LPS, lipopolysaccharide; LRRF1P1, Leucine rich repeat (in FLII) interacting protein 1; MDP, muramyldipeptide; NLR, NOD-like receptor; NOD, nucleotide binding oligomerization domain; PGN, peptidoglycan; PRR, pattern recognition receptor; RLR, RIG-I-like receptor; ssRNA, single-stranded RNA; STING, Stimulator of interferon genes; TLR, *toll*-like receptor.

Signal Transduction Pathways That Induce IFN Genes

At steady state, low levels of IFNs are produced in vivo owing to the microbiome. Autocrine loop of IFN production provides a signal to maintain basal expression of some IFN-stimulated genes (ISGs), for example STAT1. Upon encountering an infection, IFN levels are further induced through *de novo* transcription. Following their synthesis IFNs are secreted into the extracellular environment to suppress the spread of pathogens. Infectious pathogens are surveyed by specific receptors. The pattern recognition receptors (PRRs) monitor extracellular, endosomal and cytosolic compartments for signatures of pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) and non-self molecules to activate innate immune responses, which subsequently collaborate with the acquired immune response system to protect the host (**Table 1**). Four major families of receptors, the Toll-like receptors (TLRs), RIG-I like receptors (RLRs), cytosolic DNA sensors (CDSs) and NOD-like receptors (NLRs) act as sentinels to sense the danger. Plasmacytoid Dendritic Cells (pDCs) produce large quantities of type I IFNs upon engagement of TLR7 and TLR9, which detect viral or bacterial RNA and DNA molecules, respectively, that have been endocytosed or sequestered through autophagy. Our current understanding of IFN gene induction primarily comes from the IFN-β gene. The IFN-β gene is regulated by a complex enhancer that binds multiple transcription factors including NF-kB (p65/50), IRF3, c-Jun and ATF2. Of these, IRF3 is the most critical one, which undergoes signal-induced phosphorylation. IRF3 then binds to the critical IFN-gene Regulatory Element (IRE) found in IFN-β gene promoter as a dimer. IRF3 belongs to the IRF family that includes 10 structurally-similar but genetically distinct transcription factors. Virus infection, the most common stimulus, causes the activation of transcription factors NF-kB and IRF3. Following a typical RNA virus infection, the viral genomic RNA is released into the cytosol. Such RNA is readily recognized by the RNA helicases Retinoic acid Inducible Gene-I (RIG-I, also known as DDX58) or Melanoma differentiation antigen-5 (MDA-5, also known as IFIH1). These two proteins with another protein LGP2 (also known as DHX58) comprise the RLRs. LGP2 contains a RNA binding domain but lacks the caspase-recruitment domain (CARD) and is believed to act as a negative regulator of RIG-I and MDA-5 induced IFN synthesis. LGP2 dimerizes with MDA5 in some cells for positively regulating the IFN-β gene. Studies with

Lgp2-/- mice suggest that it may also act as a positive regulator of IFN induction in some tissues. The RLR proteins, RIG-I and MDA5, contain two N-terminal CARDs, a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain. They are localized to the cytoplasm and are essential sensors of cytosolic RNAs. The RNA-bound RIG-I or MDA-5 then associates with a mitochondrion associated protein MAVS (also known as IPS-1). Following this, MAVS recruits a molecular complex consisting of TRAF3-TANK-NAP1 proteins, which in turn recruits the critical serine/threonine kinase TBK1. TBK1 then phosphorylates IRF3, which forms a dimer that migrates to the nucleus and stimulates transcription of IFN-β gene (Fig. 1). Multiple sites on IRF3 are phosphorylated (S^{385}, S^{386}, S^{396}, S^{398}, S^{402}, T^{404}, S^{405}) that induce its transcriptional activity. A majority of these sites are present in the transcription activation domain (TAD) of IRF3 and are required for optimal transcription.

In the case of other viruses that undergo endosome-mediated internalization and de-capsidation, the viral nucleic acids released into the endosomal lumen are sensed by the resident TLRs viz., TLR3 (binds viral dsRNA), TLR7 and TLR8 (bind viral ssRNA) and TLR9 (viral DNA). These TLRs recruit the intracellular adaptors MyD88, TRIF, TRAM, which then serve as docking sites for TRAF3. The latter then binds and stimulates TBK1. Notably, the endosomal TLRs -7, -8, and -9 preferentially use MyD88, whereas TLR3 employs TRIF as intracellular adaptors. As described above TBK1 phosphorylates IRF3 to stimulate IFN-β gene expression (Fig. 1). Interestingly, the tumor suppressive phosphatase PTEN promotes IRF3 import into the nucleus through a removal of an inhibitory phosphorylation at the S^{97} residue of IRF3.

The non-self or viral dsDNA are sensed by multiple CDSs, amongst which is a nucleotidyl transferase, cGAS, that generates an unusual second messenger, cyclic GAMP (cGAMP), consisting of Guanosine and Adenosine linked by a characteristic 2′-3′ phosphodiester bond. cGAMP binds to an endoplasmic reticulum (ER)-resident protein, STimulator of INterferon Genes (STING; also known as ER IFN Stimulator (ERIS), Transmembrane Protein 173 (TMEM173)). Following cGAMP binding, STING moves into a peri-nuclear endosome, after passing through the Golgi apparatus, where it recruits TBK1 to stimulate IRF3 phosphorylation.

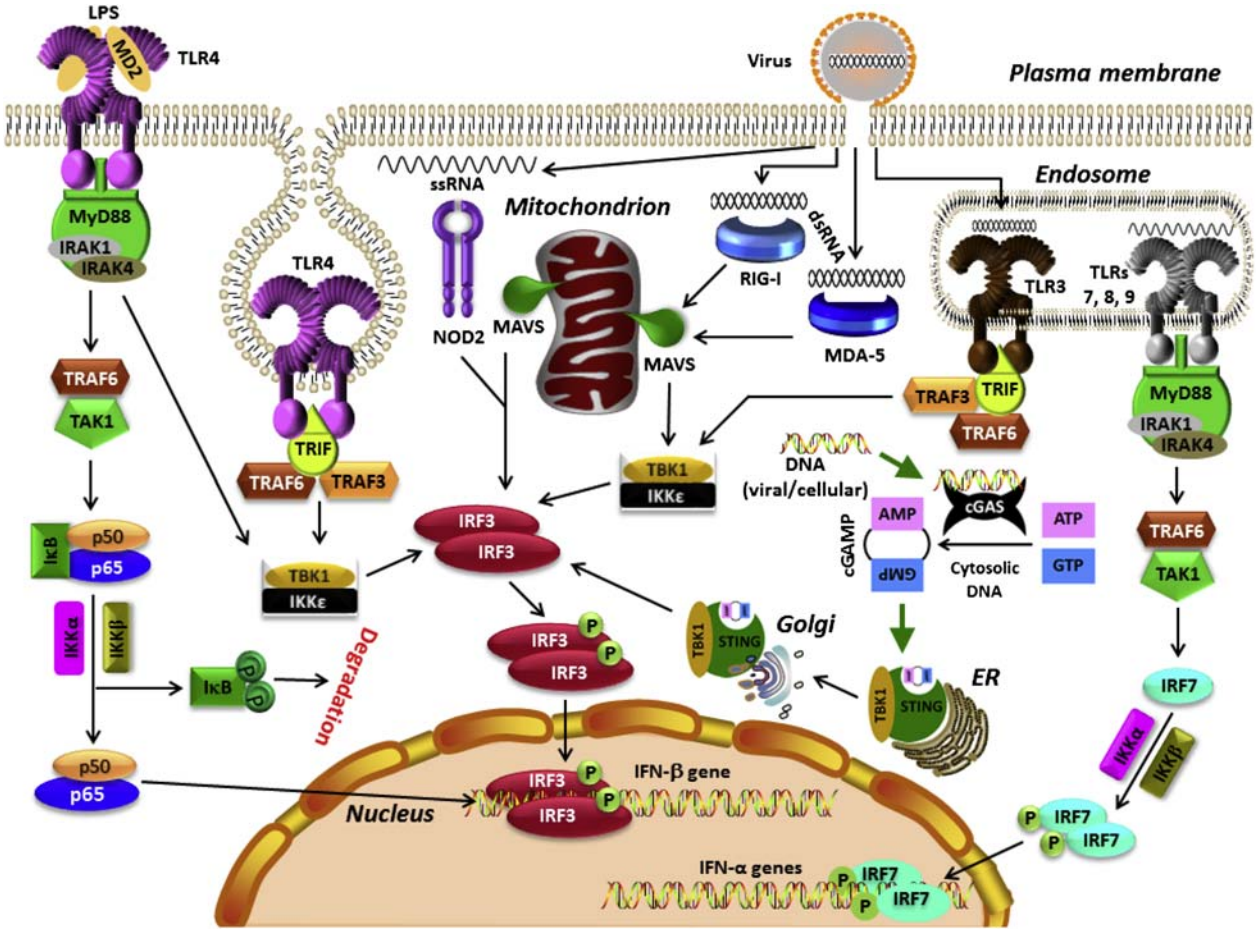

Fig. 1 Signal transduction pathways that induce type I IFNs. A number of exogenous PAMPs are recognized by resident receptors in the plasma membrane, endosome, cytoplasm, non-self nucleic acids (derived from the endogenous retrotransposons) and sensed as danger leading to the upregulation of IFN-β, the first responder cytokine. TBK1 (a signal transducing kinase) and IRF3 (a transcription factor) play a central role in these responses. IRF7 is a major inducer of IFN-α genes. IKK: IkB kinase; LPS: Lipopolysaccharide, a cell-wall phosphoglycolipid synthesized by bacteria; MAVS: Mitochondrial Anti-viral Signaling Protein (also known as IFN-β Promoter Stimulator 1(IPS-1), Virus-Induced Signaling Adaptor (VISA)); NOD2: Nucleotide-binding Oligomerization Domain-containing 2; TRIF: TIR domain-containing adaptor inducing IFN-β.

Apart from cGAS, many other cytoplasmic molecules such as DAI, DDX41 and IFI16 can sense DNA and stimulate STING to activate the TBK1-IRF3-dependent pathway. IFI16 is also an ISG. LRRFIP1 is another molecule that activates IRF3-IFN-β axis in a STING-independent but β-catenin-dependent manner. Two other cytosolic DNA sensors, DHX9 and DHX36, activate IRF7 in a MyD88-dependent manner to induce type I IFNs. Lastly, Ku70, another DNA sensor activates IRF7 and IRF1 to induce type I IFNs (**Fig. 1**).

In the classical TLR4-driven pathway, bacterial LPS binds to the extracellular receptor portion, which recruits the cytoplasmic adaptor molecules MyD88 and TRIF. The former recruits IL-1 Receptor-Associated Kinase-1 (IRAK1), which then activates the NF-kB pathway. The latter activates the TBK1 complex consisting of TRAF3-TANK-TBK1 proteins. TBK1 then phosphorylates IRF3 to stimulate IFN-β gene expression (**Fig. 1**).

The IFN-α proteins are primarily produced by pDCs and macrophages in high quantities. These genes are turned on in response to virus infection through IREs in their enhancers, to which IRF7 homodimers or IRF7: IRF3 heterodimers bind and regulate gene expression. IRF7 is an ISG and it amplifies the initial IFN signal further by inducing transcription of IFN-α genes. IRF7 resides in the cytoplasm at steady state and upon cytosolic RLR stimulation, it undergoes serine phosphorylation at the C-terminus, which permits it migration to the nucleus and induction of type I IFN genes. This is a feed-forward amplifying mechanism that explains the priming effect of IFN on its own synthesis (**Fig. 1**). In contrast to most cell types, pDCs constitutively express IRF7, enabling these cells to rapidly produce high levels of IFN-α and IFN-λ1 following virus infection.

Type III IFNs (IFN-λ) are induced by a number of RNA and DNA viruses. It is generally believed that all type I IFN inducers are capable of inducing these IFNs. However, type III IFNs are expressed in a tissue-restricted manner. Human IFN-λ1 and IFN-β genes are transcriptionally regulated in a similar manner, that is, they are controlled by IRF3, whereas IFN-λ2-4 genes, like most IFN-α genes, are dependent on IRF7. In humans, either IFN-β or IFN-λ1 can prime cells for virus-induced IFN-α and IFN-λ2-4 synthesis by upregulating IRF7 levels.

IFN-γ

The human IFN-γ gene is located on chromosome 12q14. It is expressed and induced only in cells of immune system such as antigen-activated T cells, NK cells, NKT cells and DCs. LPS and certain viral infections induce IFN-γ indirectly. Among cytokines, IL-12 and IL-18 are potent inducers of IFN-γ. Calcium ionophores like ionomycin and phorbol esters are also known to induce IFN-γ. Transcription factors Stat4, Runx3, and T-bet, and epigenetic factors have been implicated in the transcriptional regulation of IFN-γ gene. A constantly low level of IFN-γ is produced by NK cells owing to the action of transcription factors EOMES and T-bet. IL-12 and IL-18 employ STAT4 and NF-kB for inducing IFN-γ gene, respectively. Recent studies show that a long non-coding RNA, *Tmevpg1*, coded by the opposite strand of IFN-γ gene, positively regulates IFN-γ gene expression. However, *Tmevpg1* still requires transcription factors STAT4 and T-bet for inducing IFN-γ. Upon its synthesis IFN-γ forms a dimer. The NLRs upon engaging with bacterial peptidoglycan or viral components form a complex with an adaptor protein ASC and caspase-1. The latter, a cysteine protease, cleaves the inhibitory peptide from IL-18 precursor releasing the mature IL-18.

IFN Receptors

IFNs bind to cell surface receptors for exerting their biological effects. There are three major receptors in mammals: type I, type II and type III. A typical IFN receptor is constituted by two distinct subunits, coded by separate genes. Despite differences in their ligand preferences, genes coding for the subunits of type I (*IFNAR1*, *IFNAR2*), type II (*IFNGR2*) and type III (*IFNLR2* or *IL10RB*) are all clustered on human chromosome 21q22.1, suggesting their common evolutionary origin and a possible functional collaboration. Exceptionally, the *IFNGR1* is located on human chromosome 6q23.3. Almost all cells express functional type I and type II receptors, whereas the type III receptor is most abundantly expressed by epithelial cells. As mentioned above type I IFNs bind to a well characterized heterodimeric receptor that is comprised of a low-affinity IFN-α receptor subunit 1 (IFNAR1) and a high-affinity receptor subunit 2 (IFNAR2). The *IFNAR2* gene codes for three protein isoforms via exon-skipping, alternative splicing and differential usage of polyadenylation sites. The most commonly referred IFNAR2 is derived from the *IFNAR2c* transcript, the longest isoform with an intact intracellular domain and is fully competent to transduce signals. A shorter membrane bound form lacking the intracellular signaling domains is derived from the *IFNAR2b* transcript. A soluble IFNAR2a protein is similar to IFNAR2b but it has additional 11 amino acids in its carboxyl terminus. IFNAR2a and IFNR2b are generally considered as dominant negative inhibitors of type I IFN receptor-driven signaling. The type I IFN receptor is quite plastic as it interacts with nearly 17 different ligands to exert its biological effects. All type I IFNs bind to their receptor as monomers.

The type II IFN receptor (IFNGR) is exclusively used by IFN-γ. It is composed the ligand-binding IFNGR1 and the signal-transducing IFNGR2 subunits. Since IFN-γ exists and binds to the receptor as a dimer, IFNGR is a functional tetramer, that is, two each of IFNGR1 and IFNGR2 subunits. A ligand-induced assembly of the IFNGR complex initiates signal transduction.

The type III IFN receptor (IFNLR) is a very unique heterodimer that is comprised of the ligand-binding IFNLR1 and the signal-transducing IFNLR2 subunits. The latter subunit, also known as IL10RB, is also employed by IL-10, IL-22 and IL-26 for signal transduction. Like the type I receptor, the type III receptor engages with monomeric IFN-λs. Interestingly, even though the receptors and their ligands are structurally and genetically distinct, both the type I and type III IFN receptors engage similar intracellular signal transduction components (see below), suggesting a co-evolution of these molecules. Despite this similarity, neither type I receptor

engages IFN-λs nor type III receptor engages type I IFNs under physiological conditions. Tissue-restricted action of type III IFNs is so far the most important difference between type I and type III IFNs. Expression of IFNLR1 is restricted primarily to epithelial cells and some specific subsets of immune cells, which explains its tissue tropism. Fibroblasts and endothelial cells do not respond to IFN-λs, owing to a very low level of IFNLR1.

IFN-Induced Signal Transduction Pathways

Once engaged with their receptors, multiple ISGs are induced by IFNs with different kinetics. A variety of signaling pathways are employed for inducing ISGs. One of the best understood pathways leading to ISG induction is the Janus tyrosine kinase (JAK)-Signal transducer and activator of transcription (STAT) pathway. JAKs are non-receptor tyrosine kinases characterized by their conserved JAK-homology (JH) domains and two protein kinase domains (JH1 and JH2), only one of which (located in JH1 domain) is enzymatically functional (Fig. 2). The JH2 domain is a 'pseudokinase', for it lacks critical residues required for catalysis. Because of an analogy between two protein kinase domains of JAKs and the mythical two heads of Janus, the Roman god of gateways, these are known as Janus Kinases. A Src homology 2 (SH2) domain, present within JH3 and JH4 domains, allows JAKs to bind specific phospho-tyrosine containing motifs. JH5-JH7 domains form the FERM domain, which target JAKs to membrane-resident receptors. The acronym FERM stands for band 4.1 protein, Ezrin, Radixin and Moesin, respectively, the original four proteins in which this domain was found. FERM domain-containing proteins localize at the interface between the plasma membrane and the cytoskeleton. There are 4 JAKs: TYK2, JAK1, JAK2 and JAK3 coded by distinct genes in the mammalian genome. Through a set of elegant studies that involved derivation of IFN unresponsive cell mutants and genetic complementation, the laboratories of George Stark, Sandra Pellegrini and Ian Kerr provided the first unequivocal evidence for the role of JAKs in IFN signaling pathways. Following this landmark discovery, many labs showed that various cytokines and their receptors utilize various combinations of JAKs to transduce signals. Subsequent to ligand engagement with the receptors, JAKs not only undergo autophosphorylation but also phosphorylate the receptor subunits and the STATs each at a critical tyrosine residue.

STAT proteins constitute a family of 7 structurally-similar but genetically-distinct gene products (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) which function as signal transducers and transcription factors. At steady state, these proteins are dormant in the cytoplasm. Following ligand engagement these proteins are tyrosyl phosphorylated at the receptor by JAKs, form dimers, and migrate to the nucleus to stimulate the transcription of target genes. The first evidence for the role of STAT proteins

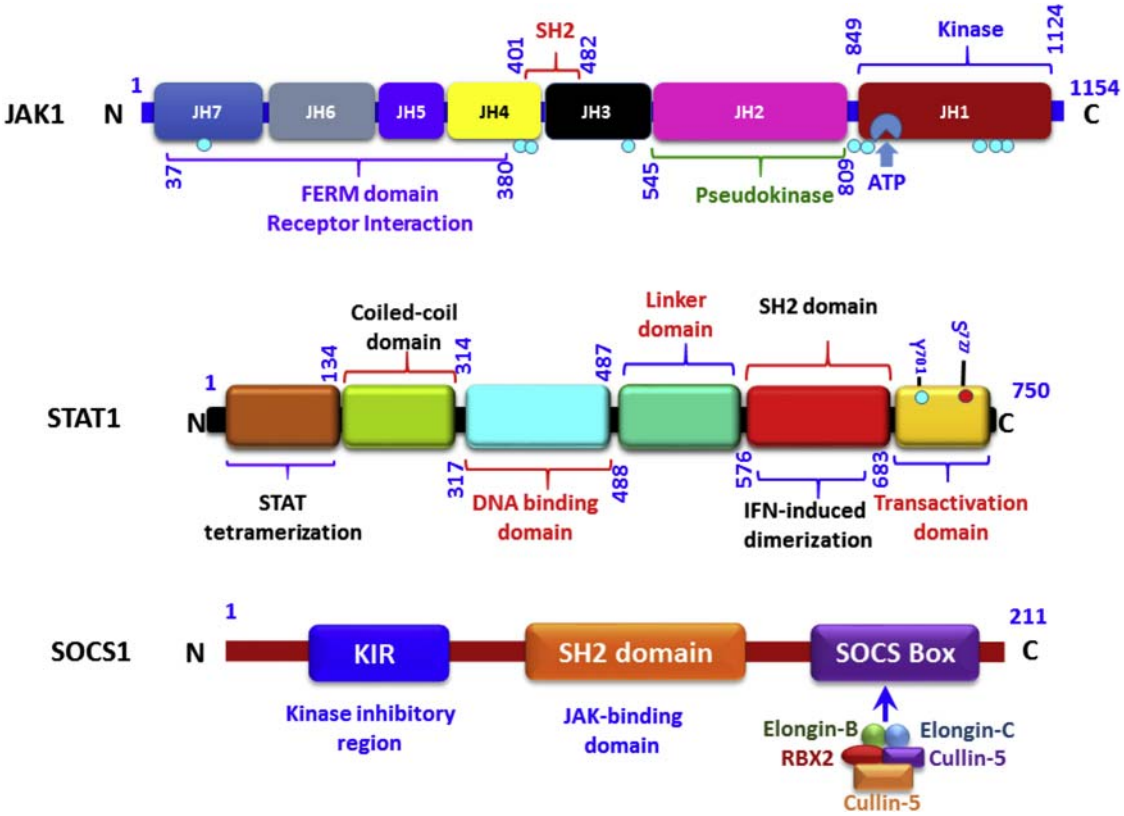

Fig. 2 Modular representation functional domains in JAK1, STAT1 and SOCS1 proteins. N: Amino terminal; C: Carboxyl terminal. Numbers indicate residues in primary sequence. Blue and red circles represent tyrosine and serine/threonine residues, respectively, that undergo phosphorylation.

(STAT1 and STAT2) in IFN signaling pathways came from the laboratory of James Darnell. The critical role of these STATs was established through genetic complementation of IFN unresponsive cell mutants generated by the Stark and Kerr laboratories. These ground breaking studies paved the way for the discovery of the other STATs and their roles in driving responses initiated by ~50 different cytokines and growth factors. A typical STAT (Fig. 2) is characterized by an N-terminal oligomerization domain, a coiled-coil domain, a central DNA binding domain, a linker domain, an SH2 domain and a C-terminal TAD. Sandwiched between the SH2 domain and TAD is a critical tyrosine, which undergoes JAK-induced phosphorylation and allows STAT dimerization. In the case of STAT1 and STAT2 such tyrosine (Y) residues are located respectively at 701 and 690 positions in their primary sequence. In addition to this, STAT1 undergoes phosphorylation at a serine residue (S^{727}) in its TAD, which occurs independently of JAKs but is required for transcriptional activation. Mutation of these critical Y and/or S residues blunts STAT-dependent transcriptional responses. That said, some recent studies show that phosphotyrosine-deficient STAT1 (notated as U-STAT) also activates a set of genes in the steady state.

In the type I and type III IFN signaling pathways (Figs. 3 and 4), TYK2 and JAK1 are essential for driving anti-viral responses and gene expression. In the case of the type I IFN receptor, TYK2 and JAK1 are associated with IFNAR1and IFNAR2 subunits, respectively, in the steady state. Without such pre-association, IFNAR1 becomes unstable on the cell surface. Ligand binding induces dimerization of the receptor subunits and juxta-positioning of TYK2 and JAK1, which permits cross-phosphorylation (required for activation) and then phosphorylation of a critical Y^{466} residue of IFNAR1 (Fig. 3). Phosphorylated Y^{466} serves as a docking site for the SH2 domain of STAT2, which permits the phosphorylation of STAT2 at its Y^{690} residue. Tyrosyl phosphorylated STAT2 then serves as a binding site for the SH2 domain of STAT1 and permits its phosphorylation at Y^{701}residue. The STAT1:STAT2 complex dissociates form the receptor, and the heterodimer migrates to the nucleus to induce transcription of ISGs. In the type I and type III signaling pathways, IRF9 (also known as p48 or ISGF3γ), a non-STAT protein, is also required for DNA binding and transcriptional activation by the STAT1:STAT2 dimer. The STAT1/STAT2/IRF9 complex (popularly known as the IFN-stimulated gene factor 3 or ISGF3) binds to the IFN-stimulated response elements (ISRE) found in the promoters of many ISGs. ISRE, a 15-bp long conserved element with the following canonical sequence 5′-AGGTTTCNNTTTCCT-3′, exhibits remarkable similarity to the IRE (found in the IFN gene enhancers) at its core. As a result, ISRE could drive IRF-driven transcription, independently of the JAK-STAT pathway, under the influence of dsRNA, a known inducer of IFN genes. Such response neither requires IFN synthesis nor IFN receptor. This redundancy allows an efficient management of innate anti-pathogen responses under situations where dysfunctioning of JAK-STAT pathway or the receptors occurs. In some cells, IFN-α also activates the tyrosyl phosphorylation of STAT3. Phosphorylated STAT3 forms a complex a transcriptional suppressor Sin3A, which inhibits induction of direct STAT3 target genes by promoting deacetylation of STAT3 and histones. This mechanism prevents the development of pro-inflammatory diseases, and dampening of IFN-induced anti-viral gene expression.

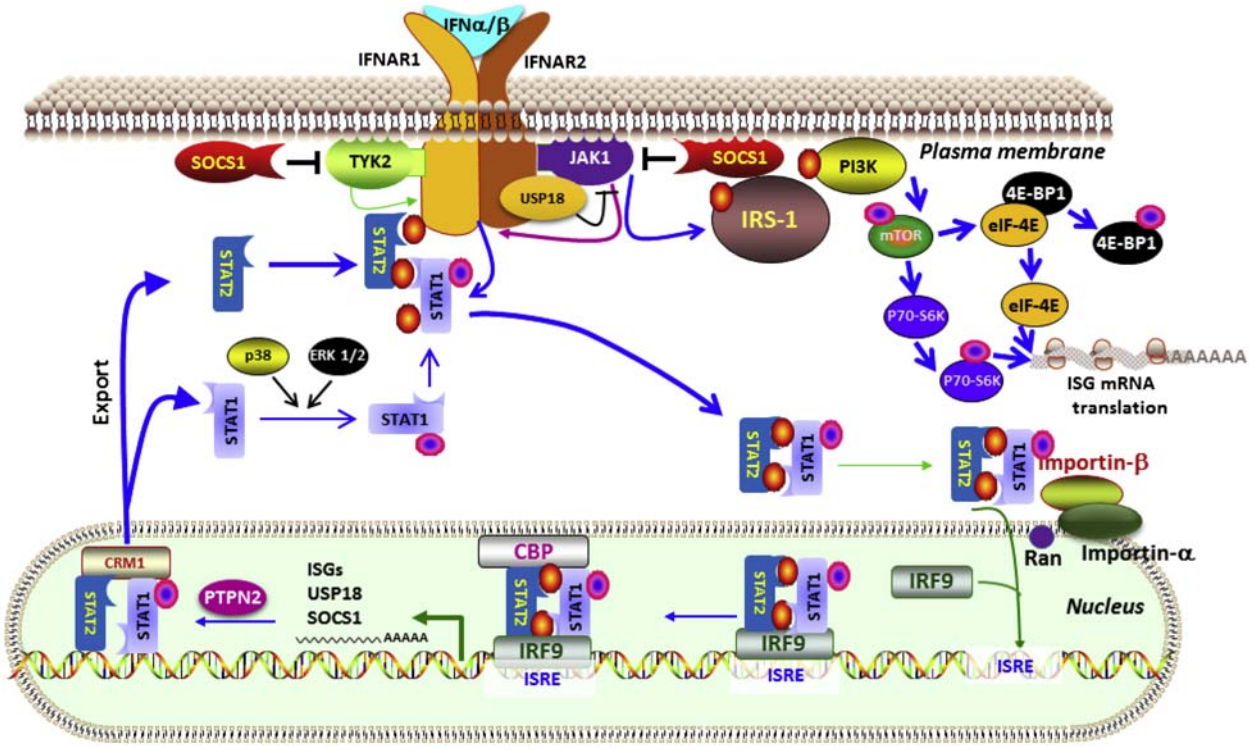

Fig. 3 Type I IFN signaling pathways. CBP: CREB-binding protein (a histone acetyl transferase that serves as a transcriptional co-activator: CRM1: Chromosome Region Maintenance 1 Homolog (also known as Exportin 1).

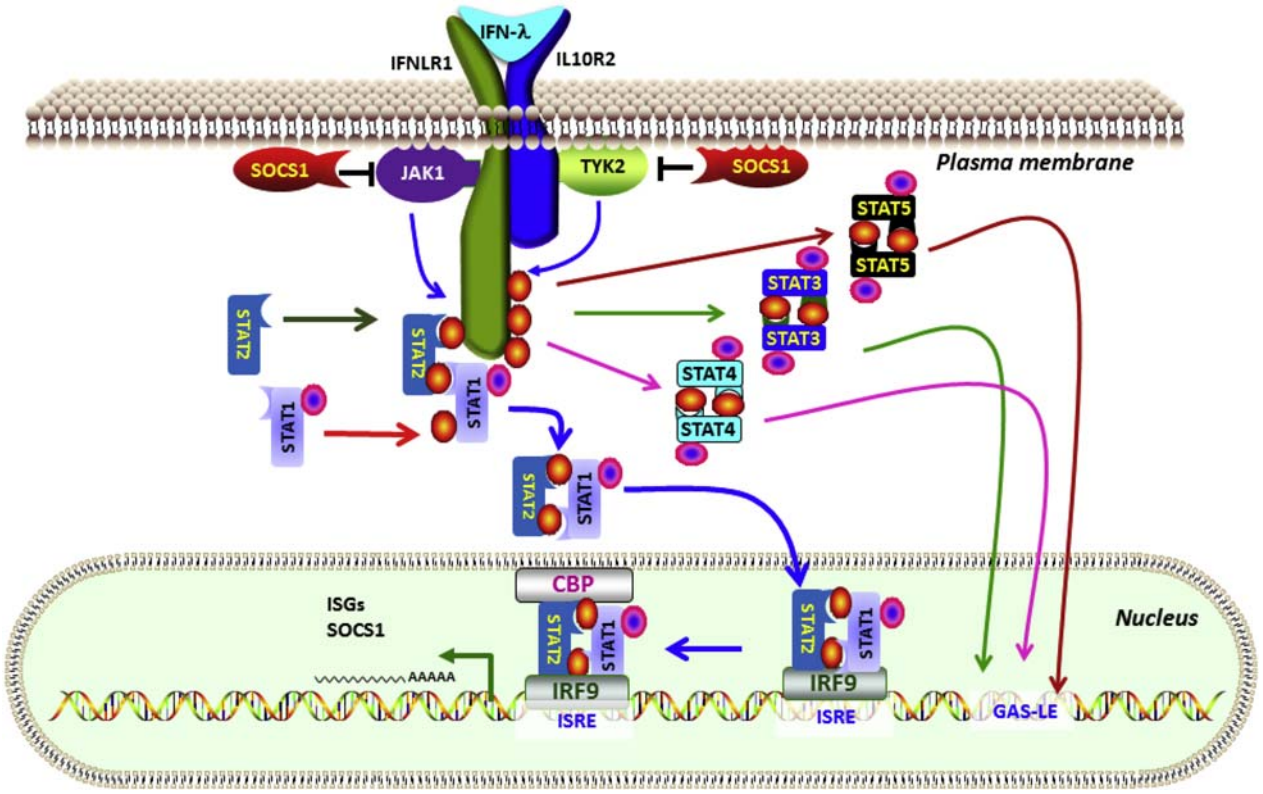

Fig. 4 Type III IFN signaling pathways. GAS: gamma-IFN activated site; GAS-LE: GAS-like elements.

Type III IFN signaling occurs in a very similar manner to that of type I IFN. IFNLR1 and IFNLR2 are pre-associated with JAK1 and TYK2 (Fig. 4). Ligand-induced aggregation of the receptor causes JAK activation and ISGF3 formation as described above. In addition, type III IFNs induce STAT3, STAT4 and STAT5, whose biological relevance is unclear.

IFN-γ-Induced JAK-STAT Signaling

In contrast to IFN-α, IFN-γ binding to its receptor primarily activates STAT1. At steady state IFNGR1 and IFNGR2 subunits of the receptor are pre-associated with JAK1 and JAK2, respectively. Ligand-induced aggregation of receptor leads to JAK activation (JAK2 activating JAK1 first). JAK1 then phosphorylates IFNGR1 on the critical Y^{440} residue (Fig. 5). This then provides a docking site for the SH2 domain of STAT1. STAT1 is then phosphorylated at its Y^{701} residue. This then serves as a docking site for a second STAT1 molecule, permitting its Y^{701} phosphorylation. STAT1:STAT1 homodimers then dissociate from the receptor, translocate to the nucleus and bind to the Gamma-IFN activated sites (GAS) of IFN-γ stimulated genes. Mutant STAT1 lacking theY^{701} residue, cannot translocate to the nucleus when stimulated with IFN-γ. STAT1 dimers are imported into the nucleus via nuclear pores where a GTP-binding protein Ran plays an important role. As already mentioned STAT1 also undergoes S^{727} phosphorylation, catalyzed by different kinases. Depending on the cell type, Mitogen-activated protein kinases (MAPKs) like the extracellular signal-regulated kinase (ERK) and p38MAPK, cyclin-dependent kinase 8 (CDK8) and Ca^{+2}/Calmodulin-dependent kinase II have been implicated in this process. IFN-γ also activates STAT3 and STAT4 proteins under certain circumstances. In fact, the IFN-γ-JAK-STAT4 pathway appears to play an important role in warding off certain viral infections.

Termination of JAK-STAT Signaling

Activated STAT1 is not retained in the nucleus more than 1 h in normal cells. A nuclear phospho-tyrosyl phosphatase TC-PTP has been shown to dephosphorylate STAT1. STAT1 is exported back to the cytoplasm and this terminates STAT1-stimulated gene expression. Two different proteins, one that targets JAKs and another that inhibits STAT1 have been reported. Upon nuclear entry and DNA binding, STAT1 induces several mRNAs. One of these mRNAs codes for SOCS1, the founding member of a large family ($n = 20$ members) of proteins containing Suppressor of Cytokine Signaling (SOCS) box motifs. SOCS1 protein has three functional

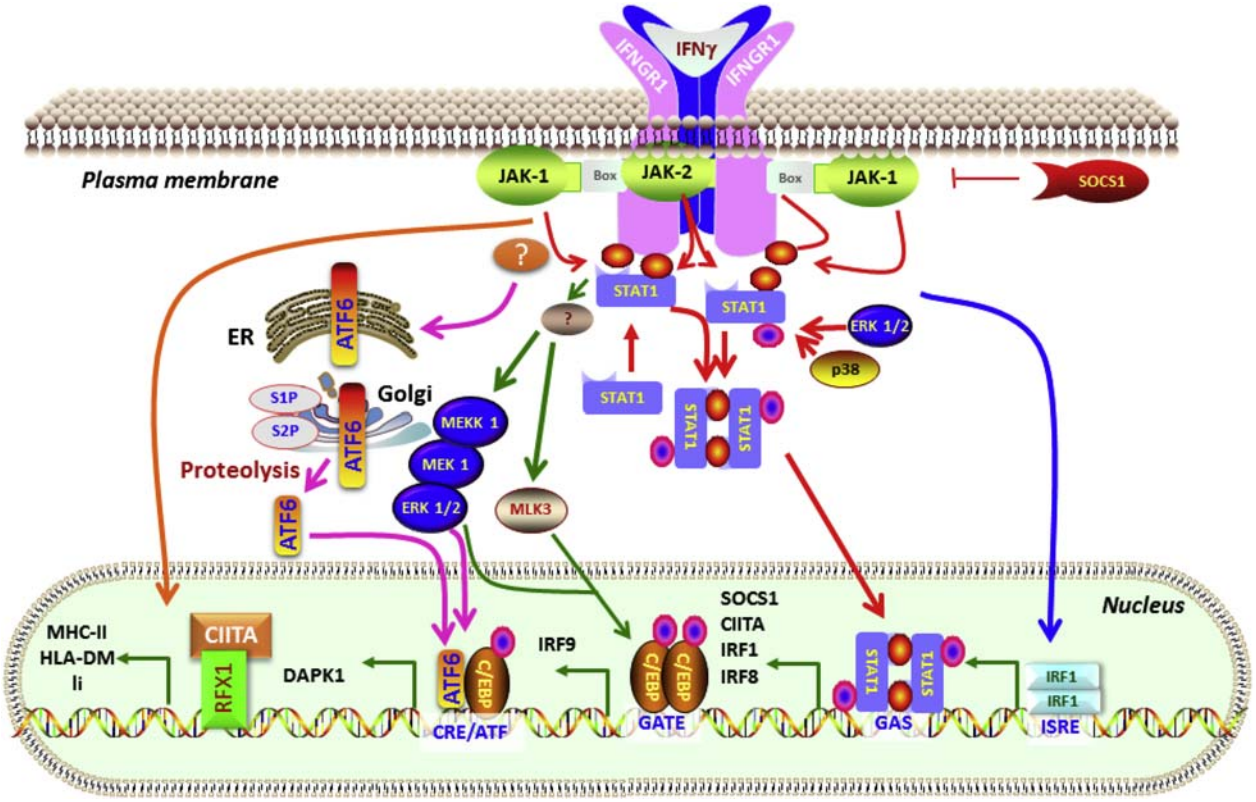

Fig. 5 Type II IFN signaling pathways. GATE: gamma-IFN activated transcriptional element; MLK3: Mixed Lineage Kinase-3; MEKK1: Mitogen-activated protein kinase kinase kinase 1; MEK1: Mitogen-activated protein kinase kinase 1; S1P: Site 1-specific protease; S2P: Site 2-specific protease. CRE: cAMP response element; ATFS: ATF6-binding site; RFX: Regulatory factor X1 (a transcription factor that binds to X-box elements in the MHC class II gene enhancers).

domains (Fig. 2): an N-terminal kinase inhibitory region (KIR), a central SH2 domain and c-terminal SOCS box. The SH2 domain of SOCS protein binds to phosphorylated JAKs, whereas the KIR domain is inserted into the kinase pocket of JAKs to block their protein kinase activity. The SOCS box interacts with a complex consisting of elongin B, elongin C, cullin-5, RING-box-2 (RBX2) and E2 ligase. This allows them to function as E3 ubiquitin ligases and mediate the degradation of proteins that they associate with through their amino-terminal regions. As a result SOCS proteins target the entire cytokine-receptor complex, JAKs and SOCS protein themselves, for proteasome-mediated degradation. Consistent with its negative regulatory role, deletion of *Socs1* resulted in fulminant inflammation and pre-weaning death of mice. Inflammation in kidneys and livers of these mice were found. These phenotypes appear to be a result of uninterrupted IFN signaling, because mice lacking both *Ifng* and *Socs1* genes do not develop such pathologies. SOCS1 also interacts with IFNAR1 to terminate type I IFN-regulated gene expression. A second mechanism that blocks STAT1 activity is the blockade of its DNA-binding function by the Protein-inhibitor of activated STAT1 (PIAS1) via physical interaction. Since, loss of PIAS1 protein resulted in upregulation of a limited number of ISGs, this suggested that it may not be a universal negative regulator of IFN-induced JAK-STAT pathways.

The type I IFN-induced signaling is also negatively regulated by targeting the IFNAR1 subunit to ubiquitin-dependent lysosome-mediated degradation. The E3 ligase complex SCF (Skp1–Cullin1–F-box) initiates IFNAR1 poly-ubiquitylation via both Lys48- and Lys63-linked chains and its degradation. The Casein kinase-1α (CK-1α)-mediated phosphorylation of IFNAR1 at S^{535} residue plays a critical role in recruiting the SCF complex to the receptor. A priming phosphorylation at S^{532} residue by the pancreatic ER kinase is required before S^{535} can be phosphorylated by CK-1α. A second mechanism that negatively regulates IFN-α signaling is the IFN-induced expression of ubiquitin-specific peptidase 18 (USP18 or UBP43) in some cells. ISG15 is an ubiquitin-like IFN-inducible protein. ISG15 is conjugated to a number of cellular proteins, although the exact biological significance is unclear. USP18, an IFN-induced cysteine protease, specifically removes ISG15 from such modified proteins. However, the phenotypic alterations caused by *Usp18* deletion in the mouse have been dissociated from its ISG15-dependent mechanisms. USP18 displaces JAK1 from IFNAR2 subunit to terminate IFN signaling. Both type I and type III IFNs induce USP18 expression, indicating that this protein may also negatively regulate type III IFN receptors. The importance of the JAK-STAT signaling pathway and downstream transcription factors in host defenses against viral infection have been confirmed using gene knockout mice (Table 2).

Table 2 Phenotypes of knockout mice lacking IFN-signaling machinery

Gene	*Defects detected*
Ciita	Loss of antigen presentation, immunodeficiency
Ifnar1	Loss of anti-viral responses
Ifngr1/2	Susceptibility to bacterial infections and carcinogens
Irf1	Immunodeficiency and increased susceptibility to viral infections.
Irf2	Loss of anti-viral effects and B-cell proliferation
Irf5	Loss of anti-viral response; less susceptible to the development of SLE
Irf7	Loss of IFN-α/β expression and susceptible to viral infections
Irf8	Immunodeficiency and increased susceptibility to viral infections
Irf9	Loss of anti-viral responses and immune system defects
Jak1	Post-natal lethality, neuronal development defects
Jak2	Lack of hematopoiesis
Socs1	Death before weaning, fatty degeneration of the liver, hyper response to viral infection
Stat1	Loss of innate immunity and susceptibility to carcinogens
Stat2	Embryonic lethality
Tyk2	Defective NK cell responses

Notes: The complex phenotypes in *Jak1*, *Jak2* and *Tyk2* deficient mice are due to the fact that these kinases participate in signaling pathways driven by other cytokines.

Post-Transcriptional Regulation of Jak-Stat Signaling

MicroRNAs (miRNAs) are small non-coding RNAs processed from their precursor RNAs (200–300 nt). Mature miRNAs (~20 nt) can either induce the degradation of the target RNAs if their sequence matches perfectly to that of target protein coding transcripts or block the translation of the protein from the target RNA, if the homology is not perfect. Two miRNAs, *miR-155* and *miR-29a* block *SOCS1* and *IFNAR1* transcripts, respectively. SOCS1 inhibition increases IFN response, whereas IFNAR1 inhibition down modulates IFN signaling. Since *miR-155* and *miR-29a* can also affect many other targets in the cell, it should be noted that the effects on IFN signaling by these miRNAs can be multi-faceted.

Non-STAT Pathways That Regulate ISG Expression

Apart from the JAK-STAT pathway a collaborative pathway is induced by IFN-α. Activated TYK2 and JAK1 regulate tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), which provides docking sites for the SH2 domains of the regulatory subunit (p85) of phosphatidylinositol 3 Kinase (PI3K). PI3K then activates the protein kinase mechanistic Target of Rapamycin (mTOR) which promotes downstream events leading to the initiation of mRNA translation (Fig. 3). mTOR stimulates ribosomal protein S6 kinase beta-1 (p70-S6K), which phosphorylates ribosomal protein S6 (RPS6), resulting in the initiation of mRNA translation. mTOR also regulates phosphorylation of the translational repressor eukaryotic translation-initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). Such phosphorylation results in its deactivation and subsequent dissociation from eIF4E, allowing the initiation of cap-dependent mRNA translation and enhances the expression of ISGs. Thus, this pathway appears to be an accessory to the transcription-activating JAK-STAT pathway, described above.

The pro-myelocytic leukemia zinc finger (PLZF) protein, coded by the *Zbtb16* gene, is essential for the expression of certain ISGs involved in anti-viral innate immune responses. PLZF, in contrast to its known gene-repressive function, acts as an inducer of gene expression in this case. PLZF binds to its putative sites in some ISG promoters, such as *Oas1g*, *CXCL10*, *Rsad2* (Viperin) and *Ifit2*. IFN-α failed to induce these genes in *Plzf* $^{-/-}$ mice. PLZF is phosphorylated at its S^{76} residue in a c-Jun N-terminal Kinase (JNK)-dependent manner in response to IFN-α. In contrast, FOXO3, a member of fork-head family of transcriptional regulators, inhibited type I IFN-induced responses by binding to its putative sites in certain ISG promoters and blocking their expression.

IFN-γ induces multiple non-STAT pathways for regulating gene expression. IFN-γ can also induce IRF1 and IRF8-driven expression of certain ISGs in myeloid compartments. The MHC class II gene is an indirect target of IFN-γ signaling. Its expression is dependent on a critical regulatory factor, the Class II Transcriptional Activator (CIITA). CIITA is an IFN-γ inducible gene that binds to the enhancer of MHC class II, HLA-DM and li genes to promote their expression (Fig. 5). CIITA expression is induced by STAT1 and IRF1. Mutations/loss of expression of CIITA causes the 'Bare-lymphocyte syndrome' a severe immunodeficiency in humans.

IFN-γ also induces the expression of the IRF9 gene through a unique element, GATE. Although GATE exhibits some partial homology to ISRE, it does not bind ISGF3 or IRF proteins. Instead it binds C/EBP-β, a member of the CAAAT/enhancer-binding protein (C/EBP) family of transcription factors. In response to IFN-γ, a MEKK1–MEK1–ERK1/2 pathway is activated, wherein the terminal enzymes ERK1/2 phosphorylate the C/EBP-β protein at a consensus motif in the regulatory domain. In addition, orphan MAPK, the mixed-lineage kinase 3 (MLK3) activates a dephosphorylation event at the N-terminal TAD of C/EBP-β, which is also required for optimal transcriptional activation (Fig. 5).

C/EBP-β also collaborates with another protein, activated transcription factor-6 (ATF6). At steady state, ATF6 exists as a precursor in the ER bound by its inhibitor BiP. ATF6 consists two major functional parts: (1) the N-terminal end, which hangs into the cytosol, serves as a transcription factor; and (2) the C-terminal end, which is in the ER lumen senses ER stress. Upon IFN-γ treatment, ATF6 undergoes ASK1–MKK3–p38MAPK-dependent phosphorylation and then migrates from the ER to the Golgi apparatus. In the Golgi it undergoes processing by the resident Site 1-specific protease (S1P) and Site 2-specific protease (S2P), which frees the N-terminal transcriptionally active part (mature form) from the C-terminal portion. The mature ATF6 protein translocates to the nucleus, where it associates with phosphorylated C/EBP-β and forms a transcriptional complex, to drive the expression of the death-associated protein kinase 1 (DAPK1), a major regulator of IFN-induced cell growth, apoptosis and autophagy (**Fig. 5**).

IFN-Inducible Genes

Nearly 5000 genes are induced by IFNs, depending on the type, dose, and cell type, species as identified by several microarray, RNA-seq and proteomic analyses. "Interferome", a web-based database that cures such data, is freely available to all investigators through the Monash University and Hudson Institute for Medical Research, Melbourne, Australia (http://www.interferome.org/interferome/home.jspx). Some of the well characterized genes and their mechanisms of action are shown in **Table 3**. Here, the mechanisms of actions of a few gene products that exert anti-viral effects are described. IFNs induce various ISGs that inhibit virus replication at various stages. The major well-defined anti-viral mechanisms include: (1) degradation of viral RNAs; (2) inhibition of viral protein synthesis; (3) prevention of viral uncoating or blockade of their localization in an infected cell (**Fig. 6**).

Table 3 Mammalian ISGs

Gene	*Function*	*Mechanism*
2′-5′ (A) synthetases	Anti-viral enzymes	Degrade viral RNA through synthesis of 2′-5′ $(A)_n$
ADAR	Inhibits viral replication	RNA editing of adenosine to inosine
APOBEC3C	Inhibits HIV replication	RNA editing cytosine deaminase
APOBEC3G	Inhibits HIV replication	RNA editing cytosine deaminase
β2-Microglobulin	Antigen presentation	Partner for MHC class I protein
CCL2 (MCP1)	Chemoattractant	Recruitment of Monocytes and leukocytes
CCL5 (RANTES)	Chemoattractant	Recruitment of Monocyte/lymphocytes
CH25H	Inhibits at early infectious cycle	Inhibits virus-host membrane fusion
CIITA	Transcriptional co-activator	Induces HLA/MHC Class II genes
CXCL10 (IP10)	Chemoattractant	Monocytes, natural killer and T-cell migration
DAPK1	Tumor suppressor	Induces apoptosis, and autophagy
DDX 60	Suppress viral replication	Increased RLR dependent signaling
FcγR1	Binds to IgG	Opsonization of microbes
IDO	Indoleamine 2,3 dioxygenase	Anti-parasitic/bactericidal
GBP1	Guanylate binding protein	Anti-viral/autophagy
GBP2	Guanylate binding protein	Anti-viral/autophagy
IGTP	GTPase	Anti-toxoplasma
IFI16	Transcription factor	Cell growth inhibition
IFIT1 (ISG56)	Blocks viral infection	Targets eIF3 to block viral IRES mediated translation
IFIT3/RIG-G	Antiviral activity	Protein-protein interaction
IFITM1	Blocks virus entry, e.g., Ebola & HCV	Alters kinetics of endosome acidification
IFITM2	Blocks virus entry, e.g., HIV-1	Increases non-specific protease activity
IFITM3	Blocks virus entry ,e.g., InfluenzaA, HIV-1	Unknown
IFITM5	Blocks virus entry	Unknown
NOS2 (iNOS)	Nitric oxide synthesis	Anti-microbial
IRGM	Macrophage Immunity-Related GTPase	Anti-bacterial/anti-protozoal
IRF1	Transcription factor/tumor suppressor	ISRE-mediated ISG expression, activates IRF3 & 7
IRF5	Transcription factor	Induces IFN-α genes; autoimmune SLE
IRF7	Transcription factor	Increased expression RIG-I and IFN-α genes
IRF8	Transcription factor	Transcription factor
ISG15	Promotes viral replication	ISGylation (Like Ubiquitination)
ISG20	Restricts viral infection	3′-5′ exonuclease activity
LRG47	GTPase	Anti-microbial/Anti-protozoal
MHC class I	Immune response	Antigen presentation
MHC class II	Immune response	Antigen presentation
MNDA	Transcription factor	Cell growth inhibition in association with p53
Mx1 (MxA)	Anti-viral GTPase	Traps incoming viral components
Mx2 (MxB)	Anti-retroviral activity/GTPase	Inhibits chromosomal integration
NOX2 (CYBB)	NAPH oxidase/ROS production	Anti-microbial/microbicidal

(Continued)

Table 3 Mammalian ISGs—cont'd

Gene	*Function*	*Mechanism*
PKR	Anti-viral Protein kinase	eIF2α phosphorylation/translation blockade
RIG-I (DDX58)	Inhibits RNA virus replication	Activates RLR signaling
RNaseL	Anti-viral endoribonuclease	2′-5′ (A) activated/promotes viral RNA degradation
PSCR1	Phospholipid scramblase	Apoptosis induction
PSMA2	Proteasome Subunit, Alpha 2	Protein degradation
PSMB8	Proteasome Subunit, Beta 8	Protein degradation
PSMB9/RING 12	Proteasome Subunit, Beta 9	Protein degradation
PSMB10	Proteasome Subunit, Beta 10	Protein degradation
PSMD8	Proteasome 26S Subunit, Non-ATPase, 8	Protein degradation
PSME1	Proteasome Activator Subunit 1	Protein degradation
SOCS1	Inhibitor of JAKs	Terminates JAK-STAT signaling
TAP1/RING4	Peptide Transporter	Antigen Processing
TGTP	GTPase	Anti-parasitic, anti-bacterial
TNFSF10/TRAIL	Ligand for cell death receptor DR2	Apoptosis
TRIM -5α	Inhibits Virus infection e.g. HIV-1	Disassembly of the capsid shell
TRIM-19 (PML)	Inhibits Virus infection	Ubiquitination, SUMOylation, ISGylation
TRIM-22	Inhibits Virus infection e.g. HIV-1	Ubiquitination, SUMOylation, ISGylation
TRIM-25	Inhibits Virus infection	Ubiquitination, Enhanced RIG-I activity
TRIM-56	Inhibits Virus infection	Ubiquitination, Activation of STING
USP-18	Ubiquitin-specific peptidase	Terminates JAK-STAT signaling binding to IFNAR
Viperin	Anti-viral activity	Alters membrane fluidity
WARS/IFP53	Tryptophanyl-tRNA Synthetase	Protein synthesis

Abbreviations: ADAR, RNA-specific adenosine deaminase; CH25H, cholesterol-25-hydroxylase; DDX, DExD/H box RNA helicase; eIF2α, eukaryotic initiation factor 2α; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFITM, IFN-inducible transmembrane; IRF, interferon regulatory factor; ISG, interferon stimulated gene; ISRE interferon-sensitive responsive element; Mx, myxovirus resistance; PKR, protein kinase R; RIG-I, retinoic acid inducible gene I; RLR, RIG-I like receptor; RNase L, 2-5A-dependent endoribonuclease latent form; TRIM, tripartite motif.

Notes: IFNs induce more than 5000 genes depending on the cell type. Only the gene products with confirmed biological activities are shown. This list includes both type I and type II IFN stimulated genes. There are no known genes that are regulated by type III IFNs only. Because of the similarity in their signal transduction pathways, it is likely that all type I IFN-induced genes are also type III IFN-inducible. This list does not include IFN-repressed genes. IFIT1, IFIT2 and IFIT3 are related at the sequence level but are functionally distinct. IFITM1, IFITM2, IFITM3 and IFITM5 are not related to IFIT proteins.

Anti-Viral Action Through Targeting Viral RNAs

Two mechanisms contribute to this of type action. In the first, IFN induces the expression of genes coding for a family of enzymes the 2′-5′ Oligoadenylate synthetases (OAS): OAS1 (40/46 kDa), OAS2 (69/71 kDa), OAS3 (100 kDa) and OASL (56/59 kDa). Although all 4 genes are IFN inducible, OASL is also directly induced by viral infection. The OAS1, OAS2 and OAS3 genes are clustered at human chromosome 12q24.1 and OASL at 12q24.2. These enzymes require dsRNA for their catalytic activity (**Fig. 6A**). Monomeric OAS1 protein is catalytically inactive. Upon dsRNA binding (viral RNAs) it tetramerizes to generate a super-activated enzyme that synthesizes an unusual class of oligonucleotides called, 2′-5′ oligoadenylate (2′-5′A) using ATP as substrate. In 2′-5′ A, nucleotides are linked by a 2′-5′ phosphodiester bond unlike the 3′-5′ linkage in DNAs and RNAs. The 2′-5′As then bind to a cognate domain of Ribonuclease L (RNaseL) to activate its nuclease activity. At steady state, RNaseL exists as a dormant monomeric endoribonuclease, with its N-terminal 2′-5′A-binding domain folded over the catalytic C-terminal domain, thus, preventing the ribonuclease activity. 2′-5′A binding to the N-terminus not only releases this inhibitory switch but also allows the dimerization of RNaseL, which dramatically increases its enzymatic activity, leading to the degradation of viral RNAs. RNaseL activity is extinguished by phosphodiesterases (PDEs) that destroy excessive 2′- 5′A, after the initial stimulation. RNaseL also generates additional dsRNA molecules through a destruction of viral RNA replication intermediates, which not only activate the RLR-dependent type I IFN synthesis but also amplifies the OAS-RNaseL loop to sustain the anti-viral effects over a prolonged period. Although RNaseL was classically described as an anti-viral enzyme, recent studies suggest that it also exerts potent anti-bacterial effects, partly through a control of phagocytosis. OAS2 and OAS3 proteins have 2 and 3 OAS domains, respectively, of which only one is enzymatically active in these proteins. These additional domain repeats are believed to recognize larger dsRNA molecules and present them correctly to the enzymatically-active domain. The OASL is unique for it not only lacks the characteristic enzymatic activity of this class but also possesses 2 ubiquitin like domains. As a result it cannot activate RNaseL, but can still exert anti-viral effects though its ubiquitin-like domains, suggesting a novel mechanism of action.

Another IFN-inducible protein that potentially contributes to the anti-viral activity is the adenosine deaminase activated by dsRNA (ADAR). This enzyme, upon activation by dsRNA, edits such RNAs to allow the production of new proteins or make the RNA unstable and target them for destruction (**Fig. 6B**). In the humans, two distinct genes *ADAR* and *ADARB1*, code for proteins with enzyme activity and their expression level is controlled in a tissue-specific manner. The *ADAR* gene, produces two isoforms of

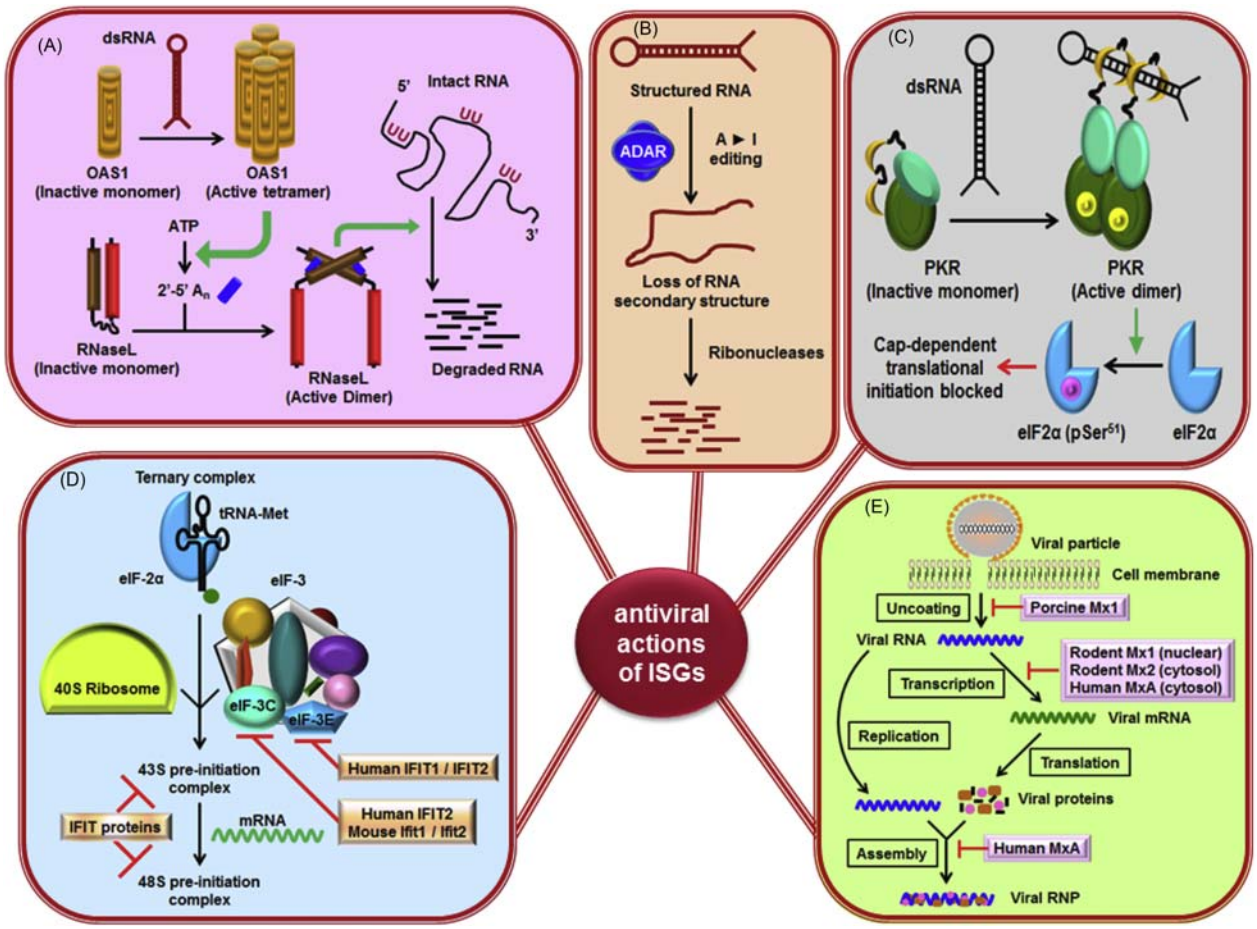

Fig. 6 Anti-viral actions of 5 different ISG pathways are shown. Most of these pathways control viral replication through post-transcriptional and post-translational mechanisms.

110 kDa (house-keeping form) and 150 kDa (IFN-inducible form) due to upstream transcriptional initiation. The 110 kDa isoform is always present in the nucleus as it lacks a nuclear export sequence that is present in the 150 kDa isoform. ADARB1 is known to edit certain host mRNAs to generate proteome diversity as well as regulate splice site choice. ADARs bind to dsRNA through their N-terminal domain. The C terminus catalyzes the Adenosine to Inosine conversion in the target RNA. This enzyme has been suggested to act as an IFN inducer, which amplifies anti-viral activity. Activated ADAR has been suggested to modify viral RNAs, which are sensed as non-self by the RLRs leading to IFN synthesis.

Anti-Viral Action Through Inhibition of Viral RNA Translation

The Protein kinase-RNA-activated (PKR) is an IFN-induced serine/threonine kinase, whose activity is stimulated by dsRNA, a classical co-factor (Fig. 6C). In the native state PKR has little or no enzymatic activity, due to auto-inhibition of its kinase activity by its N-terminal regulatory domain. Upon encountering viral dsRNA or partially dsRNA regions of viral/cellular nucleic acids, the N terminus of PKR binds to them and frees the kinase domain from repression. Two PKR molecules dimerize and autophosphorylate each other on their kinase domains. The eukaryotic translation initiation factor 2 (eIF2) is a heterotrimer consisting of α, β and γ subunits. eIF2 mediates the binding of $tRNA^{met}$ to the ribosome in a GTP-dependent manner. Once the initiation is completed, GDP-bound eIF2 is released from the ribosome as an inactive complex. To participate in another round of translation initiation, this GDP must be exchanged for GTP. Activated PKR phosphorylates the alpha subunit of eIF2 (eIF2α) at specific serine residues at positions 48 and 51. Phosphorylated eIF2α fails to assemble the ternary complex, because it does not exchange GTP to GDP. This aborts viral mRNA translation, and, hence, replication. PKR is also activated by cellular protein, PACT, independently of dsRNA. PKR is also involved in the execution of apoptosis as part of the Fas death receptor-mediated signaling.

The human ISG56 (also known as IFIT1) belongs to a family of structurally similar but genetically distinct proteins called, Interferon Induced proteins with Tetratricopeptide repeats (IFIT). The human IFIT family is constituted by 4 members viz., IFIT1

(ISG56), IFIT2 (ISG54), IFIT3 (ISG60 or IFIT4), and IFIT5 (ISG58) clustered together on chromosome 10. Two other genes *IFIT1B* (uncharacterized) and *IFIT1P1* (a pseudogene) located on chromosome 13, appear to be related members of this family. Human IFIT1 and IFIT2 interact with the 3E subunit of eukaryotic translation initiation factor 3 (eIF3), a protein complex consisting of 13 protein subunits, to regulate translation (**Fig. 6D**). The human IFIT2 and mouse Ifit1 and Ifit2 proteins bind to the eIF-3C subunit to block translation. IFIT1 inhibits protein synthesis by preventing the association of eIF3 with the 43S pre-initiation complex, thus, blunting 5′-cap-dependent viral translation. Alternatively, IFIT1 can directly recognize the type O cap (without 2′O methylation) of certain viral RNAs and prevent the formation of 43S pre-initiation complex. IFIT1 also suppresses hepatitis C virus (HCV) replication by blocking internal ribosome entry site (IRES) dependent translation. Such a translational blockade occurs independently of PKR. The murine *Ifit* family is formed by three characterized members viz, Ifit1 (Isg56), Ifit2 (Isg54) and Ifit3 (Isg49) clustered on chromosome13. *Ifit1c* (Gm14446) is an allelic variant of *Ifit1*. Two additional members of this family with unknown expression patterns and functions include, *Ifit1b* (2010002M12Rik) and *Ifit3b* (I830012O16Rik). Although, the murine Ifit1 and Ifit2 proteins exhibit sequence similarity to human IFIT proteins they are not functionally equivalent. Hence, they should treated as distinct proteins. Importantly, IFN-inducible proteins IFITM1, IFITM2, IFITM3, which are often confused to be IFIT proteins because of the name similarity, are not related to IFIT proteins. They have an unclear mode of action. Among these IFITM3 block the viral entry into cells (see **Table 3**).

The Mx proteins, although originally described as myxovirus-specific inhibitors, can target many other viruses (**Fig. 6E**). MxA, a prototypical member of this family, is a dynamin-like GTPase, whose activity is required for exerting anti-viral effects. Mx proteins form oligomers to exert anti-viral effects. Depending on the virus, different stages of viral life cycle are inhibited by the Mx proteins. Mouse Mx1 inhibits Influenza and Thogoto (THOV) viruses by blocking viral mRNA synthesis catalyzed in the nucleus by the RNA polymerase located in the viral nucleocapsids. Human MxA intercepts THOV nucleocapsids in the cytoplasm and prevents their import into the nucleus, where the virus replicates. MxA also blocks Hepatitis B virus, La Crosse virus (LACV) and other bunyaviruses by sequestering the newly synthesized viral N protein into perinuclear complexes. Human MxB blocks HIV-1 replication by preventing viral uncoating, and nuclear import of the reverse transcribed viral genome. Consistent with their critical role in anti-viral defenses, mice engineered to lack PKR, RNaseL, IFIT1 or Mx proteins are highly susceptibility to viral infections. More importantly, the anti-viral actions of ISGs are blocked by a number of viral gene products to escape from inhibition, testifying their critical role in host defenses (**Table 4**). These mechanisms include a production of soluble receptors, degradation of IRF3, inhibition of the JAK-STAT pathway, blockade of PKR and other ISGs.

Table 4 Viral Resistance to IFNs

Virus	*Component*	*Target*	*Mechanism*
Adeno	E1A protein	IRF3	Competing with IRF3 binding site
Adeno	E1A	ISGF3	Inhibits IRF9 expression/ISGF3 binding to ISRE
Adeno	VAI RNA	PKR	Blocks activation
Coxackie	3C	MAVS and TRIF	Cleavage of MAVS and TRIF
Dengue	NS4B	STAT1	Blocks STAT1 tyrosyl phosphorylation
EMCV	3C	RIG-I	Cleavage of RIG-I
Enterovirus 71	2A	IFNAR1	Cleavage of IFNAR1
Enterovirus 71	3C	RIG - I and TRIF	Sequestration of RIG-I and Cleavage of TRIF
Epstein-Barr	EBNA-2	ISG induction	Inhibits anticellular action
Epstein-Barr	EBER	PKR	Blocks activation
Epstein-Barr	BCRF-1 protein	IFN - γ gene	Inhibits IFN - γ synthesis
Epstein-Barr	BGLF4 protein	IRF3	Decrease amounts of active IRF3
HCMV	pp65 protein	IRF3	Decrease of phosphorylated IRF3, and nuclear translocation and accumulation
Hendra	V protein	STAT1, STAT2	Sequesters STATs to prevent their activation
Hepatitis A	3C protein	MAVS	Cleavage of MVAS
Hepatitis B	TP	ISG induction	Blocks signaling
Hepatitis B	ORF-C	IFN - β gene	Inhibits gene expression
Hepatitis B	X protein	MAVS	Proteasomal degradation of MAVS
Hepatitis C	NS 3-4A	MAVS	Cleavage of MVAS
HIV-1	TAR RNA	PKR	Blocks activation
HIV-1	TAR protein	PKR	Inhibits PKR
HIV-1	Protease	eIF4G,RIG-I	Cleavage of eIF4G, Sequestration of RIG-I
HPIV2	V protein	TBK1	Decoy phosphorylation substrate
HPIV2	V protein	STAT2	Ubiquitination-dependent degradation
HPV 16	E6 protein	IRF3	Direct inhibition of IRF3
HSV	2-5 A analog	RNase L	Blocks activation
HSV	ICP0	IRF3	Blocks nuclear translocation and accumulation
Influenza A	Cellular p58	PKR	Inhibits PKR

Table 4 Viral Resistance to IFNs—cont'd

Virus	*Component*	*Target*	*Mechanism*
Influenza A	NS1 protein	RIG-I	Indirect inhibition of RIG-I
KSHV	LANA-1	IRF3	Competing with IRF3 binding site
KSHV	ORF52	cGAS	Inhibits cGAS activity by direct binding
KSHV	vIRF1	p300 and ISG15	Inhibits type I IFNs by ISGylation
Measles	C protein	IRF3	Nuclear sequestration of IRF3
Measles	V protein	STAT1, STAT2	Prevents nuclear import of STATs
Mumps	V protein	TBK1	Decoy phosphorylation substrate
Mumps	V protein	STAT1	Ubiquitination-dependent degradation
Myxoma	MT2	IFN-γ receptor analog	Neutralizes IFN-γ
Nipah	W protein	IRF3	Nuclear sequestration of IRF3
Nipah	V protein	STAT1, STAT2	Sequesters STATs to prevent their activation
Papilloma	E6 protein	IFN-δ induced genes	Blocks Tyk2
Paramyxo	V protein	MDA5 and RIG-I	Inhibition of MDA5(direct) and RIG-I(indirect)
PIV 5	V protein	TBK1	Decoy phosphorylation substrate
Polio	2A protein	eIF4G	Cleavage of eIF4G
Polio	3C protein	MAVS,TRIF, p65-RelA	Cleavage of MAVS,TRIF and p65-RelA
Polyoma	T antigen	ISG induction	Blocks JAK1
Reo	σ3 protein	PKR	Sequesters dsRNA
Reo	μ2 protein	IRF9	Retains IRF9 in the nucleus and prevents ISGF3 formation.
RSV	?	STAT2	Block type I IFN signaling via STAT2 degradation
Rotavirus	NSP1	IRF3,IRF5, IRF7	Targets IRFs to degradation
Rotavirus	NSP1	STAT1, STAT2	Prevents STAT nuclear migration
Sendai	E6 protein	IRF3	Direct inhibition of IRF3
Simian Virus 5	V protein	STAT1	Ubiquitination-dependent degradation
Vaccinia	SKI	PKR	Sequesters dsRNA
Vaccinia	B19/B19R	Type I IFNs	This secretory protein acts as a soluble IFN-α/β receptor to prevent IFN-action.
Vaccinia	E3 protein	PKR	Inhibits PKR
Vaccinia	K3 protein	PKR	Analog of eIF2-α
Vaccinia	K7 protein	DDX3	Blocks IRF3 mediated IFN-b production

Abbreviations: EBER, Epstein-Barr virus encoded small RNA; EBNA2, Epstein-Barr virus nuclear antigen 2; EMCV, Encephalomyocarditis virus; HCMV, Human cytomegalovirus; HPIV2, Human parainfluenza virus 2; HPV 16, Human papilloma virus 16; HSV, Herpes simplex virus; ICP0, Infected cell protein 0; IFN, Interferon; IFNAR, Interferon alpha receptor; KSHV,Kaposi's sarcoma associated herpesvirus; LANA, Latency associated nuclear antigen; MAVS, Mitochondrial antiviral - signaling protein; MDA 5, Melanoma differentiation-associated protein 5; MT2, Myxoma virus T2 protein; NS, Non-structural protein; ORF, Open reading frame; RSV, Respiratory syncytial virus; TAR, Trans-activation response; TP, Terminal protein; PIV 5, Para influenza virus 5; TBK, TANK-binding kinase; TRIF, TIR-domain-containing adapter-inducing interferon β; TYK, Tyrosine kinase.

IFNs and Microbiota

Resident microbiota influence the homeostasis of the host immune system. IFNsα/β not only influence the host–microbiota interactions but also serve as downstream targets of these interactions, leading to further effects on immune system function. The IFN-inducible inflammatory transcriptional response was severely reduced following the loss of commensals. As a result mononuclear phagocytes from mice lacking commensals were defective at inducing anti-viral responses and failed to activate anti-viral response through NK cells. Loss of type I IFN receptor (IFNAR) in intestinal epithelial cells leads to the proliferation of Paneth cells consequently altering the intestinal microbiota composition. Microbiota-induced production of IFN-β by the resident DCs in the intestine protects mice from experimentally-induced colitis.

Anti-Microbial Actions of IFNs

IFNs acting solely as anti-viral agents is no longer a valid notion. Type I IFNs modulate the effector functions of multiple cell types involved in immune responses against bacteria, fungi, and parasites. However, depending on the duration, quantity and cell type experiencing the IFNs, both anti-microbial and host-detrimental outcomes are possible (**Fig. 7**). During the early phase of a bacterial infection, low levels of type I IFNs promote a beneficial cell-mediated immune response. In contrast, high concentrations of type I IFNs blunt B cell responses and inactivate macrophage-dependent immune responses. It should be noted that these diametrically opposing responses are also dependent on type of the pathogen. Unlike the anti-viral effects, the anti-microbial actions of IFNs sometimes are direct and at other times they are exerted through the production of cytokines. The direct anti-microbial and anti-protozoal actions of IFNs are induced by ISGs such as, inducible nitric oxide synthase (iNOS), indoleamine 2, 3 dioxygenase (IDO) and GTPases like IGTP and TGTP.

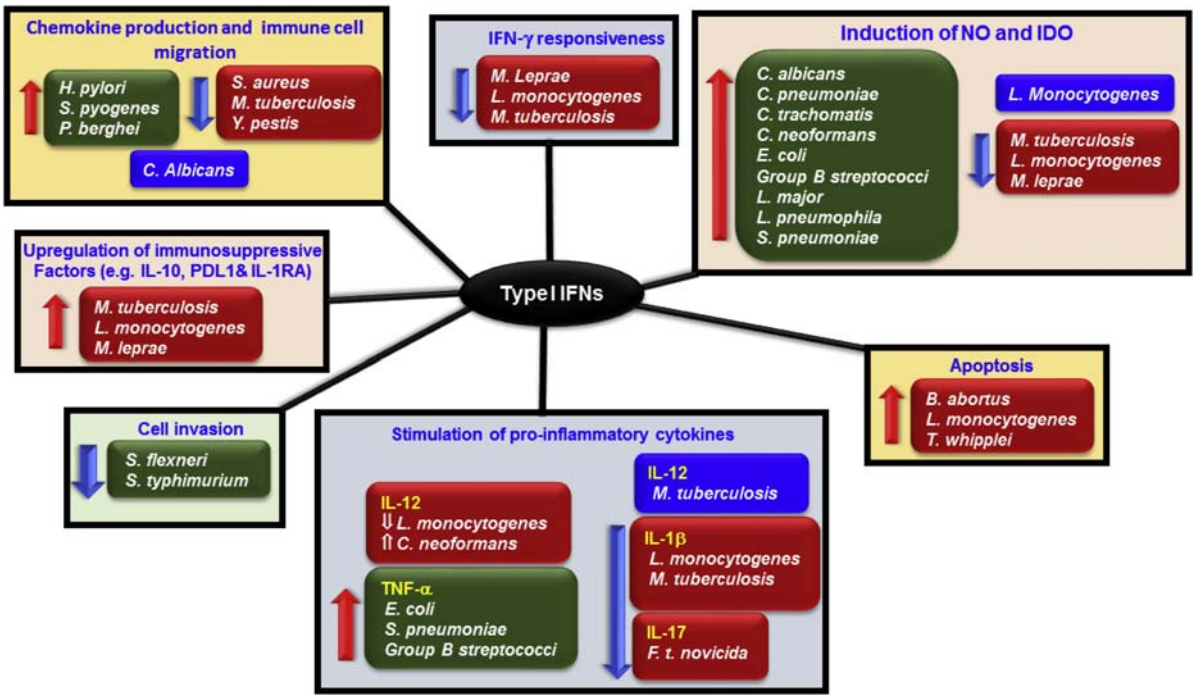

Fig. 7 Effects of type I IFNs on microbial, fungal and protozoal pathogens. Red and blue colored arrows indicate promoting and suppressing effects, respectively, on various immunity-associated processes. Green boxes: host-protective; Red boxes: host-detrimental effects; Blue boxes both host protective and detrimental effects of IFN-α/β. IL, interleukin; IL'1RA, IL'1 receptor antagonist; iNOS, inducible nitric oxide synthase; PDL1, programmed cell death 1 ligand 1; *B. abortus*, *Brucella abortus*; *C. albicans*, *Candida albicans*; *C. neoformans*, *Cryptococcus neoformans*; *C. pneumoniae*, *Chlamydia pneumoniae*; *C. trachomatis*, *Chlamydia trachomatis*; *E. coli*, *Escherichia coli*; *F. t. novicida*, *Francisella tularensis subsp. novicida*; *H. pylori*, *Helicobacter pylori*; *L. major*, *Leishmania major*; *L. monocytogenes*, *Listeria monocytogenes*; *L. pneumophila*, *Legionella pneumophila*; *M. leprae*,*Mycobacterium leprae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *P. berghei*, *Plasmodium berghei*; *S. aureus*, *Staphylococcus aureus*; *S. flexneri*, *Shigella flexneri*; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. Typhimurium*, *Salmonella enterica subsp. enterica serovar Typhimurium*; *T. whipplei*, *Tropheryma whipplei*; *Y. pestis*, *Yersinia pestis*. Adapted from McNab, F. et al. (2015) *Nature Reviews. Immunology*. **15**: 87–103.

The indirect effects of IFNs include: induction of cellular invasion, production of chemokines or pro-inflammatory cytokines, and induction of apoptosis or upregulation of autophagy. The host-detrimental actions of IFNs involve production of immunosuppressive molecules, promotion of cell invasion, suppression of microbicidal molecules such as iNOS and IDO. IFNs-α/β activate immature committed DCs, by enhancing the cell surface expression of MHC molecules and co-stimulatory molecules, such as CD80 and CD86, which robustly stimulate T cells to block spread of an infection. DCs secrete IL-12, which is critical for promoting T helper 1 (T_H1)-type responses during some bacterial infections. IL-12 induces IFN-γ production by T cells and NK cells. IFN-α down modulates the expression of pro-inflammatory cytokines IL-1, IL-12 and IL-17 during *Mycobacterium tuberculosis* and *Listeria monocytogenes* infections. Strikingly, IFN-α suppresses the beneficial anti-microbial effects of IFN-γ in the case of *M. tuberculosis*, *M. leprae, and L. monocytogenes*.

Anti-Protozoal Parasite Actions of IFNs

There are few studies on the effects of type I IFNs on parasites. As noted with bacterial infections, low doses of IFN-α/β exert a host-protective response, whereas high doses inhibit them during parasitic infections like *Leishmania major*, *Plasmodium berghei* and *P. chaubadi*. In the case of *Leishmania sp*. infections, IFN-α/β exerts a detrimental effect on the host through a blockade of macrophage functions and down regulating neutrophil number. Depending on the species and stage of infection, type I IFNs confer a negative or positive effects on host responses. IFNα/β augment infection by inhibiting $CD4^+$ T cell activity, during blood stages in mouse models of infection with *P. berghei* and *P. chabaudi*. In contrast, IFNs-α/β protect the host during *P. yoelii* infection by inhibiting reticulocytosis, a condition in which immature red blood cells accumulate in peripheral blood. During malaria infection, parasites spread into the central nervous system causing 'cerebral malaria'. IFN-α offers protection against cerebral malaria induced in mice through T_H1 cells. Parasitic RNAs are detected by MDA5 in liver leading to IFN production. Similar positive and negative effects of type I IFNs on host immunity during *Trypanosoma cruzi* infection have been reported. The IFN-induced GTPases (IRGs) viz., IGTP, TGTP, and IRGM acts as deterrents against the protozoan parasite *Toxoplasma gondii* potently inhibiting its multiplication. Some strains of *Toxoplasma* evade the anti-protozoal action of IFN-inducible immunity-related GTPase by secreting the rhoptry kinases (ROP kinases) into the host cell during infection. One such protein kinase, ROP18, phosphorylates

IRG proteins to inactivate their functions, thus, promoting virulence. *Toxoplasma* infection inhibits the expression of IFN-γ – induced secondary response genes, such as CIITA and MHC class II genes, by impairing Brahma-related gene 1 (BRG-1)-mediated chromatin remodeling of their promoters in macrophages. Other studies show that *Toxoplasma*, inhibits both type I and type II IFN-induced gene expression by preventing STAT1 nuclear-cytoplasmic cycling. Strangely, *Toxoplasma* requires IRF3 for its replication, independently of type I IFN production. It induces a Parasite-IRF3 Signaling Activation (PISA) pathway that relies on cGAS – STING – TBK – IRF3 to stimulate certain ISGs to promote its replication. Thus, there is an interesting nexus between *Toxoplasma* and IFN system.

Effects of IFNs on fungal infections

Similar to bacterial infections, IFNs-α/β exert both negative and positive effects on fungal infections. In case of *Candida albicans*, *Saccharomyces cerevisiae* and *Cryptococcus neoformans* infections, IFN-α/β exert a host-protective effect. IFN-α/β signaling is critical for various processes, including inducing the reactive oxygen species that terminate *C. albicans* in phagocytic cells, for maintaining a T_H1 like immune response (high IFN-γ, TNF, iNOS and CXCL10 levels) to *C. neoformans* and for attracting neutrophils to the site of *C. albicans* infection. However, some other models of *C. albicans* showed no effect of IFN-α/β on fungal burden, but instead, promoted a lethal immunopathology. Fig. 7 shows some of the effects of type I IFNs on various microbial infections and outcomes observed in mouse models.

Human IFN-Related Inborn Errors That Increase Infections

A number of compelling studies showed genetic mutations/variations in IFN signaling pathways or downstream products enhances sensitivity to infectious pathogens (Table 5). Earliest studies showed certain humans, who do not display any overt defects in hematopoietic system, were predisposed to infection with attenuated (e.g., BCG) and environmental mycobacteria. Defined now as'Mendelian susceptibility to mycobacterial disease (MSMD)', these rare conditions are due to genetic defects in IFN signaling pathways. Nine of the 18 genes described in MSMD mapped to critical players involved in IFN signaling such as *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *NEMO*, *CYBB* (codes for the gp91 subunit of the phagocyte NADPH oxidase) and *IRF8*. The MSMD patients do not produce *IL12RB1*, *ISG15*, *NEMO* and *IRF8* owing to defects in *IFNGR1*, *IFNGR2*, *STAT1* and *IRF8* in IFN-γ signaling. Although MSMD patients do not recapitulate all the clinical features, they are also increasingly susceptible to salmonellosis, candidiasis and tuberculosis, and rarely to infections with other intramacrophagic bacteria, fungi, or parasites and some viruses. Genetic defects (heterozygotic) in the human *IRF7* gene are also connected to life-threatening influenza infections. Cells from these individuals do amplify the initial IFN response that leads to the production of IFN-α and IFN-λ. Apart from these, autosomal recessive defects due to *TYK2* loss down regulate IFN-α/β, and IFN-λ, resulting in a hyper-susceptibility to mycobacterial, *Salmonella*, staphylococcal and viral infections. It should be noted that these phenotypes are likely to be more complex given the requirement of TYK2

Table 5 Human inborn errors that dysregulate IFN-signaling pathways and increase susceptibility to infections

Gene	*Inheritance*	*Allele*	*Cytokines*	*Disease Phenotype/Infections*
STAT1	AR	LOF, HPO	IFN-α and IFN-β, IFN-γ, IFN- λ, IL-27	mycobacteria, viruses
	AD[a]	LOF, HPO	IFN-γ	mycobacteria
IFNGR1	AR	LOF, HPO	IFN-γ	mycobacteria
	AD	LOF	IFN-γ	mycobacteria
IFNGR2	AR	LOF, HPO	IFN-γ	mycobacteria
IRF7	*AD*	LOF	IFN-α	Life threatening influenza infections
IRF8	AD	HPO	IFN-γ and others?	Mycobacteria
IL12B	AR	LOF	IL-12, IL-23	mycobacteria, *Salmonella*, CMC
IL12RB1	AR	LOF	IL-12, IL-23	mycobacteria, *Salmonella*, CMC
IKBKG	XR	HPO		multiple mycobacteria
CYBB	XR	HPO		mycobacteria
TYK2	AR	LOF	IFN-α, IFN-β, IFN-λ, IL-6, IL-10, IL-12, IL-23	mycobacteria, salmonella, viruses, *Staphylococci*, atopy[b]
STAT1	AD[a]	HPR	IFN-α, IFN-β, IFN-γ, IFN-λ, IL-27[c]	CMC
IL10RB2	AR	LOF	IL-10, IL-22, IFN-λ	colitis

AR, autosomal-recessive; AD, autosomal-dominant; XR, X-linked recessive; LOF, loss-of-function (null); HPO, hypomorphic; HPR, hypermorphic (gain of function); colitis, early-onset inflammatory colitis; CMC, chronic mucocutaneous candidiasis.

[a]Mutations were found in the DBD and tyrosine phosphorylation domain.

[b]elevated IgE.

[c]STAT1-hyperactive patients lack IL-17 T cells.

Based on Casanova, J.L. et al. (2012) *Immunity*. **36**, 515–428.

for IL-6, IL-10, IL-12 and IL-23 signaling. Patients with autosomal recessive STAT1 deficiency are broadly susceptible to viruses, including herpes simplex virus-1 (HSV-1) infections, which may cause HSV-1 encephalitis (HSE). IL-10R2-deficient patients would be expected to be unresponsive to IFN-λ, for this receptor is not utilized by either IFN-α/β or IFN-γ. Genome-wide sequencing and association studies on chronic mucocutaneous candidiasis have identified mutations in STAT1 (in its coiled-coil domain) in some patients. These mutations were also found in patients with disseminated disease caused by other fungal pathogens such as *Histoplasma capsulatum* and *Coccidioides immitis*. Strikingly, these gain-of-function and dominant mutations promote disease, suggesting a detrimental role for IFN-α/β in fungal infections, possibly through a suppression of T_H17 responses and/or an excessive differentiation of T_H1 cells. Since other cytokines, for example, IFN-γ and IL-27, also employ STAT1 for signal transduction, they may also be responsible for disease promotion.

Anti-Tumor Actions of IFN-Inducible Gene Products

IFNs affect neoplastic cell growth by two major mechanisms: (1) direct induction of anti-tumor gene expression in tumor cells, and (2) development of acquired immune response against a tumor. IFN-induced growth suppressors and the genes that participate in them are shown in **Table 6**. Deletion of *Stat1* increases susceptibility to carcinogenesis in mice. These mice also developed spontaneous $ER\alpha^+/PR^+$ breast tumors at a late age. Indeed, 45% of human $ER\alpha^+$ and 22% of ERα- breast tumors do not express detectable STAT1. STAT1 appears to mediate its anti-tumor effects through an induction of apoptosis. Caspases are proteases with an active cysteine residue that cleave a number of critical cellular proteins during apoptosis. STAT1-deficient cells do not express Caspase-1, Caspase-2 (Ich1) and Caspase-3 (Cpp32). STAT1 also regulates the expression of cyclin-dependent kinase inhibitor, p21. As a result, STAT1-deficient cells become resistant to growth inhibition and fail to undergo apoptosis. Apoptosis is also promoted by cell surface death receptors. Two such receptors Fas and DR4 are essential players in controlling lymphocyte proliferation and death of other neoplastic cells. Fas and TRAIL (a ligand for DR4) are induced by IFNs. In contrast to its known tumor-suppressive actions, STAT1 appears to function as an oncogene in certain forms of endometrial cancers, where it drives the expression of ICAM1 (an adhesion molecule), PDL1 (a molecule that induces T cell apoptosis) and c-Myc (cell proliferation).

JAK1 appears to regulate tumor suppression. In human endometrial cancers, deletions and frameshift mutations have been found in the *JAK1* gene (**Fig. 8**). Such cells fail to upregulate LPM2 and TAP1, both molecules involved in antigen processing and adaptive immune response. This is consistent with the requirement of STAT1 for immunosurveillance. Apart from these *JAK1* truncations were also found in colorectal, lung, skin and kidney cancers. In contrast, JAK2 acts as oncoprotein in some leukemias. Constitutive activation of JAK2 is noted in many myeloid leukemias, for example, polycythemia vera. A number of point mutations, deletions and frameshift mutations in *JAK2* have been reported in human leukemias. The most notable of these is the $V^{617}F$ mutation in the JH2 domain (pseudokinase) that, surprisingly, super stimulates its enzymatic activity in the absence of any cytokine signaling (**Fig.9**). Apart from these other gene translocations of *JAK2*, include a t(9;22) (p24;q11.2) which generates BCR-JAK2 fusion protein containing the N-terminal coiled-coil domain of the BCR (Breakpoint Cluster Region, a member of the Rho family of guanine nucleotide exchange factors involved in cell signaling) gene and JH1 domain of JAK2 in some chronic myelogenous leukemia. ETV6-JAK2 (also known as TEL-JAK2) is another fusion protein found in certain adult T cell leukemias, where a translocation, t(9;12) (p24;p13), between transcription factor TEL/ETV6 and JAK2 produces this fusion. The ETV6 domain in the resultant protein provides an oligomerization interface that brings the JAK2 kinase domains together to produce a constitutively active enzyme and promotes cell growth. A third fusion protein, PCM1-JAK2, is found some atypical chronic myeloid leukemias where the t(8;9) (p22;p24) translocation between the PCM1 (Peri-Centriolar Material 1) gene and JAK2 produces a chimera that possesses constitutive kinase activity. In this case the coiled-coil domain of PCM1 provides an oligomerization domain for JAK2. Based on the JAK2-$V^{617}F$ a number of kinase inhibitors have been successfully developed and implemented as a standard therapy for myeloid leukemias with constitutive JAK2 activity (**Table 7**). Although these inhibitors are developed for treating leukemias, they are also used for treating other autoimmune diseases like rheumatoid arthritis, psoriasis, and systemic lupus erythematosus, where JAK2 activities are elevated.

Table 6 IFN-induced anti-tumor effects and the genes that participate in them

Process	*IFN effects*
Cell cycle arrest	↑ $p21^{WAF1/CIP1}$, $p27^{KIP1}$, IFITM1, pRb dephosphorylation
Growth inhibition	↓ E2F1, CDKs (A, B, D2, D3, E)
	↓ HER-2/neu, c-myc
Apoptosis	↑ Fas, FasL, TRAIL, KILLER/DR5, Caspases (1, 3, 7, 8), XAF1, NO, DAPK1
Angiogenesis inhibition	↓ bFGF, MMP2, MMP9, VEGF
	↑ IP10/CXCL10
Immunosurveillance	↑ LMP2, LMP7, TAP1, TAP2, MHCI, CIITA

NO: Nitric Oxide; Up and down arrows indicate inducing and inhibitory effects of IFNs

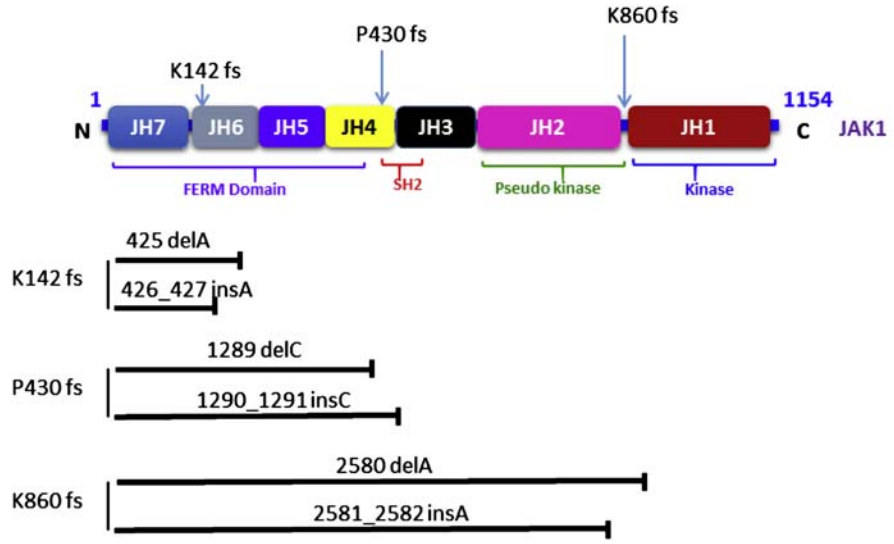

Fig. 8 JAK1 mutations in human tumors. JAK1 protein domains in modular representation and three mutational hot spots are shown on wild-type JAK1. Horizontal bars represent the truncated protein due to frameshift (fs) - mutations arising from deletion (del) and insertion (ins). Numbers indicate the position in protein and/or ORF. Adapted from Ren, Y. et al. (2013) *Science Reporter.* **3**: 3042.

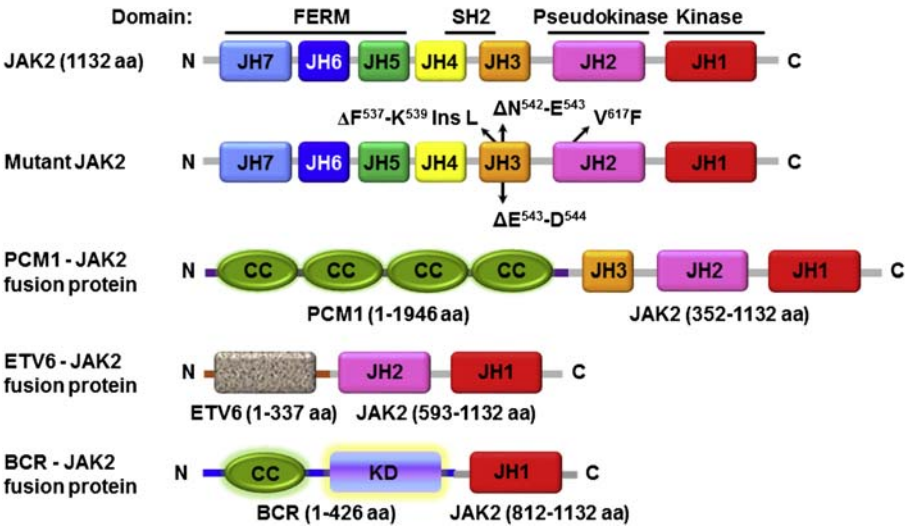

Fig. 9 JAK2 mutations in human tumors. JAK2 protein domains in modular representation showing the region where three mutational hot spots have been documented. Fusion proteins due to chromosomal translocations are also shown. Deletion (Δ) and Insertion (Ins) found in various hematological tumors. Numbers indicate the position in protein. CC: coiled-coil domain. Adapted from Bain, B.J. & Ahmad, S (2014) *British Journal of Haematology*. **166**: 809–817).

The SOCS1 protein is another ISG that acts as a tumor suppressor. SOCS1 is a STAT1-induced ISG, which turns off JAK activities in the IFN-signaling pathway. The E3 ligase activity of SOCS1 plays critical roles in its anti-tumor actions. SOCS1 exerts anti-tumor actions, independently of its anti-JAK function. For example, it associates with tumor suppressor p53 to promote growth suppressive gene expression in certain hepatocellular carcinomas (HCC) and block signaling by receptor tyrosine kinases like EGFR and FGFR3. Interestingly, the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (also known as CDKN1A) a target of p53 and an inhibitor of cell cycle arrest, acts as an oncogene in some cell types. SOCS1 targets CDKN1A to ubiquitination and its proteosomal degradation. The *SOCS1* gene is repressed by hyper-methylation in many cancers, for example pancreatic cancers, myeloid leukemias and HCC.

DAPK1 is a major IFN-inducible tumor suppressor. It was originally isolated using a genetic screen that identified IFN-γ-regulated growth suppressors. DAPK1 codes for a Ca^{+2}-calmodulin dependent serine/threonine kinase that suppresses tumor cell growth and metastasis by causing a cell cycle arrest, apoptosis and autophagy. Other stimuli such as C6-ceramide, inhibition of mitochondrial respiration, unliganded UNC5H2, TGF-β and DNA damage also require DAPK1 for inducing cell death. DAPK1 modulates cytoskeletal remodeling, membrane blebbing and autophagy. Consistent with its multifunctional nature, DAPK1 interacts with several growth-regulatory proteins. DAPK1 phosphorylates multiple substrates depending on the cell type—most notable

Table 7 JAK Inhibitors for treating cancer and autoimmune diseases

Drug	*Targeted JAK*	*Type of cancer*	*Stage of development*
Ruxolitinib	1, 2	Myelofibrosis	III
		Polycythemia vera	II
		Essential thrombocythemia	
CYT387	1, 2	Myelofibrosis	I/II
SAR302503 (TG101348)	1, 2	Myelofibrosis	I/II
Pacritinib (SB1518)	2	Myelofibrosis	II
AC-430	2	Lymphoma/Rheumatoid arthritis	Pre-clinical
R723	2	Myeloproliferative neoplasia	Pre-clinical
BMS911543	2	Myelofibrosis	Pre-clinical
AZD1480	1, 2	Glioblastoma	Pre-clinical
Tofacitinib	1, 2, 3	Rheumatoid arthritis	III
		Psoriasis	III
		Inflammatory bowel disease	III
VX-509	3	Rheumatoid arthritis	II
R-348	3	Rheumatoid arthritis	I
INCB18424 (topical formulation)	1, 2	Psoriasis	II
LY3009104 (formerly INCB-28050)	1, 2	Rheumatoid arthritis	II
		Psoriasis	IIb
GLPG-0634	1, 2, TYK2	Rheumatoid arthritis	II
CEP-33779	2	Rheumatoid arthritis	Pre-clinical
		Systemic lupus erythematosus	Pre-clinical

Based on Kontzias et al. (2012) Jakinibs: a new class of kinase inhibitors in cancer and autoimmune disease. *Current Opinion in Pharmacology*. **12**:464–470.

among these are: Beclin-1 and VPS34 via Protein kinase D (to promote autophagy network), myosin light chain, tropomyosin (to suppress cell contractility), p53 tumor suppressor, p38MAPK (for enforcing apoptosis), interacts with MARKS protein kinase to promote the phosphorylation of Tau (promotes microtubule instability and toxicity) and PAK4-LIM-cofilin (actin remodeling), blocks inflammation-induced NF-kB (promotes growth), Pin-1 (causes chromosomal instability and centrosome amplification) and integrins (metastasis suppression via adhesion) to suppress growth. Lastly, DAPK1 is also involved in translational control of inflammatory gene expression in myeloid cells through a novel IFN-regulated element called GAIT, and NMDA-driven brain damage in stroke. Consistent with its growth-suppressive properties, *DAPK1* expression is suppressed (40-90%) in a variety of human cancers.

cGAS-STING also participates in tumor surveillance. Cellular stress, such as DNA damage, induces NKG2D ligands, which allows damaged cell recognition and destruction by NK and T cells. In certain lymphoma cells the expression of NKG2D ligand, Rae1, is induced by the STING-IRF3 pathway. Expression of Rae1, in tumor cells, aids in NK cell-mediated tumor clearance. The IRF1 and IRF8 proteins are associated with the development of leukemias. IRF1 gene mutations are found in myelodysplasia and other myeloproliferative diseases. IRF8 expression is suppressed in human myelogenous leukemias. Indeed, *Irf8*−/− mice develop a chronic myelogenous leukemia-like disease. IRF7 and the dependent IFN gene signatures are lost in certain forms of human metastatic breast cancers.

In an immunocompetent host, neoplastic cells undergo immunoediting in three distinct phases: elimination, equilibrium, and escape. During the elimination phase, host immune cells (CTLs, type 1 macrophages and NK cells) kill cancer cells to reduce tumor burden. Such killing reaches a phase of equilibrium where tumors enter into a state of dormancy. During dormancy some tumor cells lose tumor antigens, stop expressing MHC molecules, acquire mutations due to genome instability, and reprogram the microenvironment through secretion of immunosuppressive factors like TGF-β, IL-10 and chemokines which recruit and activate type 2 macrophages, immature myeloid-derived suppressor cells, T^{reg} cells, which effectively block any potential immune response and creating paths for the escape of tumor cells from immune scrutiny. IFNs play a major role in shaping the elimination phase of immune response against tumors. The IFN-γ receptor and STAT1 are critical for immunosurveillance of neoplastic cells. Mice lacking these genes are highly susceptible for tumor development. Type I IFNs stimulate CTLs by various mechanisms (**Fig. 10**): (1) promote cross-priming to augment the maturation of DCs, by enhancing their capacity to process and present dead cell-associated antigens, and by promoting their migration towards lymph nodes; (2) by upregulating the expression of perforin 1 and granzyme B, they augment immune effector activity and (3) promote the survival of memory CTLs. Type I IFNs can block the elimination of antigen-activated $CD8^+$ CTLs by NK cells and stimulate the secretion of pro-inflammatory cytokines interleukin-1 beta (IL-1β) and IL-18 by macrophages. Type I IFNs inactivate regulatory T (T^{Reg}) cells, which blunt immune response, through the activation of PDE4 and the consequent depletion of cAMP.

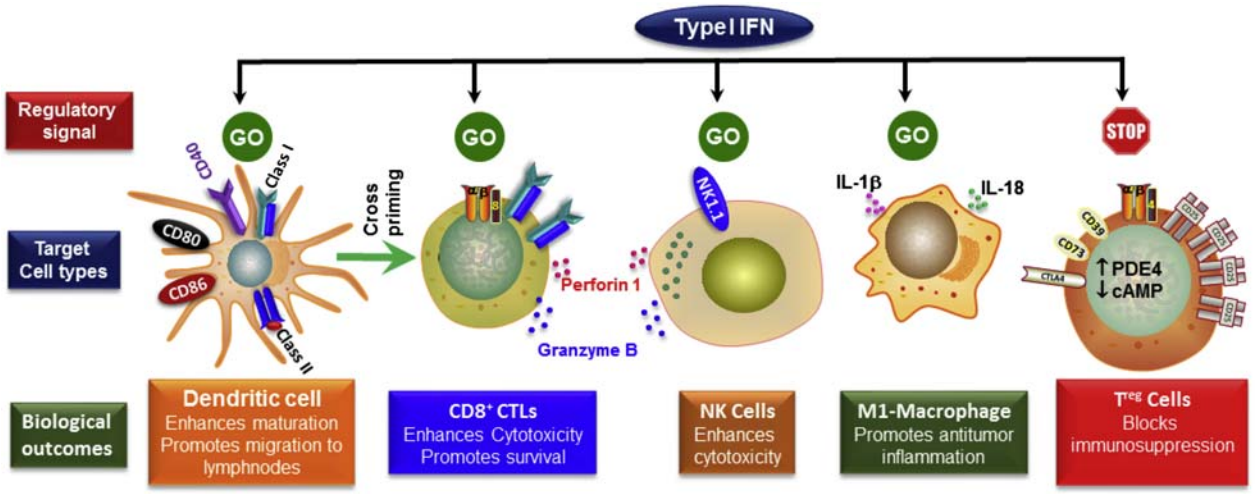

Fig. 10 Type I IFN-stimulated immune mechanisms that contribute to their anti-tumor actions. Various cell types and the effects on them are shown. Granzyme B and Perforin, cytotoxic proteins secreted by $CD8^+$ T cells, kill the target cells. PDE4: phosphodiesterase 4. Adapted from Zitvogel, L., et al. (2015) *Nature Reviews. Immunology*. **15**: 405–414.

IFNs and Autophagy

Autophagy is a type of cellular self-digestion. Although discovered originally as a cell protective mechanism in yeast cells that protects against starvation, autophagy participates in multiple physiological responses including innate immunity against viruses and bacteria, and tumor cell growth regulation. During autophagy, invading pathogens or damaged/superfluous organelle are encased into double membrane vesicles, derived from endoplasmic reticulum, called autophagosomes. These vesicles fuse with lysosome where the cargo is digested out. The resultant peptides are presented to the immune system to generate immune response. DAPK1 promotes autophagy, by phosphorylating multiple targets involved in the autophagic machinery. In addition, transcription factors C/EBP-β and ATF6 are also required for driving IFN-γ-induced autophagy. Mice and cells lacking these factors are increasingly susceptible to intracellular bacterial infections. A cellular protein p62 (also known as SQSTM) serves as a carrier of protein cargo into autophagic vesicles to promote their destruction via lysosomes. Although p62 is dispensable for clearing *T. gondii*, it is essential for generating CTL responses against this pathogen. Autophagy or autophagy-related proteins also contribute to the synthesis of type I IFN as well as to anti-viral IFN-γ signaling. Autophagy directly inhibits the replication of number of viruses. In contrast, HCV inhibits type I IFN production by promoting autophagy. Lastly, autophagy attenuates innate immune response to promote acquired immunity.

Interferon-gene Regulatory Factors (IRFs)

A total of 10 structurally similar and genetically distinct transcription factors constitute this family. These include: IRF1, IRF2 (ISGF2), IRF3, IRF4 (PIP/LSIRF/ICSAT), IRF5, IRF6, IRF7, IRF8 (ICSBP), IRF9 (also known as ISGF3γ) and IRF10. In addition, 4 other IRF-like proteins, vIRF1, vIRF2, vIRF3 and vIRF4, are coded by the human Herpes Virus 8 (HHV8 or KSHV). Mammalian IRFs are characterized by an N-terminal DNA binding domain with several tryptophan-rich (W) repeats, which allow it to fold into a winged Helix-Turn-Helix structure (Fig. 11). Their carboxy termini contain a regulatory domain, which allows IRF-association domain (IAD) and a TAD that permits dimerization among family members and gene induction, respectively. IRF heterodimers can both positively and negatively regulate gene expression depending on the cell type, signal and target gene. The founding member of this family IRF1 was isolated as a protein can regulate IFN-β gene. IRF1, IRF2, IRF3, IRF5, IRF7, IRF8 and IRF9 are intimately involved with the induction of IFNs and ISGs. IRF1, IRF5, IRF7, IRF8 and IRF9 themselves are ISGs, which amplify initial IFN response in various cell types. Some of these proteins, IRF1, IRF3, IRF5, and IRF7 undergo a signal-induced phosphorylation, in their TAD, obligatory for target gene induction. A classic example for this is IRF7. In response to PRR, RLR, dsRNA-driven signals IRF3 is activated, which turns on IFN-β gene. IFN-β produced in this manner turns on the first-round transcription of ISGs, including IRF7. The latter then binds to IFN-α gene promoters and turns on their expression, which then upregulate more ISG expression leading to the amplification of IFN-induced innate defenses. The IRFs are involved in various host-defense and anticogenic processes. For example, the *Irf1*−/− mouse embryonic fibroblasts (MEFs) are readily transformed by the *Ha-ras* oncogene owing a defect in apoptosis. Indeed, aberrations in *IRF1* gene, owing to exon-skipping, are found in human myelodysplasias and certain forms of myeloid leukemia. *Irf1*−/− mice are defective in T_H1 immune response, contain fewer CD8 cells and are highly susceptible to intracellular pathogen infections. IRF2 is required for driving T_H1 and NK cell responses, its loss leads to susceptibility to viral and parasite infections and skin diseases. Loss of IRF3 causes increased susceptibility to viral infections in mice. IRF5 also

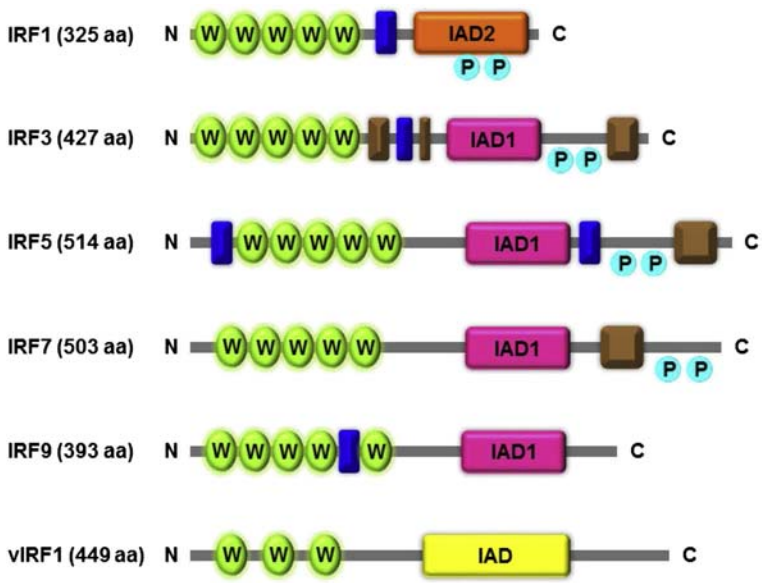

Fig. 11 Modular representation of protein domains in cellular IRFs and viral IRF. All IRFs possess a N-terminal DNA-binding domain (in *green*) characterized by a series of five tryptophan-rich (W) repeats and a C-terminal IRF-association domain (IAD in *orange*, *pink* and *yellow*), a regulatory domain. Some IRFs contain repression domain(s) (in *brown*) and a nuclear localization signal(s) (in *blue*). Phosphorylated residues involved in regulation and/or activity are shown as circles (in *turquoise*).

plays an important role in inducing IFN genes. Loss of IRF5 is associated with reduced responses to IL-6, IL-12 and TLR ligands, and metastasis in human breast cancer. Defective expression of IRF7 and ISGs is associated with metastatic breast cancers. Loss of IRF8 causes immunodeficiency and chronic myelogenous leukemia-like disease in mice. IRF8 promotes T_H1 responses, and differentiation of macrophages and DCs. Indeed, its expression is down regulated significantly in human myeloid leukemia. IRF9 is required for ISG expression and anti-viral defenses. The biological activities of IFN-regulated IRFs and the functional consequences of their loss are shown in Table 8. As mentioned previously, the HHV-8 genome codes for 4 vIRF proteins. The best studied of these, vIRF1, lacks a TAD. Although the exact functions of all 4 proteins in the context of this virus are unclear, cells expressing vIRF1 exhibit defective IFN-α gene expression and ISG expression, owing to its ability to antagonize the transcriptional activity of IRF1 and IRF3 proteins.

Interferonopathies

Although IFNs are generally involved in host-defense against the development of infections and cancers, it is becoming clear in recent reports that excessive type I IFN production, owing to defects in upstream or downstream signaling molecules, leads to several autoimmune diseases. These diseases are now termed as "Interferonopathies" (Table 9). The Aicardi-Goutières syndrome (AGS) is one such childhood interferonopathy that involves hyper-production of type I IFN. The DNA 3′ repair exonuclease 1 (TREX1) degrades short ss and dsDNA molecules generated due the reverse transcriptase activity of endogenous viruses or transposable genetic elements. Patients with AGS possess TREX1 defects, which results in an accumulation of unwanted DNA species, which serve as activating signals for the cGAS and production of cGAMP that turns on constitutive type I IFN via the STING–TBK1–IRF3 pathway. The deoxynucleoside triphosphate triphosphohydrolase with SAM and HD domains 1 (SAMHD1) hydrolyses dNTPs and degrades RNA. SAMHD1 deficiency also leads to elevated levels of IFN through cGAS and may cause AGS. Gain-of-function mutations in STING also induce an interferonopathy. IFN-induced helicase C domain-containing protein 1 (IFIH1 or MDA5) normally senses exogenous viral dsRNA. Hyperactive IFIH1 mutations require a lower threshold of dsRNA for stimulating IFN response and may also contribute to AGS. As ADAR is an ISG, mutations in both IFIH1 and ADAR may also promote the MAVS–TBK1–IRF3 signaling axis owing to their link to cytosolic RNA species. Ribonuclease H2 (RNaseH2), a trimeric enzyme constituted by RNASEH2A, RNASEH2B and RNASEH2C, degrades RNA in RNA–DNA hybrids. Tartrate-resistant acid phosphatase (TRAP, encoded by *ACP5*) a lysosomal phosphatase and PSMB8 encodes the proteasome subunit β5i are also associated with AGS. How mutations in *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *ACP5* and *PSMB8* promote hyper-IFN induction in a pathological context is unclear at this stage. Depending on the gene involved a number of clinical phenotypes are found in patients. Some of the neurological phenotypes include developmental delays, intracranial calcification, white matter disease, cerebral atrophy, spastic paraparesis, large vessel disease (stenosis, moyamoya or aneurysms) and bilateral striatal necrosis. In other patients skin-related phenotypes such as digital vasculitis and/or necrosis (chilblains); livedo and/or skin mottling, panniculitis, necrotic cheek lesions, and lipoatrophy are reported. Other clinical features of interferonopathies include: recurrent (sterile) fevers, autoimmune features, glaucoma, neonatal

Table 8 Mammalian IRFs and their functions

IRF	*Interacting partners*	*Expression*	*Function (activator or repressor)*	*Key functions*	*Phenotype of knockout mouse*
IRF1	IRF8	(Constitutive) and Inducible	Activator, regulated by phosphorylation	Promotes apoptosis and $T_H1$responses, loss may promote leukemia	Less CD8+ cells and susceptible to bacterial and viral infections
IRF2	IRF8	Constitutive and Inducible	Activator or repressor depending on presence of co-repressors	Antagonizes IRF1 and IRF9, promotes $T_H1$responses and NK-cell maturation	Susceptible to viruses, Leishmania major and skin diseases, Impaired hematopoiesis and B lymphopoiesis
IRF3	IRF3 and IRF7	Constitutive	Activator, regulated by phosphorylation	Induces early type I IFNs in response to viruses and TLR ligands	Susceptible to viruses, impaired IFN-α/β production
IRF5	IRF3, IRF5, and IRF7	Constitutive and Inducible	Activator, regulated by phosphorylation	Induces type I IFNs	Reduced IL-6, IL-12 and TNF production in response to TLR ligands
IRF7	IRF3, and IRF7	Constitutive and Inducible	Activator, regulated by phosphorylation	Induces type I IFNs and promotes macrophage differentiation	Vulnerable to viruses, reduced IFN-α and IFN-β in response to viruses and TLR ligands
IRF8	IRF1, IRF2, IRF4, and PU.1	Constitutive and Inducible	Activator or repressor depending on presence of co-activators	Promotes $T_H1$responses and macrophage and DC differentiation	CML-like syndrome, defective antiviral responses, and susceptible to intracellular pathogens
IRF9	STAT1 and STAT2	Constitutive and Inducible	Activator, depending on presence of co-activators	Induces IRF2 and IRF7, and mediates IFN effects	Defective antiviral responses, impaired IFN-α/β production

Only those IRFs known to function in the IFN-system are shown.
Based on Lohoff, M. & Mak, T.W. (2005). *Nature Reviews. Immunology*. **5**:125–135.

Table 9 Interferonopathies

Gene	*Inheritance*	*Human phenotypes*	*Protein function*
TREX1	AR or AD	AGS, FCL, SLE and RVCL	3′–5′ DNA exonuclease
RNASEH2A	AR	AGS	A catalytic subunit of the RNase H2 that degrades RNA in the RNA–DNA hybrids and removes ribonucleotides embedded in DNA
RNASEH2B	AR	AGS and spastic paraparesis	A non-catalytic subunit of the RNase H2
RNASEH2C	AR	AGS	A non-catalytic subunit of the RNase H2
SAMHD1	AR	AGS, FCL and CLL	dNTP triphosphohydrolase triphosphatase and ribonuclease
ADAR	AR or AD	AGS, DSH, BSN and spastic paraparesis, as well as CNP	adenosine to inosine conversion in dsRNA
IFIH1	AD	Various neuroimmunological and non-neurological phenotypes, including AGS, spastic paraparesis, CNP and SMS	Cytosolic sensor of dsRNA
DDX58 (*RIGI*)	AD	Atypical SMS	dsRNA cytosolic sensor
TMEM173	AD	SAVI (skin and lung)	Cytosolic DNA-sensor
ISG15	AR	MSMD and intracranial calcification (with seizures in some cases)	A negative regulator of type I IFN production by stabilization of USP18
PSMB8	AR	JMP, NNS, JASL or CANDLE (fever, contractures, neutrophilic dermatitis, lipoatrophy and panniculitis)	Part of a multi-subunit protease complex responsible for regulating proteolysis in eukaryotic cells
ACP5	AR	SPENCD, spastic paraparesis and various autoimmune phenotypes (particularly SLE)	Lysosomal acid phosphatase activity

ACP5: Acid Phosphatase 5, Tartrate Resistant (also known as TRAP) AD: autosomal dominant; AR: Autosomal Recessive; ADAR, adenosine deaminase acting on RNA (also known as DRADA, IFI4); AGS, Aicardi-Goutières syndrome; BSN, bilateral striatal necrosis; CANDLE, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature; CLL, chronic lymphocytic leukemia; CNP, complete non-penetrance; DDX58: DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 58 (also known as RIG-I); dNTP, deoxynucleotide triphosphate; DSH, *dyschromatosis symmetrica hereditaria*; dsRNA, double-stranded RNA; ENU, N-ethyl-N-nitrosourea; FCL, familial chilblain lupus; IFIH1, IFN-induced helicase C domain-containing protein 1 (also known as MDA5); IFN, interferon; ISG15; IFN-stimulated gene 15; JASL, Japanese auto-inflammatory syndrome with lipodystrophy; JMP, joint contractures, muscle atrophy, microcytic anemia, panniculitis and lipodystrophy; MSMD, Mendelian susceptibility to mycobacterial disease; NNS, Nakajo- Nishimura syndrome; PSMB8, proteasome subunit-β type 8; RNASEH2, ribonuclease H2; RVCL, retinal vasculopathy with cerebral leukodystrophy; SAMHD1, deoxynucleoside triphosphate triphosphohydrolase and ribonuclease SAM domain and HD domain 1; SAVI, STING-associated vasculopathy with onset in infancy; SMS, Singleton–Merten syndrome; SPENCD, spondylochondromatosis; TREX1, DNA 3′ repair exonuclease 1; TMEM173: Transmembrane Protein 173 (also known as STING) USP18, Ubl carboxy-terminal hydrolase 18.
Based on Crow, Y. J. & Manel, N. (2015) *Nature Reviews. Immunology*. **15**, 429–440.

thrombocytopenia and/or bone marrow suppression, hypertrophic cardiomyopathy, myositis, enchondromata, lung involvement, chronic lymphocytic leukemia, premature dental loss, aortic calcification and joint contractures.

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disease caused by the hyper-activated immune system in response to nuclear self antigens. One of the characteristics of this disease is the high steady-state serum IFN-α levels and a dysregulated expression of some ISGs. Autoantibodies such as anti-nuclear antibodies (ANA), anti-Ro, anti-La, anti-phospholipid, anti-dsDNA, anti-Smith and anti-nuclear ribonucleoproteins are commonly found in patients. These antibodies deliver self-nucleic acids to endosomal TLRs following F_c receptor-mediated internalization of immune complexes. This leads to hyper-induction of type I IFNs. In fact elevated levels of type I IFN in serum and dysregulated hyper expression of certain ISGs (dubbed as IFN signature) is commonly seen in 50% of these patients. Hyperactivation of IRF5 is commonly seen in this disease. The central region connecting the DBD and the IAD of IRF5 is rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues and constitutes thus a PEST domain, normally present in proteins that undergo a rapid turnover. The IRF5 gene, located on chromosome 7q32, is regulated in a complex manner by multiple gene promoters and several splice variants of its mRNA. For example a polymorphic 30-nucleotide insertion in the PEST domain produces a high-risk allele. Such IRF5 transcripts and the resultant proteins are highly stable, resulting in a persistent activation of IFN-α genes. IRF5 also activates the expression of IL-12, IL-17, IL-23 and TNF-α in human DCs and high IL-12, IL-17, IL-23 are found in plasma of SLE patients. In mammals, two major types of macrophages, the M1 and M2, mediate inflammatory and tissue repair responses, respectively. The increased cytokine levels polarize macrophages toward the M1 type to enhance inflammatory responses. Indeed, IRF5 is a key transcription factor involved in M1-macrophage differentiation. In addition, IRF5 plays a major role in the differentiation of B lymphocytes into plasma cells (which produce serum antibodies in high amounts) by promoting the expression of B lymphocyte-induced maturation protein 1 (BLIMP1). IRF5 is also involved in class switch of IgG subtypes during pathogenesis in B lymphocytes. Thus, the IRF5 – IFN axis plays a major role in driving SLE pathogenesis. Apart from SLE, IRF5 single nucleotide variations and a 5-bp insertion-deletion are also associated with disease progression in multiple sclerosis (MS), a neurodegenerative disease. Aging-associated cognitive decline is affected by external and internal factors of the brain. The choroid plexus, an interface between the brain and the peripheral blood, of aging mice and human brains expresses a type I IFN signature. In mice, this signature was stimulated by brain-derived signals, present in the cerebrospinal fluid. Neutralization of type I IFN signaling within the aged brain partially restored cognitive function and hippocampal neurogenesis and reestablished IFN-γ-dependent choroid plexus activity, which is lost in aging. Thus, IFNs play a major role in cognition-associated pathologies.

Clinical use of IFNs

The type I IFNs are efficacious in the treatment of malignancies, viral infections and autoimmune diseases (**Table 10**). The efficacy of IFN therapy is dependent on a number of factors including cell types, genetics, immune status, and microbiome etc. (**Fig. 12**). IFN-α plays a role in the management of different neoplasias, particularly those of hematological origin, that is of a myeloid and lymphoid etiology. IFN-β has been shown to have beneficial effects in the treatment of relapsing-remitting MS. IFN-γ is an absolute requirement for resistance against acute infection with *T. gondii* and development of Toxoplasmic Encephalitis during the late stage of infection. Thus, this unique repertoire of biological activities of IFNs has generated considerable interest in its potential for the treatment of viral infections, cancers and immunological disorders. Indeed, administration of IFNs is now the treatment of choice for some cancers, particularly leukemias and Kaposi Sarcoma, MS, rheumatoid arthritis and viral infections such as chronic Hepatitis-B and C (**Table 10**). However, many other types of cancer and viral infections are only partially responsive to IFN therapy. The molecular mechanisms, which determine the anti-viral and anti-proliferative sensitivity or resistance to IFNs, are yet to be fully elucidated.

Type I IFNs and Anti-Cancer Therapies

Some diseases, where IFN is a disease promoter, for example, Lupus, HIV and Interferonopathies, treatment with neutralizing antibodies may alleviate the negative effects (**Fig. 13**). However, the clinical effectiveness of such therapies need to be examined. The STING-activating ligands, and oncolytic viruses are the new IFN-based emerging therapies that have shown some promising. A number of recent reports indicate that the success of conventional chemotherapeutics, targeted anti-cancer agents, radiotherapy and immunotherapy relies on type I IFN signaling. IFNAR1 neutralization abolishes the therapeutic effect of monoclonal antibodies that are specific for human epidermal growth factor receptor 2 (ERBB2; popularly known as HER2/neu) or those specific for epidermal growth factor receptor (EGFR) in mouse models of cancer. Anthracyclines are used for treating a number of human cancers. Recent studies show that these drugs induce type I IFN production. Consistent with these, increased expression of MxA in breast tumor biopsies from patients who received anthracycline treatment predicts a better response to therapeutic response. The efficacy of anthracyclines as anti-cancer agents is suppressed by IFNAR1-neutralizing antibodies.

Radiation therapy induces IFN-β production by the myeloid (not by the tumor) cells in melanoma models. The anti-tumor effect of radiation is lost in *Ifnar1*−/− mice, as well as in mice transplanted with *Ifnar1*−/− hematopoietic cells. Type I IFN signaling is also critical for the robustness of immunotherapies. Imiquimod, a synthetic agonist of TLR7 and TLR8 promotes the IFNAR1-dependent recruitment of pDCs into the tumor bed of skin cancers. pDCs secrete type I IFNs which activate an autocrine loop

Table 10 IFN formulations in clinical practice

Type of IFN	*Chemical modification*	*Structure*	*Source*	*Recommendation*
Lymphoblastoid IFN-α N1	None	Mixture of natural human IFN-α subtypes	Lymphoblastoid cells	Hairy cell leukemia, juvenile laryngeal papillomatosis, condylomata acuminata, chronic hepatitis B or C
Natural human leukocyte IFN-α	None	Mixture of natural human IFN-α subtypes	Human leukocytes	Hairy cell leukemia, multiple myeloma, non-Hodgkin lymphoma, follicular lymphoma, chronic myelogenous leukemia, malignant melanoma, AIDs-related Kaposi's sarcoma, chronic hepatitis B or C
Human IFN-α-Le	None	Human leukocytic IFN-α (1, 2b, 8, 10, 14, 21)	Human leukocyte	Malignant melanoma; treatment of patients who initially respond to recombinant IFN-a, but for whom treatment subsequently fails, most likely as the result of neutralizing antibodies
IFN-α2b	None	165 aa (19 kDa) Arginine at position 23 deletion at position 44	Recombinant *E. coli*	Chronic hepatitis B or C, hairy cell leukemia, follicular lymphoma, condylomata accuminata, AIDs-related Kaposi's sarcoma and malignant melanoma
IFN-α2a	None	165 aa (19 kDa) Lysine at position 23 deletion at position 44	Recombinant *E. coli*	Chronic hepatitis B or C, hairy cell leukemia, chronic myelogenous leukemia
Consensus IFN (IFN-α con-1)	≈ 89% homology with IFN-α and 30% homology with IFN-β	166 aa (19.4 kDa)	Recombinant *E. coli*	Chronic hepatitis C, hairy cell leukemia
Peg IFN-α2b	12-kDa linear PEG covalently linked	165 aa (19 kDa)	Recombinant *E. coli*	Chronic hepatitis C
Peg IFN-α2a	40-kDa branched PEG covalently linked	165 aa (19 kDa)	Recombinant *E. coli*	Chronic hepatitis B and chronic hepatitis C
Peg IFN-α2a	20-kDa linear PEG covalently linked	165 aa (19 kDa)	Recombinant *Hansenula polymorpha*	Chronic hepatitis C
Peg IFN-α2b	31-kDa linear PEG covalently linked	165 aa (19 kDa)	Recombinant *E. coli*	Melanoma after surgery
Albinterferon (Albuferon)	r-Human albumin modified IFN-α2b	165 aa (19 kDa)	Recombinant *Kluyveromyces*	Chronic hepatitis C
IFN-α2b	polyActive technology-based controlled-release recombinant formulation	165 aa (19 kDa)	Recombinant *E. coli*	Chronic hepatitis C
IFN-α2b	MedUSA technology - based recombinant formulation	165 aa (19.4 kDa)	Recombinant *E. coli*	Chronic hepatitis C
IFN-β1a and 1b	None	165 aa (18.5 kDa)	Mammalian cells and Recombinant *E. coli*	Relapsing-remitting Multiple Sclerosis (RRMS)
IFN-γ1b	None	166 aa (18.5 kDa)	Recombinant *E. coli*	Chronic granulomatous disease, Severe malignant osteopetrosis

Based on Antonelli, G., et al. (2015) *Cytokine & Growth Factor Reviews*. **26**: 121–131

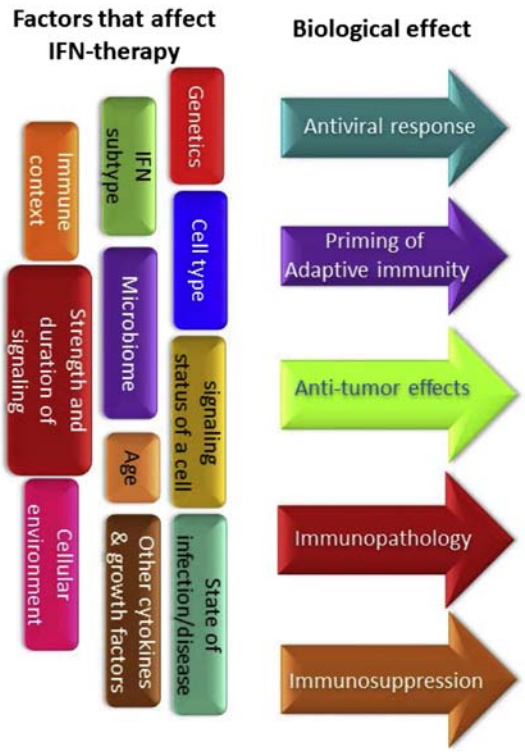

Fig. 12 Factors that affect the therapeutic effectiveness of type I IFN responses in vivo.

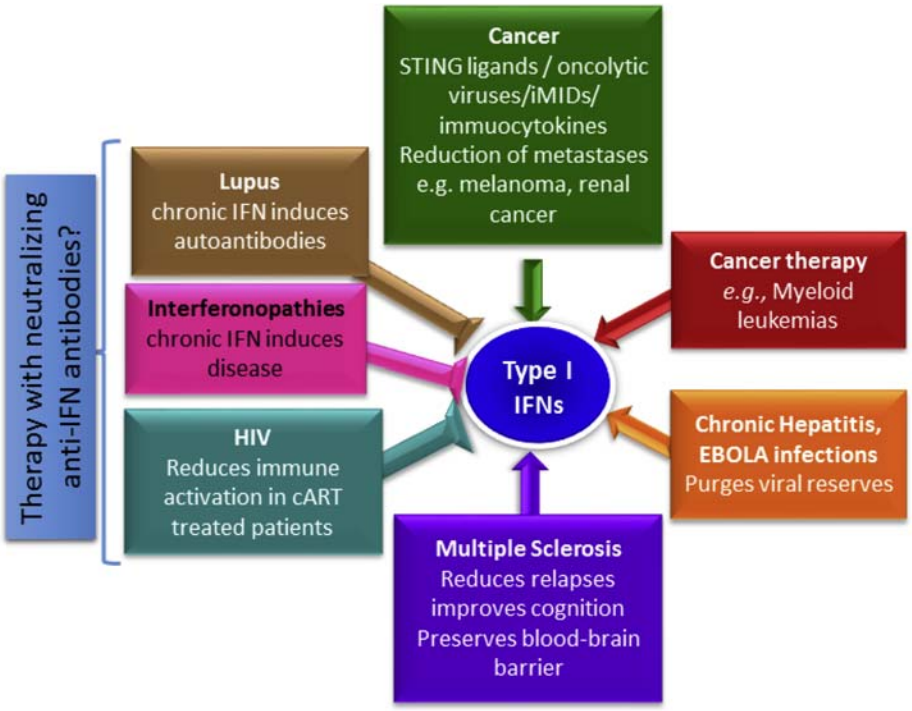

Fig. 13 Current clinical therapies based on type I IFN biology.

that promotes tumor killing by pDCs. iMiDs are immunomodulatory molecules (for example thalidomide, lenalidomide and pomalidomide) approved for the treatment of erythema nodosum leprosum (a complication of leprosy), multiple myeloma and myelodysplastic syndrome. iMiDs promote the expression of IRF7.

Emerging Targeted Type I IFN-Based Immunotherapies

Systemic administration of IFN, although produces strong anti-tumor effects, is associated with side effects such as fatigue, anorexia, hepatotoxicity, flu-like symptoms and severe depression. One approach solve this problem is to deliver the drug into tumor microenvironment. Immuno-cytokines are new therapeutics in development, where anti-tumor cytokines are fused to specific antibodies to achieve effective suppression tumor growth without wide-spread systemic toxicities. IFN-α2b coupled to hL243 (a humanized monoclonal antibody that is specific for HLA-DR) is effective against human myeloma and lymphoma xenografts. Similarly, IFN-β fused to Cetuximab (a clinically approved EGFR-targeting monoclonal antibody) robustly suppresses the growth of EGFR-expressing tumors that failed to respond to unmodified Cetuximab. IFN-α or IFN-β fusion proteins with HER2-specific monoclonal antibody efficiently target HER2-expressing neoplasms better than a therapeutic regimen based on the unmodified monoclonal antibody. Notably, the therapeutic effects of type I IFN-containing immunocytokines targeted to EGFR seem to rely on adaptive immune responses involving CTLs, DCs and IFNAR1. An anti-CD20-IFN-α fusion displays potent biological activity against CD20-expressing lymphoma cells. Other strategies to potentiate IFN-induced therapies are modifying the hematopoietic stem cells with IFN-α/β genes such that they express in differentiated cells such as monocytes, which suppress tumor growth in the microenvironment. The cGAS/cGAMP is another major target for inducing IFN-dependent tumor suppression in cancer models. Recent studies from many labs have reported highly encouraging results, which showed a strong tumor suppressor activity. Another new area that is emerging is the use of oncolytic viruses that exploit mutations in cancer cells to enable their replication. To avoid the growth-inhibitory effects of endogenous IFNs, cancer cells lose the ability to induce IFNs and ISGs. Viruses also use strategies to avoid the IFN-induced anti-viral actions (**Table 4**). Such IFN resistance becomes an Achilles' heel for cancer cells, because oncolytic viruses replicate efficiently in IFN-deficient cells. A number of oncolytic virus-based therapies have shown promising results in experimental models. Recently, the first oncolytic viral product, Talimogene laherparepvec, was approved by the FDA for human cutaneous, subcutaneous and nodal lesions in patients with melanoma recurrent after initial surgery. Thus, oncolytic viruses offer another IFN-based therapeutic strategy for controlling tumor growth.

Conclusions and Perspectives

It is clear that IFNs provided trailblazing insights into various diverse biological processes, such as transcription, post-transcriptional regulation, protein translation, self- and non-self nucleic acid recognition, innate defenses against infectious pathogens, autoimmune diseases, and anti-cancer mechanisms. That JAK-STAT pathways regulate IFN-induced gene expression was a ground breaking discovery, which led to the discovery that about 50 different cytokines, peptide hormones, growth factor receptors employ this system. IFNs act as sentinels in the 'immunosurveillance' of cancer is also well established. Apart from certain viral vaccines, IFNs are the first recombinant biological therapeutics to enter clinics for the treatment of various viral, neurological and neoplastic diseases. The success of IFNs as cancer therapeutics is comparable to those of most chemotherapeutics. Although some target-specific therapeutics such as Imatinib (which blocks the BCR-ABL kinase in CML) and a new small molecule polymerase inhibitor Solvari (HCV) are being increasingly preferred over IFN (because of their severe side effects), clinical resistance to these small molecules is a major concern and it is more likely to occur with these drugs than with IFNs. Pegylated IFNs and 'Immuno-cytokines' (IFNs coupled to specific antibodies) and IFN modulators will alleviate some of the concerns over the side-effects of IFNs. Thus, IFNs will continue to be drugs of choice in the future because of their multi-level effector functions.

Acknowledgments

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Further Reading

Antonelli, G., Scagnolari, C., Moschella, F., Proietti, E., 2015. Twenty-five years of type I interferon-based treatment: A critical analysis of its therapeutic use. Cytokine and Growth Factor Reviews 26, 121–131.
Billiau, A., Matthys, P., 2009. Interferon-γ: A historical perspective. Cytokine and Growth Factor Reviews 20, 97–113.
Bordon, Y., 2014. Tumour immunology: Anticancer drugs copy bugs. Nature Reviews. Immunology 14, 766–767.
Bronte, V., 2014. Tumors STING adaptive antitumor immunity. Immunity 41, 679–681.
Cai, X., Chiu, Y.H., Chen, Z.J., 2014. The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. Molecular Cell 54, 289–296.
Casanova, J.L., Holland, S.M., Notarangelo, L.D., 2012. Inborn errors of human JAKs and STATs. Immunity 36, 428–515.

Crow, Y.J., Manel, N., 2015. Aicardi-Goutieres syndrome and the type I interferonopathies. Nature Reviews. Immunology 15, 429–440.
Egli, A., Santer, D.M., O'Shea, D., Tyrrell, D.L., Houghton, M., 2014. The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections. Emerging Microbes and Infections 3, e51.
Fensterl, V., Sen, G.C., 2015. Interferon-induced Ifit proteins: Their role in viral pathogenesis. Journal of Virology 89, 2462–2468.
Fish, E.N., Platanias, L.C., 2014. Interferon receptor signaling in malignancy: A network of cellular pathways defining biological outcomes. Molecular Cancer Research 12, 1691–1703.
Fuchs, S.Y., 2013. Hope and fear for interferon: The receptor-centric outlook on the future of interferon therapy. Journal of Interferon and Cytokine Research 33, 211–225.
Fuertes, M.B., Woo, S.R., Burnett, B., Fu, Y.X., Gajewski, T.F., 2013. Type I interferon response and innate immune sensing of cancer. Trends in Immunology 34, 67–73.
González-Navajas, J.M., Lee, J., David, M., Raz, E., 2012. Immunomodulatory functions of type I interferons. Nature Reviews. Immunology 12, 125–135.
Haller, O., Staeheli, P., Schwemmle, M., Kochs, G., 2015. Mx GTPases: Dynamin-like antiviral machines of innate immunity. Trends in Microbiology 23, 154–163.
Hertzog, P.J., 2012. Type I interferons as primers, activators and inhibitors of innate and adaptive immune responses. Immunology and Cell Biology 90, 471–473.
Hoffmann, H.H., Schneider, W.M., Rice, C.M., 2015. Interferons and viruses: An evolutionary arms race of molecular interactions. Trends in Immunology 36, 125–138.
Horvath, C.M., 2004. Silencing STATs: Lessons from paramyxovirus interferon evasion. Cytokine and Growth Factor Reviews 15, 117–127.
Ivashkiv, L.B., Donlin, L.T., 2014. Regulation of type I interferon responses. Nature Reviews. Immunology 14, 36–49.
Kalvakolanu, D.V., 2003. Alternate interferon signaling pathways. Pharmacology and Therapeutics 100, 1–29.
Kalvakolanu, D.V., Choi, K., Borden, E.C., 2010. Interferons and hematopoeitic cytokines. In: Mendelsohn, J., Howley, P.M., Israel, M.A., Liotta, L.A. (Eds.), The molecular basis of cancer. W.B. Saunders Company, Philadelphia, pp. 503–534.
Kontzias, A., Kotlyar, A., Laurence, A., Changelian, P., O'Shea, J.J., 2012. Jakinibs: A new class of kinase inhibitors in cancer and autoimmune disease. Current Opinion in Pharmacology 12, 464–470.
Lazzari, E., Jeffries, C., 2014. IRF5-mediated signaling and implications for SLE. Clinical Immunology 153, 343–352.
Levine, R.L., Pardanani, A., Tefferi, A., Gilliland, D.G., 2007. Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nature Reviews. Cancer 7, 673–683.
Lohoff, M., Mak, T.W., 2005. Roles of interferon-regulatory factors in T-helper-cell differentiation. Nature Reviews. Immunology 5, 125–135.
McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A., 2015. Type I interferons in infectious disease. Nature Reviews. Immunology 15, 87–103.
Richetta, C., Faure, M., 2013. Autophagy in antiviral innate immunity. Cellular Microbiology 15, 368–376.
Schmid, D., Munz, C., 2007. Innate and adaptive immunity through autophagy. Immunity 27, 11–21.
Schneider, W.M., Chevillotte, M.D., Rice, C.M., 2014. Interferon-stimulated genes: A complex web of host defenses. Annual Review of Immunology 32, 513–545.
Sen, G.C., Sarkar, S.N., 2007. The interferon-stimulated genes: Targets of direct signaling by interferons, double-stranded RNA, and viruses. Current Topics in Microbiology and Immunology 316, 233–250.
Shiloh, R., Bialik, S., Kimchi, A., 2014. The DAPK family: A structure–function analysis. Apoptosis 19, 286–297.
Stark, G.R., Darnell Jr., J.E., 2012. The JAK-STAT pathway at twenty. Immunity 36, 503–514.
Taylor, G.A., Feng, C.G., Sher, A., 2004. P47 GTPases: Regulators of immunity to intracellular pathogens. Nature Reviews. Immunology 4, 100–109.
Todd, J.A., 2014. Constitutive antiviral immunity at the expense of autoimmunity. Immunity 40, 167–169.
Vesely, M.D., Kershaw, M.H., Schreiber, R.D., Smyth, M.J., 2011. Natural innate and adaptive immunity to cancer. Annual Review of Immunology 29, 235–271.
Wack, A., Terczynska-Dyla, E., Hartmann, R., 2015. Guarding the frontiers: The biology of type III interferons. Nature Immunology 16, 802–809.
Zitvogel, L., Galluzzi, L., Kepp, O., Smyth, M.J., Kroemer, G., 2015. Type I interferons in anticancer immunity. Nature Reviews. Immunology 15, 405–414.

Jaws Cancer: Pathology and Genetics

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Abbreviations

CFD Craniofacial fibrous dysplasia
CGCG Central giant cell granuloma
COF Cemento-ossifying fibroma
FD Fibrous dysplasia
JTOF Juvenile trabecular ossifying fibroma
JPOF Juvenile psammomatoid ossifying fibroma
OS Osteosarcoma

Ameloblastoma

Definition

Ameloblastoma is the second most common benign odontogenic tumor following odontoma and generally arises inside bone. The conventional subtype accounts for 80%–85% of cases and has also been designated as solid/multicystic type due to its histologic pattern of growth. Left alone, the tumor grows progressively and expands the gnathic bones which can result in severe facial deformity and a characteristic polylobulated lytic ("soap bubble") appearance in conventional X-rays and CT scans. Desmoplastic ameloblastoma is defined by pronounced desmoplasia and has been considered a separate subtype in the WHO classification of 2005. According to the current classification released in early 2017, however, it is now regarded as a histologic variant of conventional ameloblastoma. Unicystic ameloblastoma is a distinct subtype presenting as a single cystic cavity with or without luminal proliferation (intraluminal vs luminal type) and accounts for 5%–15% of cases. Infiltration of the cystic wall by tumor cells defines the mural variant of unicystic ameloblastoma and there has been considerable debate as to whether this variant should be considered part of the spectrum of conventional or unicystic ameloblastoma. Rarely, ameloblastoma can develop primarily in the soft tissues of the gingiva or edentulous alveolar areas which is classified as extraosseous or peripheral ameloblastoma. Lastly and even rarer, conventional ameloblastoma can metastasize despite appearing completely benign histologically (so-called metastasizing ameloblastoma). Ameloblastic carcinoma is the overtly malignant variant of ameloblastoma and can develop without (primary) or within a preexisting or recurrent ameloblastoma (secondary).

Burden

Odontogenic tumors are very rare in general and epidemiologic data are difficult to gather. For ameloblastoma, an annual incidence of about 0.5 cases per million population has been estimated. Conventional ameloblastoma most frequently develops in the posterior mandible (80%), is uncommon in the first two decades and reaches its peak in the fourth and fifth decade of life. Desmoplastic variants are found most commonly in the anterior mandibular region. Unicystic tumors can occur earlier in life with 50% of cases developing in the second decade. The posterior mandible is again the most common site (80%) and particularly when associated with an impacted tooth, tumors can be indistinguishable from dentigerous cysts or odontogenic keratocysts clinically and radiographically. Malignant ameloblastomas (including metastasizing ameloblastoma and ameloblastic carcinoma) have been estimated to occur with an incidence of 1.79 cases per 10 million population in the United States.

Risk Factors

There are no established risk factors for developing ameloblastoma.

Pathology

Ameloblastoma originates from rests of the dental lamina which can also be found outside bone (and can give rise to peripheral ameloblastoma). All tumors share common morphologic features resembling the enamel organ, including a basal layer of columnar to cuboidal cells with hyperchromatic, palisaded and polarized nuclei and subnuclear vacuolization (also referred to as Gorlin–Vickers criteria). The shift of nuclei away from the basal lamina has been termed "reverse polarity." In the central areas,

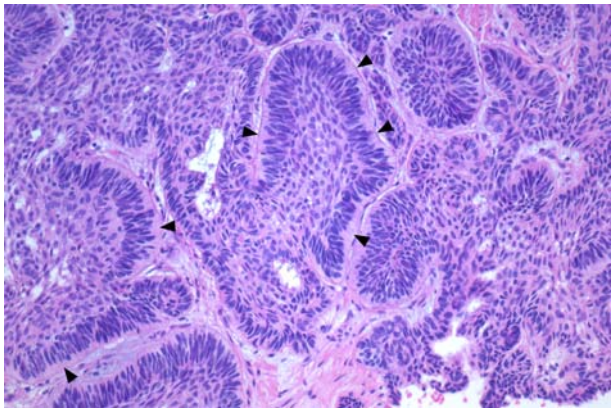

Fig. 1 Ameloblastoma: Interconnected islands of epithelial cells with peripheral palisading and subnuclear vacuoles *(arrowheads)*. The more central parts are less well arranged and resemble the stellate reticulum of the enamel organ.

the tumor cells are loosely arranged and resemble the stellate reticulum (Fig. 1). They can further show spindle, basaloid and granular cell morphology and/or squamous metaplasia and often undergo cystic degeneration. Occasionally, the peripheral cell layer lies on a thick eosinophilic band which is, however, not specific for ameloblastoma.

The conventional subtype (historically also designated as solid/multicystic subtype) primarily shows two patterns of growth: islands or follicles of epithelial cells define the follicular type, complex and continuously anastomosing strands the plexiform type. Both patterns can occur in the same tumor and the epithelial proliferations are generally embedded in a mature fibrous stroma. Although important for the pathologist to recognize, histotyping of growth patterns is of no clinical relevance. In the desmoplastic variant, the stromal component is strikingly rich in collagen fibers and seems to compress the epithelial islands from the outside. The classic Gorlin–Vickers criteria can sometimes be blurred but are usually present at least focally. Myxoid changes of the stromal component can occasionally be observed immediately adjoining the odontogenic epithelium.

Unicystic ameloblastoma presents as a well-defined, often large and generally mono-cystic cavity with a lining that is at least focally composed of ameloblastic epithelium. Commonly, the epithelial layer appears flat and can therefore resemble dentigerous (or other odontogenic) cysts. Secondary metaplasia caused by non-specific inflammatory changes or superinfection can further obscure the defining histologic criteria and render a diagnosis on bioptic material challenging to sometimes impossible. If the tumor appears purely cystic, a luminal variant of unicystic ameloblastoma should be considered, intraluminal projections define the intraluminal variant. Infiltration of ameloblastic epithelium into the cystic wall are diagnostic for the mural variant. Extraosseous or peripheral ameloblastoma is morphologically identical to the conventional subtype and is only defined by its site of origin.

Ameloblastic carcinoma appears cytologically malignant with nuclear enlargement and polymorphism as well as increased nuclear hyperchromasia and mitotic activity. Abnormal mitotic figures, necrosis, perineural and/or vascular invasion may occur but borderline lesions can be difficult to diagnose. In general, the ameloblastic differentiation is still recognizable, at least focally, but nearly undifferentiated spindle cell lesions have been described.

Immunophenotyping is of no diagnostic value in ameloblastoma and individual subtypes.

Molecular Pathology and Genetics

Conventional ameloblastomas are characterized by MAPK pathway and FGFR2 mutations as well as several non-MAPK mutations, including SMO, SMARCB1, CTNNB1, and PIK3CA. The most common alteration is the BRAF V600E mutation which can be identified in roughly 2/3 of cases and is usually confined to mandibular tumors. It is more frequent in patients of younger age. In maxillary ameloblastomas, SMO mutations are the most common aberrations. KRAS, NRAS, HRAS, and FGFR2 (activator of RAS) mutations are generally mutually exclusive to BRAF mutations and have been reported in 2/3 of V600E wild type cases, sometimes in addition to SMO mutations. Taking into account all genes described, somatic mutations in known oncogenes can be detected in roughly 90% of ameloblastomas. Specific BRAF inhibitors (e.g., vemurafenib) have been shown to be effective in ameloblastoma cell lines and in selected patients harboring the V600E mutation. The experience is so far, however, limited to case reports and systematic studies have not been conducted so far. SMO inhibition in hedgehog-driven tumors or antagonizing EGFR signaling which is often overactive in ameloblastoma might be promising alternatives but efficacy has also been demonstrated only in cell lines yet. Data on unicystic ameloblastoma and ameloblastic carcinoma are still limited to small series and case reports but BRAF V600E mutations seem to be the most prevalent alteration in those tumors as well.

Notably, V600E BRAF mutations have been identified also in the epithelial component of other odontogenic tumors, including ameloblastic fibroma and precursor lesions of developing odontoma (ameloblastic fibroodontoma), pointing to common pathway alterations in the molecular pathogenesis of these lesions.

Microenvironment Including Immune Response

There are no established data on the microenvironment of ameloblastoma. On pure morphologic basis, tumor infiltrating inflammatory cells are not a common feature in these tumors.

Staging and Grading

As ameloblastoma is considered a benign tumor, staging is confined to local tumor extent and there is no grading system. Ameloblastic carcinoma is not graded either, staging, however, should be complemented to exclude pulmonary metastases.

Prognostic and Predictive Biomarkers

The single most important prognostic factor in ameloblastoma is the accuracy of surgery aiming for free margins which may be obtained by careful enucleation and curettage for small lesions whereas larger lesions may require more radical treatment. For the same reason it is difficult to appraise biomarker studies in the literature, since early recurrence might not be due to a mutation or an overexpressed marker protein but simply caused by incomplete surgery. BRAF V600E mutations were found to be associated with a favorable outcome but there are conflicting data reported in the literature as well. Whether BRAF and SMO mutations and/or EGFR overexpression might serve as predictive markers in the future has to be elucidated in larger studies.

Odontogenic Myxoma/Myxofibroma

Definition

Odontogenic myxoma is considered the third most common benign odontogenic tumor following odontoma and ameloblastoma and generally arises inside bone (peripheral variants are exceptionally rare). It belongs to the group of benign mesenchymal odontogenic tumors and lacks an epithelial component. Greater amounts of collagens define odontogenic myxofibroma although the distinction is arbitrary and without clinical relevance. The tumors usually present lytic in conventional X-rays and CT scans and cause a painless swelling or asymmetry of the jaws over time. Similar to ameloblastoma, they can be polylobulated resulting in a "soap bubble" or honeycomb appearance with a delicate bony trabeculation. Tumor extent can be difficult to assess radiologically and should include MRI scans. Root displacement is common, root resorption can occur but is rather infrequent ($<10\%$).

Burden

The lesions are rare and account for 2%–5% of all odontogenic tumors. Roughly 2/3 of cases develop in the mandible, tumors of the maxilla tend to infiltrate the maxillary sinus. Most cases are located in the molar and premolar areas. Women seem to be more commonly affected then men (ratio 1.5:1). The age distribution is broad but 3/4 of cases develop within the second to fourth decade of life.

Risk Factors

There are no established risk factors for developing odontogenic myxoma.

Pathology

Odontogenic myxomas are locally aggressive, non-encapsulated and non-metastasizing neoplasms that diffusely infiltrate the marrow spaces. The neoplastic cells are evenly distributed in abundant extracellular ground substance rich in acid mucopolysaccharides and show stellate, spindle-shaped or round cell morphology. Thin cytoplasmic processes interconnect the tumor cells, the cytoplasm is pale to eosinophilic (Fig. 2). In case of odontogenic myxofibroma, the amount of collagen is more pronounced, but the myxoid character of the lesion remains recognizable. The lesional cells can show mild atypia and scattered mitotic figures, occasionally, epithelial remnants can be found within the tumor. Immunohistochemically, the tumor cells express vimentin, orosomucoid 1 and nestin with non of the marker proteins being specific for odontogenic myxoma.

The main differential diagnosis are normal structures. Both the dental papilla and the dental follicle of a developing tooth show evenly dispersed spindle cells within a myxoid stroma that can closely mimic odontogenic myxoma. During development of the dental pulp, the cells in the periphery of the papilla condensate to form odontoblasts and are sometimes lined by a layer of inner enamel epithelium. The follicle surrounding the tooth germ can furthermore become hyperplastic and contain various derivates of the odontogenic apparatus including cementum, dystrophic calcifications and epithelial remnants. Whenever normal structures are identified in close vicinity to tissue resembling myxoma, the differential diagnosis of tissue related to a developing tooth should always be considered. Furthermore, correlation with clinical and radiologic findings is mandatory since odontogenic myxomas are mass-forming lesions that should appear as such and are not diagnosed as incidental findings.

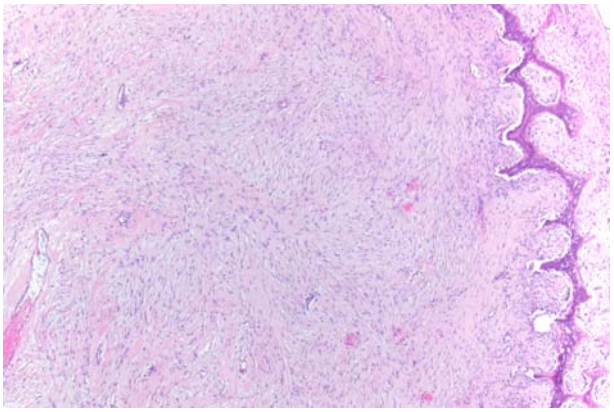

Fig. 2 Odontogenic myxoma: Monomorphic stellate-like spindle cells delineated by a layer of newly formed woven bone *(right side)*.

Molecular Pathology and Genetics

Odontogenic myxoma has been linked to tuberous sclerosis and Gorlin syndrome in single case reports but data on the molecular pathogenesis of the disease is scarce. Mutations in the PRKAR1A gene, which is commonly mutated in cardiac myxomas (both in sporadic cases and in the setting of Carney complex), have been reported in 2/17 odontogenic myxomas. Common oncogenes and tumor suppressors, however, do not seem to play a major role in tumor development, as targeted sequencing of 50 frequent cancer genes did not identify a single (pathogenic) mutation in a set of nine odontogenic myxomas.

Microenvironment Including Immune Response

There are no established data on the microenvironment of odontogenic myxoma. On pure morphologic basis, tumor infiltrating inflammatory cells are not a common feature in these tumors.

Staging and Grading

As odontogenic myxomas are benign neoplasms, staging is confined to local tumor extent which can be difficult to assess due to diffuse infiltration into bone and the surrounding structures. The tumors are not graded.

Prognostic and Predictive Biomarkers

Similar to ameloblastoma, complete resection with free margins is the single most important prognostic factor in odontogenic myxoma. Recurrences are reported to occur in 25% of cases. For small cases with a more fibrous nature, enucleation and curettage may be a therapeutic option.

Osteosarcoma of the Jaws

Definition

Osteosarcoma (OS) is a malignant mesenchymal tumor in which the tumor cells produce bone. Gnathic tumors differ in several aspects from their peripheral counterparts warranting a separate discussion in this article. Symptoms are generally nonspecific and include pain, swelling and loosening of teeth. Radiologically, OS present as mixed radiolucencies reflecting the kind, extent and mineralization of neoplastic matrix formation. Aggressive features such as periosteal reaction and/or cortical permeation are commonly present in high-grade lesions.

Burden

Although OS generally develops in the metaphyses of long bones during skeletal growth, the fourth most common site of origin are the jaws, accounting for approximately 6% of cases. The disease is nevertheless exceedingly rare with an estimated annual incidence of about 1–2 cases per 10 million population. Contrary to the peripheral skeleton, gnathic tumors develop after skeletal maturity and preferentially in the third and fourth decade of life. Men and women are equally affected, there is a slight predilection for the mandible.

Risk Factors

Risk factors are similar to extragnathic OS including prior radiotherapy, M. Paget or several cancer predisposition syndromes (e.g., Li Fraumeni and Retinoblastoma syndrome).

Pathology

Histologically, jaw tumors do not differ from their peripheral counterparts and usually show pleomorphic tumor cells producing a lace-like osteoid matrix (Figs. 3 and 4). The degree of atypia, mitotic activity and necrosis varies but is usually less pronounced compared to extragnathic tumors. Chondroblastic variants are more common in the jaws which can cause diagnostic problems, particularly in core needle biopsies. It therefore needs to be kept in mind that pure chondrogenic tumors (chondroma or chondrosarcoma) are exceedingly rare in the jaws, and diagnosis cannot be made with certainty unless an IDH1/2 mutation has been identified or the complete tumor has been histologically screened for smaller foci of osteoid formation. On the other hand, whenever cartilage is present in a biopsy from the jaws and there is no history of prior fracture with callus formation, an OS should always be within the differential diagnosis.

MDM2 amplification can be found in about 79% of parosteal, 29% of low-grade central and in $<10\%$ of conventional high-grade OS. Immunohistochemical expression of MDM2 can serve as a surrogate but needs confirmation using FISH due to limited specificity. Since parosteal OS is virtually nonexistent in the jaws and data on MDM2 amplification of gnathic OS is lacking, the diagnostic value of MDM2 testing remains unclear. In general, the analysis only helps in case an amplification is detectable but normal copy numbers of the gene do not argue against OS. Additional immunohistochemistry with antibodies directed against SATB2, pro-collagen I and osterix can help to confirm an osteoblastic lineage differentiation but are usually not required and also not specific for the disease.

Molecular Pathology and Genetics

Osteosarcomas generally show highly complex karyotypes with abundant numerical and structural aberrations. Only recently, the tumors have been shown to acquire homologous recombination repair insufficiency as a common trait in the majority of cases ($>85\%$ of cases) that might be therapeutically exploitable by the use of PARP inhibitors. Up to now, genetic analyses of osteosarcoma have been limited to tumors of the peripheral skeleton and have not included gnathic OS. The molecular basis for the differences in biologic behavior and prognosis is therefore still unknown.

Microenvironment Including Immune Response

There are no established data on the microenvironment of gnathic OS. On pure morphologic basis, tumor infiltrating inflammatory cells are not a common feature in these tumors.

Staging and Grading

There are no commonly accepted guidelines for staging procedures in gnathic OS but besides the assessment of local tumor extent, pulmonary metastases should be ruled out by a CT scan of the chest. Grading is generally performed according to tumors of the peripheral skeleton.

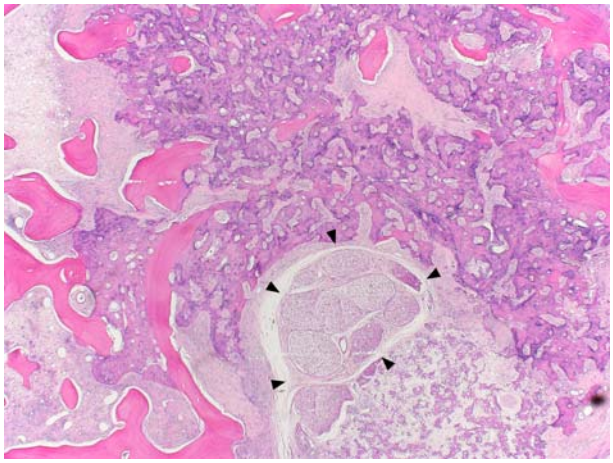

Fig. 3 Osteosarcoma: Neoplastic matrix formation permeating through preexisting bone and encircling the inferior alveolar nerve *(arrowheads)*.

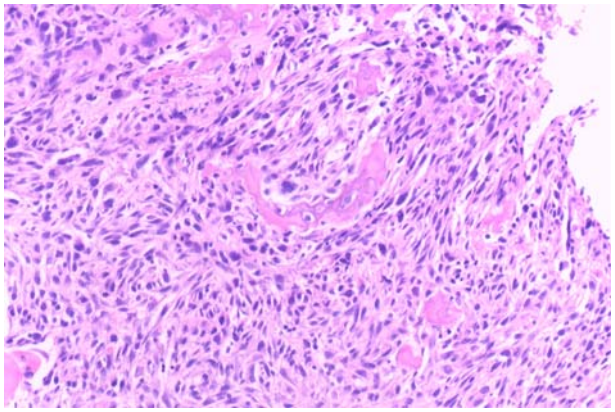

Fig. 4 Osteosarcoma: Pleomorphic and hyperchromatic spindle cells with immature and neoplastic new bone formation.

Prognostic and Predictive Biomarkers

For yet unknown reasons, gnathic OS metastasize far less frequently (6%–21% of cases) and later in the course of the disease compared to OS of the peripheral skeleton, rendering complete resection with clear margins the single most important prognostic factor for affected patients. If achievable, 10-year survival rates exceed 80% and the prognosis remains excellent even if clear margins can only be accomplished after repeated surgery. The role of (neo-)adjuvant chemotherapy is thus still controversial and difficult to investigate systematically due to the rarity of the disease. There are, however, tumors that follow an aggressive course which might not be obvious morphologically. Notably, the favorable outcome of most OS is restricted to tumors developing in the jaws, whereas tumors of the skull or facial bones behave similar to extragnathic tumors.

Craniofacial Fibrous Dysplasia

Definition

Fibrous dysplasia (FD) is a skeletal anomaly caused by a post-zygotic missense mutations of the GNAS gene encoding the activating alpha-subunit of the stimulatory G-protein. The mutation results in the proliferation of undifferentiated bone marrow related stem cells that transform into functionally impaired bone forming progenitors. Marrow spaces are replaced by a fibroblastic spindle cell stroma containing immature trabeculae of woven bone. Fibrous dysplasia might involve single (monostotic) or multiple bones (polyostotic) and can occur together with a range of endocrinopathies and skin lesions (Café-au-lait spots) as McCune Albright syndrome or along with intramuscular myxoma as Mazabraud's syndrome. In the craniofacial bones, multiple adjacent bones are characteristically affected which is still considered as a monostotic involvement and designated as craniofacial fibrous dysplasia (CFD). Symptomatic patients generally notice painless diffuse swellings of the affected region which might cause significant cosmetic deformity and compression/obstruction of vital structures (e.g., the optic nerve or foramina of the skull base). Radiographically, early FD is primarily lytic but develops the characteristic homogeneous ground-glass appearance over time. Lesions are not well defined and gradually blend into the adjacent normal bone. The radiologic and clinical picture is highly characteristic so the diagnosis can often be made without requiring histologic proof (Fig. 5).

Burden

Exact figures on the incidence of FD or CFD are unknown because many patients are asymptomatic. Monostotic involvement is 6–10 times more common than polyostotic disease, craniofacial involvement is frequent (>50% of cases). CFD most frequently involves the maxilla and adjacent bones but can develop in virtually any (craniofacial) bone.

Risk Factors

There are no established risk factors for developing crandiofacial dysplasia.

Pathology

Morphologically, CFD is similar to FD in the peripheral skeleton and reveals a monomorphic and cytologically bland spindle cell stroma containing islands or trabeculae of woven bone. In early stages, the stroma appears immature and richly cellular although mitotic activity is low. Directly evolving from the spindle cells, varying amounts of metaplastic woven formation are recognizable that typically lack osteoblastic rimming. The architecture of the trabeculae can be curvilinear and has been described to resemble

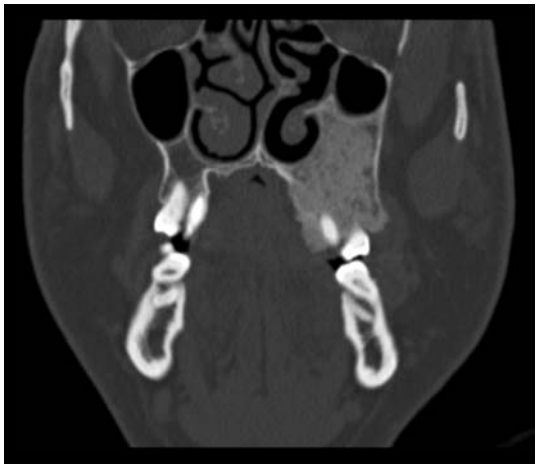

Fig. 5 Fibrous dysplasia: Fibrous dysplasia presenting as an ill-defined expansion of the left maxilla with homogenous ground-glass appearance (CT scan, coronal plane).

Chinese script letters (Fig. 6). Although nonspecific, the kind of matrix formation is almost unique and diagnostic for FD, especially in the hand of an experienced pathologist. Using special stains (e.g., van Gieson), a typical pattern of Sharpey's fibers radiating perpendicularly from the trabeculae into the surrounding stroma can furthermore be seen. Over time, the stromal cellularity decreases and the woven bone matures slowly into lamellar bone that can also show some degree of osteoblastic rimming due to an ongoing remodeling. Occasionally, FD can undergo infarction which can further blur the diagnostic changes and further complicate the histologic diagnosis. Like in other tumors and tumor-like lesions of bone, diagnosis should thus always include the correlation with clinical and radiologic findings, particularly in long-standing disease.

Molecular Pathology and Genetics

Point mutations in the GNAS gene can be detected in the vast majority (>90%) of cases. Over time, however, the lesional cells progressively decrease in number and the amount of mutant gene copies can fall below the detection threshold of the diagnostic method used. Particularly in older or matured lesions, lack of GNAS mutations therefore does not necessarily argue against the diagnosis.

Microenvironment Including Immune Response

There are no established data on the microenvironment of craniofacial fibrous dysplasia. On pure morphologic basis, tumor infiltrating inflammatory cells are not a common feature in these tumors.

Staging and Grading

Fibrous dysplasia is a benign condition and does not require staging procedures besides the assessment of local extent and individual symptoms. There is no grading system either.

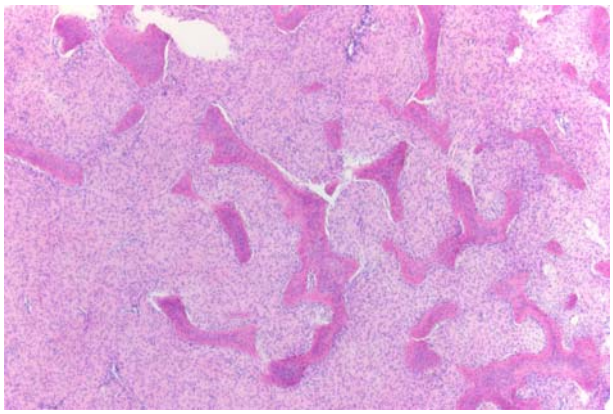

Fig. 6 Fibrous dysplasia: Irregularly shaped woven bone formation embedded in a monomorphous fibroblastic spindle cell stroma lacking atypia.

Prognostic and Predictive Biomarkers

In most patients, CFD stabilizes or even undergoes some degree of remission after skeletal maturity. Surgical intervention should only be considered in severe cases to preserve function and optimize esthetics. Although there is no unified consensus on the exact timing or even the indication for surgery, it is usually postponed until the patient has reached the end of puberty. There are no prognostic or predictive biomarkers to anticipate the clinical course of individual patients. Malignant transformation, most commonly into high-grade OS, is exceedingly rare.

Ossifying Fibroma

Definition

Ossifying fibroma together with (craniofacial) fibrous dysplasia and cemento-osseous dysplasia belong to the descriptively defined group of fibro-osseous lesions of the jaws. Among three variants that can be distinguished, the most common type is the cemento-ossifying fibroma (COF), a tumor of odontogenic origin that exclusively develops in the tooth-bearing areas of the jaws. Juvenile trabecular ossifying fibromas (JTOF) and juvenile psammomatoid ossifying fibromas (JPOF) are rare bone tumors that can develop also in extragnathic sites. All variants generally present as painless intraosseous swellings that can reach considerable and disfiguring dimensions if left untreated. Early lesions are radiologically lytic but over time matrix mineralization increases, resulting in a mixed lytic and sclerotic appearance; usually, a lytic rim remains encompassing the lesion. JTOF and JPOF can grow rapidly and are therefore also designated as juvenile aggressive subtypes, but in general, ossifying fibroma are slowly growing, well circumscribed and expansile lesions that lead to cortical thinning. Aggressive periosteal reactions or wide destruction of preexisting bone are generally not present and are important aspects in the differential diagnosis of osteosarcoma. Occasionally, the lesions are associated with secondary aneurysmal bone cysts.

Burden

Cemento-ossifying fibroma preferentially develops in women in the third to fourth decade of life (female to male ratio = 4:1). Although any tooth-bearing area of the jaws might be affected, the mandible and primarily the molar/premolar region is the most common site of origin. The juvenile variants occur at younger age (8.5–12 years mean age for JTOF and 16–33 years for JPOF) but the age distribution is broad and ranges up the seventh decade. JTOF are most common in the maxilla but can rarely occur also at extragnathic sites, JPOF only infrequently affect the jaws and are more prevalent in extragnathic locations, preferentially in the periorbital frontal and ethmoid bones. All subtypes generally develop as solitary lesions with the exception of multifocal manifestations in the exceedingly rare hyperparathyroidism jaw tumor syndrome. Multifocal involvement of lesions with a similar morphology have also been reported as (familial) gigantiform cementoma.

Risk Factors

There are no established risk factors for developing ossifying fibroma.

Pathology

Ossifying fibromas are characterized by varying amounts and kinds of hard tissue formation embedded in a moderately cellular and fibroblast-like spindle cell stroma. The latter usually appears monomorphic and without significant atypia although hyperchromatic nuclei can be present. Mitotic activity is usually low. In COF, the matrix component consists of woven bone trabeculae with prominent osteoblastic rimming and different stages of maturation (Fig. 7). Additionally, hypo- or acellular cementum-like material can usually be encountered. Over time, the mineralized matrix increases and can coalesce to form more complex structures, that are also visible radiologically as enlarging areas of sclerosis. JTOF can infiltrate into the surrounding marrow spaces and typically demonstrates delicate strands of woven bone that lack evidence of maturation. The matrix and stroma component blend into each other resulting in very immature appearing bone (Fig. 8). In JPOF, spherical and paucicellular matrix deposits reminiscent of psammoma bodies/cementum-like material predominate that can coalesce over time and secondarily induce new bone formation (Fig. 9).

Fibrous dysplasia and particularly cemento-osseous dysplasia can mimic ossifying fibroma histologically, underlining the need for a thorough radiologic and clinical correlation of findings. Cemento-osseous dysplasia is usually not expansile and often occurs multifocally, fibrous dysplasia is less well defined, can affect several adjacent craniofacial bones and typically lacks osteoblastic rimming.

Molecular Pathology and Genetics

Due to the rarity of the disease and the intense mineralization requiring decalcification procedures, molecular and genetic analyses are restricted to few and smaller case series so far. Mutations in the CDC73 gene causes the hyperparathyroidism jaw tumor syndrome and has been detected also in sporadic cases. Recent studies have suggested juvenile ossifying fibromas to represent

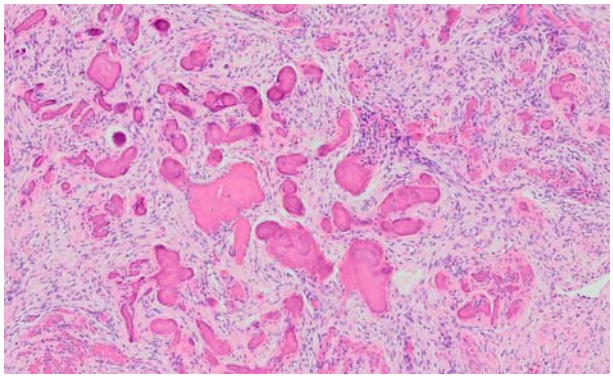

Fig. 7 Cemento-ossifying fibroma: Bony particles of varying size and shape embedded in a monomorphous fibroblastic spindle cell stroma lacking atypia.

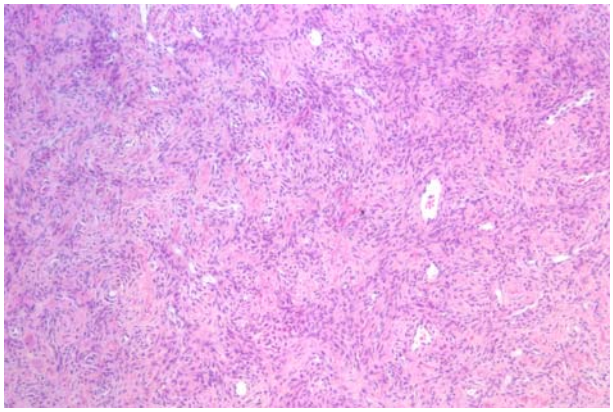

Fig. 8 Juvenile trabecular ossifying fibroma: Immature and slender strands of woven bone directly emerging from and blending into a fibroblast-like spindle cell stroma without atypia.

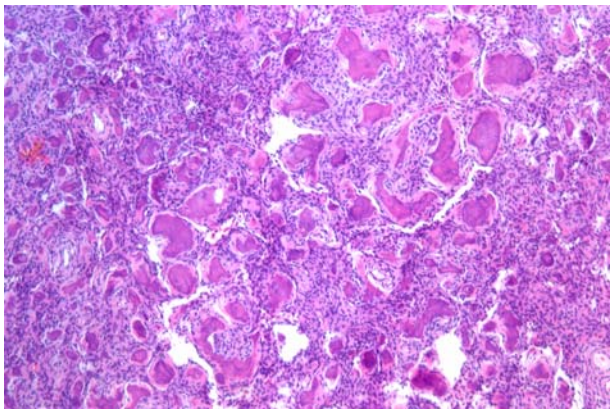

Fig. 9 Juvenile psammomatoid ossifying fibroma: Multiple psammoma-body like ossicles embedded in a cellular spindle cell stroma without significant atypia.

potential precursor lesions of osteosarcoma of the jaws due to an increased amount of MDM2 and RASAL1 templates detected by qPCR, both located on the long arm of chromosome 12. Whereas MDM2 and RASAL1 flank nearly the complete chromosomal arm, the amplification typical for parosteal osteosarcoma and a smaller subset of low-grade central osteosarcoma (approximately 30% of cases) is confined to a very small chromosomal region encompassing the MDM2 and CDK4 genes that are located in close proximity to each other. Although interesting, the data published is not sufficient to conclude a relationship between ossifying fibroma and osteogenic sarcoma or even to consider juvenile ossifying fibroma as a potential precursor of osteosarcoma. GNAS mutations typical for fibrous dysplasia do not occur in ossifying fibroma.

Microenvironment Including Immune Response

There are no established data on the microenvironment of ossifying fibroma. On pure morphologic basis, tumor infiltrating inflammatory cells are not a common feature in these lesions.

Staging and Grading

As ossifying fibromas are uniformly benign, staging is confined to the assessment of local tumor extent. There is no grading system applicable to these lesions.

Prognostic and Predictive Biomarkers

There are no established prognostic or predictive biomarkers in ossifying fibroma. If excised completely, recurrences are rare. Malignant transformation has not been convincingly described.

Central Giant Cell Granuloma

Definition

Central giant cell granulomas (CGCG) are localized and benign but locally aggressive tumors occurring exclusively in the jaws. The majority of lesions grow slowly and present as asymptomatic expansions of bone but one third of patients experience rapid growth, pain, resorption and displacement of teeth, bone errosion (or destruction), and/or soft tissue infiltration. Generally, CGCG are solitary lesions but can occur multifocally, particularly when associated with neurofibromatosis type 1 or Noonan−/LEOPARD syndrome (so-called RASopathies). Radiographically, CGCG appear lytic and polylobulated. They can reach considerable dimensions with cortical thinning and sometimes show central septae of woven bone resulting in a typical honeycomb pattern. Aggressive permeative growth or periosteal reactions are usually absent.

Burden

CGCG represent about 10% of all benign tumors in the jawbones. They occur more commonly in women and usually develop under the age of 20. The anterior mandible is the most frequently affected site.

Risk Factors

There are no established risk factors for developing CGCG.

Pathology

Histologically, CGCG show a lobular proliferation of mononuclear cells with a storiform pattern of growth. The cells appear spindle-shaped or polygonal and lack cytologic atypia (Fig. 10). Mitoses are frequently seen but are not atypical. Intermingled and partly clustered around vascular spaces and hemosiderin deposits, a varying number of rather small multinucleated giant cells

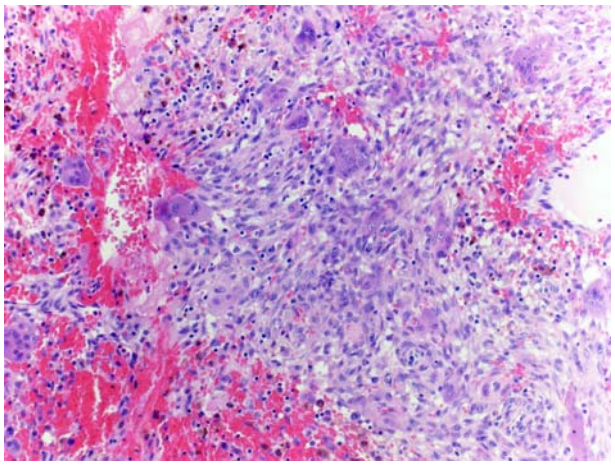

Fig. 10 Central giant cell granuloma: Monomorphic spindle cells arranged in a storiform pattern with intermingled multinucleated giant cells.

(< 15 nuclei per section) can be observed. In some cases, they can be present only focally but they generally do not predominate the histology like in classic giant cell tumor of bone. The lobules of tumor cells are typically incompletely separated by fibrous septae that can contain metaplastic new bone formation.

Brown tumors related to hyperparathyroidism can appear morphologically identical to CGCG so laboratory tests should be complemented to rule out such a condition. The solid variant of aneurysmal bone cyst is another differential diagnosis that can histologically closely mimic CGCG and molecular tests can be useful to exclude these lesions (see below). Conventional giant cell tumors of bone generally do not occur in the jaws with very rare convincing exceptions affecting the condylar process. Syndrome-related giant cell lesions are morphologically identical to sporadic cases but can show a striking predominance of spindle-shaped mononuclear cells. Another syndrome with a similar histologic appearance is cherubism that typically affects all quadrants of the jaws and leads to a marked and sometimes disfiguring expansion of bone.

Molecular Pathology and Genetics

Although the genetic background of sporadic CGCG is still unknown, molecular tests can help to rule out histological mimics. Giant cell tumors of bone typically harbor point mutations in the *H3F3A* gene (p.G34W) which have not been detected in CGCG. These lesions are therefore not only histologically but also molecularly distinct. Primary aneurysmal bone cysts show rearrangements of the *USP6* gene in about 70% of cases that can be detected by FISH analyses. Although a negative FISH result does not exclude the diagnosis, aneurysmal bone cysts usually have larger pseudocystic areas that should make a distinction from CGCG easily possible in the majority of cases.

Microenvironment Including Immune Response

There are no established data on the microenvironment of CGCG. On pure morphologic basis, tumor infiltrating inflammatory cells are not a common feature in these tumors.

Staging and Grading

As CGCG is considered a benign tumor, staging is confined to local tumor extent and there is no grading system.

Prognostic and Predictive Biomarkers

Most CGCG can be cured by local curettage but recurrences occur, particularly in clinically aggressive and syndrome-related lesions. The role of adjuvants including antiresorptive drugs (e.g., RANK ligand inhibitors and bisphosphonates) is currently still unclear and should be restricted to patients in which a complete removal of the lesion is surgically difficult or unfeasible.

Malignant Odontogenic Tumors

The odontogenic tissues occasionally are the source of malignant neoplasms occurring within the jaw bones. They may be epithelial, mesenchymal, or mixed. The following malignant epithelial types (odontogenic carcinomas) are recognized: ameloblastic carcinoma, primary intraosseous carcinoma, sclerosing odontogenic carcinoma, clear cell odontogenic carcinoma and ghost cell odontogenic carcinoma. Mesenchymal malignancies are labeled as odontogenic sarcoma and those of a mixed nature are known as odontogenic carcinosarcoma. Their extreme rarity precludes more extensive discussion; for more information the reader is referred to specialized texts on odontogenic tumors.

Prospective Vision

The field of genetics of odontogenic tumors is yet largely unexplored. It is to be expected that within the near future, elucidation of the pathways involved in these tumors will offer new tools for diagnosis and treatment.

Further Reading

Baumhoer, D., Brunner, P., Eppenberger-Castori, S., et al., 2014. Osteosarcomas of the jaws differ from their peripheral counterparts and require a distinct treatment approach. Experiences from the DOESAK Registry. Oral Oncology 50, 147–153.

Behjati, S., Tarpey, P.S., Haase, K., et al., 2017. Recurrent mutation of IGF signalling genes and distinct patterns of genomic rearrangement in osteosarcoma. Nature Communications 8, 15936.

Heikinheimo, K., Kurppa, K.J., Elenius, K., 2015. Novel targets for the treatment of ameloblastoma. Journal of Dental Research 94, 237–240.

Maiorano, E., Slootweg, P.J., 2016. Maxillofacial skeleton and teeth. In: Cardesa, A., Slootweg, P.J., Gale, N., Franchi, A. (Eds.), Pathology of the head and neck, 2nd edn. Springer, Heidelberg, pp. 179–227.

Odell, E.W., Muller, S., Richardson, M., et al., 2017. Odontogenic and maxillofacial bone tumours. In: El-Naggar, A.K., Chan, J.K.C., Grandis, J.R., Takata, T., Slootweg, P.J. (Eds.), WHO classification of head and neck tumours, 4th edn. Lyon, IARC, pp. 204–260.

Presneau, N., Baumhoer, D., Behjati, S., et al., 2015. Diagnostic value of H3F3A mutations in giant cell tumour of bone compared to osteoclast-rich mimics. Journal of Pathology: Clinical Research 1, 113–123.

Salinas-Souza, C., De Andrea, C., Bihl, M., et al., 2015. GNAS mutations are not detected in parosteal and low-grade central osteosarcomas. Modern Pathology 28, 1336–1342.

Santos, J.N., Sousa Neto, E.S., Franca, J.A., et al., 2017. Next-generation sequencing of oncogenes and tumor suppressor genes in odontogenic myxomas. Journal of Oral Pathology and Medicine (Epub ahead of print).

Slootweg, P.J., 2009. Lesions of the jaws. Histopathology 54, 401–418.

Tabareau-Delalande, F., Collin, C., Gomez-Brouchet, A., et al., 2015. Chromosome 12 long arm rearrangement covering MDM2 and RASAL1 is associated with aggressive craniofacial juvenile ossifying fibroma and extracranial psammomatoid fibro-osseous lesions. Modern Pathology 28, 48–56.

Vered, M., Wright, J.M., 2017. Update from the 4th edition of the World Health Organization classification of head and neck tumours: odontogenic and maxillofacial bone tumors. Head and Neck Pathology 11, 68–77.

Kidney Cancer: Diagnosis and Treatment

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Abbreviations

ccRCC Clear cell renal cell carcinoma
CN Cytoreductive nephrectomy
CT Computed tomography
FISH Fluorescence in situ hybridization
HRCC Hereditary renal cell carcinoma
IARC International Agency for Research on Cancer (WHO)
ICD-O International Classification of Diseases for Oncology
ISUP International Society of Urological Pathology
LND Lymph node dissection
MRI Magnetic resonance imaging
PN Partial nephrectomy
PRCC Papillary renal cell carcinoma
RCC Renal cell carcinoma
TCGA The Cancer Genome Atlas
TNSA Total number of aberrations
VEGF Vascular endothelial growth factor
VEGFR Vascular endothelial growth factor receptor
VHL von Hippel-Lindau (gene or syndrome)
WHO World Health Organization

Definition and Classification

Kidney tumors include tumors of the renal cortex and renal pelvis. Approximately 90% of renal tumors are renal cell carcinomas (RCC). Of those, about 80% are clear cell tumors (ccRCC; ICD-O code: 8310/3) which are a heterogeneous group of malignant neoplasms composed of cells with clear or eosinophilic cytoplasm. They have a typical vessel formation and a characteristic molecular background with *VHL* inactivation and the upregulation of *HIF* (see the section "Pathology and Genetics").

Other, less common, variants include papillary (ICD-O code: 8260/3), chromophobe (8317/3), MiT family translocation (8311/3), and Bellini duct (collecting duct) renal cell carcinoma (8319/3). Medullary renal carcinoma (8510/3) is a variant of collecting duct renal carcinoma and was initially described as occurring in sickle-cell trait-positive patients. Renal cancer associated with end-stage acquired cystic kidney disease is regarded as an independent histological subtype.

Presentation and Diagnosis

The two most common renal cell carcinomas are clear cell and papillary type. Clear cell renal cell carcinomas (ccRCCs) are typically sporadic solitary globular tumors protruding from the renal cortex in either kidney. The border with the kidney is usually sharp, with a pseudocapsule. Multifocality and/or bilaterality are rare, and—together with an early age onset—are characteristic for familial cancer syndromes, such as von Hippel-Lindau (VHL) syndrome. Papillary renal cell carcinoma (PRCC) occurs in the renal cortex and may be multifocal. Multiple tumors may occur in association with renal scarring. Multiple and/or bilateral tumors are also characteristic for hereditary disease. PRCC is often well circumvented with a pronounced pseudocapsule and varies in color, depending on the degree of intratumoral hemorrhage. It usually has a friable consistency. Larger tumors may contain fibrosis as well as foci of necrosis and/or cystic degeneration.

The most common RCC symptoms are hematuria and flank pain. However, the classic triad of flank pain, visible hematuria, and palpable abdominal mass is rare (6%–10%) in earlier disease stages. About 60%–80% of kidney tumors are detected incidentally on ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI), many of them at an asymptomatic stage.

Laboratory evaluation includes a complete blood count and a comprehensive metabolic panel which may include serum corrected calcium, serum creatinine, liver function studies, and urinalysis. CT of the abdomen, with or without pelvic CT, and chest x-ray are essential studies in the initial workup. For metastatic evaluation, chest radiography must be performed at the very least,

although chest CT is more accurate than radiograph for chest staging. Abdominal MRI is used to evaluate the inferior vena cava if tumor involvement is suspected, or it can be used instead of CT for detecting renal masses and for staging when contrast material cannot be administered. As the recommended abdominal imaging studies provide high diagnostic accuracy, a needle biopsy is not always necessary before surgery.

Histological diagnosis includes, besides difining the RCC type, an evaluation of the nuclear grade, sarcomatoid features, vascular invasion, tumor necrosis, and invasion of the collecting system and peri-renal fat as well as defining pT and pN categories. A variety of grading systems for renal cell neoplasia have been proposed, the most widely used being the Fuhrman grading system. However, none of these systems has been validated for many of the most recently described renal cell carcinoma morphotypes and using the four-tiered Wolrd Health Organization/International Society of Urological Pathology (WHO/ISUP) grading system is therefore recommended.

Epidemiology and Risk Factors

Kidney Cancer Burden

Kidney cancer is the 13th most common cancer in the world and the 9th most frequent in men. In 2012, 214,000 new cases were reported in men and 124,000 in women. Approximately 70% of cases occurred in countries with high and very high levels of socioeconomic development. Renal cell carcinoma is more frequent in men than in women and is rare in children. With 143,000 deaths from kidney cancer (91,000 in men and 52,000 in women) in 2012, it was the 16th cause of cancer death worldwide (Fig. 1).

Both incidence and mortality rates have been increasing in many countries, across different levels of socioeconomic development. The number of new kidney cancer cases is predicted to rise by 184,064 by the year 2030, and the number of associated deaths by 86,754.

Etiology and Risk Factors

Renal cell carcinoma is more common in men than in women (approximately 2:1), with the peak incidence between 60 and 70 years of age. Approximately 2%–4% of renal carcinomas are hereditary and having a first-degree relative with RCC roughly doubles the risk of developing the disease. Hereditary clear cell RCCs are rare. Chromosome 3p14.2 was suggested as a genomic region containing a kidney cancer susceptibility gene as chromosomal translocations involving this region have been found in

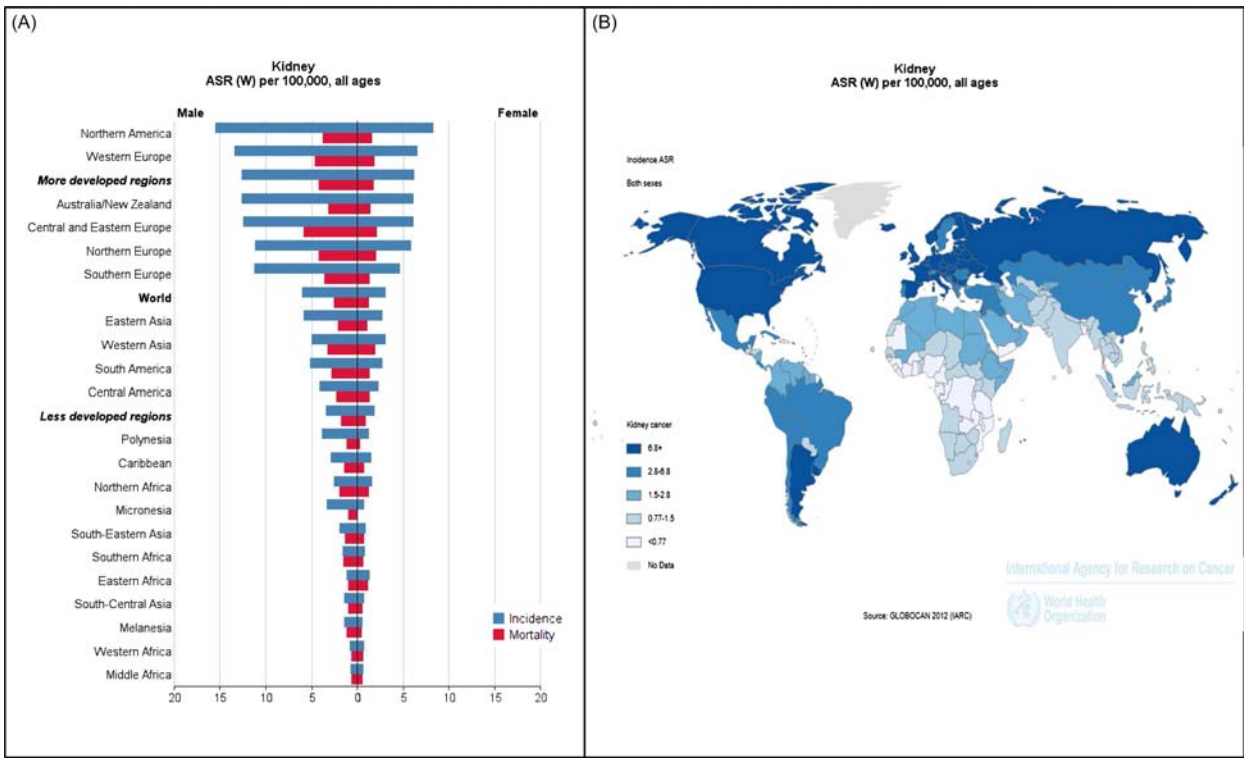

Fig. 1 Incidence and mortality of kidney cancer worldwide. (A) Age-standardized incidence and mortality rates (ASR) by gender and geographical area. (B) Incidence distribution worldwide. From Ferlay, J., et al. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]*. Lyon, France: International Agency for Research on Cancer. Available from http://globocan.iarc.fr (accessed February 20, 2018).

Table 1 Risk factors for renal carcinoma

Carcinogenic agents classified by IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (volumes 1–120)	
	Agents of sufficient evidence for kidney carcinogenicity in humans
	Tobacco smoking
	X-radiation and gamma-radiation
	Trichloroethylene
	Plants containing aristolochic acid
	Phenacetin and phenacetin-containing analgesic mixtures
	Agents of limited evidence for kidney carcinogenicity in humans
	Arsenic and inorganic arsenic compounds
	Cadmium and cadmium compounds
	Perfluorooctanoic acid
	Printing processes
	Welding fumes
	Aristolochic acid
Other carcinogenic agents and lifestyle risk factors	
	Obesity
	Hypertension and the use of antihypertensive drugs

a small proportion of families with hereditary renal cell carcinoma (HRCC) and a high prevalence of somatic loss of 3p heterozygosity was identified by tumor studies. However, no causative gene has been identified so far. Several familial syndromes are associated with an increased risk of renal tumors. The most common is the von Hippel-Lindau (VHL) syndrome associated with germline mutations in the *VHL* gene (3p25.3), which predisposes to multiple bilateral renal cell carcinomas and renal cysts. The gene for the hereditary nonpapillary renal cell carcinoma, however, is thought to be different form the *VHL* gene.

Smoking, obesity (body fatness) and hypertension are established risk factors for the development of RCC. The proportion of all renal cancer cases attributable to overweight and obesity in the United States and in European countries has been estimated to be approximately 40%. Hypertension and the use of antihypertensive medication are associated with an increased risk of RCC independently of obesity. Additionally, a number of occupational, lifestyle and health-related exposures have been suggested as potential risk factors for RCC development (Table 1). Finally, the incidence of renal cancer among patients with end-stage acquired cystic kidney disease is reported to be markedly increased.

Pathology and Genetics

The gene which is by far most frequently altered in kidney cancer is the *von Hippel-Lindau* (*VHL*) gene which is a recessive tumor suppressor gene controlling cellular oxygen sensing. Clear cell renal cell carcinoma (ccRCC), the most common type of RCC, is closely associated with *VHL* mutations which lead to stabilization of hypoxia-inducible factors (HIF-1α and HIF-2α) in both sporadic and familial forms. In sporadic ccRCC, this gene has also been found to be inactivated by epigenetic silencing and loss of the 3p chromosome. Overall, this gene is biallelilcally inactivated in a majority of ccRCCs and the loss of the VHL protein function appears to be a critical event in renal carcinogenesis, contributing to tumor initiation, progression and metastasis.

Alterations in genes controlling the maintenance of chromatin states are also frequent in ccRCC. *PBRM1*, a subunit of the PBAF SWI/SNF chromatin remodeling complex has been reported to be mutated in 40%–50% of ccRCCs, whereas *histone deubiquitinase BAP1* and *histone methyltransferase SETD2* in 15% and 12% of cases, respectively. *BAP1*-mutant tumors are typically high-grade and have poor survival.

Whole-exome sequencing of ccRCC tumors from 417 patients has identified 19 significantly mutated genes, with *VHL*, *PBRM1*, *SETD2*, *KDM5C*, *PTEN*, *BAP1*, *MTOR*, and *TP53* being the eight most commonly altered genes.

At the level of chromosome aberrations, deletions of chromosome 3p have been shown to be very frequent in ccRCC (91% of samples in a recent study by the Cancer Genome Atlas (TCGA) Research Network). This locus encompasses all the four most commonly mutated genes: *VHL*, *PBRM1*, *BAP1* and *SETD2*. Arm level losses on chromosome 14q associated with loss of *HIF1A* are also frequent (45% of ccRCCs samples in the recent TCGA study) and have been associated with a more aggressive disease and a worse prognosis. Gains of 5q copy numbers are found in approximately 70% of ccRCC cases. Additional focal amplifications refined the region of interest to 60 genes in 5q35. Focally deleted regions included among others the tumor suppressor genes *CDKN2A* at 9p21 and *PTEN* at 10q23. The PI3K/AKT pathway was also shown to be altered by specific DNA methylation events.

Overall, the most recent data have highlighted the importance of the well-known VHL/HIF pathway, the newly emerging chromatin remodeling/histone methylation pathway, and the PI3K/AKT pathway in ccRCC. The latter presents a strong therapeutic target, supporting the potential value of MTOR and/or related pathway inhibitor drugs for this type of cancer. Of note, despite a proven high intratumoral genetic heterogeneity of renal tumors, the most common gene-level alterations seem to be shared

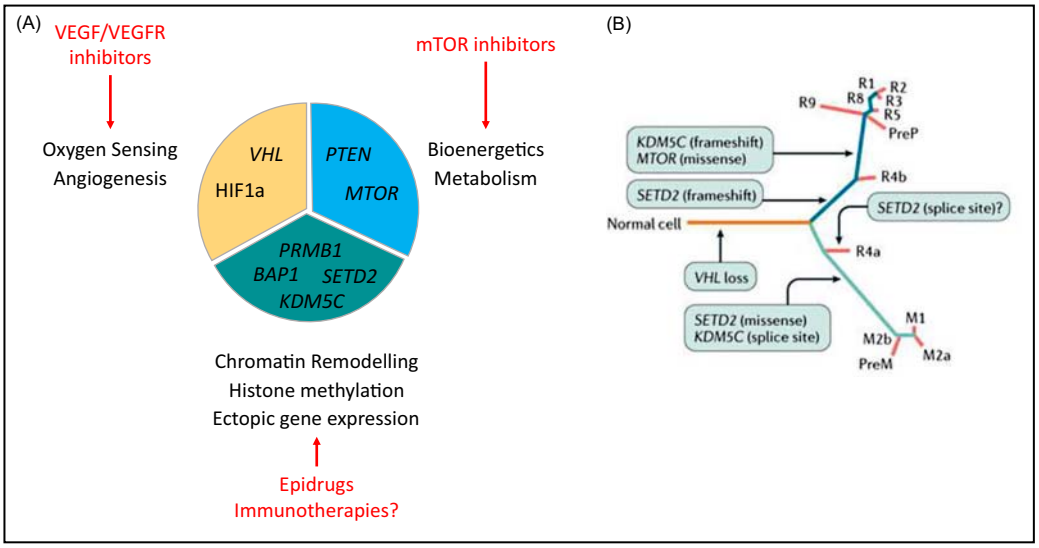

Fig. 2 Genetic alterations in renal cell carcinoma (RCC). The tumor develops by accumulating alterations in three major categories of genes, each of them containing potential therapeutic targets (A). Some alterations (e.g., *VHL* inactivation) occur early in the tumorigenesis and are common to all cells within a tumor. However, further progression results in the development of multiple clones with different alterations and an overall high intratumoral heterogeneity of the tumor (B).

between different areas of the same tumor (Fig. 2). However, studies evaluating the heterogeneity of tumors with regard to specific alterations will be needed to validate their utility as biomarkers or therapeutic targets.

Compared to ccRCC, the genetic background of other, less common, subtypes of renal cell carcinoma is less known. PRCC is typically associated with trisomy and tetrasomy of chromosome 7, trisomy of chromosome 17, and loss of the Y chromosome. Chromosome 7 contains the locus of the *MET tyrosine kinase gene* (7q32) whose heterozygous germline mutations are associated with the hereditary form of PRCC. The *MET* gene may also be altered by other mechanisms—somatic *MET* mutations have been reported in a proportion of sporadic PRCC cases. Moreover, a number of chromosomal aberrations other than the most typical ones have also been shown in PRCCs, with the type 1 and type 2 tumors showing different alterations (Table 2).

Biomarkers

The range of histologies and tumor phenotypes a renal mass can represent is a challenge for the diagnostics and treatment. Renal tumors are genetically diverse with variable prognoses and treatment response rates even within one histological subtype. Therefore, precise molecular differentiation of renal carcinomas would be key to proper treatment. However, while imaging biomarkers (see the section "Presentation and diagnosis") are reliable tools for early detection of kidney tumors, identifying prognostic and predictive biomarkers which would characterize the tumor aggressiveness and metastatic potential on an individual level remains an unmet need. Until now, risk stratification in localized tumors is based on T-category and grading but this does not reliably predict metastases.

Many putative prognostic and predictive biomarkers have been published for ccRCC, including in particular molecules involved in angiogenesis, inflammatory response and the regulation of cell cycle. Baseline serum levels of vascular endothelial growth factor (VEGF) which is key to the angiogenesis of RCC and is targeted by many anticancer drugs have been shown to be prognostic of overall survival in some studies. It has also been suggested it might be a predictive biomarker for sorafenib treatment benefit in RCC patients. High baseline levels of plasma carbonic anhydrase IX (CA-IX), tissue inhibitor of metalloproteinase 1 (TIMP-1), and Ras p21 were shown to be prognostic of reduced overall survival in metastatic RCC patients but not predictive of the sorafenib benefit. Low levels of IL-8, hepatocyte growth factor (HGF), osteopontin and TIMP-1 were all shown to be associated with significantly longer progression-free survival in patients treated with pazopanib. In patients receiving placebo, IL-6, IL-8 and osteopontin were all strong prognostic markers, while IL-6 levels appeared to be predictive of pazopanib treatment benefit. Moreover, Hypoxia-inducible factor (HIF), Ki67 (proliferation), p53, phosphatase and tensin homolog (PTEN; cell cycle), E-cadherin, and other molecules detectable in serum, urine, or circulating free DNA obtained from peripheral blood have also been investigated. However, none of all these biomarkers has been shown to clearly improve the predictive accuracy of current prognostic systems, none has been externally validated, and as of now their routine use in clinical practice is not recommended.

It becomes clear that we need to use molecular signatures rather than single-molecule biomarkers. A 16-gene signature based on the analysis of the expression of genes involved in angiogenesis (*APOLD1*, *EDNRB*, *NOS3*, *PPAP2B*), cell growth/division

Table 2 Gene loci that are most frequently altered in renal carcinoma

Pathway	*Gene (locus)*	*Alteration type*	*Estimate*
–	Loss of chromosome 3p		Up to 91% of ccRCCs
–	Gains of 5q copy numbers		Approximately 70% of ccRCCs
–	Loss of chromosome 14q, associated with *HIF1A* deletion		Up to 45% of ccRCCs
HIF-1 signaling (response to hypoxia)	*VHL* (3p25.3)	Biallelic inactivation by germline or somatic mutations, loss of chromosome, and epigenetic silencing by promoter methylation	Somatic mutations in nearly 40% of all kidney tumors and in 83% of RCCs
Chromatin remodeling/histone methylation	*PBRM1* (3p21.1)	Somatic mutations	40%–50% of ccRCCs
	BRCA1-associated protein *BAP1* (3p21.1)	Somatic mutations	15% of ccRCCs; associated with higher grade and poor prognosis
	SETD2 (3p21.31)	Somatic mutations	12% of ccRCCs
	Lysine-specific demethylase KDM5C (Xp11.22)	Somatic mutations	4%–9% of ccRCCs
P13K/PTEN/Akt: control of cell metabolism	*PTEN* (10q23)	Somatic mutations, deletions	6%–25% of ccRCCs
	MTOR (1p36.22)	Somatic mutations	7% of ccRCCs
C-Met signaling	*MET* protooncogene (7q31.2)	Hyperactivation by point mutations and/or chromosome amplifications	Somatic mutations in approximately 13% of sporadic PRCC cases

ccRCC, Clear cell renal cell carcinoma; *PRCC*, Papillary renal cell carcinoma; *RCC*, Renal cell carcinoma.

(*EIF4EBP1*, *TUBB2A*, *LMNB1*), immune response (CEACAM1, CX3CL1, CCL5), and inflammation (*IL-6*), plus reference genes (*AAMP*, *ARF1*, *ATP5E*, *GPX1*, *RPLP1*) elaborated by Rini and collaborators has been reported to predict renal carcinoma recurrence and reliably stratify patients within disease subgroups by recurrence risk. This assay is particularly promising as there seem to be little intratumoral heterogeneity in the expression of these 16 genes at the RNA level.

Brannon and collaborators have described a gene signature which allows to individually classify ccRCC tumors into two distinct molecular subtypes: ccA and ccB, based on their gene expression profiles which are associated with different clinical outcomes and could potentially be used to guide treatment decisions. The ccA group was shown to relatively overexpress genes associated with hypoxia, angiogenesis, fatty acid metabolism, and organic acid metabolism, whereas ccB tumors overexpressed a more aggressive panel of genes that regulate the epithelial-mesenchymal transition (EMT), the cell cycle, and wound healing. A recent study has validated these signatures as being independently associated with survival, with ccA patients having a markedly better prognosis. Based on these results, a 34-gene classifier (ClearCode34) for assigning ccRCC tumors to the two subtypes has been developed and validated as having an added prognostic value in patients with localized tumors. Combining this molecular profiling with standard clinical tumor evaluation criteria may allow to stratify patients according to the recurrence risk. However, prospective studies with large cohorts of patients will be needed to fully refine the integrated prognostic algorithm. Moreover, a substantial intratumoral heterogeneity so as to these two molecular subtypes has been shown, which implies that multiple biopsies from the same tumor will have to be used to identify clones of poor prognosis. Therefore, the accuracy of the putative prognostic signatures depending on the tumor size and the usefulness of biomarkers based on biopsy material should be evaluated. Finally, the clinical relevance of this model to metastatic tumors has yet to be investigated.

Another group has suggested using the so called total number of aberrations (TNSA) score, a signature of four copy number alterations evaluated using a fluorescence in situ hybridization (FISH) test to identify primary tumors with a high metastatic potential. This putative biomarker has been validated in two independent cohorts and has been shown to independently predict metastasis and correlate with survival. A prospective analysis of a larger multicenter cohort is necessary to define the degree of influence of each specific aberration on the different survival end-points and to develop a multivariate prognostic model for ccRCC, integrating each genomic aberration and well-established prognostic parameters such as T-stages, tumor size, nodal status, and histological grade.

Our knowledge on putative molecular biomarkers in RCC subtypes other than clear cell type is very limited. In papillary RCC, we have to distinguish two subtypes: type 1 with good prognosis and type 2 which is much more aggressive and associated with a shorter survival. Recently, it has been suggested that the aggressive subtype 2 of the PRCC is associated with hypermethylation, 9p alterations, specific miRNA changes and—to some extent—*FH* mutations. However, the development of corresponding prognostic biomarkers remains at an early investigational stage.

Overall, it seems possible to identify aggressive renal cell carcinomas by analyzing the molecular signatures in tumor samples and so stratify patients into groups with different prognosis based on a specific molecular background. However, more studies are needed to validate the putative biomarkers suggested so far and develop clinically relevant algorithms for integrating them into prognostic patient stratification models. In addition, the issue of intratumoral heterogeneity needs to be meticulously addressed. Liquid biopsies containing free nucleic acids as well as extracellular vesicles may help overcome this problem but their utility as prognostic biomarkers in addition to their diagnostic potential also needs to be evaluated.

Management and Therapy

For patients with surgically resectable RCC, the standard of care is surgical excision with a curative intent. For early stage (T1) localized RCC, the treatment of choice is partial nephrectomy (PN). Compared with radical nephrectomy, this nephron-sparing surgery better preserves kidney function and limits the development of metabolic and cardiovascular disorders in a long term. If there is clinical evidence of the adrenal gland invasion, adrenalectomy should simultaneously be performed. In patients with adverse clinical features (a large diameter of the primary tumor or sarcomatoid histological features), extended lymph node dissection (LND) should be considered. Patients with T2 tumors and localized masses not treatable by partial nephrectomy as well as patients not fit for PN for other reasons (e.g., due to the use of anticoagulants) are offered laparoscopic radical nephrectomy.

Elderly and comorbid patients with incidental small renal masses, who have a low risk of RCC-specific mortality and a significant competing-cause mortality risk, may be managed by alternative (nonsurgical) approaches. Active surveillance defined as the initial monitoring of tumor size by serial abdominal imaging (ultrasound, CT, or MRI) should be considered at first, with delayed intervention using ablative therapies (cryoablation or radiofrequency ablation performed laparoscopically or percutaneously) reserved for tumors showing clinical progression during follow-up.

In patients with locally advanced nonresectable tumors, embolization can control symptoms, such as visible hematuria or flank pain. The use of neoadjuvant targeted therapy to downsize tumors is still experimental and cannot be recommended outside clinical trials. In case of clinically positive lymph nodes, lymph node dissection is always justified and can add staging information. LND is also recommended for staging purposes in case of enlarged lymph nodes. However, the extent of lymph node dissection remains controversial.

Tumor resection is curative only if all tumor deposits are excised. For most patients with metastatic disease, cytoreductive nephrectomy (CN) is palliative and systemic treatments are necessary. However, metastatic RCC is often resistant to conventional chemotherapy. Therefore, inoperable or metastatic RCCs are typically managed by systemic treatment with targeted agents and/or immune checkpoint inhibitors. Over the past decade, a number of targeted therapies have been shown to provide a benefit in the treatment of advanced RCC. These therapies include small molecules or monoclonal antibodies inhibiting signaling through the vascular endothelial growth factor (VEGF) and its receptor (VGFR): sunitinib, sorafenib, bevacizumab, pazopanib, axitinib, cabozantinib and lenvatinib. These treatments, as mono-agent or combined regimens, are the current standard of care as first or second-line systemic therapy for advanced RCC. Single-agent treatment with inhibitors of mTOR signaling, such as everolimus and temsirolimus, is an approved second-line treatment.

Current clinical trials are investigating combinations of anti-VEGF therapy with immunotherapy using checkpoint inhibitors, such as monoclonal antibodies against the programmed cell death protein 1 (PD1; e.g., nivolumab, pembrolizumab) and its ligand (PDL1; avelumab, atezolizumab). Another combination under investigation is nivolumab with ipilimumab, an antibody inhibiting the cytotoxic T lymphocyte-associated protein 4 (CTL4). Blocking T-cell inhibitory signals by these antibodies promotes T-cell activation and antitumor response, providing an overall survival benefit in patients who have failed to respond to first- or second-line therapy with small drug inhibitors.

Prospective Vision

In the early 2000s, there were very limited options for the treatment of metastatic kidney cancer and the median patient survival was about 15 months. Introducing small drug inhibitors of VEGF-VEGFR and mTOR pathways in the past decade has caused the median survival to double. Using combinations of these drugs with checkpoint immunotherapies which are being developed is expected to further improve these significant benefits, achieving durable remission in a high number of patients. Progressing towards this objective will, however, require further advances in diagnosing and managing high-risk patients as well as development of systemic therapies. A major gap in the current knowledge is the lack of suitable biomarkers for predicting treatment responses and informing therapeutic decisions. One of the main areas for research to this effect is exploiting the data generated by integrative approaches combining genomics, transcriptomics and epigenomics in order to identify reliable predictive biomarkers that take into account the dynamics of intratumoural heterogeneity. This would enable a better management of off-target effects causing severe toxicities as well as provide biomarker-based rationales for combination regimens allowing to overcome drug resistance.

See also: Renal Cell Cancer: Pathology and Genetics.

Further Reading

Brannon, A.R., et al., 2010. Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. Genes & Cancer 1, 152–163.

Brooks, S.A., et al., 2014. ClearCode34: A prognostic risk predictor for localized clear cell renal cell carcinoma. European Urology 66 (1), 77–84.

Cancer Genome Atlas Research Network, 2013. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 499 (7456), 43–49.

European Association of Urology. Guidelines on Renal Cell Carcinoma, 201x: http://uroweb.org/guideline/renal-cell-carcinoma/ (accessed on February 5, 2018).

Farber, N.J., 2017. Renal cell carcinoma: The search for a reliable biomarker. Translational Cancer Research 6 (3), 620–632.
Moch, H., Humphrey, P.A., Ulbright, T.M., Reuter, V.E. (Eds.), 2016WHO classification of tumors of the urinary system and male genital organs, 4th edn, vol. 8. International Agency for Research on Cancer, Lyon, France.
National Comprehensive Cancer Guidelines (NCCN). NCCN Clinical Practice Guidelines in Oncology. Kidney Cancer. Version 2.2018 – November 30, 2017: https://www.nccn.org/professionals/physician_gls/default.aspx#site.
Pastore, A.L., et al., 2015. Serum and urine biomarkers for human renal cell carcinoma. Disease Markers 2015, 251403.
Rni, B., et al., 2015. A 16-gene assay to predict recurrence after surgery in localized renal cell carcinoma: Development and validation studies. The Lancet Oncology 16 (6), 676–685.
Sanjmyatav, J., et al., 2014. Identification of high-risk patients with clear cell renal cell carcinoma based on interphase-FISH. British Journal of Cancer 110, 2537–2543.
Serie, D.J., et al., 2017. Clear cell Type A and B molecular subtypes in metastatic clear cell renal cell carcinoma: Tumor heterogeneity and aggressiveness. European Urology 71 (6), 979–985.

Relevant Websites

http://www.auanet.org/—American Urological Association.
www.euroweb.org—European Association of Urology.

Laryngeal Cancer: Diagnosis and Treatment

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Glossary

Glottis The glottis extends from an artificial horizontal plane extending bilaterally across the apex of the laryngeal vestibule to approximately 1 cm below the true vocal folds.

Laryngectomy Partial (partial laryngectomy) or complete (total laryngectomy) surgical removal of the larynx, usually as a treatment for cancer of the larynx.

Laryngoscopy A procedure to look at the larynx and pharynx for abnormal areas. A mirror or a rigid or flexible laryngoscope (a thin, tube-like instrument with a light and a lens for viewing) is inserted through the mouth or the nose (fibroscope) to see the larynx. A special tool on the laryngoscope may be used to take samples of tissue.

Supraglottis The supraglottis extends from the tip of the epiglottis to an artificial horizontal plane extending bilaterally across the apex of the laryngeal vestibule, including the epiglottis, the false vocal cords (ventricular bands), the ventricles, the aryepiglottic folds and the arytenoids.

Subglottis The lower part of the larynx between 1 cm below the true vocal folds and the trachea (windpipe).

Tracheostomy The surgical formation of an opening into the trachea through the neck to allow the passage of air. The term "tracheotomy" refers to the incision into the trachea that forms a temporary or permanent opening, which is called a "tracheostomy," however; the terms are sometimes used interchangeably.

Introduction

Cancers of the larynx represents approximately 0.8% of the total cancer risk and is the second most frequent head and neck cancer. The number of new cases of cancer (cancer incidence) is 3.1 per 100,000 men and women per year (based on 2010–14 cases). The American Cancer Society's most recent estimates for laryngeal cancer in the United States for 2017 are that about 13,360 new cases of laryngeal cancer (10,570 in men and 2790 in women) will be diagnosed and about 3660 people (2940 men and 720 women) will die from laryngeal cancer, which is 0.6% of all cancer deaths.

Cancer of larynx is strongly related to cigarette smoking. The role of alcohol in inducing laryngeal cancer remains unclear, although it may be play a role specially in supraglottic cancer.

More than 95% of laryngeal tumors are squamous cell carcinomas (SCC). Other laryngeal malignancies include: verrucous SCC, papillary SCC, nonkeratinizing HPV-positive SCC, spindle cell carcinomas, basaloid SCC, lymphoepithelial carcinomas, acantholytic SCC, adenosquamous carcinomas, salivary tumors and sarcomas, among others.

Glottic, supraglottic, and subglottic cancers represent approximately two-thirds, one-third, and 2% of laryngeal cancers, respectively. Tumors that extend past the ventricle from either a glottic or supraglottic primary are considered transglottic tumors.

Nearly 75% of patients with glottic carcinoma present with hoarseness and often they are diagnosed at an early stage. However, nearly 70% of patients with supraglottic and subglottic laryngeal cancers usually present with advanced disease due to a paucity of symptoms, propensity for local extension, and rich lymphatics resulting in a high incidence of lymph node metastases (mainly supraglottic tumors). Early diagnosis can lead to a reduction in mortality.

Diagnosis

The initial evaluation of patients with suspected laryngeal carcinoma needs to include a detailed medical history to assess clinical risk factors (history of tobacco and alcohol use and environmental exposures) and symptom severity. Moreover, a complete head and neck physical examination should undergo, including examination of all mucosal sites of the upper aerodigestive tract, due to the frequent occurrence of multiple primary tumors in patients with a head and neck tumor. It is advisable an assessment of speech and swallowing function, communication needs, nutrition, health behaviors and availability of social support for what is typically prolonged period of treatment and rehabilitation. Finally, pretreatment dental evaluation is also recommended for patients who will undergo radiation, given the risk of dental infection, damage, and treatment-induced osteoradionecrosis.

Then, if a malignant tumor is suspected, it must be confirmed by obtaining a biopsy of the lesion for histopathological study. Finally, radiologic imaging is performed to obtain precise anatomical details regarding the tumor localization and extension. **Fig. 1** represent an algorithm for diagnostic evaluation of a laryngeal cancer.

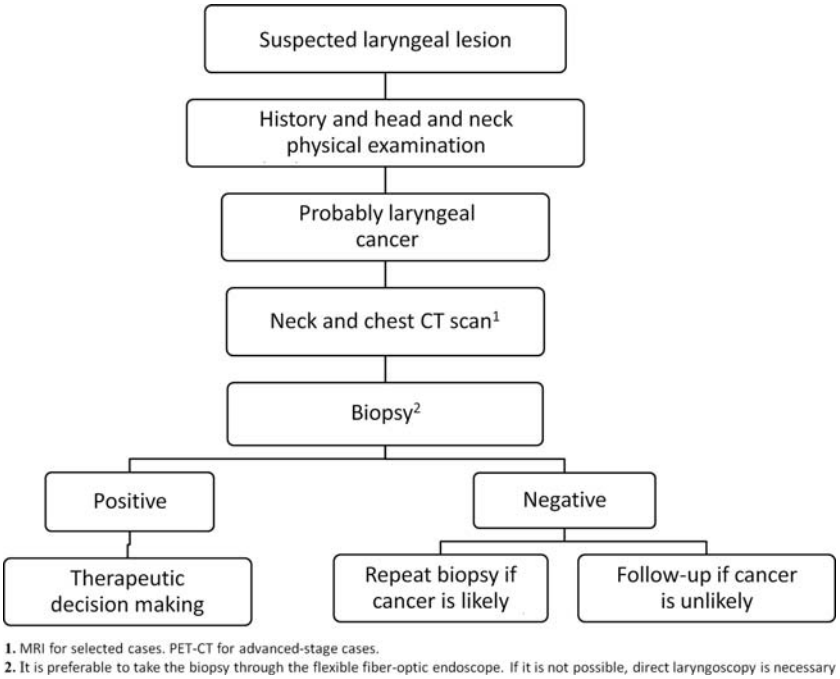

Fig. 1 Algorithm for diagnostic evaluation of a laryngeal lesion. *CT*, computed tomography; *MRI*, magnetic resonance imaging; *PET*, positron emission tomography.

Clinical Evaluation

Patients with primary early-stage tumors arising on the true vocal cords usually present with complaints of dysphonia and hoarseness; sore throat, odynophagia, dysphagia, referred otalgia, pain localized to the thyroid cartilage, hemoptysis, airway obstruction, stridor and neck adenopathy are features of advanced-stage glottic lesions. Early diagnosis is crucial for both improved survival and laryngeal preservation. In general, persistent hoarseness (>15 days) in an adult patient requires endoscopic visualization of the larynx to rule out a tumor.

Supraglottic tumors are usually more advanced than glottic cancer at presentation and patients may experience the following symptoms or signs: discomfort or sensation of a lump in the throat (the most frequent initial symptom), dysphagia, odynophagia, the sensation of something stuck in the throat, occasional respiratory obstruction, hemoptysis, referred pain to the ipsilateral ear (by way of the vagus and auricular nerves) or a mass in the neck; however, hoarseness is not a prominent symptom of supraglottic cancer until the lesion becomes quite extensive.

Primary subglottic tumors are usually asymptomatic at an early stage and they do not present until more advanced in size where dyspnea ad stridor are frequent. Dysphonia is relatively common in advanced subglottic tumors due to the involvement of the true vocal cord or direct extension to the recurrent laryngeal nerve. An emergent tracheotomy is not uncommon in these patients.

Late symptoms of laryngeal cancer include weight loss, dysphagia, foul breath and aspiration.

Physical Examination

After the medical history, a thorough clinical examination using a rigid or flexible fiber-optic endoscope is performed (Fig. 2A). It allows an adequate assessment of the surface extent of the primary tumor, the mobility of the vocal cords and performing an initial tumor staging. Accurate evaluation of the primary tumor prior to initiating treatment is vitally important, especially if any voice-sparing surgical procedure is a consideration. It is advisable to document the lesions of the larynx by a photograph or to depict them on a drawing to describe the site of origin of the primary tumor with its local extension to adjacent sites within the same region of the larynx or from one region to another region. Bulky lesions may extend beyond the larynx into the adjacent base of the tongue, pyriform sinus, or retrocricoid region and this should be assessed. The relation to the anterior commissure is very important in glottic tumors and in lesions involving the laryngeal surface of the epiglottis. Determination of the mobility of the vocal cords is critical because there are subtle distinctions between mobile, partially fixed and fixed cords. Fixation of the vocal cord from laryngeal cancer is caused by invasion or destruction of the vocal cord musculature, invasion of the cricoarytenoid muscle or joint or invasion of the recurrent laryngeal nerve. Ulceration of the infrahyoid epiglottis, fullness of the vallecula or palpation of a diffuse, firm fullness above the thyroid notch with widening of the space between on the hyoid and the thyroid cartilages are indirect signs of preepiglottic space invasion. Retrocricoid invasion may be suspect when the laryngeal click disappears on examination or when the thyroid cartilage protrudes interiorly, producing a fullness of the neck. Pain or tenderness to palpation or a small bulge over the thyroid cartilage is suggestive of thyroid invasion.

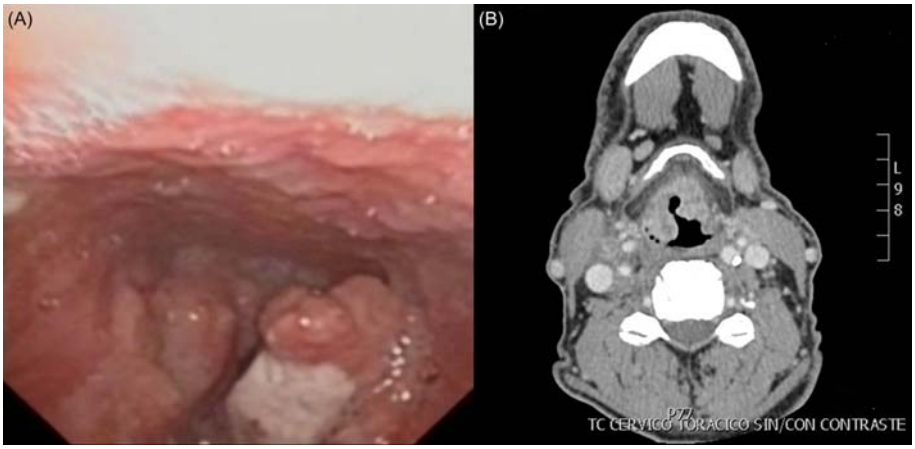

Fig. 2 (A) Endoscopic view of the larynx showing a tumor of the supraglottic. (B) Axial contrast enhanced CT showing an advanced supraglottic carcinoma. Moderately thick lesion arising from laryngeal surface of epiglottis with involvement of preepiglottic space.

Nowadays, apart from the endoscopy with conventional white light, narrow band imaging (NBI) endoscopy should be use in the routine pharyngo-laryngeal examination because it helps to make an early diagnosis of tumors of the larynx. Moreover, it is a useful tool in the postoperative surveillance to detect recurrences. NBI may be used in both flexible fiberoptic or rigid laryngoscopes. NBI is an imaging technique for the depiction of tumor-specific neoangiogenesis. A higher contrast between the mucosal epithelium and blood vessels is achieved in NBI endoscopy using filtered light comparing to white light observations. This allows detection of small suspicious mucosal changes or small tumors, few millimeters in diameter, which are not observable using white light. NBI also allows to define better the extension of tumors, which is crucial for perform targeted biopsy and for determination of resection margins in cancer surgery. NBI is increasingly used for follow-up of patients after treatment for head and neck malignant tumors, when early detection of possible recurrence is crucial.

Other imaging techniques such as auto fluorescence, contact endoscopy and optical coherence tomography (OCT) are increasingly used in ENT practice. However, these techniques have not been reproduced and is consequently not a standardized method.

Sometimes it is necessary to carry out a direct laryngoscopy under general anesthesia. The ventricles, subglottic area, apex of the pyriform sinus and retrocricoid area must be carefully examined when these areas are not well examined by the above techniques and they are at risk of being affected by the tumor. Through the laryngoscope may be introduced rigid telescopes (0, 30,45 or 70 degrees) or microscopic examination can be performed.

A detailed examination of the neck is mandatory whenever a laryngeal tumor is suspected. Regional metastases are the most important negative prognostic factor, decreasing the survival rate by 50%. The subdigastric basin (level II) is the most frequently involved. The submandibular area (level I) is rarely affected and the risk of spinal accessory lymph node (level V) involvement is small. Anterior commissure and anterior subglottic invasion are associated with involvement of the Delphian node. In tumors of the vocal cord, the incidence of clinically positive lymph nodes at diagnosis approaches 0% for T1 lesions and 2% for T2 lesions. This incidence increases from 20% to 65% for T3 and T4 lesions. The supraglottic tumors tend to have a higher rate of lymphatic metastasis and the incidence of clinically positive nodes is up to 60% at the time of diagnosis and 15% are bilateral. Spread to the pyriform sinus, vallecula and the base of tongue increases the risk of lymph node metastases.

The incidence of distant metastases is generally thought to be 10% or less. Although this increases with locoregional extent of disease and is more common with supraglottic (and subglottic) tumors, distant metastases are still found in only a small number of patients diagnosed with laryngeal cancer (15% or less). If spread through the bloodstream does occur, the lungs are the most common site of metastasis, followed by the bones.

Histopathological Diagnosis

Biopsy should be performed at the time of diagnosis, ideally after imaging, but it should not delay the treatment. A correct histologic diagnosis is critical due to the impact on therapeutic approach and prognosis and highlights the need for both a high index of clinical suspicion and adequate representative biopsies. A generous biopsy specimen is taken from the suspicious areas, looking to take tissue samples in depth and avoiding areas of necrosis. The biopsy should be performed through a flexible fiberoptic endoscope or, if this is not possible, by direct laryngoscopy.

Imaging

When malignancy is suspected, computed tomography (CT) scan with contrast enhancement (Fig. 2B) and/or magnetic resonance imaging (MRI) must be performed to define the submucosal extent and deeper margins of the tumor. CT is preferred because it is

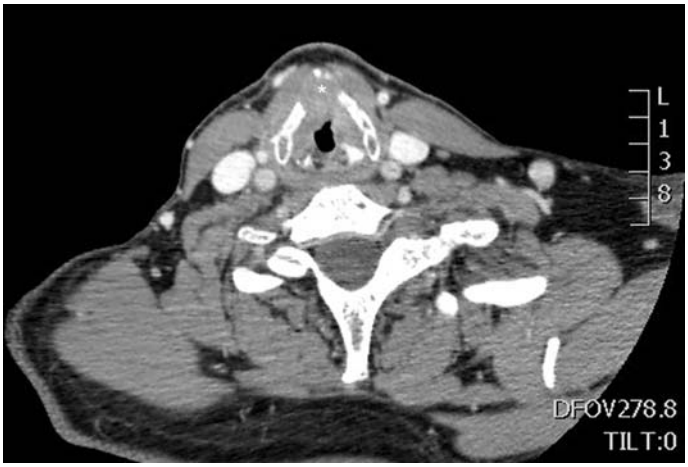

Fig. 3 Axial contrast enhanced CT performed in a patient with a tumor invading the anterior commissure and penetrating through the cartilage.

much faster than MRI. Small and superficial mucosal tumors may not be appreciated at CT or MRI and hence, it is mandatory that an endoscopy is done prior to any imaging study. Integration of cross-sectional imaging with endoscopy findings significantly improves the accuracy of T staging. The accuracy of clinical T staging alone for laryngeal cancer to be 57.5%, but as high as 80% when combined with contrast-enhanced CT. CT and/or MRI are valuable to obtain precise anatomical details regarding the tumor localization and extension (preepiglottic and paraglottic space involvement, subglottic extension, cartilage destruction and submucosal and extralaryngeal extension, which may not be obvious on clinical examination) (Fig. 3).

CT and/or MRI of the entire neck provide information on nodal disease that may not be clinically evident. A minimum axial diameter more than 10 mm, round or spherical shape, a necrotic node of any size and a node with indistinct spiculated margins (suggesting extra nodal disease spread) are the generally accepted radiological criteria to diagnose malignant nodes at CT and MRI. The sensitivity and specificity of CT to detect nodal disease using these criteria are 90% and 75% respectively.

It is important to note that imaging should be performed before biopsy so that abnormalities that may caused by the biopsy are not confused with tumor.

The general radiological criteria used for tumor involvement include asymmetric soft tissue prominence or thickening, abnormal contrast enhancement, a bulky mass, obliteration of the normal fat planes and spaces, or a combination of these.

A high-resolution CT scan with contrast enhancement (1–2 mm thick sections through the larynx) is generally preferred. Contrast enhancement helps to outline the blood vessels and the tumor. CT delineates the extent of disease and the presence and extent of lymphatic involvement. CT offers high spatial resolution; discriminates among fat, muscle, bone, and other soft tissues; and surpasses MRI in the detection of bony erosion. Nevertheless, MRI is probably better to define subtle extralaryngeal extension and to assess early thyroid cartilage destruction. MRI has a high sensitivity (89%–95%) but lower specificity (74%–84%) as compared to CT for the detection of cartilage invasion. The negative predictive value of MRI to exclude cartilage invasion is also very high, at around 94%–96%. T2-weighted or postcontrast T1-weighted cartilage signal intensity greater than that of the adjacent tumor is considered to indicate inflammation, and signal intensity similar to that of the adjacent tumor was considered to indicate neoplastic invasion. Supraglottic tumors may arise in the epiglottis or in the aryepiglottic folds and false cords. Epiglottic tumors primarily invade into the preepiglottic space. While the tumors arising from the mobile portion of the epiglottis may spread from the preepiglottic space further into the base of tongue and laterally into the paraglottic space, those arising from the petiole often invade the low preepiglottic space and via the anterior commissure, reach the glottis or subglottis. The primary sign of preepiglottic space invasion at imaging is replacement of the normal fat by abnormal enhancing soft tissue. The sensitivity of CT and MRI to detect invasion of the preepiglottic space is 100% and the corresponding specificities are 93% and 84–90%. Tumors arising in the aryepiglottic folds present as exophytic or infiltrative masses. They expand the aryepiglottic fold and spread into the paraglottic space. They may spread further anteriorly into the preepiglottic space or posteriorly to invade the piriform sinus. Tumors originating in the false vocal cords are lateral masses with a strong predilection for submucosal spread into the paraglottic space. More extensive tumor may destroy the thyroid cartilage and spread transglottically into the glottis and subglottis. Tumor spread to the paraglottic space on CT or MRI is seen as replacement of the normal paraglottic fat by the enhancing tumor tissue. Both CT and MRI have a high sensitivity of about 95% to detect paraglottic tumor spread, the specificity, however, ranges between 50 and 75% as peritumoral inflammation may mimic tumor resulting in false positive assessments.

Glottic tumors commonly arise from the anterior half of the vocal cord and spread into the anterior commissure. Anterior commissural disease is seen on CT or MRI as soft tissue thickening of more than 1–2 mm. The accuracy of CT in predicting anterior commissure involvement is about 75%. From the anterior commissure, the tumor may spread further anteriorly into the contralateral cord and the thyroid cartilage or posteriorly into the posterior commissure, the arytenoids, cricoarytenoid joint and the cricoid. While vocal cord mobility is best assessed at endoscopy, disease in the cricoarytenoid joint and interarytenoid region have been described as imaging correlates for vocal cord fixation. The tumor may spread superiorly to access the preepiglottic space and

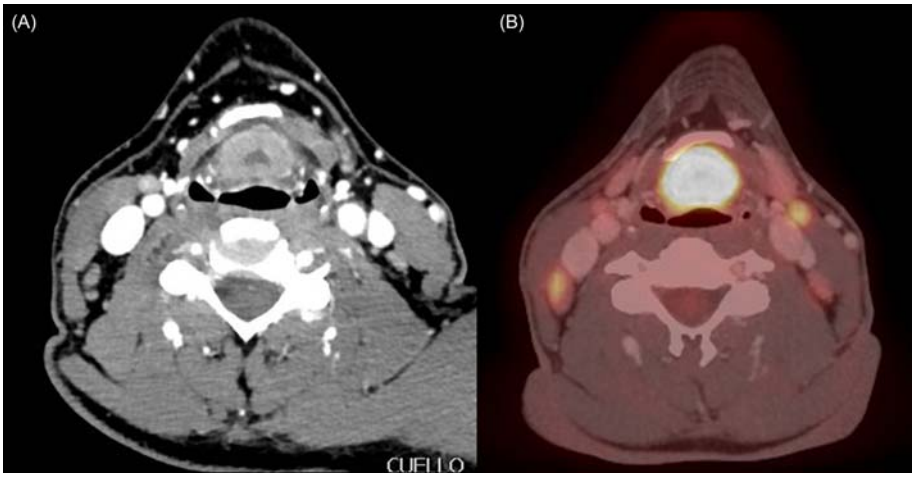

Fig. 4 (A) Axial contrast enhanced CT depicting a supraglottic tumor invading the preepiglottic space. (B) PET-CT showing a hypermetabolic focus of active tumor occupying the preepiglottic space that corresponds to the CT abnormality.

the paraglottic space, or inferiorly to reach the subglottis. Subglottic spread below the anterior commissure is seen as an irregular thickening of the cricothyroid membrane. Tumor may gain access into the extralaryngeal tissues through the cricothyroid membrane.

Subglottic cancer is diagnosed if any tissue thickening is noted between the airway and the cricoids ring. Due to their late presentation, invasion of the cricoids cartilage, trachea and the cervical esophagus with extralaryngeal spread are common findings in these patients at imaging.

Laryngeal tumors encroaching on both, the glottis and supraglottis, with or without subglottic component and when the site of origin is unclear, is termed as transglottic tumor. This tumor spread is frequently through the paraglottic space and is readily identified on CT or MRI. Transglottic carcinoma is frequently accompanied by metastatic lymphadenopathy. Coronal images are particularly helpful in assessing transglottic extension of tumor.

Nowadays, to assess the possibility of diagnosing distant disease and second primary tumors, a chest CT should be routine. Second primary malignancies have been reported in up to 20% of patients with laryngeal cancer.

Moreover, the use of positron emission tomography (PET) scanning with ^{18}F-fluorodeoxyglucose (FDG) and combined CT with PET imaging allow to combine functional and anatomical studies (**Fig. 4**). FDG imaging has the potential to distinguish between benign and malignant processes, grade tumors, identify metastases, and diagnose tumor recurrence. Although standardized uptake values (SUV) of FDG uptake has been used by some authors to identify tumoral tissue, to date, no universally accepted SUV threshold has been determined to differentiate benign from malignant nodal disease. In head and neck cancer, FDG imaging is useful for detecting clinically occult recurrences and for determining residual disease in the neck following definitive radiotherapy. CT-PET can detect occult disease and use alter the treatment plan in up to 30% of patients. CT-PET has a high negative predictive value but the specificity is not very high. The overall accuracy of in identifying nodal disease is higher than that of CT alone, by almost 20%. However, CT-PET is not useful to exclude the presence of metastases in the clinically N0 neck because PET cannot detect very small tumors (<5–7 mm).

Staging

Staging is based on TNM criteria developed by the AJCC/UICC (8th edition) (**Tables 1–4**). This classification incorporates all information available prior to treatment, including the clinical examination, endoscopy, endoscopic biopsy and cross-sectional imaging. The guidelines rely heavily on the use of cross-sectional imaging for the T staging; however, no recommendation is made regarding the preference of one technique over the other. Laryngeal cancer staging is dependent on tumor location and subsite involvement. For supraglottic tumors, the subsites that are important for staging include the suprahyoid and infrahyoid epiglottis, aryepiglottic folds, arytenoids and ventricular bands (false vocal cords). The glottic subsites include the true vocal cords including the anterior and posterior commissure. Other important factors involved in laryngeal cancer staging include the presence of impaired true vocal cord mobility or frank paralysis, base of the tongue involvement, preepiglottic space involvement, paraglottic space involvement and thyroid or cricoid cartilage invasion. It is important to note the fact that a tumor that erodes only the internal cortex of the thyroid cartilage is classified as T3, while if the tumor passes through the cartilage is considered as a T4 tumor.

Those tumors that cause vocal cord fixation, involve base of the tongue preepiglottic or paraglottic spaces, show thyroid or cricoid cartilage invasion, invade tissues beyond the larynx (trachea, soft tissues of the neck, esophagus thyroid gland, strap muscles or extrinsic muscle of the tongue) or are associated with regional lymphatic metastases, are considered as advanced disease. Cancers that invade the prevertebral space, encase the internal carotid artery or extend into the mediastinum are considered incurable with surgery.

Table 1 Primary tumor (T) according to TMN 8th edition

TX	*Primary tumor cannot be assessed*
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Supraglottis	
T1	Tumor limited to one subsite of supraglottis with normal vocal cord mobility
T2	Tumor invades mucosa of more than one adjacent subsite of supraglottis or glottis or region outside the supraglottis without fixation of the larynx.
T3	Tumor limited to the larynx with vocal cord fixation and/or invades any of the following: postcricoid area, preepiglottic space, paraglottic space, and/or inner cortex of thyroid cartilage
T4a	Moderately advanced local disease. Tumor invades through the thyroid cartilage and/or invades tissues beyond the larynx
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures
Glottis	
T1	Tumor limited to the vocal cord(s) with normal mobility
T1a	Tumor limited to one vocal cord
T1b	Tumor involves both vocal cords
T2	Tumor extends to supraglottis and/or glottis, and/or with impaired vocal cord mobility
T3	Tumor limited to the larynx with vocal cord fixation and/or invasion of the paraglottics space, and/or inner cortex of the thyroid cartilage
T4a	Moderately advanced local disease. Tumor invades through the outer cortex of the thyroid cartilage and/or invades tissues beyond the larynx
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures
Subglottis	
T1	Tumor limited to the subglottis
T2	Tumor extends to vocal cord(s) with normal or impaired mobility
T3	Tumor limited to the larynx with vocal cord fixation
T4a	Moderately advanced local disease. Tumor invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures

Table 2 Regional lymph nodes (N) according to TMN 8th edition

	Clinical (cN)	*Pathological (pN)*
Nx	Regional lymph nodes cannot be assessed	
N0	No regional lymph nodes metastasis	
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension	
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension	
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension	
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension	
N3a	Metastasis in a lymph node more than 6 cm in greatest dimension without extranodal extension	Metastasis in a lymph node more than 6 cm in greatest dimension without extranodal extension
N3b	Metastasis in a single or multiple lymph nodes with clinical extranodal extension (skin involvement or soft tissue invasion with deep fixation/tethering to underlying muscle or adjacent structures or clinical signs of nerve involvement)	Metastasis in a lymph node more than 3 cm in greatest dimension with extranodal extension or, multiple ipsilateral, or any contralateral or bilateral node(s) with extranodal extension

Table 3 Distant metastasis (M) according to TMN 8th edition

M0	No distant metastasis
M1	Distant metastasis

Table 4 Stage groups according to TMN 8th edition

Stage 0	Tis N0 M0
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0 M0
	T1–3 N1 M0
Stage IVA	T4a N0–1 M0
	T1-4a N2 M0
Stage IVB	T4b Any N M0
	Any T N3 M0
Stage IVC	Any T Any N M1

Treatment

The primary goal of treatment in head and neck cancer is to achieve optimal oncological outcomes while preserving function and quality of life as much as possible. This is particularly important in the treatment of laryngeal cancer. The larynx has important functions—including breathing, voice making and swallowing, and therefore both the disease and its treatment may significantly affect quality of life. As previously mentioned, 95%–98% of malignant neoplasms of the larynx are squamous cell carcinomas (SCC), so we will only refer to this histological type.

In the treatment of these cancers we have several options available (surgery, radiation therapy, chemotherapy and molecular targeted therapies), which can be used alone or in combination. In general, patients in early stages (I–II) are treated with a single modality therapy, either surgery of radiotherapy. But approximately 40% of laryngeal cancers occur at advanced stages (III–IV) at diagnosis, requiring aggressive treatment that usually involves combining several treatment modalities. Primary treatment decisions may depend on patient desires, tumor extent and location, experience of the treatment team, the adequacy of follow up surveillance, medical comorbidities and long-term voice and swallowing expectations. Since most cases of laryngeal cancer are associated with high consumption of tobacco and alcohol, co-morbidities are common, which not only determine the choice of treatment and compliance, but are an important determinant of overall patient survival.

Treatment of Early Stage (T1–T2) Tumors

Treatment options for T1–T2N0 laryngeal SCC include radiotherapy (RT), transoral laser surgery (TLS), and open partial laryngectomy (see below). Functional results after open partial laryngectomy are usually worse compared to RT and TLS so that this alternative is rarely employed. Given the high probability (>20%) of occult nodal metastasis in supraglottic cancers, even in these initial stages, the neck must be included in the treatment plan of the tumors on this location, and it is usually treated with the same modality therapy (surgery or RT) than the primary tumor.

The decision whether to select RT or TLS (with neck dissection in supraglottic cancers) depends on a number of factors, including the location and extent of the tumor, anatomical circumstances (probability of exposure of the tumor in TLS), the medical condition of the patient, the likelihood of tumor control after treatment, anticipated functional outcome (voice quality), the expertise of the attending physicians and logistical considerations. In this complex decision-making process, we should also include patient preference, after an informed discussion of the pros and cons of each treatment modality.

The likelihood of local control after RT or TLS is equivalent and is approximately 85%–95%. An advantage of RT is that it is applicable to all patients with T1–T2N0 SCCs, whereas TLS could not be used in patients with inadequate exposure of the tumor (due to the location of the tumor or anatomical circumstances). Patients with significant medical comorbidities who are poor candidates for anesthesia may be better treated with RT. On the other hand, in glottic tumors, we must keep in mind that the more of the glottis that is involved with SCC, requiring a wider resection, the poorer the voice quality after TLS. Therefore, RT may be preferred treatment option for patients with more demanding requirements for voice quality. When RT is used, the dose of radiation is from 63 Gy (2.25 Gy/fraction) to 66 Gy (2.0 Gy/fraction) for T1 and 65.25 Gy (2.25 Gy/fraction) to 70 Gy (2.0 Gy/fraction) for T2 disease.

Patients with anterior commissure involvement will provide technical challenges and, even in experienced hands, may have local control rates that are somewhat lower compared with T1–T2 SCCs without anterior commissure invasion. Efficacy of RT is not affected by involvement of the anterior commissure. In addition, voice quality is likely to be worse after TLS in these cases.

One advantage of TLS is that it can be repeated several times in contrast to RT. The ability to repeat TLS may contribute to the fact that the likelihood of laryngeal preservation may be higher when TLS can be offered as initial treatment. Many patients with recurrences after RT will undergo total laryngectomy, although laryngeal preservation may be feasible with salvage open partial laryngectomy or TLS in selected patients after radiation failure.

Another advantage of TLS is the shorter length of treatment. In cases where the neck is not dissected, TLS can be done on an outpatient basis whereas RT is delivered once daily on weekdays over 5–7 weeks. In relation to this, an important point to be taken

into consideration is that TLS is the most cost-effective treatment of early laryngeal SCC, radiation therapy being 2–4-fold more expensive. The presence and extent of a cost differential will vary with the medical system.

Regardless of the modality chosen, physicians should track their own patient's functional and survival data rather than rely on the best reported results from the most experienced institutions. Analysis of outcomes should include tumor control, survival, functional outcomes (quality of voice) and larynx preservation rates.

Treatment of Advanced Stage (T3–T4) Tumors

For years, total laryngectomy (with postoperative radiotherapy in high-risk cases) was the only treatment option for patients with intermediate to advanced laryngeal cancer (T3–T4). In addition, either ipsilateral or bilateral neck dissections should be performed in these cases, based on tumor location and extent of nodal metastasis (if present).

Over the past two decades, great progress has been made in the management of this disease, with multimodality approaches aimed at laryngeal preservation reshaping the treatment landscape. In response to the common use of total laryngectomy, the nonsurgical approaches have often been referred to as "organ preservation" strategies. In many institutions, it appears that there are organ preservation strategies and then there is surgery. In contrast, we think there are both nonsurgical organ preservation strategies and surgical organ preservation strategies (Fig. 5). The key is that in both approaches the goal is to spare the functions of the larynx. A new paradigm has emerged in which both the surgical and nonsurgical approaches have equal value in functional laryngeal preservation. However, in T4 tumors laryngeal preservation rates are much lower and often present complications which compromise laryngeal function. In addition, patients with T4 tumors treated with up-front surgery had superior overall survival than those treated with CRT, and therefore in these cases total laryngectomy continues to be recommended.

Surgical options for functional laryngeal preservation

In addition to the time-honored approaches of vertical partial laryngectomy and horizontal or supraglottic laryngectomy, the options for conservation laryngeal surgery have significantly improved over the past two decades. Transoral minimally invasive surgery and supracricoid partial laryngectomy (SCPL) have emerged as important function-preserving approaches for patients with laryngeal cancer.

After either partial laryngectomy or open resection, absence of adverse features, including extra nodal spread, positive margins, perineural invasion, or lymphovascular invasion, allows for observation of the patient with no need for adjuvant therapy. When these high-risk features are present, adjuvant RT is recommended.

Vertical partial laryngectomy (VPL)

VPL (or vertical hemylaryngectomy) encompasses a spectrum of procedures ranging from laryngofissure with cordectomy to extended hemi-laryngectomy. Common to all these procedures is vertical transection of thyroid cartilage and glottic resection extending into the paraglottic space (Fig. 6). In VPL, vertical incisions are made through the thyroid cartilage near the anterior commissure and just anterior to the posterior edge of the thyroid cartilage. The resulting resection includes the true vocal cord and immediate sub glottis, ventricle, false vocal cord, and arytenoepiglottic fold, and usually crosses just in front of the vocal process of the arytenoid posteriorly. This area can extend around the anterior commissure to involve the anterior one-third of the opposite vocal cord if required. When the anterior commissure is removed, the procedure is termed a frontolateral hemylaryngectomy.

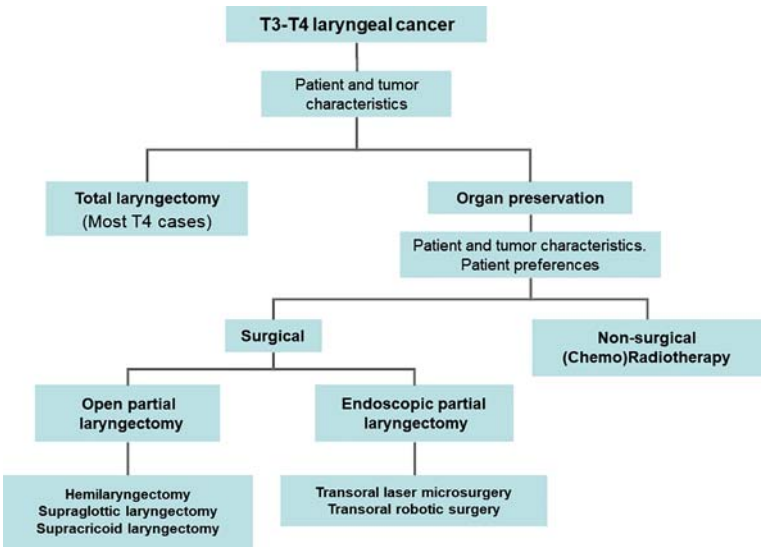

Fig. 5 Algorithm for treatment of advanced laryngeal cancer.

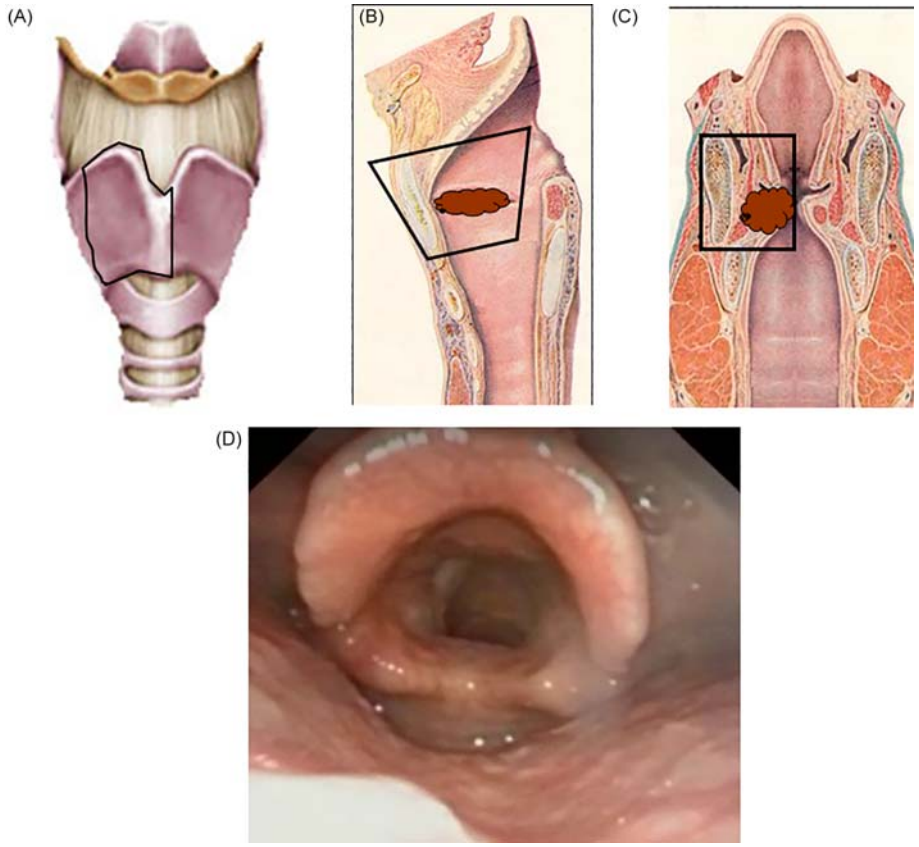

Fig. 6 Vertical partial laryngectomy. (A) Incisions in laryngeal framework. (B) and (C) Sagittal and coronal views showing the resected portions of the larynx. (D) Endoscopic picture of the larynx after a right hemilaryngectomy.

It is a well-established procedure for T1 and T2 glottic cancers. Some authors believe that patients with fixation of the true vocal cord (T3) caused by direct invasion of the cancer into the thyroarytenoid muscle are still candidates for a vertical hemylaryngectomy. However, in patients with vocal cord fixation caused by cricoarytenoid joint invasion a hemilaryngectomy should not be considered. Other contraindications are involvement of the posterior commissure or the thyroid cartilage, and extension superiorly to the aryepiglottic fold.

With this technique overall local control and laryngeal preservation rates, between 82 and 95% have been reported for T1–T2 cases, and 5-year survival rates were greater of 90%. The local control and 5-year survival rates were lower for T3 cases, with reported local control rates between 73% and 85%. These results reflect the continuing value of the VPL in selected cases. However, currently, with the advancement of laser surgery, the role of the VPL is questionable. For most patients with lesions amenable to VPL, laser surgery provides equal local control rates, with superior voice and swallowing function and less complications. And because the relatively high recurrence rates in T3 cases, VPL was replaced by supracricoid laryngectomy for these cases in many centers.

Supraglottic laryngectomy

Supraglottic laryngectomy involves resection of the epiglottis, the false vocal cords, the aryepiglottic folds, the hyoid bone (in most cases), the upper aspect of the thyroid cartilage, and the contents of the preepiglottic space (Fig. 7). The resection can be extended to include one arytenoid, the tongue base or pyriform sinus.

The result is that the patient has a nearly normal voice but a significant challenge in developing normal swallowing caused by the loss of the protective mechanisms of the epiglottis and false cords. Rehabilitation, which involves temporary tracheostomy in all patients and a temporary feeding tube (usually nasogastric), is achieved in most patients within 1 month of surgery with removal of the feeding tube and tracheostomy. The rehabilitative process is complicated by either preoperative or postoperative radiation therapy as well as extension of the surgical resection to include the tongue base, arytenoid cartilage, or pyriform sinus.

Supraglottic laryngectomy is indicated in all T1–T2 supraglottic cancers, but also in patients with T3 and T4 supraglottic tumors that involve preepiglotic space or one arytenoid, or that extend into the pyriform sinus or the tongue base. Massive tumors with cartilage erosion, subglottic extension, or involvement of the lateral wall of the pyriform sinus remain subject to total laryngectomy. In addition, the patient must have adequate pulmonary function to be a candidate for a supraglottic laryngectomy.

Local control was better for those with tumor confined to the endolarynx (>90%), but was over 80% for all sites, and laryngeal preservation rates described are also over 80% cases. Overall five-year survival rates are comparable to that obtained with total

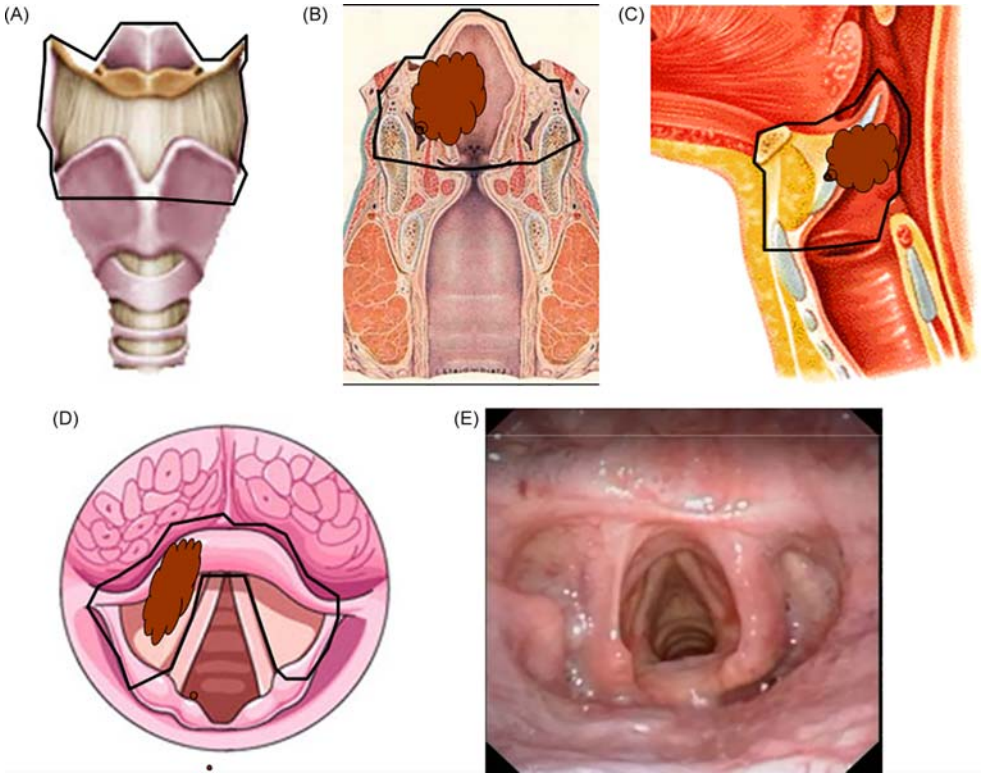

Fig. 7 Supraglottic laryngectomy. (A) Incisions in laryngeal framework. (B–D) Coronal, sagittal, and axial views showing the resected portions of the larynx. (E) Endoscopic picture of the larynx after a supraglottic laryngectomy.

laryngectomy, and range from 67% to 90%. These rates are over 85% for stage I–II tumors, between 75% and 80% for stage III, and between 55% and 70% for stage IV patients. However, in these tumors survival rates depends more from the presence and extension of nodal metastases than the size of the primary tumor.

The laryngeal preservation rates with this technique are very good, with overall reported rates over 85% of patients. However, these rates were lower (60%–80%) in T3–T4 tumors. In addition, functional results were good to fair, with more than 90% of patients achieving decannulation and oral diet.

Nowadays, conventional supraglottic laryngectomy is being replaced by laser supraglottic laryngectomy because the oncological results of transoral laser surgery for early and moderately advanced laryngeal cancer appear to be comparable to those of classic supraglottic laryngectomy, and the endoscopic approach offers functional advantages.

Supracricoid laryngectomy (SCPL)

The SCPL is an alternative to (chemo) radiation therapy, supraglottic laryngectomy, and near total and total laryngectomy in selected cases of supraglottic and transglottic carcinoma. This procedure is a true functional preservation technique and should be considered as a conservative laryngeal technique as it preserves physiological rehabilitation of speech, swallowing, and respiration without a permanent tracheostomy.

SCPL involves resection of the following structures: the true vocal cords, the false vocal cords, the aryepiglottic folds, the epiglottis (to a variable degree), the subglottic to the superior aspect of the cricoid cartilage, the thyroid cartilage, and the contents of the pre- and paraglottic spaces. The resection can include one arytenoid but must preserve the hyoid bone (**Fig. 8**). Two different reconstructions are possible depending on the extent of disease involving the epiglottis. In cases in which only the inferior portion of the epiglottis is involved, the suprahyoid epiglottis can be preserved and used in the reconstruction (cricohyoidoepiglottopexy, CHEP). In cases in which it is not oncologically feasible to preserve the epiglottis, the reconstruction will involve the impaction of the base of tongue/hyoid complex to the cricoid cartilage (cricohyoidopexy, CHP).

Supracricoid laryngectomy with cricohyoidoepiglottopexy (SCPL-CHEP) is indicated in glottic tumors: T2 (especially with anterior commissure involvement), T3 and selected T4 (limited thyroid cartilage invasion). Is contraindicated in cases with fixation of the cricoarytenoid joint, invasion of the posterior commissure, cricoid invasion, extralaryngeal spread of tumor or poor pulmonary function.

Supracricoid laryngectomy with cricohyoidopexy (SCPL-CHP) is also indicated in T2–T4 laryngeal tumors: supraglottic tumors extended onto the vocal cord or anterior commissure and in transglottic tumors. Limitations are the same than with SCPL-CHEP, and also invasion of the hyoid bone.

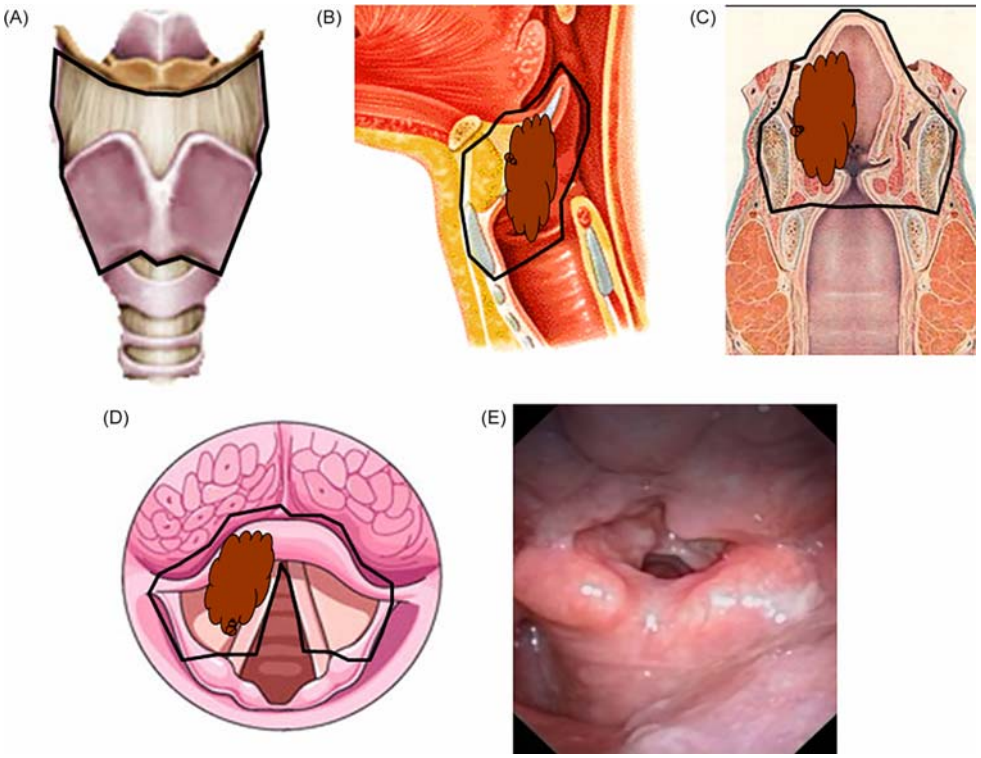

Fig. 8 Supracricoid laryngectomy (with crico-hyoidopexy). (A) Incisions in laryngeal framework. (B–D) Sagittal, coronal, and axial views showing the resected portions of the larynx. (E) Endoscopic picture of the larynx after a supracricoid laryngectomy.

Local control and organ-preservation rates with SCPL as primary therapy in patients with T2 and selected T3 lesions exceed 90% and are comparable with—if not better than—rates seen with chemotherapy and radiation and with total laryngectomy.

With respect to functional outcomes, although speech and swallowing are restored following SCPL, voice quality is substantially different postoperatively, although subjective voice analysis rank voice as globally "acceptable" by the patient and the physician. Restoration of normal swallowing may take several weeks and requires intensive rehabilitation; nonetheless, more than 80%–90% of patients can be expected to have swallowing function restored within the first year.

Transoral laser surgery

Transoral laser surgery (TLS) is minimally invasive and is performed under suspension-direct laryngoscopy, with an operating microscope, microsurgical instruments, and the surgical CO_2 laser. In conjunction with SCPL, this has been one of the two areas of greatest development in conservation laryngeal surgery in recent years.

The approach challenges a basic surgical tenet, as the tumor is transected and removed piecemeal through a laryngoscope. However, transection reveals the depth of tumor penetration and allows for clear visualization of tumor margins during the procedure.

In contrast to open laryngeal surgery, the cartilaginous laryngeal framework and the infrahyoid muscles are preserved during endoscopic resections, which is believed to improve postoperative function. Additionally, the concept of adequate margins is viewed differently for endoscopic resections: the goal is preservation of as much adjacent normal tissue as possible while ensuring a clear margin.

With respect to patient selection, exposure through the laryngoscope dictates which tumors can be managed by TLS. It is a well-established procedure for T1–T2 glottic or supraglottic carcinomas, as previously indicated. Additionally, some authors indicated this technique in selected T3 glottic tumors (fixation of the true vocal cord caused by direct invasion of the cancer into the thyro-arytenoid muscle), T3 supraglottic tumors (with limited preepiglotic space invasion) and also in some T4 cases (limited base of tongue invasion).

Several reports have shown good oncologic results in intermediate and advanced laryngeal cancer. The 5-year local control with laser alone and laryngeal preservation rates are approximately 95% and 98% for T1 tumors, 85% and 95% for T2 tumors, and 70% and 75% for T3 tumors. These results compare well to those of standard supraglottic laryngectomy. Regardless of the surgical technique employed, negative margins are essential in limiting local recurrence. Tumor involvement of the surgical margin after TLS has been associated with higher rates of local recurrence and distant metastasis, lower specific survival rate, and the necessity of salvage surgery.

Functional outcomes following TLS for laryngeal cancers are excellent. By maintaining one valve of the larynx, the airway is protected and voice and swallowing can be resumed with appropriate rehabilitation. Aspiration occurred in most patients soon after surgery, but recovered within 1–6 months, with recovery being faster in partial resections. Functional results depend on the extent of the resection. The return of voice quality also depends on the depth and extent of resection.

The functional results of TLS are generally superior to those of the conventional open approach, in terms of the time required to restore swallowing, tracheotomy rate, incidence of pharyngocutaneous fistulae, and shorter hospital stay. These functional advantages can be attributed to the more conservative nature of the endoscopic procedure, because normal tissues are not disrupted during the procedure. With open procedures, the thyroid cartilage, soft tissues and infrahyoid and suprahyoid muscles are divided, and the hyoid bone is frequently resected. There is invariably airway compromise and a need for a temporary tracheotomy. With endoscopic resection, tracheotomy is almost never indicated. Avoidance of tracheotomy and preservation of the strap muscles may facilitate faster return and ensure improved long-term swallowing function.

Transoral robotic surgery

The concept of robot-assisted surgery is gaining popularity for multiple different specialties, and more recently in minimally invasive head and neck surgery. The overriding advantages from the proponents of robot-assisted surgery are the excellent 3-dimensional visualization and 2- or 3-handed surgery through minimally invasive approaches that are afforded by the instrument. The wider angle of vision and angled lenses increases the range of the endoscopic visual surgical field compared with the "line of sight" visual field gained by microscopes. The 2-dimensional visualization provided by single channel optical systems in current endoscopes lacks the depth perception of 3-D vision provided by the binocular optical systems used in standard microsurgery. The 5-mm robotic endoscope commonly used has a dual-channel optical system coupled with a dual charge-coupled device, which allows for 3-D visualization of the surgical field at the surgeon's console. Another advantage of the technology used in the da Vinci robotic instrumentation is its ability to provide movement at the instrument tip with 7° of freedom and 90° of articulation and motion scaling. This allows the surgeon, who sits at the console with an adjustable arm, support to perform precise tremor-free movement in a deep and confined space, with working angles usually not achievable with nonrobotic instruments.

Weinstein and O'Malley have previously reported on the development and refinement of a novel procedure called transoral robotic surgery (TORS) in preclinical experimental models. These foundational studies established the technical feasibility of TORS to gain access to the oral cavity, oropharynx, hypopharynx, supraglottis, and glottis, and they also introduced basic concepts of patient safety and methods for controlling active bleeding.

Although the present literature reports early findings, without long-term oncologic outcomes, the results are consistently encouraging. Indeed, some institutions have shown that transoral robotic surgery programs can be successfully established yielding excellent clinical results.

Nonsurgical organ preservation protocols

In the 1990s, organ preservation treatment protocols combining chemotherapy and radiotherapy were introduced as an alternative to total laryngectomy with the objective to preserve a functional larynx without compromising oncological outcome.

The effectiveness of concomitant chemoradiation (CRT) as an effective organ preservation strategy was initially established by The Department of Veterans Affairs (VA) Laryngeal Cancer Study Group in 1991. Their conclusions were subsequently confirmed by the GETTEC and RTOG 91–11 trials and two individual data meta-analyses.

According to the different treatment guidelines (European Society of Medical Oncology -ESMO-, National Comprehensive Cancer Network -NCCN-, American Society of Clinical Oncology -ASCO-) the current standard of treatment for patients with T3 laryngeal carcinoma who desire a nonsurgical organ preservation treatment is concomitant radiotherapy with cisplatin, with salvage surgery in cases of persistent disease, or induction chemotherapy followed by definitive radiation/chemoradiation or surgery depending on clinical response. The dose considered standard for cisplatin by most researchers is 100 mg/m^2 administered on days 1,22, and 43 of radiotherapy, and radiation therapy is administered with a conventional fractionation (2 Gy/day to give 70 Gy in 7 weeks). With these treatments, the larynx is preserved in approximately 2/3 of patients.

Although initially not addressed by the VA protocol, it was recognized later that instead of organ preservation, functional preservation is a more relevant outcome. This functional preservation is defined as an "in situ" larynx without need for permanent tracheostomy and permanent gastrostomy at 2 years after finishing the treatment. Despite the various randomized trials that have all confirmed that the CRT approach achieves survival rates similar to treatment with total laryngectomy, none have shown improvement in survival rates with an organ preservation approach. Furthermore, some investigators have been concerned about the long term toxic effects of CRT treatment on laryngeal function and the decreases in overall survival rates with nonsurgical treatment reported more recently from large tumor registries and long-term follow-up of original trials. This raises a critical question of whether the results of a complex multidisciplinary treatment approach developed in controlled clinical trials by skilled investigators can be effectively generalized to standard practice.

Patients specifically included in the pivotal trials were predominantly patients with T3 tumors located at supraglottis, half of them without vocal cord fixation and a small proportion of patients with T4 tumors with minimal invasion to the cartilage, which do not represent the entire spectrum of "advanced larynx cancer." When the results of CRTs were extrapolated and applied in clinical practice and low volume nonteaching centers, selection criteria may not have been applied as strictly as suggested, and many patients who would not have been candidates to receive CRT (most T4 cancers) were included in this type of treatment. There

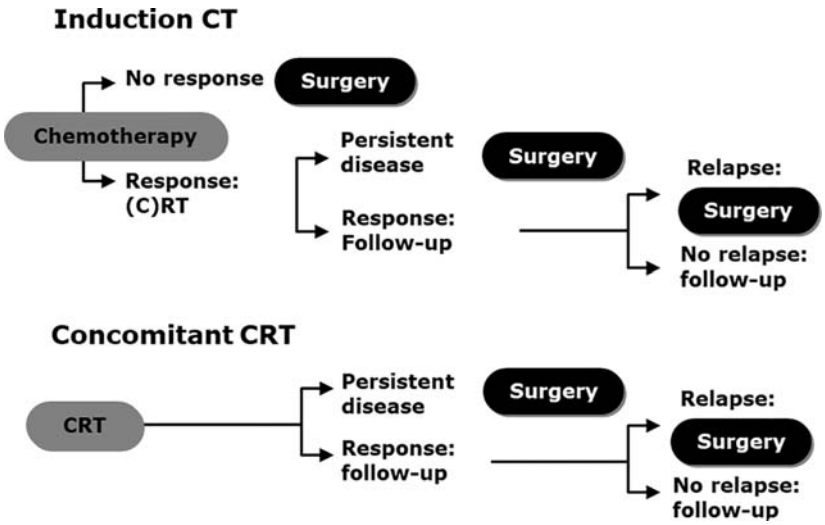

Fig. 9 Role of the salvage surgery in the context of nonsurgical treatment protocols of laryngeal cancer.

are few data about the rate of cartilage invasion or vocal cord fixation in these large cohort studies, but it could be expected to be higher than those reported in pivotal trials.

Therefore, despite state of the art treatment facilities, survival may be decreased due to an incomplete treatment dose, lack of supportive care and treatment of adverse effects of these treatments, loss of follow up, etc. Moreover, if salvage laryngectomy, which is part of an organ preserving strategy (Fig. 9) to obtain comparable survival rates between nonsurgical and surgical treatments, is not offered to patients in case of recurrence, survival is hampered in this patient category. It is highly probable that salvage surgery is not offered due to the lack of organized strategies to assess primary response to CRT, to the delay in the detection of early recurrences, to the lack of surgical experience in resection and reconstruction and for the lack of support from health systems to manage postoperative complications and rehabilitation.

For advanced laryngeal cancers, as laryngectomy is seen as a "mutilating" treatment and organ preservation is an important achievement in treatment, organ preservation protocols will be used more frequently and laryngectomy will be avoided as primary treatment or even when salvage is necessary. In the VA trial, salvage laryngectomy was more frequently done in patients with glottic tumors, vocal cord fixation, and gross invasion of the cartilage and T4 tumors, which indicates early evidence of worse therapeutic response to CRT in these patients. However, CRT was still offered to all patients under the assumption that it will offer better quality of life in comparison with laryngectomy.

There is enough evidence to say that treatment of T4 advanced larynx cancer should consider total laryngectomy since survival outcomes appear better than with CRT in most reports. For patients with T3 tumors, definitive CRT strategies are acceptable on the condition that all resources for the administration of the treatment, follow-up and surgical salvage are available. The entire factors mentioned gives some light to the contradictory results but also offer information for physicians who treat patients with advanced larynx cancer, in order to consider not only the results derived from CRT, but to consider geographical, cultural and socioeconomic conditions before offering the treatment.

The introduction of new systemic therapies should be analyzed carefully before applying them in patients with advanced larynx cancer in order to avoid indiscriminate administration to patients that are not going to get clinical advantages from its use. Customization of treatment is also critically important if overall survival rates are to be improved upon. Future developments of more precise functional imaging capabilities to monitor the response to nonsurgical therapies and possible persistence/recurrence of cancers could allow earlier intervention leading to changes of therapy or salvage surgery which would be expected to enhance survival results. The development of useful biomarkers reflecting disease characteristics such as chemo- or radiosensitivity could be useful in more precise decision making, thereby reducing redundancy of treatments and toxicities.

In summary, decision making for treatment of patients with advanced laryngeal cancer has never been more complex. Primary treatment decisions may depend on patient desires, tumor extent, experience of the treatment team, the adequacy of follow up surveillance, medical comorbidities and long-term voice and swallowing expectations.

Prognosis

Some authors have explored prognostic factors that affect survival in larynx cancer and identified that age, sex, functional status, comorbidities, tumor stage and subsite, type of therapy and treatment of recurrences are closely related with this outcome.

Five-year disease-specific survival is excellent (>95%) in early stage (I–II) patients, and can be considered good (70%–80%) in intermediate stage (III) patients. In advanced disease stages (stage IV) 5-year survival rates drop to 50%–60%. The main cause of death is local-regional recurrence, with distant metastasis being infrequent (less than 10%).

See also: Larynx Cancer: Pathology and Genetics.

Further Reading

American Society of Clinical Oncology, Pfister, D.G., Laurie, S.A., Weinstein, G.S., et al., 2006. American Society of Clinical Oncology clinical practice guideline for the use of larynx-preservation strategies in the treatment of laryngeal cancer. Journal of Clinical Oncology 24, 3693–3704.

Ferlito, A., Rinaldo, A., Silver, C.E., et al., 2008. Neck dissection for laryngeal cancer. Journal of the American College of Surgeons 207, 587–593.

Haigentz Jr, M., Silver, C.E., Hartl, D.M., et al., 2010. Chemotherapy regimens and treatment protocols for laryngeal cancer. Expert Opinion in Pharmacotherapy 11, 1305–1316.

Holsinger, F.C., Nussenbaum, B., Nakayama, M., et al., 2010. Current concepts and new horizons in conservation laryngeal surgery: An important part of multidisciplinary care. Head & Neck 32, 656–665.

Lefebvre, J.L., 2006. Laryngeal preservation in head and neck cancer: Multidisciplinary approach. Lancet Oncology 7, 747–755.

López, F., Williams, M.D., Skálová, A., et al., 2017. How phenotype guides the treatment of the most common malignant salivary neoplasms of the larynx? Advances in Therapy 34, 813–825.

López, F., Williams, M.D., Cardesa, A., et al., 2017. How phenotype guides management of non-conventional squamous cell carcinomas of the larynx? European Archives of Otorhinolaryngology 274, 2709–2726.

Mendenhall, W.M., Takes, R.P., Shah, J.P., et al., 2015. Current treatment of T1N0 squamous cell carcinoma of the glottic larynx. European Archives of Otorhinolaryngology 272, 1821–1824.

Rodrigo, J.P., Coca-Pelaz, A., Suárez, C., 2011. The current role of partial surgery as a strategy for functional preservation in laryngeal carcinoma. Acta Otorrinolaringologica Española 62, 231–238.

Rodrigo, J.P., Suárez, C., Silver, C.E., et al., 2008. Transoral laser surgery for supraglottic cancer. Head & Neck 30, 658–666.

Sanabria, A., Chaves, A.L.F., Kowalski, L.P., et al., 2017. Organ preservation with chemoradiation in advanced laryngeal cancer: The problem of generalizing results from randomized controlled trials. Auris Nasus Larynx 44, 18–25.

Suárez, C., Rodrigo, J.P., Silver, C.E., et al., 2012. Laser surgery for early to moderately advanced glottic, supraglottic, and hypopharyngeal cancers. Head & Neck 34, 1028–1035.

Yesensky, J., Agrawal, N., Bayan, S., et al., 2017. AHNS series-do you know your guidelines? Principles of treatment for glottic cancer: A review of the National Comprehensive Cancer Network guidelines. Head & Neck 39, 1729–1732.

Relevant Websites

https://www.asco.org/practice-guidelines/quality-guidelines/guidelines/head-and-neck-cancer—American Society of Clinical Oncology (Head and neck cancer).
http://www.esmo.org/Guidelines/Head-and-Neck-Cancers—European Society of Medical Oncology (Head and neck cancer).
https://www.nccn.org/store/login/login.aspx?ReturnURL=https://www.nccn.org/professionals/physician_gls/pdf/head-and-neck.pdf—National Comprehensive Cancer Network.
https://www.cancer.gov/types/head-and-neck/patient/laryngeal-treatment-pdq—National Cancer Institute.
http://www.cancerresearchuk.org/about-cancer/laryngeal-cancer—Cancer research UK (laryngeal cancer).
https://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0032515/—NCBI (laryngeal Cancer treatment).

Larynx Cancer: Pathology and Genetics

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Glossary

Epithelial-mesenchymal transition Process in which epithelial cells change to mesenchymal cells. It is essential in normal embryonal development; in later life, it contributes to wound healing, development of fibrosis and progression of cancer.

Invasion Direct extension and penetration of cancer cells through the epithelial basement membrane into the underlying stroma/lamina propria.

Invasive front Deepest part of the tumor, at tumor-host interface, the most important part of the tumor for invasion and progression.

Precancerosis Lesions with morphologic atypia, associated with an increased risk of cancer development.

Cancer of the Larynx; Pathology and Genetics

Cancer of the larynx is the second most common respiratory cancer, after lung cancer. The vast majority belongs to squamous cell carcinoma (SCC) and its variants. Other carcinoma types are rare and include neuroendocrine carcinomas and adenocarcinomas, particularly adenoid cystic carcinoma and mucoepidermoid carcinoma. They are diagnosed, graded and classified similarly to their counterparts in other organs. Sarcomas are also uncommon, the most important being chondrosarcoma. Malignant lymphomas and malignant melanomas can rarely arise in the larynx, as well as metastases from various primary tumors. Only SCC and its variants will be described in details.

Squamous Cell Carcinoma of the Larynx

Definition

Squamous cell carcinoma (SCC) is a malignant tumor showing evidence of squamous differentiation, e.g., intercellular bridges and/or keratinization. It arises from squamous epithelium or from respiratory epithelium that has undergone squamous metaplasia.

Burden

SCC is the most common malignant tumor of the larynx, accounting for approximately 95% of all malignant tumors at this location. The majority are conventional type SCC.

Laryngeal cancer accounts for 1.6%–2% of all malignant tumors in men, and for 0.2%–0.4% of malignant tumors in women, and for approximately 1.0% of all cancer deaths. Its incidence is on the rise in some countries, particularly in women, presumably related to increased incidence of smoking. It occurs most frequently in the 6th and 7th decades, with a strong male predominance. It rarely occurs in children.

Risk Factors

Cigarette smoking and alcohol consumption, especially in combination, are the most important risk factors in laryngeal cancer. Among them, smoking is more important. Avoiding of smoking and alcohol consumption could prevent about 90% of laryngeal carcinomas. Some other factors, such as gastroesophageal reflux, radiation exposure, diet and nutritional factors have been also related to an increased risk of laryngeal cancer, particularly in patients who lack the major risk factors.

Much attention has been recently paid to the possible role of infection with human papillomavirus (HPV) in the pathogenesis of laryngeal cancer. The results of recent studies, using modern techniques, such as in situ hybridization for HPV E6/E7 mRNA, which enable to detect transcriptionally active, integrated virus, suggest that HPV, particularly type 16, plays a role in the development of 5%–10% of laryngeal SCCs.

Pathology

Macroscopic features

SCC of the larynx may present as a flat lesion with raised edges, as an exophytic lesion frequently with a central ulceration, or an ulcerative endophytic lesion. Most of them occur in the supraglottic and glottic region, while subglottic SCC is very rare.

- Supraglottic SCC arises most commonly in the epiglottis, followed by the false vocal cords, aryepiglottic folds, ventricles and the arytenoids. It tends to spread to oropharynx and pyriform sinus, but it rarely invades the glottis and thyroid cartilage.
- Glottic SCC arises mostly from the anterior half of the vocal cord or from the anterior commissure. Because of poor lymphatic supply, glottic SCC tends to remain localized for a long period. As SCC progresses, it invades the vocal muscle resulting in the fixed vocal cord which is an ominous clinical sign. In late stages of the disease, it may extend to the opposite true vocal cord, to the supraglottis and subglottis; it may also extend through the thyroid cartilage and invade the soft tissue of the neck.
- Subglottic carcinoma involves the region extending 1 cm below the true vocal cord, between the lower edge of the true vocal cord and the first tracheal cartilage (Fig. 1).

Microscopic features

SCC is characterized by invasive growth and variable degrees of squamous differentiation. Invasive growth is manifested by interruption of the basement membrane and the growth of islands, cords or single (dyscohesive) tumor cells in the subepithelial stroma. Large tumors may extend into deeper structures, i.e., muscle, cartilage or bone. Perineural invasion and invasion of lymphatic and blood vessels may be present, which is a reliable proof of an invasive cancer. Squamous differentiation is manifested by intercellular bridges (desmosomes) and/or keratinization, with keratin pearl formation.

SCC is traditionally graded into well-, moderately- and poorly differentiated SCC. The criteria for grading are: the degree of differentiation, nuclear pleomorphism and mitotic activity. Well differentiated SCC resembles closely normal squamous epithelium and contains varying proportions of large, differentiated keratinocyte-like cells and small basal-type cells, which are usually located at the periphery of the tumor islands. There are intercellular bridges and usually full keratinization; mitoses are scanty. Moderately differentiated SCC exhibits more nuclear pleomorphism and more mitoses, including abnormal mitoses; there is usually less keratinization. In poorly differentiated SCC, basal-type cells predominate, with a high mitotic rate, including abnormal mitoses, barely discernible intercellular bridges and minimal, if any keratinization. Keratinization should not be considered an important histological criterion in grading SCC (Fig. 2).

Tumor growth at the invasive front can show an expansive pattern, an infiltrative pattern or both. An expansive growth pattern is characterized by large tumor islands with well defined pushing margins, whereas an infiltrative pattern is characterized by scattered small cords or single tumor cells, with poorly defined, diffusely spreading, infiltrating margins.

Differential diagnosis

The diagnosis of SCC does not usually present a diagnostic problem for pathologists. Nevertheless, well differentiated SCC must be distinguished from pseudoepitheliomatous hyperplasia, which is a benign condition that consists of deep, irregular tongues of epithelium that lack atypia and abnormal mitoses, and is associated with granular cell tumor or infections. Other differential diagnoses include verrucous carcinoma and papillary carcinoma, discussed later.

Poorly differentiated SCC must be differentiated from malignant melanoma, lymphoma, neuroendocrine carcinoma and adenocarcinoma, using immunohistochemistry and special stains for demonstration of mucin production. Melanoma is distinguished

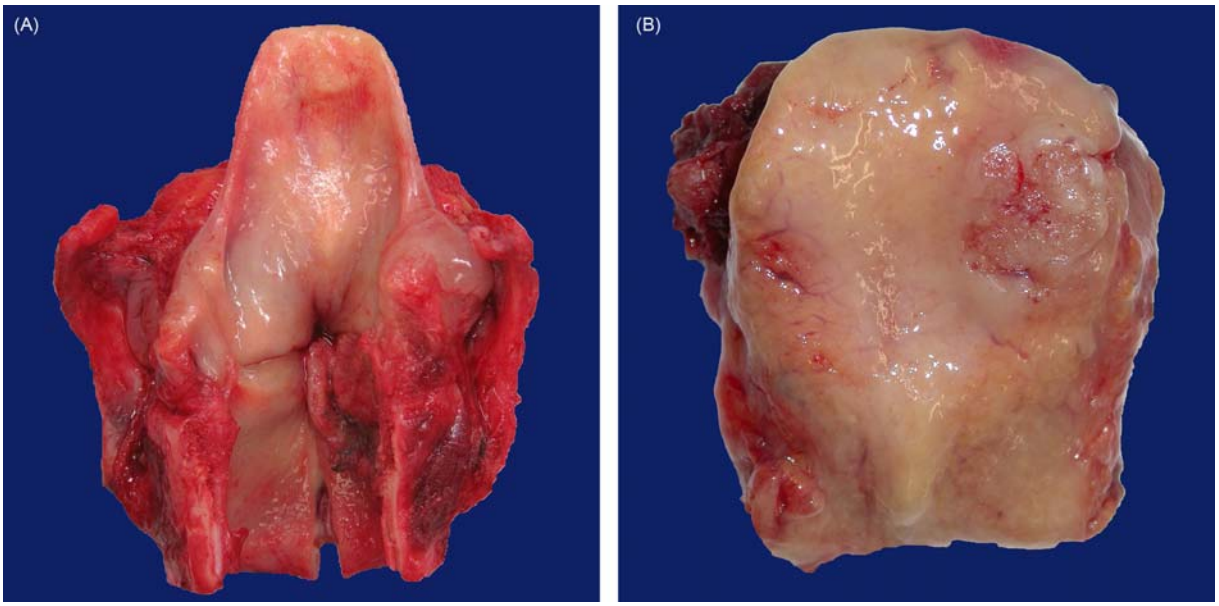

Fig. 1 Squamous cell carcinoma of the larynx. (A) Large exophytic tumor of the glottis extending to the supraglottic and subglottic regions. (B) Small flat tumor of the epiglottis with slightly raised edges.

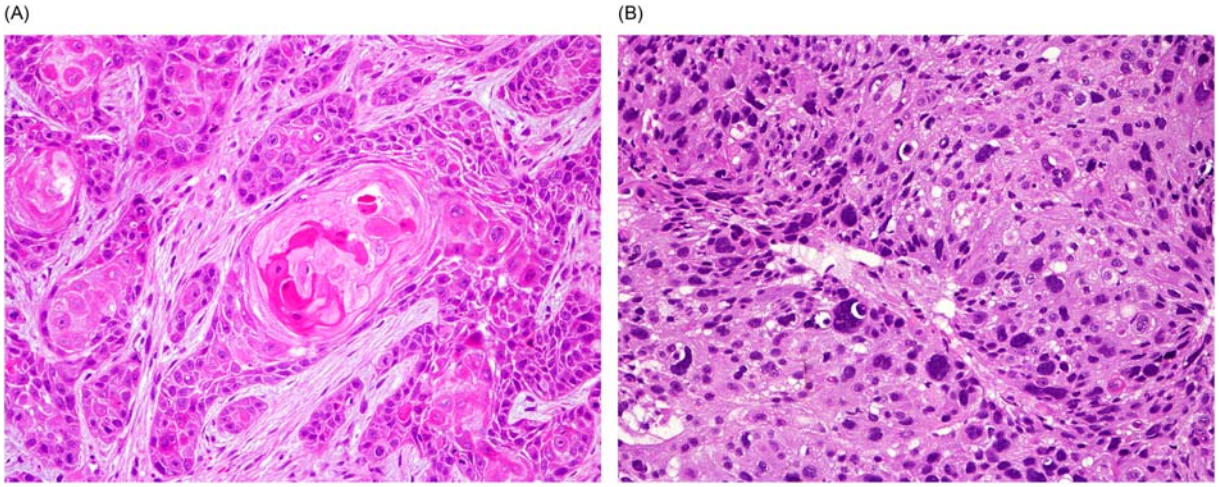

Fig. 2 (A) Moderately differentiated keratinizing squamous cell carcinoma: moderate nuclear and cellular pleomorphism, rare mitotic figures. (B) Poorly differentiated nonkeratinizing squamous cell carcinoma: marked nuclear and cellular pleomorphism, high mitotic rate.

from SCC by expression of S100, HMB45, melan A, MITF. Neuroendocrine carcinoma expresses neuroendocrine markers (synaptophysin, chromogranin, CD56). Lymphoma is distinguished from SCC by the presence of CD45 and markers of B-cell or T-cell differentiation.

Molecular Pathology and Genetics

SCCs of the head and neck including laryngeal SCCs are believed to be among the most highly mutated malignant tumors. Loss of heterozygosity (LOH) and comparative genomic hybridization studies have shown gains of 3q, 5p, 8q, 11q13, and 18p with losses at 3p, 5q, 8p, 9p, 11q23–24, 13q, and 18q.

Cyclin-dependent kinase inhibitor 2A (CDKN2A) and TP53 are among the most commonly affected tumor suppressor genes. Epidermal growth factor receptor (EGFR), vascular endothelial growth factor A (VEGFA), prostaglandin-endoperoxide synthase 2 (PTGS2), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) and matrix metalloproteinases are among the most commonly amplified and mutated oncogenes.

EGFR, for example, regulates cell growth, migration and survival, and is overexpressed in 80–90% of cases, increasing progressively from precancerous lesions to SCC. Anti-EGFR treatment is now available but has not proven effective in the majority of patients with laryngeal SCC.

P53 is involved in apoptosis and cell cycle regulation. Loss-of-function mutations have been found in laryngeal SILs and SCC. In SCC, the presence of mutations is associated with poor survival and may predict the response to treatment.

PIK3CA plays a role in signaling pathway affecting tumor cell growth, metabolism and survival. Mutations are frequent in SCC of the larynx and is a potential target for treatment with PIK3CA inhibitors.

Much progress has been achieved in our understanding of genetic abnormalities in precancerosis and SCC of the larynx. Hopefully, this will be used in the future as diagnostic criteria for assessing risk of carcinoma development in precancerous lesions and in surgical margins, and the risk of unfavorable course in SCC. Importantly, they are also potential targets for new treatment modalities. Some modern drugs have already been introduced, but patients with head and neck SCC, including laryngeal SCC, respond poorly, and we are still waiting for new targeted drugs to increase survival of patients with laryngeal SCC.

Microenvironment Including Immune Response

Invasive SCC of the larynx is associated with desmoplastic stromal reaction, which consists of proliferation of myofibroblasts, excessive deposition of extracellular matrix and neovascularisation. Together with inflammatory and immune cells, cytokines and other signaling molecules, they constitute tumor microenvironment. It resembles in many ways the processes of inflammation and wound healing and has been compared to a wound which does not heal. The tumor cells and microenvironment are closely related, and the constant interaction between them is complex, playing an important role in the final outcome.

It seems that desmoplastic stromal reaction is present only in invasive SCC and never in precancerosis, regardless of the grade, and may be considered an additional marker of invasion. The desmoplastic stromal reaction tends to be pronounced in well- and moderately differentiated SCC and weak or absent in poorly differentiated SCC, as well as in carcinomas, related to infection with HPV and Epstein–Barr virus (EBV).

Inflammatory cells comprise cells of innate and adaptive immune system. Some of these cells possess antitumoral properties, while others suppress antitumor immunity and are manipulated by the tumor cells to evade the immune system. Recent studies

suggest an important role of regulatory T cells, tumor-associated macrophages and myeloid-derived suppressor cells. There is merging evidence that immune cells mediating host response contribute significantly to the highly variable outcome, providing a clue why some precancerous lesions progress to cancer and others do not, and why some SCCs progress, metastasize and kill the patient and others do not.

Targeting tumor microenvironment to reverse tumor immune escape and induce inhibitory immune cells could represent basis for the development of new treatment modalities in the future.

Staging and Grading

The TNM system (T-tumor, N-node, M-metastasis), established by the International Union Against Cancer (UICC) is widely used for staging laryngeal cancer. The TNM status is based on the extent of the tumor and infiltration of the surrounding structures and organs, and on the presence of regional and distant metastases. The nodal status is assessed on the basis of the number of metastatic nodes, their location and size (expressed as the greatest dimension of the node with metastasis, with cut-off values of 3 and 6 cm) and the presence of extranodal extension. The most common sites for distant metastases are the lungs and bones.

Stage remains the most significant predictor of survival. Consistently, the prognosis varies from excellent for patients with early disease (stage I and II), with more than 90% 5-year survival rate, to poor for patients with advanced disease (stage III and IV), with less than 60% 5-year survival.

Laryngeal SCCs are usually graded as G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated) or G4 (undifferentiated). The criteria and prognostic significance of grading are described elsewhere.

Prognostic and Predictive Biomarkers

Clinical prognostic factors

- Stage is the most significant predictor of survival. Extent of the tumor, depth of invasion and the presence of regional and distant metastases are independent predictors of survival.
- Localization is an important prognostic factor for laryngeal SCC. The most favorable course has been reported for glottic SCC; glottis has a poor lymphatic supply and glottic SCC rarely spreads to the regional lymph nodes. The supraglottic and subglottic regions have a rich lymphatic network, resulting in common metastases in the regional lymph nodes.
- Other factors that may have a significant impact on the outcome of SCC laryngeal include patient age at presentation, comorbidity and performance status.

Histopathological prognostic factors

- Differentiation: the prognostic significance of traditional grading into well-, moderately- and poorly differentiated SCC is controversial and mostly correlates poorly with outcome. The main criticism of this widely used system is related to its subjectivity and lack of objective criteria.
- Invasive front: the growth pattern at the invasive front has prognostic implication: an infiltrative pattern is associated with a more aggressive course and poorer prognosis than an expansive pattern.
- Lymphovascular invasion: tumor cells often invade the thin-walled lymphatic vessels, capillaries and veins. The finding of the penetration of tumor cells in the lymphatic and/or blood vessels is associated with a high probability of lymph node and/or distant metastases, with recurrence and poor survival. However, the presence of lymphovascular invasion should not be considered synonymous with metastasis, because many tumor cells that enter in the lymphatic system and circulation are destroyed (**Fig. 3**).
- Perineural invasion is defined as tumor cells within any of the three layers of the nerve sheath or tumor foci outside of the nerve with involvement of at least 33% of the nerve's circumference. Perineural invasion enables tumor to spread well beyond the extent of local invasion. It is a marker of increased locoregional recurrence rates, a shorter time to recurrence and decreased survival.
- Extracapsular spread (ECS), also termed extranodal extension, in lymph node metastases is defined as penetration of the lymph node capsule by tumor cells, with infiltration of the perinodal fibro-adipose tissue. It can be visible by the naked eye (macroscopic/gross ECS) or on histopathologic examination (microscopic ECS). ECS has been recognized to worsen the adverse outcome associated with nodal metastasis.
- Resection margins: the aim of cancer surgery is to remove as much neoplastic tissue and retain as much healthy tissue as possible. Total surgical removal of the tumor can be proved by clear surgical/resection margins. Margins are considered clear if there is no invasive SCC or carcinoma in situ. Although the concept of clear margins seems straightforward, the definition of adequate margins, i.e., how much healthy tissue around the tumor must be removed, is highly controversial. It is generally believed that a margin of 5 mm is adequate but some believe that even margins of 1–2 mm are adequate, particularly in glottic cancer.

Resection margins clear of tumor are associated with a lower recurrence rate and better survival. However, up to 22% of patients with histologically negative margins undergo treatment failure. In these cases, relapse may be due to a minimal residual disease or to field cancerisation, the latter being related to the presence of genetically damaged cells in microscopically normal mucosa. It has

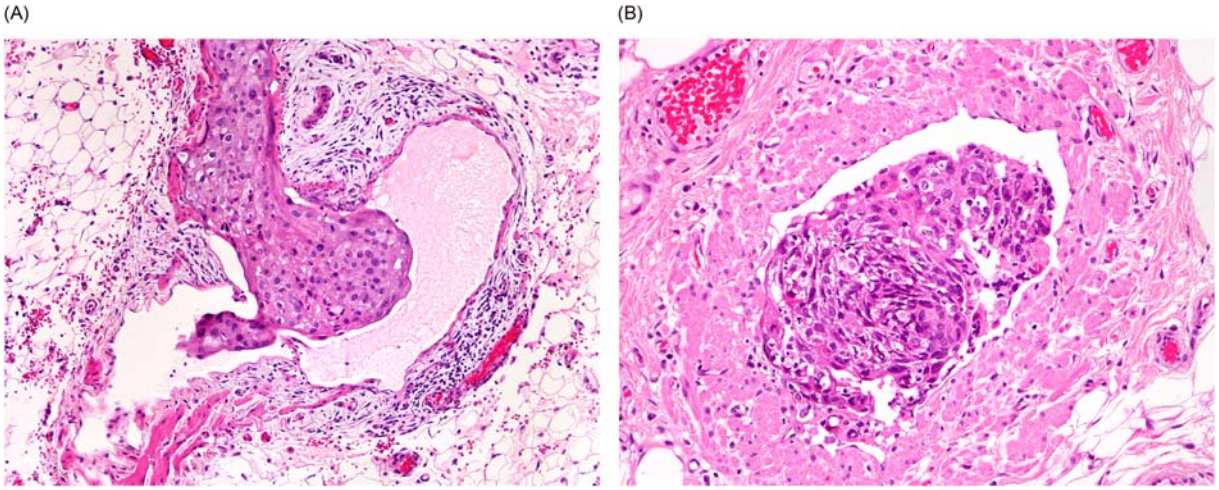

Fig. 3 (A) Lymphatic invasion: carcinoma cells in a thin-walled lymphatic vessel. (B) Vascular invasion: carcinoma cells within a small vein.

therefore been proposed to introduce novel molecular assays to detect these genetically damaged, phenotypically normal mucosal cells in resection margins. This form of margins has been termed "molecular margins." Some studies reported promising results using various genetic alterations including TP53 mutations, loss of heterozygosity, promoter hypermethylation, eIF4E proto-oncogene overexpression, mitochondrial DNA mutations etc. Nevertheless, there is no clinical experience with these methods and no consensus on how to use them in routine work.

Precancerous Lesions of the Larynx

Transition from normal laryngeal epithelium to precancerous lesions and SCC is a stepwise process, in which genetic changes progressively accumulate, followed by architectural and cytologic abnormalities. The entire spectrum of morphologic changes in this process has been referred to as dysplasia or squamous intraepithelial lesions (SILs). Several classification systems are used worldwide. Aiming to harmonize the various concepts of different classifications, the new edition of the WHO classification of head and neck tumors proposed a two-tiered system, classifying these lesions as low-grade or high-grade dysplasia (SILs). Some authors believe that carcinoma in situ must be defined as a separate category. Dysplasia (SILs) is associated with an increased risk of progression to SCC, from 1.6% for low-grade dysplasia to 12.5% for high-grade dysplasia, and up to 40% for carcinoma in situ (Fig. 4).

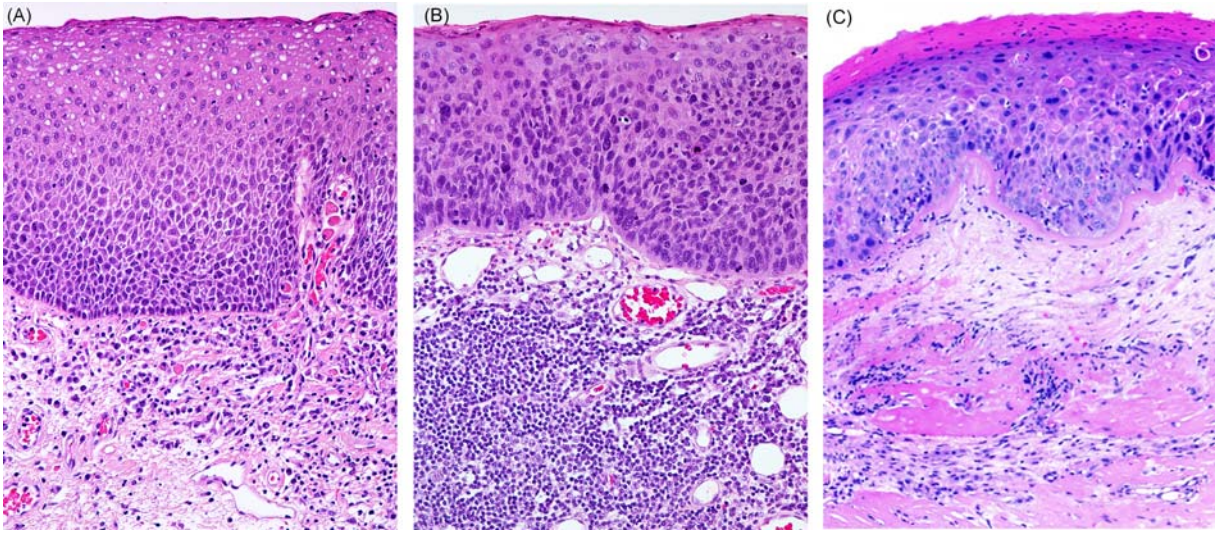

Fig. 4 (A) Low-grade dysplasia (squamous intraepithelial lesion): hyperplastic squamous epithelium with augmented basal-type cells, occupying lower half of epithelial thickness, with rare mitotic figures and no atypia. (B) High-grade dysplasia (squamous intraepithelial lesion): hyperplastic squamous epithelium with augmented basal-type cells, occupying almost the entire epithelial thickness, with marked atypia and high mitotic rate. (C) Carcinoma in situ: epithelial cells show severe atypia, with frequent mitoses, architectural disorder and preserved basement membrane.

Subtypes of Squamous Cell Carcinoma of the Larynx

Several variants of SCC in the larnyx have been described in the WHO Classification of the head and neck tumors, including verrucous carcinoma, basaloid SCC, papillary SCC, spindle cell carcinoma, adenosquamous carcinoma and lymphoepithelial carcinoma. Most of them are true clinico-pathologic entities with important prognostic implications. Their recognition also helps to distinguish them from other tumors.

Variants of SCC are rare and together account for 5%–10% of laryngeal malignant tumors. Similarly to conventional SCC, they mostly occur in the 6th and 7th decades, more frequently in males, and are often associated with alcohol abuse and tobacco.

Verrucous Carcinoma

Verrucous carcinoma (VC) is a variant of well differentiated SCC which grows slowly but can cause extensive destruction. Pure VC is believed to be unable to metastasize. The possible relation of VC to HPV infection has been controversial. Recent studies using highly sensitive and specific molecular methods suggest that VC is not related to infection with HPV.

Macroscopically, VC presents as an exophytic, broad based tumor with a warty surface.

Microscopically, it consists of thickened, club-shaped filiform projections with prominent surface keratinization and blunt stromal invaginations lined by thick, well differentiated squamous epithelium, composed of a few layers of basal cells and multiplied, voluminous spinous cells lacking cytologic criteria of malignancy. Mitoses are present only in the basal layer. The tumor invades the subjacent stroma with a well defined, pushing margin. Approximately 10% of VCs of the larynx contain foci of conventional SCC. They are referred to as hybrid (mixed) tumors and have the potential for metastasis (Fig. 5).

VC is characterized by a high frequency of initial misdiagnosis. An adequate, full-thickness biopsy specimen must be taken, when a clinician suspects VC; moreover, multiple biopsies may be needed to rule out a conventional SCC component in a VC.

Differential diagnosis includes verrucous hyperplasia, well differentiated SCC, papillary SCC and squamous papilloma. Invasion below the level of the basal cell layer of the neighboring normal squamous epithelium distinguishes VC from verrucous hyperplasia. Lack of atypia helps to rule out conventional SCC and papillary SCC. VC also lacks the well formed, wide papillary fronds of a squamous cell papilloma.

Molecular pathology and genetics of VC are largely unknown.

Pure VC has a significantly better prognosis than conventional SCC and doesn't affect overall survival of the patients when properly treated. Patients with hybrid carcinoma must be treated aggressively as if they had conventional SCC.

Basaloid Squamous Cell Carcinoma

Basaloid squamous cell carcinoma (BSCC) is a biphasic tumor composed of neoplastic basaloid cells and foci of SCC. In the larynx, BSCC is not associated with HPV infection.

Macroscopically, BSCC does not show any characteristic features, distinguishing it from conventional SCC. It usually present as slightly elevated tumor with a central ulceration and elevated edges.

Microscopically, it consists of a SCC component and basaloid cells. The SCC component can present as foci of conventional SCC or as dysplastic changes in the surface epithelium. Basaloid cells are small, with hyperchromatic nuclei without nucleoli and scant cytoplasm. They grow in a solid nests which are closely packed, with a jigsaw-puzzle pattern. Stromal hyalinization between and within the tumor nests is a characteristic feature. Large central necroses of comedo type and prominent peripheral palisading are

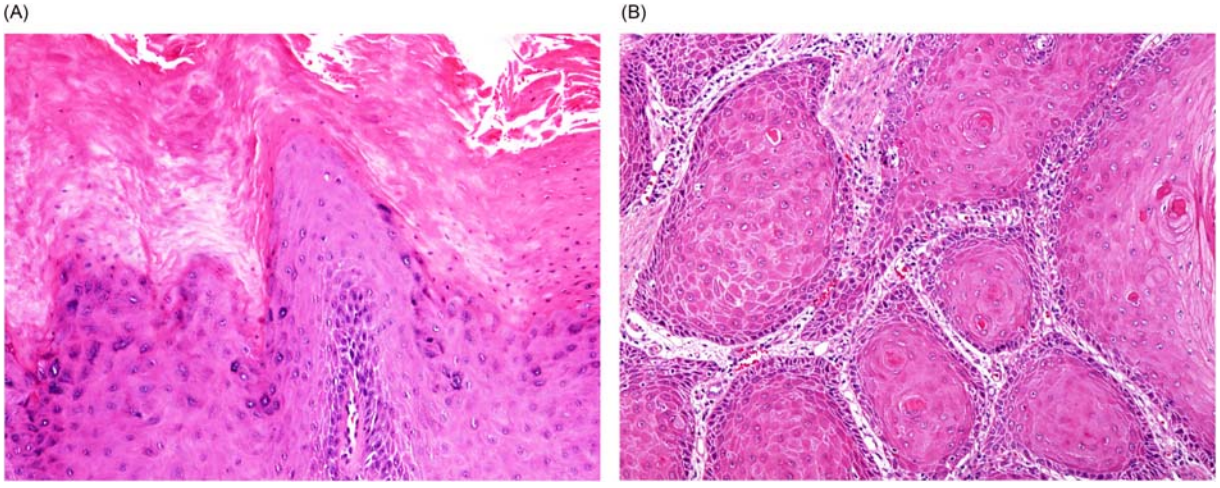

Fig. 5 Verrucous carcinoma. (A) Filiform projections of well differentiated squamous epithelium with prominent surface keratinization, with no atypia. (B) Tumor invades the subjacent stroma with well defined, pushing margins.

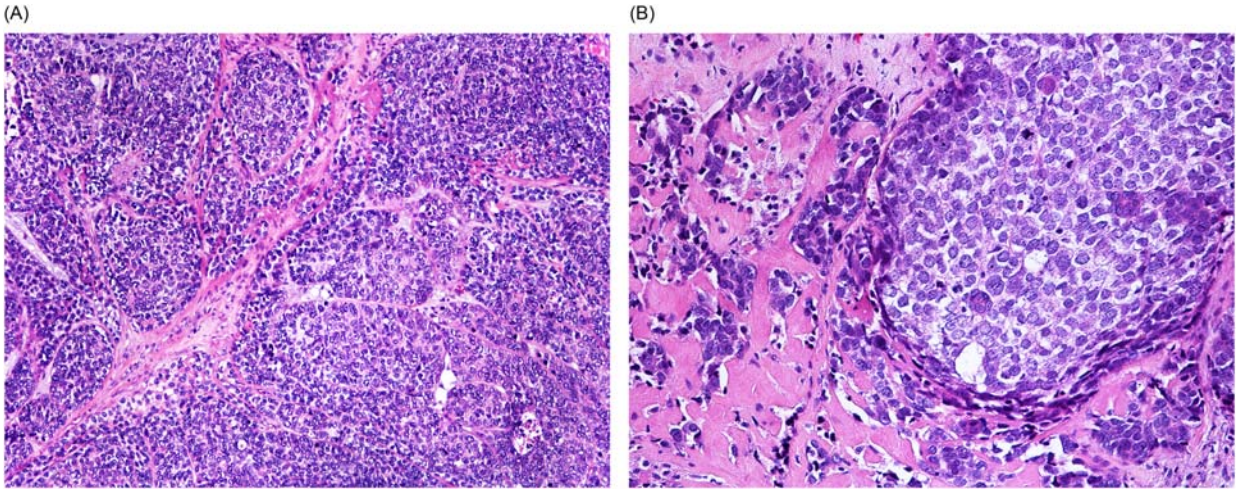

Fig. 6 Basaloid squamous cell carcinoma. (A) Islands of closely packed small cells with hyperchromatic nuclei without nucleoli and scant cytoplasm. (B) Stromal hyalinization between and within the tumor islands.

usually present. Distinctive features of basaloid SCC are small cystic spaces mimicking gland formation, containing PAS-positive material. BSCC may occasionally lack the SCC component (Fig. 6).

Immunohistochemically, BSCC expresses high-molecular-weight cytokeratins, p63 and p40, but it does not express neuroendocrine markers.

The most important differential diagnosis includes adenoid cystic and neuroendocrine carcinoma. The former lacks squamous differentiation and usually expresses S100, vimentin and focally p63. Neuroendocrine carcinoma is distinguished from BSCC by positivity for neuroendocrine markers, e.g., synaptophysin, chromogranine and/or CD56. In large tumors extending to the oropharynx, HPV-positive SCC must be considered; it is characterized by immunohistochemical overexpression of p16 and the presence of HPV16/18.

Molecular pathology and genetics of BSCC of the larynx are largely unknown.

There is some controversy regarding the prognosis of basaloid SCC. Some studies suggested that it has a worse outcome than conventional SCC, whereas others suggested that prognosis in both tumor types is similar when matched for site and stage.

Papillary Squamous Cell Carcinoma

Papillary squamous cell carcinoma (PSCC) is characterized by an exophytic, papillary growth pattern and a good prognosis. In the larynx, it is rarely associated with HPV infection.

Macroscopically, PSCC presents as exophytic, friable, soft tumors.

Microscopically, they are composed of papillary projections, which consist of a central delicate fibrovascular core covered by neoplastic squamous epithelium. The covering epithelium is nonkeratinizing or minimally keratinizing, it may be composed of immature basaloid cells or may be more pleomorphic resembling carcinoma in situ. Stromal invasion is often difficult to demonstrate in biopsy specimens (Fig. 7).

Differential diagnosis includes squamous papilloma and verrucous carcinoma. Papilloma and VC share similar architecture with PSCC, but PSCC is differentiated from both VC and papilloma by the presence of atypia of the squamous epithelium covering the papillae.

Molecular pathology and genetics of PSCC of the larynx are largely unknown.

PSCC is generally believed to have a better prognosis than conventional SCC, though recurrences are frequent.

Spindle Cell Carcinoma

Spindle cell carcinoma (SpCC), also referred to as sarcomatoid carcinoma, is a biphasic tumor composed of conventional SCC and malignant spindle cells. The characteristic spindle cell phenotype of the neoplastic cells in SpCC is the result of epithelial-mesenchymal transition. Similar to conventional SCC, SpCC has been etiologically related to cigarette smoking and alcohol consumption. It has been suggested that SpCC may develop after radiation exposure; however, some authors believe that this is not a major etiologic factor.

Macroscopically, SpCC can present either as an exophytic, polypoid lesion or as a flat, ulcerated tumor.

Microscopically, the SCC component may be well-, moderately- or poorly differentiated, keratinizing or nonkeratinizing, and transition between the two components may be abrupt or gradual. The spindle cell component usually forms the bulk of the tumor. Spindle cells are often pleomorphic, with large hyperchromatic nuclei, prominent nucleoli, and numerous mitoses. Sometimes,

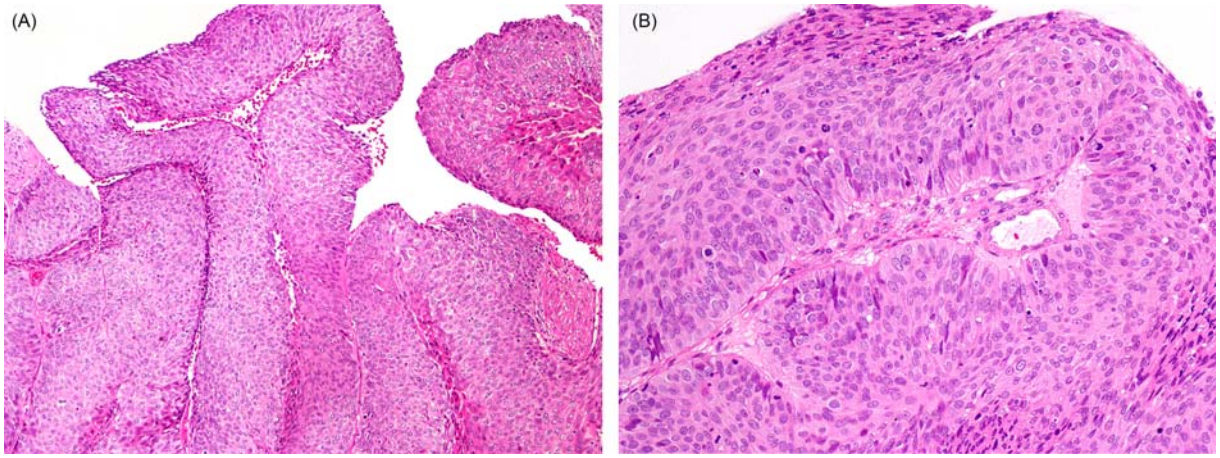

Fig. 7 Papillary squamous cell carcinoma. (A) Exophytic tumor, consisting of papillary projections, covered by nonkeratinizing squamous epithelium and fibrovascular cores. (B) The epithelium covering papillae shows nuclear and cellular atypias and increases mitotic activity.

only spindle cells are present. If such tumors are less cellular, they may mimic benign reactive lesions. Foci of osteosarcomatous, chondrosarcomatous or rhabdosarcomatous differentiation may be present, particularly in patients who had previously been treated by radiotherapy (Fig. 8).

Immunohistochemically, tumor cells in SpCC often express epithelial and mesenchymal markers. Cytokeratin and vimentin coexpression has been observed in individual tumor cells. Cytokeratin expression can be demonstrated in spindle cells in 40%–85% of cases. The most sensitive/reliable epithelial marker for SpCC seems to be keratin (AE1/AE3, K1) K1, K18 and EMA. Spindle cells always express vimentin and often other mesenchymal filaments, such as myogenic markers (smooth muscle actin, muscle specific actin, desmin).

A diagnosis of a SpCC is based on demonstration of an invasive or in situ SCC and a malignant spindle cell component. However, when a SCC component cannot be demonstrated, the diagnosis is more difficult and SpCC must be distinguished from a number of benign and malignant processes, such as spindle cell sarcomas, nodular fasciitis, inflammatory myofibroblastic tumor and malignant melanoma. In the larynx, true sarcomas (with the exception of chondrosarcoma) and benign mesenchymal tumors are very rare. It is therefore the general view, that a malignant spindle cell tumor in the mucosa of the larynx tract is probably a SpCC and not a sarcoma. A negative reaction for S100 protein and HMB45 helps to distinguish SpCC from malignant melanoma.

Molecular pathology and genetics of SpCC is complex and is similar to poorly differentiated SCC. SpCC also shows features of epithelial-mesenchymal transition, e.g., up-regulation of transcription repressors (Snail, Slug, SIP and Twist etc.) and down-regulation of microRNAs implicated in the induction of epithelial-mesenchymal transition.

Prognosis of the laryngeal SpCC is controversial, but it seems to be favorable for patients with no history of radiation, glottic location and polypoid appearance of the tumor.

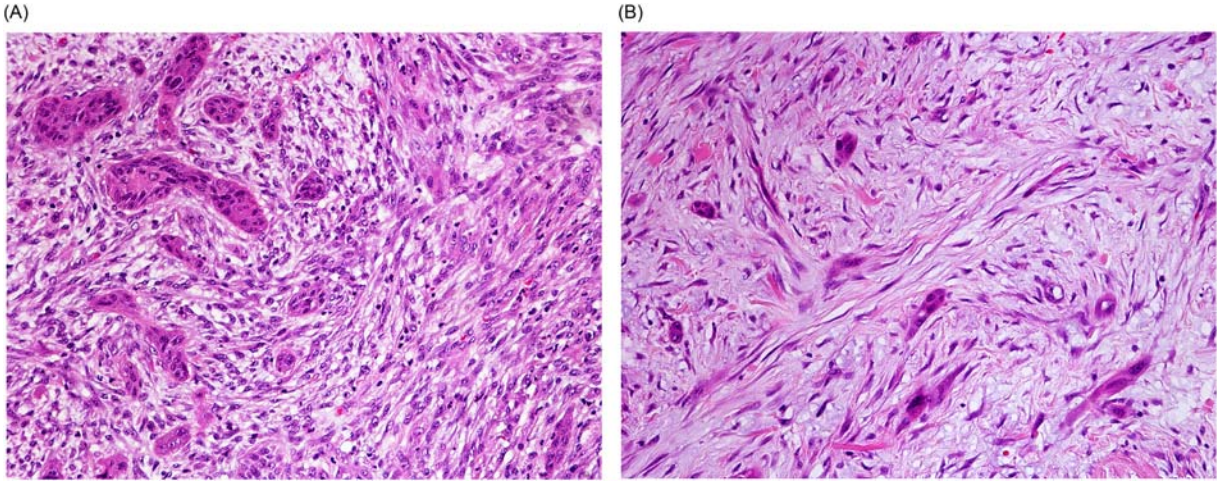

Fig. 8 Spindle cell carcinoma. (A) Small islands and cords of squamous cell carcinoma and dense proliferation of neoplastic spindle cells. (B) Spindle cell carcinoma with abundant edematous, myxoid stroma.

Adenosquamous Carcinoma

Adenosquamous carcinoma (ASC) is a malignant tumor that arises from the surface epithelium and shows both squamous and glandular differentiation.

Macroscopically, ASC cannot be distinguished from conventional SCC.

Microscopically, the two components occur in close proximity but are generally distinct and separate and are not closely intermingled as in mucoepidermoid carcinoma. The SCC component can present either as in situ or as invasive SCC, and the adenocarcinoma component is usually located in the deeper parts of the tumor, consisting of tubular, alveolar or ductal structures (Fig. 9).

Immunohistochemistry shows distinctive staining patterns in the two components: positive staining for CEA and low-molecular-weight cytokeratins, such as K7 and CAM5.2 in the adenocarcinomatous component and positive staining for p63, K5/6 and K7 in the SCC component. Both components express high-molecular-weight cytokeratins.

Molecular pathology and genetics of ASC of the larynx are largely unknown.

Differential diagnosis includes mucoepidermoid carcinoma, adenoid SCC and conventional SCC invading the normal salivary glands. It is important to differentiate ASC from mucoepidermoid carcinoma because ASC has a worse prognosis. Features favoring a diagnosis of ASC are separate and distinct areas of SCC and adenocarcinomatous components and the involvement of the surface epithelium, exhibiting either atypical hyperplasia, carcinoma in situ or invasive SCC.

The presence of mucin in the true glandular spaces helps to distinguish ASC from adenoid SCC. Conventional SCC invading or entrapping the normal salivary or mucoserous glands can be confused with ASC, especially in small biopsy specimens. In such cases, preservation of the lobular gland architecture and lack of significant atypia are observed, helping to distinguish conventional SCC from ASC.

In general, ASC has a more aggressive course than conventional SCC, with a tendency for early lymph node metastases, frequent local recurrences and dissemination.

Lymphoepithelial Carcinoma

Lymphoepithelial carcinoma (LC) is a poorly differentiated SCC or undifferentiated carcinoma, associated with a dense lymphocytic stromal infiltration. It is morphologically similar to nonkeratinizing nasopharyngeal carcinoma, undifferentiated subtype. It is extremely rare in the larynx. In contrast to nasopharyngeal LC, laryngeal LC is rarely associated with EBV infection.

Macroscopically, LC cannot be distinguished from conventional SCC.

Microscopically, LC is composed of small clusters or large syncytial masses of cells with oval or round vesicular nuclei and prominent nucleoli, and poorly defined, sparse cytoplasm. The stroma is densely infiltrated by T lymphocytes, occasionally admixed with plasma cells, follicular dendritic cells and eosinophils. Immunohistochemically, LC expresses cytokeratins distinguishing it from other malignant neoplasms, e.g., malignant melanoma and lymphoma (Fig. 10).

Molecular pathology and genetics of LC of the larynx are largely unknown.

LC is more aggressive than conventional SCC, with a higher incidence of cervical lymph node metastases and a propensity for distant metastases.

Prospective Vision

A step towards a unique classification of precancerous lesions has been made in the last WHO edition of Classification of head and neck tumors.

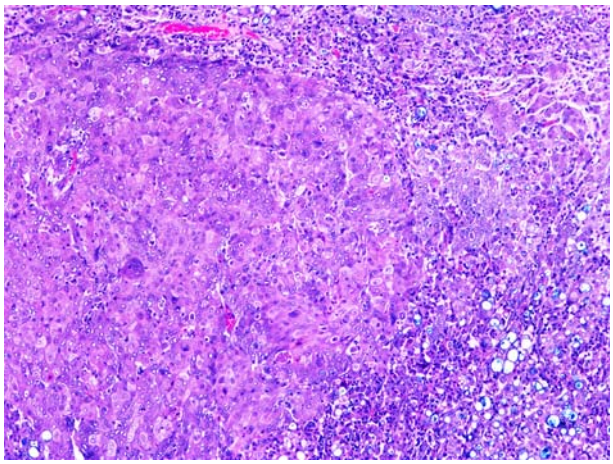

Fig. 9 Adenosquamous carcinoma. Two components, clearly separated: squamous cell carcinoma component on the left, and adenocarcinoma component on the right, with mucin production within the cytoplasm of tumor cells.

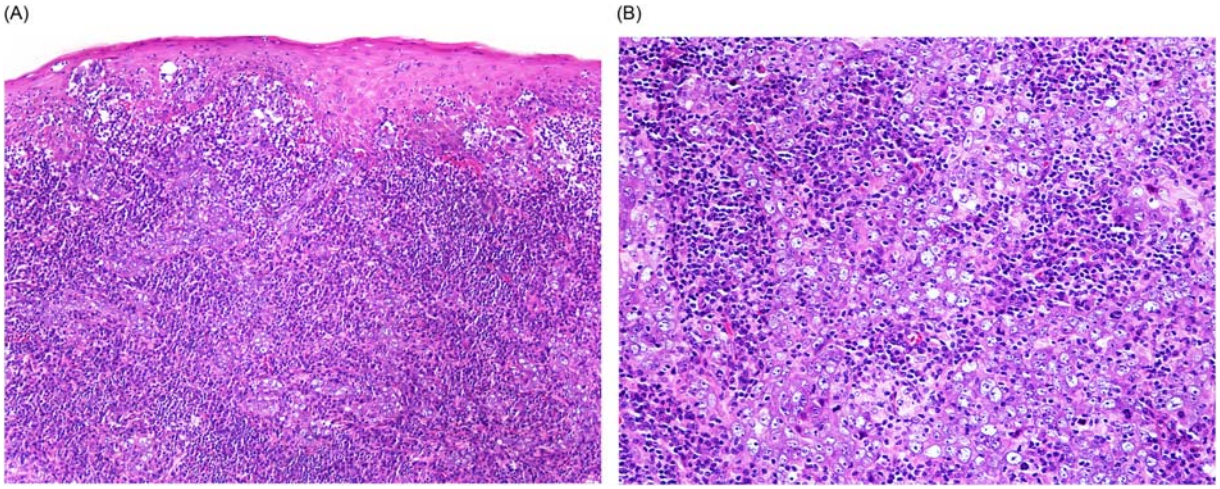

Fig. 10 Lymphoepithelial carcinoma. (A) and (B) Islands of poorly differentiated carcinoma beneath the surface epithelium, with stroma densely infiltrated by lymphocytes and occasional plasma cells.

In contrast to oropharyngeal carcinoma, no significant progress has been achieved in the understanding of etiology, pathology and treatment of laryngeal carcinoma.

Early diagnosis of precancerous lesions, proper classification and optimal choice of treatment is therefore essential for better outcomes of patients at risk for laryngeal cancer.

It is now clear that a small proportion of laryngeal SCC is related to high-risk HPV, but clinical significance of this finding, if any, is yet to be determined.

Discovery of new targets and treatment modalities are needed, possibly targeting not only tumor cells, but also other constituents of the tumor microenvironment.

See also: Laryngeal Cancer: Diagnosis and Treatment.

Further Reading

Alos, L., Castillo, M., Nadal, A., et al., 2004. Adenosquamous carcinoma of the head and neck: Criteria for diagnosis in a study of 12 cases. Histopathology 44, 570–579.
Christofori, G., 2006. New signals from the invasive front. Nature 441, 444–450.
de Miguel-Luken, M.J., Chaves-Conde, M., Carnero, A., 2016. A genetic view of laryngeal cancer heterogeneity. Cell Cycle 15, 1202–1212.
El-Naggar, A.K., Chan, J.K.C., Grandis, J.R., Takata, T., Slootweg, P.J. (Eds.), 2017. WHO classification of head and neck tumors, 4th edn.,. IARC, Lyon.
Gale, N., Gnepp, D.R., Poljak, M., et al., 2016. Laryngeal squamous intraepithelial lesions: An updated review on etiology, classification, molecular changes, and treatment. Advances in Anatomic Pathology 23, 84–91.
Hinni, M.L., Ferlito, A., Brandwein-Gensler, M.S., et al., 2013. Surgical margins in head and neck cancer: A contemporary review. Head and Neck 35, 1362–1370.
Liebig, C., Ayala, G., Wilks, J.A., Berger, D.H., Albo, D., 2009. Perineural invasion in cancer: A review of the literature. Cancer 115, 3379–3391.
Mehrad, M., Carpenter, D.H., Chernock, R.D., et al., 2013. Papillary squamous cell carcinoma of the head and neck: Clinicopathologic and molecular features with special reference to human papillomavirus. American Journal of Surgical Pathology 37, 1349–1356.
Odar, K., Kocjan, B.J., Hošnjak, L., et al., 2014. Verrucous carcinoma of the head and neck—not a human papillomavirus-related tumour? Journal of Cellular and Molecular Medicine 18, 635–645.
Puri, S.K., Fan, C.Y., Hanna, E., 2003. Significance of extracapsular lymph node metastases in patients with head and neck squamous cell carcinoma. Current Opinion in Otolaryngology & Head and Neck Surgery 11, 119–123.
Trivedi, S., Rosen, C.A., Ferris, R.L., 2016. Current understanding of the tumor microenvironment of laryngeal dysplasia and progression to invasive cancer. Current Opinion in Otolaryngology & Head and Neck Surgery 24, 121–127.
Zidar, N., Boštjančič, E., Gale, N., et al., 2011. Down-regulation of microRNAs of the miR-200 family and miR-205, and an altered expression of classic and desmosomal cadherins in spindle cell carcinoma of the head and neck—hallmark of epithelial-mesenchymal transition. Human Pathology 42, 482–488.
Zidar, N., Gale, N., Cardesa, A., Ortega, L., 2016. Larynx and hypopharynx. In: Cardesa, A., Slootweg, P.J., Gale, N., Franchi, A. (Eds.), Pathology of the head and neck, 2nd edn. Springer-Verlag, Berlin, pp. 338–386.

Li–Fraumeni Syndrome☆

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Glossary

Familial cancer A form of cancer that occurs in various members of the same family at a far higher rate than would be expected to occur by chance or that would be attributed to shared environmental conditions, for example, Li–Fraumeni syndrome.

Germline mutation A mutation which occurs in the germ cell and is therefore propagated to every cell in the body and can be inherited, in contrast to somatic mutations which are acquired.

Li–Fraumeni syndrome (LFS) A familial cancer syndrome associated with occurrence of a wide spectrum of cancers that typically develop at significantly earlier ages than their sporadic counterparts. These include breast carcinomas, soft tissue and bone sarcomas, leukemia, and brain tumors among others. For families at risk, the probability of developing some invasive form of cancer by age 30 is close to 50%.

p53 A tumor suppressor protein encoded by the *TP53* tumor suppressor gene whose primary function is to activate maintenance of genome integrity. Somatic *TP53* mutations occur in over 50% of human cancers; germline *TP53* mutations occur in ~80% of LFS patients.

Sporadic cancer Nonhereditary malignancy.

Trp53 (transgenic) mice Mice that have been genetically engineered either to be devoid of functional p53 or to express mutant alleles that compromise wild-type p53 function.

Tumor suppressor gene A gene whose protein product, when inactivated, can contribute to the malignant transformation of a normal cell to a tumor cell.

Nomenclature

LFS Li–Fraumeni syndrome

MRI Magnetic resonance imaging

yr Years

Introduction

Inherited mutations in "cancer genes" pose the most potent and penetrant oncogenic influence in human carcinogenesis, exceeding the effects of such environmental factors as ionizing radiation, tobacco, and occupational carcinogens. Hereditary cancers have been reported to occur in autosomal dominant, recessive or X-linked patterns of inheritance, and for some (most notably breast and colon) at least two different hereditary forms exist, distinguished by their clinical features. Different genetic events are thought to influence the respective phenotypic associations. Some neoplasms such as acute myelogenous leukemia occur only rarely in a familial form, whereas others such as retinoblastoma occur in the heritable form almost as frequently as in the sporadic form. There tends to be a wide degree of penetrance of the susceptibility to tumors arising among family members for each particular gene. Furthermore, the number of different types of cancer varies from family to family. For example, the only well-documented second tumor in the context of hereditary retinoblastoma is osteosarcoma. However, members of a Li–Fraumeni syndrome (LFS) family are at risk for a wide spectrum of tumors, including, but not limited to, carcinomas of the breast, lung and adrenal cortex, soft tissue sarcomas, osteosarcoma, leukemia, and a spectrum of brain tumor subtypes. Studies of hereditary cancers have identified a class of genes that is critical to both carcinogenesis and normal development. The identification of these genes facilitates the identification of increasing numbers of individuals (with or without cancer) who harbor them, as well as opportunities for development of novel therapeutic options. These advances have generated both rays of hope as well as significant challenges both for families and clinicians.

History and Definition

In 1969, Li and Fraumeni reported the results of a survey of 280 medical records and 418 death certificates of childhood rhabdomyosarcoma (RMS) patients diagnosed in the United States. In five families in whom siblings or cousins had a childhood sarcoma,

☆*Change History*: May 2018. O Michaeli and D Malkin updated all text and added new text.
This article is an update of David Malkin, Li–Fraumeni Syndrome, in Encyclopedia of Cancer (Second Edition), edited by Joseph R. Bertino, Academic Press, 2002, Pages 21–29.

 https://doi.org/10.1016/B978-0-12-801238-3.96122-1

a high concentration of cancers of diverse types was noted on the ancestral line of one parent. Soft tissue sarcomas and breast carcinomas, as well as acute leukemias, brain tumors, carcinomas of the lung, pancreas, and skin frequently occurred in first- and second-degree relatives, and adrenocortical carcinomas were often seen in siblings.

This family cancer syndrome came to be known as the Li–Fraumeni Syndrome (LFS). A subsequent prospective analysis of more than 50 similarly affected families defined the list of cancer phenotypes in affected members to include osteosarcoma, brain tumors, leukemias, and adrenocortical carcinomas, in addition to the originally described component tumors, namely soft tissue sarcomas and breast cancer (**Fig. 1**).

It was suggested in the original reports that the familial occurrence of neoplasms originating at discordant sites might represent a counterpart of the tendency for a single individual to develop multiple primary tumors. Indeed, neoplasms in LFS tend to develop in children and young adults, often as multiple primary cancers in affected individuals. Although LFS shares with other hereditary cancer syndromes the tendency for tumors to develop at unusually early ages at multiple sites, the constellation of tumors is quite distinct.

"Classic" LFS families include one member diagnosed with a sarcoma before age 45, a first-degree relative with cancer before age 45, and another first- or second-degree relative in the same ancestral line with any cancer diagnosed under 45, or a sarcoma at any age.

It was recognized that the identification of a defective gene or genes conferring a predisposition in carriers within these families would assist in clarification of the definition of the syndrome. Although LFS had been characterized in both a statistical and a classical genetic manner, identification of the gene that yielded this striking cancer predisposition remained elusive until 1990. Recognition of the limitations placed on standard linkage analysis suggested that the candidate approach to isolating the responsible gene might be productive. The class of genes most strongly associated with familial neoplasms has been the tumor suppressor genes. Of those genes known at the time, *TP53* was most likely to be linked to LFS, and indeed was found to be its cause.

Subsequent to the initial report of *TP53* mutations in classic LFS families, numerous cases were reported of individuals who did not fulfill the classical LFS definition but who either had an extensive familial history or who carried a germline *TP53* mutation. These families were termed "LFS variants" or "LFS like" with several somewhat different definitions that, in general, broadened the classic criteria. For example, the Eeles (1995) definition of LFS like families include individuals with any childhood cancer or sarcoma, brain tumor, or adrenocortical carcinoma diagnosed under 45 years of age, with one first or second degree relative, with typical LFS cancer (sarcoma, breast cancer, brain tumor, leukemia, or adrenocortical carcinoma) diagnosed at any age, plus one first or second degree relative in the same lineage with any cancer diagnosed under age 60.

In 2001, a wider set of criteria were proposed by the French LFS consortium that expanded referral opportunities for individuals for genetic testing for *TP53* mutations. The original "Chompret criteria" included the following (Chompret et al., 2001):

1. Proband affected by one of a narrow spectrum cancer (see below) before 36 years and at least one first or second degree relative affected by a narrow spectrum tumor (other than breast cancer if the proband is affected by breast cancer) before 46 years or multiple primary tumors.

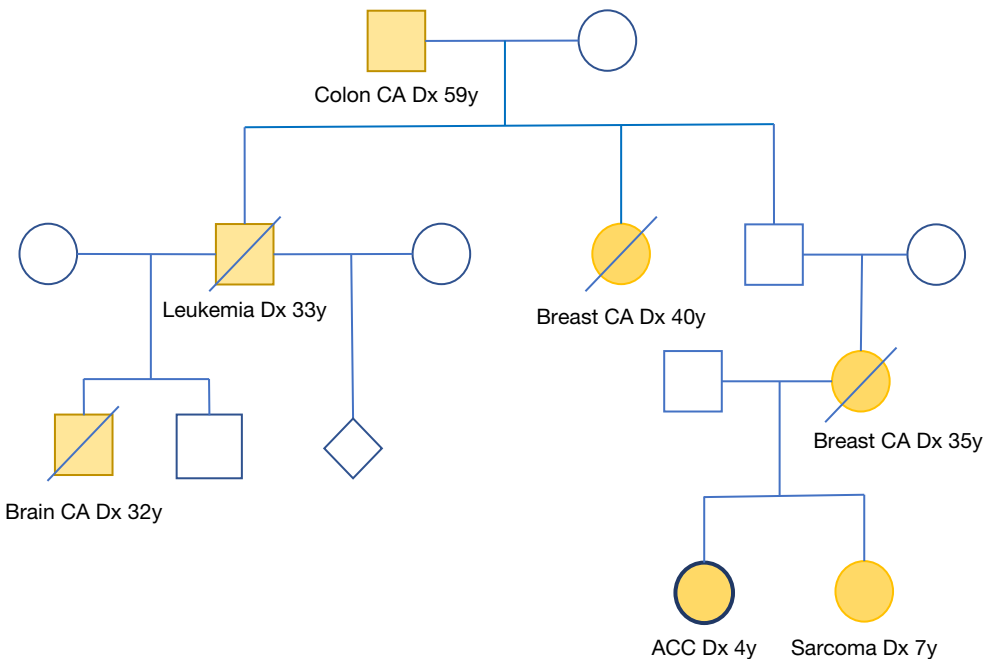

Fig. 1 Li–Fraumeni syndrome "classic" pedigree.

Table 1 Criteria for referral for TP53 mutation analysis (based on "revised Chompret criteria")

Familial presentation	Proband with tumor belonging to LFS tumor spectrum before age 46 years, AND at least one first/second-degree relative with LFS tumor (except breast cancer if proband has breast cancer) before age 56 years or with multiple tumors
Multiple primitive tumors	Proband with multiple tumors (except multiple breast tumors), two of which belong to LFS tumor spectrum and first of which occurred before age 46 years
Rare tumors	Proband with one of: adrenocortical carcinoma, choroid plexus carcinoma, anaplastic rhabdomyosarcoma, very early-onset breast cancer (<31 years), hypodiploid acute lymphoblastic leukemia, medulloblastoma (SHH subtype), irrespective of family history (at any age unless specified)

LFS tumor, premenopausal breast cancer, soft tissue sarcoma, osteosarcoma; *CNS tumor*, adrenocortical carcinoma.
CNS, central nervous system; *SHH*, Sonic Hedgehog; *LFS*, Li–Fraumeni syndrome.
Based on "2015 Version of Chompret Criteria", in: Bougeard, G. et al. (2015). Revisiting Li–Fraumeni syndrome from *TP53* mutation carriers. *Journal of Clinical Oncology* **33**(*21*), 2345–2352.

2. Proband with multiple primary tumors, two of which belong to the narrow spectrum and the first of which occurred before 36 years, whatever the family history.
3. Proband with adrenocortical carcinoma whatever the age of onset and family history.

The "narrow spectrum of tumors" includes soft tissue sarcoma, osteosarcoma, brain tumor, breast cancer, and adrenocortical carcinoma.

These criteria were further revised in 2009 (Tinat et al., 2009) and again in 2015 (Bougeard et al., 2015) (**Table 1**). These criteria can now be expanded even more, as recently several other cancers have been added to the list for whom gene testing is highly suggested (see "**Diagnosis and TP53 Germline Prevalence in Selected Cohorts**" section). The current criteria that should be generally used to guide *TP53* gene testing is depicted in **Table 1**.

The *TP53* Tumor Suppressor Gene

Alterations of the *TP53* gene or its encoded protein are the most frequently observed genetic events in human cancer; more than 50% of sporadic cancers harbor somatic *TP53* mutations and p53 is functionally dysregulated in more than 80%. The human gene, located on chromosome 17p13, encodes a 53-kDa nuclear phosphoprotein. p53 binds specific DNA consensus sequences and is a transcription factor that regulates the expression of other growth regulatory genes.

Structural analysis suggests that several highly conserved amino acids contact the minor or major grooves of the DNA helix. p53 function may be inactivated through sequence changes at these or other codons that alter the conformation of the protein and prevent it from forming tetramers or activating transcription.

Activation of p53 is the consequence of various stress signals to the cell: oncogene expression, DNA damage, hypoxia, nutrient deprivation, oxidative stress, replication defects, and more. Activation of p53 induces several downstream effects, including cell cycle arrest, senescence, apoptosis or DNA repair, through modulation of numerous genes. The exact effect will be determined according to the type of stress, the specific cell type, and other factors.

Inhibition of growth is achieved by blocking progression at a checkpoint control site prior to G1/S and at the G2/M restriction point. p53, commonly referred to as the "guardian of the genome" is important in maintaining the fidelity of DNA repair, particularly in response to double-stranded (ds) DNA breaks induced by ionizing radiation or certain chemotherapeutic drugs. Wild-type p53 mediates apoptosis when overexpressed in cultured cells in the absence of appropriate differentiation or proliferation signals.

Transcriptional targets of p53 are many and include Mdm-2 which is involved in a negative regulatory system that terminates the response of a cell to DNA damage; GADD45, a DNA repair protein; CDKN1a, which is critical for G1 cell-cycle arrest and cell senescence; PUMA and NOXA, mediators of apoptosis; and miR34, a microRNA which regulates genes that promote apoptosis, cell-cycle progression and differentiation.

In a cell with normal p53, levels of the protein rise in response to DNA damage, and the cell arrests prior to the G1/S transition, where genomic repair or apoptosis ensues with the mechanism being determined by the transforming oncoprotein(s). In a cell in which p53 is inactivated, G1 arrest does not occur and damaged DNA is replicated. During mitosis, the presence of damaged DNA results in mutation, aneuploidy, mitotic failure, and cell death.

As "the guardian of the genome" the functions of p53 are therefore very complex. Indeed, p53 has additional roles that involve autophagy, modulation of reactive oxygen species levels, ferroptosis and metabolism, as preventing tumorgenesis can be achieved by controlling aberrant cellular metabolism. Moreover, *TP53* alterations are necessary but not sufficient, for the ultimate cancer that arises from these malignant clones, as other genetic events are clearly required. While the scope of this chapter focuses on germline TP53 mutations and LFS, we suggest further reading of *TP53* tumor suppressor gene itself.

Germline *TP53* Mutations

In 1990 (Malkin et al., 1990), five LFS families were studied to determine whether *TP53* played any role in the occurrence of cancer in affected family members. Base pair mutations were identified in the germline of affected members in each of the five families

studied. Although the missense mutations were initially observed between codons 252 and 258 in exon 7, within one highly conserved region of the gene, further analysis of one family revealed a 2-bp deletion at codon 184 in exon 5 instead of the codon 252 mutation. The wild-type allele was deleted in tumors of affected individuals. Several unaffected relatives were mutant gene carriers, suggesting that they might be at risk to develop cancer at a later date.

Since that original report, germline *TP53* mutations were reported in additional LFS families, with other constitutional mutations in the same region of the gene, as well in different ones.

However, while most LFS families harbor a *TP53* alternation, there is a lack of 100% concordance with the "classic" LFS phenotype. In fact, only 60%–80% of "classic" LFS families harbor germline *TP53* mutations. The other clinical criteria as mentioned above are broader and less specific; therefore, only approximately 40% of "LFS-Like" families and 30% of individuals meeting the 2009 version of the Chompret criteria, carry mutations. This genotype–phenotype discordance, in which not even all "classic" LFS exhibit *TP53* mutation, may be explained in several ways. These include posttranslational p53 alterations, endogenous undetected promoter defects leading to aberrant expression of the *TP53* message, complete *TP53* deletion, effects of modifier genes, or alterations of other genes that may influence the phenotype generated by the presence of a specific germline *TP53* mutation.

Nevertheless, the high frequency of germline *TP53* mutations in LFS families together with the tight association of tumor formation in *Trp53*-deficient or *Trp53*-mutant mice (see "**Animal Models of Li–Fraumeni Syndrome**" section) do confirm a causal association of germline *TP53* alterations and cancer predisposition.

Currently, hundreds of germline *TP53* mutations have been reported. For the most part, these correspond to the somatic mutations and can be found across the gene. However, the most common mutations include those found in the original hotspots along with several additional ones, located in the highly conserved DNA binding domain of the gene, in exons 5–8 (**Fig. 2**). According to the recent version of International Association for Research on Cancer (IARC. R18, April 2016), the most common mutation hotspots, excluding a unique one at codon 337 (see below), are at codons 248, 273, 175, 245, 282, 213, 125, and 158, in decreasing order of frequency. Point mutations are the most prevalent (70%–96%), specifically missense mutations. Other, less common alternations are splice site and nonsense mutations that encode a truncated p53 protein, as well as rare frameshift mutations, intragenic deletions, in-frame insertion/deletions, and also intronic mutations.

Animal Models of Li–Fraumeni Syndrome

Transgenic animals carrying distinct deregulated oncogenes develop tumors that appear to be cell-type specific. To better study p53 in vivo, mice have been created that either lack functional p53 or express dominant-negative mutant alleles that inhibit wild-type p53 function.

In one of the early studies by Donehower et al. (1992), homozygous knockout mice with a germline *Trp53* null mutation ($p53^{-/-}$) were created. These mice were highly susceptible to malignant lymphomas and sarcomas, which occurred in >75% before 6 months of age. Tumor development, primarily sarcomas, was delayed in heterozygotes mice ($p53^{+/-}$); however, by 18 months of age, 50% of them develop tumors. Multiple primary tumors were noted in ~30% of tumor-bearing $p53^{-/-}$ mice, while virtually none of the $p53^{+/+}$ mice had developed tumors by 18 months. The heterozygous mice are a closer genetic model for LFS, since affected LFS individuals are heterozygous for mutant p53 rather than homozygous, and a substantial fraction of the germline mutations are functionally null for p53. Developmentally, the mice were normal, implying lack of crucial function of p53 in the evolving embryo. In a different study, the $p53^{-/-}$ state was again compatible with normal development. However, the yield of $p53^{-/-}$ offspring varied from 16.6% to 23%. The apparent increase in fetal loss is related to presence of fetal exencephaly, although increased fetal loss is not seen in human LFS families.

The genetic background of mice (C57BL/6 mice vs. 129/Sv), determined by strain-associated modifier genes, was shown in the aforementioned study to influence the tumor spectrum in the setting of p53 functional deficiency. The importance of genetic background in influencing the tumor type is as well exemplified by the occurrence of unusual cancers, including pineoblastomas and islet cell tumors in $p53^{-/-}$ mice crossed with mice heterozygous for an *RB1* mutation. In a study by Kuperwasser et al. (2000), which looked at female mice and breast carcinomas, high rate of mammary tumors developed in p53-deficient mice in a BALB/c background, but not in other genetic backgrounds (C57Bl/6 or 129/Sv). Notably, BALB/c-$p53^{-/-}$ mice did not develop high rates of mammary tumors, but this was likely due to early death from other tumors, especially lymphomas. Indeed, when mammary glands of BALB/c-$p53^{-/-}$ female mice were transplanted into wild-type (BALB/c-$p53^{+/+}$) hosts, 75% developed mammary tumors.

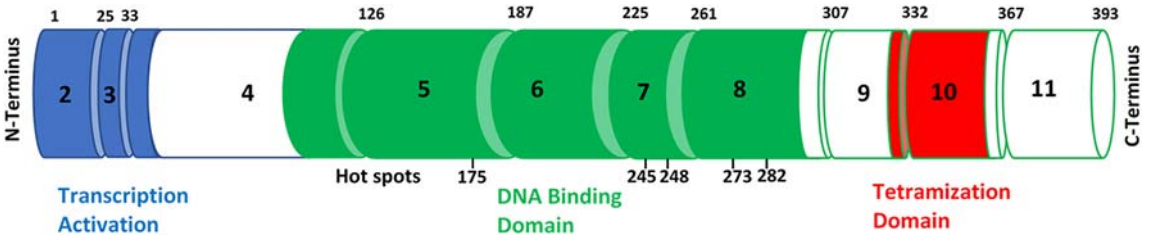

Fig. 2 Structural organization of p53.

Mice deficient for p53 are more sensitive to the effects of certain carcinogenic agents. $p53^{+/-}$ mice exposed to dimethylnitrosamine developed liver hemangiosarcomas more rapidly than treated $p53^{+/+}$ animals. $p53^{-/-}$ mice treated with an initiator, dimethylbenzanthracene, and a promoter, 12-*O*-tetradecanoyl-phorbol-13-acetate, show a more rapid rate of malignant progression of skin papillomas to carcinomas. These studies help distinguish whether p53 mutations play rate-limiting or tissue-specific roles in the tumor progression pathway. p53-deficient and p53-Tg mice (mice carrying mutant transgene) exposed to sublethal doses of γ-irradiation develop tumors, usually sarcomas, earlier than in untreated animals. This susceptibility is associated with a twofold increase in the accumulation of radiation-induced dsDNA breaks compared to that seen in $p53^{+/+}$ animals. These studies confirm that p53 prevents the accumulation of cells sustaining radiation-or chemically induced DNA damage.

More than 80% of the *TP53* germline mutations in humans are missense mutations in the conserved regions of the p53 DNA binding domain, whereas only few are nonsense mutations that result in no p53 or a truncated p53 protein. Therefore, it was crucial to test missense mutations in mice, and not only null p53 models. A model by Liu et al. (2000) looked at heterozygous mice with *TP53* R172H mutation ($p53^{R172H/-}$), which corresponds to the *TP53* R175H hotspot mutation in human cancers, and demonstrated different spectrum of tumors. Although mice with this mutation expressed low levels of mutant p53, there was high prevalence of metastatic disease compared to heterozygous null mice ($p53^{+/-}$). Notably, most of the tumors from these mice had an intact *TP53* allele, suggesting that loss of wild-type *TP53* was not important to tumor development.

Actually, in LFS patients, approximately 40% of tumors retain an intact wild-type *TP53* allele, in contrast to sporadic tumors, in which mutations or loss of both *TP53* alleles occur more frequently. In humans with germline missense *TP53* mutation, when p53 is functionally null, all the tumors will show LOH (loss of heterozygosity), meaning loss of the wild-type allele. In mouse models, however, tumors from mice with one null *TP53* allele ($p53^{+/-}$) were analyzed, and over half were found with an intact, wild-type *TP53* allele, which was functionally active.

Provocative studies have tested whether the tumorigenic activity of a mutant p53 is altered by the presence or absence of wild-type p53 in vivo. Mice carrying the 135Ala > Val mutant transgene were crossed with p53-deficient mice. The mutant p53-Tg accelerated tumor formation in $p53^{+/-}$, but not in $p53^{-/-}$ mice, suggesting that this loss-of-function mutation had a dominant-negative effect with respect to tumor incidence and cell growth rates. Although the tumor spectrum was similar in transgenic and nontransgenic mice, the transgenics showed a predisposition to lung adenocarcinomas. Thus, a given p53 alteration may have distinct tissue specificity with respect to tumorigenic potential.

The dominant-negative effect, by which mutant p53 functionally inactivates the wild-type p53 allele, was demonstrated by Chène (1998) in a mouse model with two types of mutations. The first, Arg175His, is a structural mutation, which affects the global configuration of the p53 protein, hence interfering with its ability to bind DNA. The p53 protein is active as a tetramer. When wild-type p53 forms a tetramer with such a mutant protein to form heterotetramers, that heterotetramer does not bind DNA due to its defective configuration. The second, Arg248Trp (and similarly-Arg 273Leu), is an active site mutation, which affects the actual site of DNA binding on the p53 protein, and not its configuration. This is also a dominant negative mutation: the complete lack of ability to bind DNA causes the wild type subunit of the heterotetramer to act improperly so that all parts of the heterotetramer cannot bind DNA. However, a different kind of mutation, which does not allow any tetramerization with the wild type protein, (e.g., Arg175His; Leu330Ala) will not cause such a dominant negative effect. Likewise, an active site mutation which causes low affinity of DNA binding but does not completely preclude it (e.g., Arg273His) also do not cause such dominant negative effects, since some binding is possible.

The role of those two different types of point mutations were examined in study by Olive et al. (2004). *Trp53* R270H is a missense mutation which corresponds to the *TP53* R273H mutation in humans and is an active site mutation. A different mutation, *Trp53* R172H, corresponds to the *TP53* R175H in humans. Heterozygous mice ($p53^{R270H/+}$ and $p53^{R172H/+}$) developed different tumor spectra when compared to $p53^{+/-}$ mice, to each other (in the same genetic background) and to $p53^{-/-}$ mice. Tumors arising in $p53^{-/-}$ mice were primarily carcinomas (specifically lung adenocarcinomas), most of which were metastatic or invasive, as well as B cell lymphomas. In contrast, $p53^{R172H/+}$ mice showed a higher incidence of sarcomas—particularly osteosarcomas, with frequent metastatic disease. Thus, at least in mice, different genotypes produce different phenotypes.

Lang et al. (2004) also created $p53^{R172H/+}$ mice. These mice developed osteosarcomas and carcinomas but with a much higher frequency of metastases than $p53^{-/-}$ mice. Importantly, both studies demonstrated the "gain-of-function" effect common to many p53 missense mutations, meaning that the mutant p53 exhibits additional inherent tumorigenic activity. Both also highlighted possible tumor suppressor roles of p63 and p73. These other p53 "family members" were functionally inactivated by the mutant $p53^{R172H/+}$ in mouse embryonic fibroblasts, providing an explanation for their role in the "gain of function" effect.

Owing to the fact that p53 has several functions, one can speculate that different mutations will cause distinct altered functions. An interesting study evaluated mice harboring a R172P mutation, which corresponds to the human R175P. This mutant retains cell cycle arrest while abrogating initiation of apoptosis. Consequently, cells of mice with a homozygous mutation ($p53^{R172P/R172P}$) were able to induce cell-cycle arrest and maintain a stable genome. Notably, the mice had delayed tumor onset compared with homozygous null mice ($p53^{-/-}$). This finding suggests that delayed cell-cycle arrest contributes to tumor suppression.

These mouse models and many others shed light on the critical functions of p53 and the phenotypic manifestations of different *TP53* mutations. The continuing expansion of our knowledge through these models is still crucial to further understand the phenotype: genotype correlations in LFS. However, although mice are powerful models with which to study various aspects of p53 biology, they are still somewhat imperfect representations of LFS and cannot be entirely relied upon to explain the clinical and genotype associated phentoypes of the human disease.

Clinical Aspects and Epidemiology of Li–Fraumeni Syndrome

Prevalence

Currently, more than 700 families have been reported in the database of the International Association for Research on Cancer (IARC). However, estimations of the actual prevalence of the syndrome are much higher, until recently estimated at approximately 1:5000, as awareness of cancer predispositions is increasing and many cases are not reported. In addition, newer ways of determining the prevalence are emerging (i.e., by surveying genetically larger cohorts and through the use of next generation sequencing technologies) which suggest that the *TP53* mutation population carrier rate may be as high as 1:500.

Penetrance

Penetrance is thought to be very high, although it is challenging to determine as cancer development in an individual is not entirely dependent on the specific mutation. As genetic tests are becoming more prevalent, more and more people are identified with *TP53* germline mutations without having a rich familial history or even having cancer. Some are identified by the occurrence of cancer in a family relative. Moreover, others are discovered incidentally, for instance by genetic testing which was performed for reasons other than a cancer diagnosis.

Studies have demonstrated a high penetrance rate with estimates of a lifetime cancer risk of 93% in females and 75% in males. Some reports attribute 100% penetrance by the age of 70 years.

This cancer risk is determined by various variables including gender and age. Hwang et al. (2003) looked at 56 carriers of germline *TP53* mutations and found that 12%, 35%, 52%, and 80% developed cancer by ages 20, 30, 40, and 50 years, respectively. In comparison, Bougeard et al., 2015 found an earlier age of onset, with 22% of carriers diagnosed with a cancer by age 5 years and 41% by age 18 years. While these differences are attributed to different cohorts with different inclusion criteria, it is notable that cancer risk is substantially higher than the general population. Notably, risk of cancer begins from infancy, as one of these studies reports that 4% of participants developed a malignancy during the first year of life.

Tumor Spectrum

LFS is a cancer predisposition syndrome with no other phenotypic features. The cancer spectrum is vast and virtually any cancer can occur. However, the "core cancers" that were included in Li and Fraumeni's original report are indeed more prevalent. In the report by Bougeard et al., 2015, 322 affected carriers were described. The five core cancers were the most frequent, with breast cancer more prevalent than any other, occurring in 60% of females. Soft tissue sarcoma and osteosarcoma followed, affecting 27% and 16% of patients, respectively. ACC and brain tumors affected 13% of individuals each. IARC reports 1644 tumors in *TP53* carriers, of which 27% are breast cancers, 13% STS, 12% brain cancers, 11% are adrenal and 10% involve the bones (**Fig. 3**).

Other cancers that occur in *TP53* carriers with some frequency are leukemia, lymphoma, embryonal cancers, various carcinomas, other kinds of sarcomas, neuroblastomas, and more. Interestingly, several tumor types that frequently harbor a somatic *TP53* mutation are less common among individuals with LFS, though when they do occur, they tend to happen at a lower age of onset than

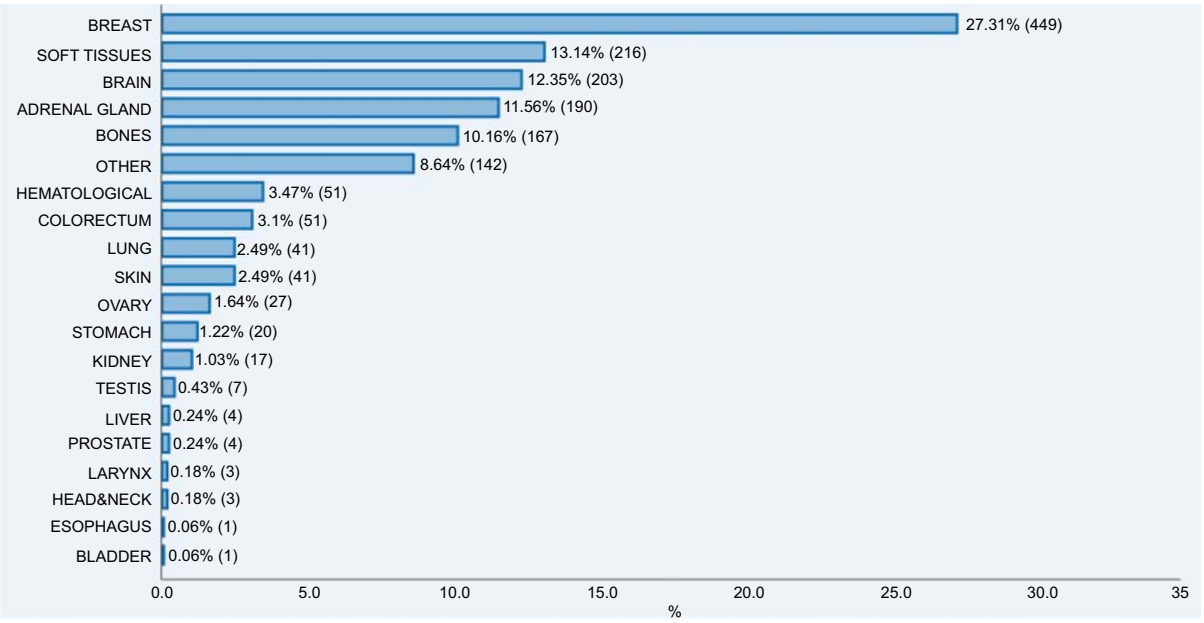

Fig. 3 Tumors associated with *TP53* germline mutations ($n = 1644$).

their sporadic counterparts. For example, colorectal cancer occurred in only 3% of *TP53* carriers, though on average at least two decades earlier than the median age of onset for sporadic CRC.

Type of cancer naturally changes in relation to age. Although adults with LFS are more commonly affected by breast cancer and soft tissue sarcomas (STS), in adolescents the most common tumors are osteosarcomas and young children are noted for adrenocortical carcinoma (ACC), brain tumors, and STS.

In regards to gender differences, since LFS is an autosomal dominant disorder, males and females have an equal chance of carrying the affected gene. However, penetrance differs and women appear to have a higher chance of developing cancer. This difference is attributed in part to the relative abundance of breast cancer, although several studies report that additional factors are involved. In a study by Olivier et al., 2003, among 494 tumors in individuals with germline *TP53* mutations, there was an excess in males of brain tumors, hematopoietic cancers, and stomach cancer. Excess of females was observed for skin cancer and adrenocortical carcinoma. This distribution is in concordance with gender predilection in sporadic tumors, though the excess of females with ACC is exceptional. Males and females were equally affected by soft tissue and bone sarcoma, lung cancer, and colorectal cancer. Mai et al. (2016) report that among females, the cumulative incidence rates by age 70 years were 54% for breast cancer, 15% for STS, 6% for brain tumors, and 5% for osteosarcoma. Among males, the incidence rates were 22% for STS, 19% for brain tumors and 11% for osteosarcoma.

Females not only have a greater chance for cancer, but also tend to be affected at an earlier age. A difference of 11 years between median age of cancer onset was reported among females (28.0 years) and males (17.0 years). This difference was still present after excluding breast cancers, though it was narrowed to 4 years—tumor onset in females was 13 years, compared to 17 years in males.

Multiple Cancers

A striking feature in LFS kindreds is the high frequency of affected members who develop multiple primary neoplasms. One multicenter study demonstrated germline *TP53* mutations in leukocyte DNA from 4 of 59 patients (6.8%) who had survived second cancers but did not have family histories compatible with the LFS.

The increased risk for multiple cancers is pronounced in patients who had their first cancer at a young age or were previously treated with radiation. While early studies state a relatively low incidence of second primaries, more recent studies report a higher incidence, perhaps due to different inclusion criteria, differences in treatment, and survival. For example, in a study by Hisada et al. (1998) among patients diagnosed between 1968 and 1986, the incidence of second, third and fourth primary cancers were 15%, 4%, and 2%, respectively. Notably, 71% of the multiple cancers developed were the LFS core tumors. In contrast, Mai et al. (2016) report in 2016 that almost 50% of LFS patients develop second primary tumors after a median of 10 years following their initial cancer. Breast cancer was the most commonly occurring second malignancy, followed by STS and brain and lung cancer. Interestingly, females had a higher risk of second cancer when compared with males, but lower risk of death. Similarly, Bougeard et al., 2015 reported multiple primary tumors in 43% of mutation carriers, metachronous presentation being more common than synchronous.

This higher chance of multiple primary cancers cannot be attributed solely to therapy, as the incidence is also increased when compared to patients with a history of sporadic cancers. According to a study by Hwang et al. (2003), there is 12-fold higher risk to develop a second primary cancer among carriers, when compared to the general population. In this report, among LFS patients the second cancer occurred at a median of 9.3 years after the first malignancy, which is longer than the median time for treatment-related secondary malignancy in noncarriers in this cohort.

As survivorship increases and surveillance is implemented (see "**Management**" section), more instances of patients surviving multiple cancers are reported, several of which are quite remarkable with documentation of up to 17 primary cancers in a single patient. As mentioned, the estimates of second cancer risk will continue to evolve as more data from cases of *TP53* carriers becomes available, mostly of individuals that do not fulfill the classical LFS criteria, or have any familial history of cancers.

Germline *TP53* Aberrations and Phenotype: Genotype Correlations

Understanding genotype: phenotype correlations in individuals with LFS can aid clinicians in determining more precise estimates of the likelihood of occurrence of specific tumors according to age group, gender, and genotype. Ideally, surveillance and management can be tailored according to those characteristics. Although not fully understood, several important correlative associations have been described.

One important observation is that dominant-negative *TP53* missense mutations within the DNA-binding domain are associated with the highest cancer risk and young age of onset. Such mutations were detected most commonly in pediatric cases of LFS with brain tumors, osteosarcoma and rhabdomyosarcoma (RMS). In contrast, the majority of pediatric cases with adrenocortical carcinoma (ACC) harbor other types of alterations. Other studies have noted that gain of function mutations were associated with younger age of onset when compared to loss of function mutations.

A unique association between genotype and phenotype is described for the germline *TP53* mutation R337H. This mutation is abundant in Brazil, where this founder mutation results in a high prevalence of LFS. The specific mutation (c.1010G > A) is located at codon 337 in exon 10, within the oligomerization domain of the p53 protein. Interestingly, the pH level is a determinant of the mutant protein's function, so that only in the physiological pH range (up to 7.5), does the protein form normal oligomers. At higher pH, and higher temperatures, the mutant protein is unable to dimerize and therefore does not assemble into a functional protein.

The *TP53* R337H mutation is estimated to be present in 0.3% of residents of the South/Southeastern regions of Brazil, resulting in estimate of more than 300,000 carriers. The spectrum of cancers is similar to that observed with other mutations; however, there is a greater than 15-fold increase in childhood ACC in the Brazilian population. In addition, there is high occurrence of papillary thyroid cancer, renal cancer, and lung adenocarcinoma. The age of tumor onset is somewhat older so that the penetrance in the younger age group is lower: cancer occurs before age 30 in only 15%–20%, compared with 50% in carriers of other *TP53* mutations. The lifetime penetrance is, however, as high as in "classic" LFS.

The phenotype: genotype correlations are complicated by various alterations that modify the function of p53. One example are specific *TP53* polymorphisms, such as a duplication within intron 3 (PIN3). When this duplication is found in a *TP53* R337H mutation carrier, the malignancies are more likely to appear at a later age, compared to carriers of the same *TP53* mutation that do not harbor the PIN3 duplication. PIN3 also confers a higher susceptibility to breast and colorectal cancer.

Another genetic modifier is a specific polymorphism of MDM2, an important regulator of p53. MDM2 ubiquinates p53 so that it is targeted for degradation. Bond et al. (2004) found that a T>G substitution at nucleotide 309 (SNP309) in the promoter of the first intron of MDM2 leads to increased levels of MDM2 and hence accelerated degradation of p53. As a result, it is associated with a higher rate of tumor formation in *TP53* mutation carriers and was shown to correlate with younger age of tumor onset. This higher risk is augmented when, in addition to the MDM2 SNP309 polymorphism, the *TP53* mutation carrier also harbors a *TP53* polymorphism at codon 72Pro (Arg72Pro). If a 72Arg variant of the p53 protein exists instead of 72Pro, a higher affinity of p53 to MDM2 will be achieved, causing greater degradation of p53 and younger age of cancer.

A different mechanism of functional modification of *TP53* is through differential methylation of other targets. One example are the microRNAs (miRs), short noncoding RNA molecules that modulate protein expression by promoting RNA degradation and inhibiting mRNA translation. miR-34a is a regulatory microRNA of p53. It was found that miR-34a promotor is hypomethylated in *TP53* mutation carriers, thus making it more active as a "cover" mechanism for the absent p53 tumor suppressor activity. On the other hand, hypermethylation of miR-34a, which decreases its function, is associated with decreased overall survival. This finding was demonstrated in choroid plexus carcinomas (CPC), a typical LFS tumor. Investigators found increased hypermethylation across three CpG sites of the miR-34a promoter, when compared with wild type *TP53* CPCs. Moreover, patients with CPCs that harbored miR-34a promoter hypermethylation had a lower median survival, a finding consistent with the fact that *TP53* mutant CPCs are known to have a more clinically aggressive course, with poorer survival.

An additional microRNA that was found to modify the LFS phenotype is miR-605, which regulates the p53–MDM2 loop. MDM2 as mentioned is a cellular inhibitor of p53, and is correlated with earlier age of tumor onset when increased. A variant of miR-605 gene, the G allele, was shown to be associated as well with an earlier age of tumor onset in LFS patients, in almost all tumor types. In vitro, when nucleofected as pre-miR 605 plasmids to RMS cell lines, the G allele showed decreased processing from its precursor form to its mature form, as compared to the "normal" A allele. Furthermore, in the context of mutant p53, overexpression of miR-605 resulted in a significant reduction in cell viability and oncogenic properties, including cellular proliferation and migration potential.

An intriguing genotype: phenotype phenomenon in LFS is anticipation, wherein successive generations manifest with earlier onset of malignancies. While the exact mechanisms remain unknown, there is probably a role for accelerated telomere attrition. Tabori et al. (2007) compared the telomere length in peripheral blood leukocytes of *TP53* mutation carriers and noncarriers. Carriers who were affected with cancer had shorter telomeres than unaffected carriers or healthy controls. The same was true when comparing affected children with cancer in comparison to their unaffected family members.

Excess DNA copy number variation (CNV) was also found to be one of the characteristics of the LFS phenotype. In a study by Shlien et al., 2008, of individuals with LFS compared the general population, *TP53* mutation carriers had a significant increase in CNVs across their genome, particularly if they had a family history of cancers. This is postulated to be the consequence of the underlying genomic instability caused by the mutant p53. In addition, some LFS individuals had only a few CNVs but of exceptionally large deletions or duplications. Subsequently, large deletions that encompass the entire *TP53* gene, appear to be associated with a distinctive phenotype of congenital anomalies without a predisposition to cancers.

While such genetic modifiers continue to be explored, it is now acknowledged that phenotype: genotype correlations are the result of complex genetic interactions including genetic modifiers within and outside of *TP53* itself.

Diagnosis and *TP53* Germline Prevalence in Selected Cohorts

LFS can be defined as an inherited form of cancers caused by germline mutations of the *TP53* gene; therefore, its diagnosis is best confirmed by the identification of a pathogenic variant in the gene. However, prior to the genetic test, the diagnosis in most cases has to stem from a high index of suspicion.

The revised Chompret criteria described above should aid in the decision of which cancer patient should be referred for genetic testing. As mentioned, these criteria are broad and more sensitive for identifying individuals with LFS who do not have familial cancer. In fact, estimations of de novo mutation frequency in LFS patients range between 7% and 20%, though the number might be even higher as more individuals are now being referred for genetic testing. In particular, the revised criteria focus also on specific cancer diagnoses which confer high risk of *TP53* germline mutation, regardless of the familial history. For instance, 9% of children diagnosed with RMS harbor germline *TP53* alterations. This fraction increases to 23% for patients younger than 4y and up to 73% of children with RMS with diffuse anaplasia. The incidence of *TP53* germline alternations in other sarcomas vary according to specific

type; for example, up to 10% of children with osteosarcoma harbor germline *TP53* mutations. Among children with ACC, about 50% have *TP53* germline alterations, and newer estimates range as high as 70%. However, in adult ACC patients, germline *TP53* mutations are exceedingly rare.

Two studies of patients with CPC found that about 40% carry *TP53* mutations. This rate is increased when cohorts focus on patients with somatic *TP53* mutation or on patients with a familial history of cancer. More than 10% of children with the Sonic Hedgehog (SHH) subgroup of medulloblastoma (SHH-MB) harbor *TP53* germline mutations, though the number increases to 30% when children between 8 and 17 are studied. Furthermore, 56% of children with the subgroup SHH/TP53-MB (SHH-MB with somatic *TP53* mutation) carry such a mutation.

While most patients with childhood acute lymphoblastic leukemia (ALL) do not have cancer predisposition, an uncommon subset of them with hypodiploid ALL, have substantial risk to harbor *TP53* germline mutation. Studies show that 19%–43% of such children are in fact carriers.

The prevalence of *TP53* germline carriers among breast cancer patients substantially changes with age. At older age they represent a minority. However, up to 10% of very early onset breast cancer (< 30 years of age) have LFS, similar to the rate of *BRCA 1/2* mutations in this population.

While all of these types of malignancies do confer high risk of underlying constitutional *TP53* mutation, it is important to bear in mind that for all of these cohorts, ascertainment bias is likely to lead to an overestimation of tumor risk in individuals with LFS. Regardless, germline testing is still recommended in these clinical contexts.

Suspicion of an underlying predisposition for cancer should also be raised with other features of a given malignancy. For instance, detection of specific tumor signatures should promote germline testing. In a report by Rausch et al., 2012, the phenomenon of chromothripsis—massive genomic rearrangements—was associated with *TP53* germline mutations in SHH MB. When looking at medulloblastomas, the vast majority displayed low numbers of single nucleotide variant (SNV) per chromosome. However, 13 of 98 tumors showed high number of SNVs, consistent with chromothripsis. Eleven of these thirteen were SHH-MBs, of whom 10 harbored *TP53* mutations, whereas none of the wild-type SHH-MBs showed this phenomenon. Therefore, the finding of chromothripsis in medulloblastoma, should raise the suspicion of an underlying *TP53* germline mutation. Likewise, comparing SNV rate in a given tumor to the expected SNV rate in the same "reference" sporadic tumor, can point to the need of germline testing.

All these features of a specific cancer diagnosis should advance the suspicion for an underlying predisposition. Other "universal criteria" that should raise suspicion of LFS include multiple primary tumors (synchronous or metachronous) and familial history of cancer, though not necessarily as depicted in the "classic" LFS criteria.

In an era of increasing genetic tests, many individuals are being diagnosed without even being affected. Individuals can be identified following a family member's diagnosis of LFS or incidentally.

Once a referral for genetic testing has been made, the type of genetic testing is determined according to the indication. A known familial variant usually leads to testing for this variant only. Where the mutation is not known, single gene testing by Sanger sequencing is often the initial test of choice with the addition of copy number analysis for intragenic deletions/duplications as well as examination of the intron/exon borders. This however may not identify certain types of variants (e.g., deep intronic variants).

Updated data regarding the different variants, including their interpretation as pathogenic or not, can be found in the website of the International Agency for Research on Cancer (IARC) *TP53* mutation database (listed below). It compiles *TP53* mutation data that have been reported in the published literature since 1989, and is updated annually. In addition to somatic *TP53* mutations, it includes the largest datasets on individuals carrying a sequenced *TP53* germline mutation or affected by cancer and belong to an "LFS family."

Management

Surveillance

Implementation of a surveillance protocol for germline *TP53* mutation carriers enables the presymptomatic detection of malignancies. Such early tumor detection can potentially allow definitive localized treatment approaches that may obviate or minimize exposure to systemic or radiation treatment.

In 2011, and later in 2016, Villani et al. (2016) first reported feasibility and potential survival benefit of a comprehensive surveillance strategy in patients with LFS. This surveillance protocol, termed the "Toronto Protocol," consisted of regular physical examinations together with scheduled imaging and biochemical studies, and was later proven to be beneficial in terms of survival. Among the initial cohort of 59 patients who chose to undertake this surveillance protocol, 40 neoplasms were detected pre-symptomatically in 19 patients, over a median follow-up period of 32 months. Two additional cancers were diagnosed between surveillance assessments in one patient. In contrast, 61 neoplasms presented clinically in 43 of 49 patients who did not undergo surveillance. Furthermore, 25 of 40 neoplasms on the surveillance "arm" were low grade or premalignant at the time of detection, suggesting that early detection through surveillance may identify lesions before malignant transformation. Five years OS was 88.8% in the surveillance arm, versus 59.6% in individuals not undergoing surveillance. Several other studies have since suggested improved clinical outcomes for *TP53* mutation carriers with intensive screening, specifically with the utility of WBMRI.

Table 2 Recommended LFS screening protocol

Adults
General assessment
• Complete physical examination every 6 months
• Prompt assessment with primary care physician for any medical concerns
Breast cancer
• Breast awareness (age 18 years onward)
• Clinical breast examination twice a year (age 20 years onward)
• Annual breast MRI screening[a] (ages 20–75)
• Consider risk-reducing bilateral mastectomy
Brain tumor (age 18 years onward)
• Annual brain MRI (first MRI with contrast; thereafter without contrast if previous MRI normal)
Soft tissue and bone sarcoma (age 18 years onward)
• Annual WBMRI[a]
• US of abdomen and pelvis every 12 months
Gastrointestinal cancer (age 25 years onward)
• Upper endoscopy and colonoscopy every 2–5 years
Melanoma (age 18 years onward)
• Annual dermatologic examination
Children (birth to age 18 years)
General assessment
• Complete physical examination every 3–4 months, including blood pressure, growth curve, Cushingoid appearance, signs of virilization, and full neurologic assessment
• Prompt assessment with primary care physician for any medical concerns
ACC
• US of abdomen and pelvis every 3–4 months
• In case of unsatisfactory US, blood tests[b,c], may be performed every 3–4 months: total testosterone, dehydroepiandrosterone sulfate, and androstenedione
Brain tumor
• Annual brain MRI (first MRI with contrast; thereafter without contrast if previous MRI normal and no new abnormality)
Soft tissue and bone sarcoma
• Annual WBMRI

WBMRI, whole-body MRI; *US*, ultrasound; *ACC*, adrenocortical carcinoma.

[a]Breast MRI/US of abdomen and pelvis to alternate with annual WBMRI (at least one scan every 6 months).

[b]The efficacy of biochemical surveillance for detection of adrenocortical carcinoma has not been shown.

[c]Serial specimens obtained at the same time of day and processed in the same laboratory.

Kratz, C.P., Achatz, M.I. and Brugières, L. (2017). Cancer screening recommendations for individuals with Li–Fraumeni syndrome. *Clinical Cancer Research* **23**(*11*), e38–e45. http://clincancerres.aacrjournals.org/content/23/11/e38.

In 2016, international consensus surveillance recommendations were generated for individuals carrying a pathogenic *TP53* variant, and those fitting the classic clinical definition of LFS. These were based on the "Toronto protocol" with slight modifications and is depicted in **Table 2**. Based on prediction of timing of cancer initiation for the various tumor types, the surveillance approach is proposed according to age groups. Also considered are adverse effects of "over" surveillance including use of contrast, risks from invasive medical procedures or anesthesia, and false positive or false negative results. The importance of regular thorough physical examination and targeted history is emphasized, as well as the prompt assessment of any medical concern.

For early identification of ACC, ultrasound is recommended until 18 years of age, with or without ACC-specific blood tests every 3–4 months. Given its low incidence in adults, there is no need for specific ACC surveillance after childhood. The lifelong brain tumor risk justifies annual brain MRI in all age groups. In order to minimize the potential risk for gadolinium accumulation in individuals undergoing multiple enhanced MRIs, if the initial MRI performed with a gadolinium-based contrast agent (GBCA) shows normal results, the following MRIs may be conducted without GBCA unless an abnormality is seen. For solid tumor surveillance, specifically the lifelong risk of sarcoma, an annual rapid whole body MRI (WBMRI) including limbs, is recommended to all individuals. In addition, abdominal and pelvic ultrasound is recommended annually in adults (while children will have such imaging every 3–4 months as above). The adult abdominal imaging should be every 6 months—alternating between WBMRI and ultrasound. The annual WBMRI cannot replace dedicated brain MRI, as was demonstrated by Ballinger et al. (2017) in a meta-analysis, and it is generally suggested that the two examinations should alternate. In adults, as the risk for gastrointestinal cancers increases, a dedicated periodic exam (colonoscopy/upper endoscopy) is indicated starting at age 25. Annual dermatology exams should start no later than at 18 years of age, as are breast cancer prevention strategies.

While this protocol is currently widely accepted, changes will evolve with more universal uptake and as more experience is reported. Specifically, genotype: phenotype correlations will be clarified, permitting differential recommendations by specific organ risk and disease penetrance.

Treatment of Malignancies in the Context of LFS

In multiple tumor types, somatic *TP53* mutation was proven to confer poorer prognosis in comparison to wild type. Likewise, the prognosis of some specific tumors was reported to be worse among *TP53* germline carrier in comparison to the same tumors when occurring sporadically.

Since the key role of p53 is in response to DNA damage, one could make an argument of the need to minimize exposure of these patients to radiotherapy and some chemotherapy. Indeed, the deleterious effect of radiation in animal models of LFS is well established. Data among LFS patients is not vast, yet is growing. There are several case reports and small case series that integrate a growing volume of evidence suggesting an increased risk of second malignancies in the radiation field of patients with LFS. For example, Bougeard et al. (2015) reported development of tumors within the radiotherapy fields in 30% of mutation carriers. Hisada et al. (1998) studied 200 LFS patients of whom 30 had multiple primary cancers. Nine of those 30 had radiation in the past, in six of which the secondary tumor was within the radiation field. Hence, trying to avoid radiation therapy when feasible, especially in high dose and large volumes, is recommended. However, it should be noted that in the context of treatment discussions for large or high-grade malignancies, omitting radiation may not be a viable therapeutic option. More knowledge of the effects of radiation as well of chemotherapy and other agents in this setting is needed.

An exciting new direction in treatment options for LFS patients is coming from studies of specific treatments aimed at restoring the function of p53. PRIMA-1 (p53 reactivation and induction of massive apoptosis) and its derivate, APR-246 (PRIMA-1^{MET}), were studied for their ability to restore some of the functions of mutant p53. In various tumor cell lines they were shown to convert p53 to its active conformation, inhibit proliferation and promote apoptosis. Antitumor activity has been demonstrated in several xenograft models and the first human studies showed a good safety profile. Other promising agents such as pyrazoles (e.g., PK7088) bind to specific p53 mutated proteins, and restore their conformation and activity to some extent. Zinc-metallochaperones can transport zinc to specific zinc-deficient p53 mutant proteins, so that the protein's folding and function are restored.

Metformin, a commonly used antidiabetic drug, was shown to impact several pathways in cancer, one of which is p53. Metformin is known to inhibit mitochondrial respiration, which is essential to cancer progression. Wang et al. (2017) took mice with a known *Trp53* germline mutation and generated an additional mutation in their mitochondria, causing a decreased oxygen consumption rate. These mice had increased cancer free survival as compared to the other *Trp53* mutated mice. Metformin was given to the mutant mice leading to the same effect on mitochondria as the double mutant ones, of reduced oxygen consumption and induction of antiproliferation signaling cascade. Metformin given to LFS (human) patients, resulted in decreased oxygen consumption rate in mononuclear cells, with decreased mitochondrial activity and similar anticell proliferation signaling. These are all examples of the evolving approach to make p53 a targetable genetic lesion, that will hopefully change the current treatment of LFS specifically, and cancer in general.

Ethical and Psychological Considerations

The potential ethical and psychosocial impact of being able to predictively screen unaffected family members or children for disease predisposition raises a number of complex issues.

While this article is not meant to discuss these in detail, one would be remiss to ignore their significance. Predictive testing for germline *TP53* mutations (or any other disease-predisposing gene) must be based on theoretical principles of respect for the autonomy of the patient and freedom from coercion, benefit to the patient, universal accessibility and freedom from discrimination, and maintenance of confidentiality of results. In the evolving flood of disease and cancer-predisposing genes that are being made available for mutation screening, guidelines addressing the following issues must now be established using *TP53* testing as a model: (1) who is to have access to the information, (2) what safeguards are to be established to protect employees and others from discrimination, (3) at what stage in the development of these genetic tests should mass screening be advocated, and (4) when might mandatory genetic screening be acceptable practice?

For several reasons, testing cannot at present be offered to the general population. Even within the general cancer population the carrier rate is demonstrably low. However, mass screening can be considered in populations that carry a high mutation rate. Such an exceptional example is the southern Brazilian population. Indeed, in a Brazilian study, neonatal screening for the p.R337H mutation followed by surveillance in mutation carriers led to the detection of lower stages of ACCs. The sensitivity and specificity of screening methods to identify mutations are yet not well known and both false-positive and false-negative results have been encountered. The sequence-confirmed presence of a base pair alteration itself must be interpreted carefully to ensure that its protein product has significance in that in some way it inactivates normal p53 function.

Furthermore, effective ways must be developed to provide informed consent to those being screened for a *TP53* germline mutation, about potential beneficial and harmful psychologic and social sequelae. Multidisciplinary services should be incorporated, including oncology, psychiatry, psychology, genetic counseling, medical ethics, and molecular and medical genetics. Such services require skills in communicating risk information and how to motivate adherence to cancer prevention and early detection.

Yet ethical considerations should be taken into account: Is there an obligation to inform family members of individuals who are identified as having such a genetic susceptibility? How aggressively should people at genetic risk for cancer be recruited into surveillance protocols, or clinical trials?

The issue of prenatal testing is also challenging. Risks for future children in the family should be addressed, and discussion with families should include options for preimplantation genetic diagnosis, prenatal diagnosis or testing of infants after birth. The

potential to reduce risk of losing a child or young adult makes prenatal testing compelling to some families. For other parents, prenatal diagnosis will not change the fate of the pregnancy but can be beneficial in term of very early initiation of surveillance protocol. Furthermore, the option of preimplantation genetic diagnosis (PGD) is also a possible with the use of in vitro fertilization (IVF) techniques, and should be discussed when feasible.

The psychological burden of the identification of a cancer predisposition syndrome has to be taken into consideration. While some individuals who are de novo carriers do not have personal of familial experience of cancer, others are very familiar with malignancies and their outcomes. The perceptions are hence different. While some individuals will prefer to disregard such diagnosis, other advocate for a more active route. Although surveillance confers physical, psychosocial, and financial challenges, and sometimes increased anxiety, many individuals believe in the value of this approach. In fact, some of the patients going through surveillance report enhanced sense of control, security, and empowerment facing this very poor prognostic syndrome.

Concluding Remarks

Decades after its first description, and years after the identification of its cause, our knowledge of Li–Fraumeni syndrome has expanded dramatically. However, the prognosis for individuals with LFS is still grim. Comprehensive data of the molecular causes, clinical manifestations and better understanding of genotype: phenotype interactions will allow for better prediction of disease. Improving surveillance, which in turn will allow for earlier detection of tumor will greatly influence the longevity and quality of life of the patient. Most importantly, discovering ways to make p53 a drugable target will make the most substantial contribution. Not only will we be able to treat premalignant lesions in the setting of germline mutations, the impact on all cancers, sporadic and predisposed, will be enormous.

See also: Cell Responses to DNA Damage. TP53.

References

Ballinger, M.L., et al., 2017. Baseline surveillance in Li-Fraumeni syndrome using whole-body magnetic resonance imaging: A meta-analysis. JAMA Oncology 3 (12), 1634–1639. https://doi.org/10.1001/jamaoncol.2017.1968.

Bond, G.L., et al., 2004. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 119 (5), 591–602.

Bougeard, G., et al., 2015. Revisiting Li–Fraumeni syndrome from TP53 mutation carriers. Journal of Clinical Oncology 33 (21), 2345–2352. https://doi.org/10.1200/JCO.2014.59.5728.

Chompret, A., et al., 2001. Sensitivity and predictive value of criteria for p53 germline mutation screening. Journal of Medical Genetics 38 (1), 43–47.

Chène, P., 1998. In vitro analysis of the dominant negative effect of p53 mutants. Journal of Molecular Biology 281 (2), 205–209.

Donehower, L.A., et al., 1992. Mice deficient for *p53* are developmentally normal but susceptible to spontaneous tumors. Nature 356, 215.

Eeles, R.A., 1995. Germline mutations in the TP53 gene. Cancer Surverys 25, 101–124.

Hisada, M., et al., 1998. Multiple primary cancers in families with Li-Fraumeni syndrome. Journal of the National Cancer Institute 90 (8), 606–611.

Hwang, S.J., et al., 2003. Germline p53 mutations in a cohort with childhood sarcoma: Sex differences in cancer risk. American Journal of Human Genetics 72, 975–983, 975.

Kuperwasser, C., et al., 2000. Development of spontaneous mammary tumors in BALB/c p53 heterozygous mice. A model for Li-Fraumeni syndrome. American Journal of Pathology 157 (6), 2151–2159.

Lang, G.A., et al., 2004. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell 119 (6), 861–872.

Li, F.P., Fraumeni Jr., J.F., 1969. Rhabdomyosarcoma in children epidemiologic study and identification of a familial cancer syndrome. Journal of the National Cancer Institute 43, 1365–1373.

Liu, G., et al., 2000. High metastatic potential in mice inheriting a targeted p53 missense mutation. Proceedings of the National Academy of Sciences 97 (8), 4174–4179.

Mai, P.L., et al., 2016. Risks of first and subsequent cancers among TP53 mutation carriers in the National Cancer Institute Li-Fraumeni syndrome cohort. Cancer 122 (23), 3673–3681.

Malkin, D., et al., 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 250 (4985), 1233–1239.

Olive, K.P., et al., 2004. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell 119 (6), 847–860.

Olivier, M., Goldgar, D.E., Sodha, N., 2003. Li–Fraumeni and related syndromes: Correlation between tumor type, family structure, and TP53 genotype. Cancer Research 63, 6643–6650.

Rausch, T., et al., 2012. Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. Cell 148 (1–2), 59–71. https://doi.org/10.1016/j.cell.2011.12.013.

Shlien, A., et al., 2008. Excessive genomic DNA copy number variation in the Li–Fraumeni cancer predisposition syndrome. Proceedings of the National Academy of Sciences of the United States of America 105 (32), 11264–11269. https://doi.org/10.1073/pnas.0802970105.

Tabori, U., et al., 2007. Younger age of cancer initiation is associated with shorter telomere length in Li-Fraumeni syndrome. Cancer Research 67 (4), 1415–1418.

Tinat, J., et al., 2009. 2009 version of the Chompret criteria for Li–Fraumeni syndrome. Journal of Clinical Oncology 27, 108–109.

Villani, A., et al., 2016. Biochemical and imaging surveillance in germline TP53 mutation carriers with Li–Fraumeni syndrome: 11 year follow-up of a prospective observational study. The Lancet Oncology 17 (9), 1295–1305. https://doi.org/10.1016/S1470-2045(16)30249-2.

Wang, P.Y., et al., 2017. Inhibiting mitochondrial respiration prevents cancer in a mouse model of Li–Fraumeni syndrome. The Journal of Clinical Investigation 127 (1), 132–136. https://doi.org/10.1172/JCI88668.

Further Reading

Ariffin, H., et al., 2014. Whole-genome sequencing analysis of phenotypic heterogeneity and anticipation in Li–Fraumeni cancer predisposition syndrome. Proceedings of the National Academy of Sciences of the United States of America 111 (43), 15497–15501. https://doi.org/10.1073/pnas.1417322111.

Deb, S., Deb, S.P., 2014. Mutant p53 and MDM2 in cancer. Springer, Berlin.
Duffy, M.J., Synnott, N.C., Crown, J., 2017. Mutant p53 as a target for cancer treatment. European Journal of Cancer 83, 258–265. https://doi.org/10.1016/j.ejca.2017.06.023.
Hainaut, P., Olivier, M., Wiman, K.G., 2013. p53 in the clinics. Springer, Berlin.
Kool, M., et al., 2014. Genome sequencing of SHH medulloblastoma predicts genotype-related response to smoothened inhibition. Cancer Cell 25 (3), 393–405. https://doi.org/10.1016/j.ccr.2014.02.004.
Kratz, C.P., et al., 2017. Cancer screening recommendations for individuals with Li–Fraumeni syndrome. Clinical Cancer Research 23 (11), e38–e45. https://doi.org/10.1158/1078-0432.CCR-17-0408.
Zhang, J., et al., 2015. Germline mutations in predisposition genes in pediatric Cancer. New England Journal of Medicine 373 (24), 2336–2346. https://doi.org/10.1056/NEJMoa1508054.
Zhukova, N., et al., 2013. Subgroup-specific prognostic implications of TP53 mutation in medulloblastoma. Journal of Clinical Oncology 31 (23), 2927–2935. https://doi.org/10.1200/JCO.2012.48.5052.

Relevant Websites

http://p53.iarc.fr/TP53GeneVariations.aspx—IARC p53 updated database.
https://www.lfsassociation.org—Li–Fraumeni association.
http://p53.fr/—The TP53 website.
http://exac.broadinstitute.org/gene/ENSG00000141510—The Exac browser.

Lipid Metabolism

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Introduction

This is widely recognized that tumors display an increased ability to synthesize lipids, and that this lipogenesis is tightly coupled to glucose metabolism. The endogenously synthesized lipids fuel membrane biogenesis in rapidly proliferating cancer cells. Lipids may also play substantial role in cell signaling, operating as second messengers and hormones. Changes in lipid metabolism may also affect cell growth, proliferation, differentiation, and motility. Major lipid synthesis pathways include *de novo* fatty acid synthesis pathway and mevalonate pathway; that leads to the synthesis of cholesterol and isoprenoids. The significance of these pathways in tumorigenesis is widely reported.

De Novo Fatty Acid Synthesis Pathway

De novo fatty acid synthesis or *de novo* lipogenesis is a coordinated series of enzymatic reactions that moderates the flow of carbons from different sources to fatty acids (Fig. 1). The first step of *de novo* lipogenesis is the conversion of cytoplasmic citrate to acetyl-CoA by ATP-citrate lyase (ACLY). The resulting acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACACA). Acetyl-CoA and malonyl-CoA are then coupled to the acyl-carrier protein domain of the rate-limiting enzyme fatty acid synthase (FASN).

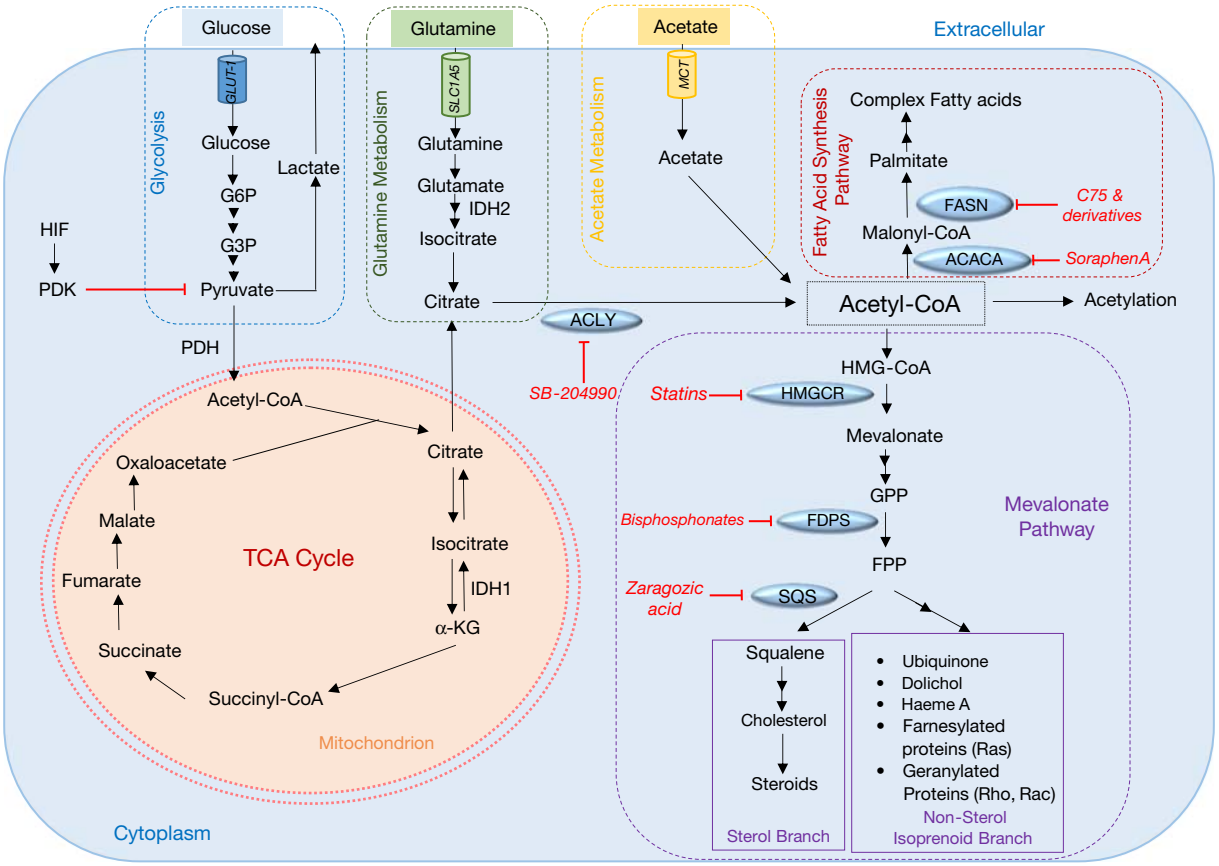

Fig. 1 Overview of cellular lipid metabolism. *De novo* lipogenesis and mevalonate pathway are fueled by acetyl-CoA derived from glucose, glutamine and/or acetate. Intermediates of these pathways yield diverse biosynthetic precursors for macromolecule production necessary for cell proliferation and survival. Key metabolic enzymes known to be upregulated during cancer are represented in blue. Some of the common pharmacological inhibitors of these pathways are indicated in *red*. Many reactions and their ability to be reversed are omitted for simplicity. Abbreviations used: *α-KG*, α-ketoglutarate; *G3P*, glyceraldehyde-3-phosphate; *G6P*, glucose-6-phosphate; *HIF*, hypoxia-inducible factor; *MCT*, monocarboxylate transporter; *IDH*, isocitrate dehydrogenase; *ACACA*, acetyl-CoA carboxylase; *ACLY*, ATP-citrate lyase; *FASN*, fatty acid synthase; *HMGCR*, 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase; *FDPS*, farnesyl disphosphate synthase; *FPP*, farnesyl pyrophosphate; *GLUT*, glucose transporter; *PDH*, pyruvate dehydrogenase; *PDK*, pyruvate dehydrogenase kinase; *SLC1A5*, solute carrier family 1 member 5; *TCA*, tricarboxylic acid.

Repeated condensations of acetyl groups generate palmitic acid (16:0)—a basic 16'carbon saturated fatty acid. Palmitic acid is further elongated and desaturated to generate complex fatty acids (e.g., stearate).

Cancer cells are shown to display up-regulated *de novo* lipogenesis, whereas most nonmalignant counterparts preferentially acquire fatty acids from exogenous sources. Certain types of nonmalignant cells/tissues—including adipocytes, hepatocytes, hormone-sensitive cells and fetal lung tissue—also display active *de novo* lipogenesis. However, in general *de novo* lipogenesis is suppressed in most nonmalignant cells.

Several lines of evidence suggest that activation of the *de novo* fatty acid synthesis pathway is required for carcinogenesis. Chemical or RNA interference-mediated inhibition of the key enzymes involved in fatty acid synthesis, including FASN, ACACA and ACLY, has been shown to attenuate cancer cell growth and to induce cancer cell death. Moreover, enhanced expression of FASN is correlated with poor prognosis in cancer patients.

The mammalian cells have a limited ability to synthesize polyunsaturated fatty acids *de novo*, because they lack the Δ12 desaturase—an enzyme which introduces a second *cis* double bond into monounsaturated fatty acids. Therefore, enhanced *de novo* lipogenesis enriches the cancer cell membranes with saturated and/or mono-unsaturated fatty acids. As these fatty acids are less prone to lipid peroxidation than polyunsaturated acyl chains, *de novo* fatty acid synthesis is suggested to make cancer cells more resistant to oxidative stress-induced cell death. Moreover, as saturated lipids pack more densely, the increased lipogenesis also alters lateral and transverse membrane dynamics that may limit the uptake of drugs, rendering the cancer cells more resistant to therapy. The altered saturation index of cancer cell membrane also affects fundamental cellular processes including signal transduction, gene expression and ciliogenesis. Furthermore, *de novo* fatty acid synthesis may also regulate the formation and composition of membrane structures that orchestrate signal transduction and motility (e.g., lipid rafts, invadopodia, and blebs). Lipogenesis can also control biosynthesis of lipid signaling effectors implicated in cancer genesis and progression, including phosphatidylinositols, lysophosphatidic acid, and prostanoids.

In nonmalignant cells fatty acids are also used to supply energy via a highly regulated process-lipolysis. Three lipases—adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL)—act in a stepwise series of reactions in order to generate diacyl glycerol (DAG), monoacylglycerol (MAG), and glycerol respectively, with subsequent release of three fatty acids. Free cytoplasmic fatty acids are then activated, by coupling to coenzyme A followed by exchange of acyl chain to carnitine via carnitine acyltransferase, to be transported into the mitochondrial matrix. Repeated rounds of this process (known as β-oxidation) guarantees the balance between lipogenesis and lipid digestion in nonmalignant cells. Certain types of tumors, including prostate tumors, display increased dependence on β-oxidation of fatty acids as their main source of energy. Human leukemia cells are also shown to require β-oxidation for their proliferation and survival.

Mevalonate Synthesis Pathway

The mevalonate pathway leads to the synthesis of sterols and isoprenoids that are shown to be crucial for tumor-growth. Multiple enzymes of this pathway are recognized to be essential for proliferation and survival of various types of cancer cells. The first committed step of the mevalonate pathway is conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonic acid (mevalonate) by HMG-CoA reductase (HMGCR) (**Fig. 1**). In the next step of the pathway mevalonate is metabolized to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Farnesyl pyrophosphate synthase catalyzes sequential condensation reactions of DMAPP with two units of IPP to form farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate synthase catalyzes yet another condensation reaction to form geranylgeranyl pyrophosphate (GGPP). FPP is the branch point for several pathways leading to various end-products including cholesterol, steroid and dolichols. Additionally, FPP can also be converted into geranylgeranyl pyrophosphate (GGPP) by GGPP synthase. Squalene synthase catalyzes the first reaction of the pathway committed exclusively to cholesterol biosynthesis and plays a crucial role in directing intermediates to either sterol or non-sterol branches of this metabolic pathway.

The oncogenic potential of the mevalonate pathway has also been investigated. Mevalonate pathway is shown to induce proliferation in primary leukemia cells. HMGCR is also suggested to be a candidate metabolic oncogene. It was shown that ectopic expression of HMGCR accentuates growth of transformed and non-transformed cells and cooperates with RAS to drive the transformation of primary mouse embryonic fibroblasts cells. Moreover, direct administration of mevalonate via miniosmotic pumps into mice harboring breast cancer cell line xenografts resulted in increased tumor-growth. Higher expression of different genes of the mevalonate pathway has also been correlated with poor prognosis in breast cancer patients.

As mentioned above the mevalonate pathway generates various metabolites that are implicated in carcinogenesis. For instance, cholesterol is shown to be involved in cancer cell proliferation and protection of cancer cells against immune surveillance as well as various therapeutic agents. In addition to that cholesterol serves as the precursor for synthesis of steroid hormones and oxysterols that are suggested to play various roles in cancer progression.

Farnesyl-diphosphate and geranylgeranyl-diphosphate are respectively involved in farnesylation and geranylgeranylation of a variety of proteins. Farnesylation and geranylgeranylation are required for the ability of Ras and Rho proteins to induce malignant transformation, invasion, and metastasis. Dolichol is an essential component of the N-glycosylation of nascent polypeptide. Protein N-glycosylation is often altered in cancer and may contribute in tumor formation progression. On the other hand, complex branching of N-glycans also leads to tumor-suppressive properties in some cancers. Coenzyme Q is crucial for ATP production in cancer cells that rely on oxidative phosphorylation to produce energy.

Therapeutic Targeting

Studies on significance of *de novo* lipid synthesis pathways in cancer progression suggest that the enzymes involved in these pathways would be rational therapeutic targets for cancer treatment. Multiple studies on therapeutic targeting of *de novo* fatty acid synthesis pathways mainly focused on pharmacological inhibition of FASN (Fig. 1). The effects of FASN-inhibitors have been examined in diverse preclinical tumor models. These inhibitors are effective in chemoprevention of breast cancer in HER2/neu transgenic mice. They also reduce incidence of chemically induced lung tumorigenesis. Several studies have shown that inhibition of other key enzymes involved in fatty acid synthesis pathway—including ACACA and SCD—limit the growth and proliferation of cancer cells. Silencing of ACLY has also been reported to prevent cancer cell growth both in vivo and in vitro.

The significance of inhibitors of the mevalonate pathway as antitumor agents has also been investigated. Statins—potent competitive inhibitors of HMGCR—have been shown to induce growth arrest and apoptosis in cancer cells both in vitro and in vivo. Moreover, bisphosphonates—inhibitors of FPP synthase—are also approved for the treatment of multiple myeloma breast and prostate cancer. Zaragozic acid A—inhibitor of squalene synthase—attenuates proliferation and induces death of prostate cancer cells. Table 1 provides a list of pharmacological inhibitors of *de novo* lipid synthesis in clinical or preclinical trials.

Table 1 List of pharmacological inhibitors of lipid metabolism pathways

Compound/inhibitor	*Target*	*Drug development stage*	*Cancer type*	*Selected references*
Fatty acid synthesis pathway				
SB-204990	ACLY	Preclinical	Solid and non solid tumors	Aisenberg (1961), Alli et al. (2005), Baenke et al. (2013), and Baron et al. (2004)
TOFA	ACACA	Preclinical	Lung, colon, and breast cancer	Aisenberg (1961), Alli et al. (2005), Bauer et al. (2005), Beckers et al. (2007), and Beloribi-Djefaflia et al. (2016)
Soraphen A	ACACA	Preclinical	Prostate cancer	Aisenberg (1961) and Boroughs and DeBerardinis (2015)
Metformin	ACACA	FDA approved	Solid tumors	Chajes et al. (2006)
GSK2194069	FASN	Preclinical	Prostate cancer	Aisenberg (1961), and Das and Hoefler (2013)
Triclosan	FASN	Preclinical	Prostate cancer, breast cancer	Aisenberg (1961), Alli et al. (2005), DeBerardinis et al. (2008), Effert et al. (1996), and Fackler and Grosse (2008)
Fasnall	FASN	Preclinical	NA	Fritz et al. (2010)
FAS31	FASN	Preclinical	Ovarian cancer	Aisenberg (1961) and Gao and Zhang (2008)
C247	FASN	Preclinical	Non-small-cell lung cancer and breast cancer	Aisenberg (1961), Hanai et al. (2011), and Hatzivassiliou et al. (2005)
BZ36	SCD	Preclinical	Prostate cancer	Aisenberg (1961) and Kridel et al. (2004)
A939572	SCD	Preclinical	Solid tumors	Aisenberg (1961), Alli et al. (2005), Kuhajda (2006), Kuhajda et al. (2000), and Kusakabe et al. (2000)
Transcriptional regulators				
Fatostatin	SREBPs	Preclinical	Prostate cancer	Aisenberg (1961), Liu et al. (2010), and Mashima et al. (2009)
Betulin	SREBPs	Preclinical	Multiple cancers	Aisenberg (1961) and Mason et al. (2012)
Mevalonate pathway				
Statins	HMGCR	Clinical and Preclinical	Solid tumors	Alli et al. (2005) and Menendez and Lupu (2007)
Simvastatin	HMGCR	Preclinical	Prostate cancer	Alli et al. (2005) and Mullen et al. (2016)
FAs oxidation				
Etomoxir	CPT1	Preclinical	Prostate cancer, Leukemia and myeloma	Aisenberg (1961), Alli et al. (2005), Orita et al. (2008), Pavlova and Thompson (2016), and Price et al. (2002)
Perhexiline	CPT1	Preclinical	Leukemia	Aisenberg (1961) and Rohrig and Schulze (2016)
Triacscin C	ACS	Preclinical	Solid tumors	Aisenberg (1961) and Rysman et al. (2010)
Lipolysis				
JZL184	MAGL	Preclinical	Solid tumors	Fritz et al. (2010) and Samudio et al. (2010)
CT-32501	AGPAT	Preclinical	Solid tumors	Fritz et al. (2010) and Stylli et al. (2008)

Key: *ACACA*, (acetyl-CoA carboxylase A); *ACLY* (ATP-citrate lyase); *ACP*, (acyl carrier protein); *ACS*, (acyl-CoA synthetases); *AGPAT*, (acylglycerolphosphate acyltransferase); *AMPK*, (AMP-activated protein kinase); *CPT1* (carnitine palmitoyltransferase); *FASN*, (fatty acid synthase); *HMGCR*, (3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase); FDPS, (farnesyl disphosphate synthase); *MAGL*, (monoacylglycerol lipase); *SCD*, (stearoyl-CoA desaturase); *SCAP*, (SREBP cleavage-activating protein); *SREBPs*, (sterol regulatory element binding protein); *TOFA*, (5-tetradecyl-oxy-2-furoic acid).

See also: Dietary Factors and Cancer.

References

Aisenberg, A.C., 1961. The glycolysis and respiration of tumors. Academic Press, New York, pp. pp. 12–13.

Alli, P.M., Pinn, M.L., Jaffee, E.M., McFadden, J.M., Kuhajda, F.P., 2005. Fatty acid synthase inhibitors are chemopreventive for mammary cancer in neu-N transgenic mice. Oncogene 24, 39–46.

Baenke, F., Peck, B., Miess, H., Schulze, A., 2013. Hooked on fat: The role of lipid synthesis in cancer metabolism and tumor development. Disease Models and Mechanisms 6 (6), 1353–1363.

Baron, A., Migita, T., Tang, D., Loda, M., 2004. Fatty acid synthase: A metabolic oncogene in prostate cancer? Journal of Cellular Biochemistry 91, 47–53.

Bauer, D.E., Hatzivassiliou, G., Zhao, F., Andreadis, C., Thompson, C.B., 2005. ATP citrate lyase is an important component of cell growth and transformation. Oncogene 24, 6314–6322.

Beckers, A., Organe, S., Timmermans, L., Scheys, K., Peeters, A., et al., 2007. Chemical inhibition of acetyl-CoA carboxylase induces growth arrest and cytotoxicity selectively in cancer cells. Cancer Research 67, 8180–8187.

Beloribi-Djefaflia, S., Vasseur, S., Guillaumond, F., 2016. Lipid metabolic reprogramming in cancer cells. Oncogenesis 5, e189.

Boroughs, L.K., DeBerardinis, R.J., 2015. Metabolic pathways promoting cancer cell survival and growth. Nature Cell Biology 17 (4), 351–359.

Chajes, V., Cambot, M., Moreau, K., Lenoir, G.M., Joulin, V., 2006. Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. Cancer Research 66, 5287–5294.

Das, S.K., Hoefler, G., 2013. The role of triglyceride lipases in cancer associated cachexia. Trends in Molecular Medicine 19, 292–301.

DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G., Thompson, C.B., 2008. The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. Cell Metabolism 7 (1), 11–20.

Effert, P.J., Bares, R., Handt, S., Wolff, J.M., Bull, U., et al., 1996. Metabolic imaging of untreated prostate cancer by positron emission tomography with 18fluorine-labeled deoxyglucose. The Journal of Urology 155, 994–998.

Fackler, O.T., Grosse, R., 2008. Cell motility through plasma membrane blebbing. The Journal of Cell Biology 181, 879–884.

Fritz, V., Benfodda, Z., Rodier, G., Henriquet, C., Iborra, F., et al., 2010. Abrogation of *de novo* lipogenesis by stearoyl-CoA desaturase 1 inhibition interferes with oncogenic signaling and blocks prostate cancer progression in mice. Molecular Cancer Therapeutics 9, 1740–1754.

Gao, X., Zhang, J., 2008. Spatiotemporal analysis of differential Akt regulation in plasma membrane microdomains. Molecular Biology of the Cell 19, 4366–4373.

Hanai, J., Doro, N., Sasaki, A.T., Kobayashi, S., Cantley, L.C., et al., 2011. Inhibition of lung cancer growth: ATP citrate lyase knockdown and statin treatment leads to dual blockade of mitogen-activated protein Kinase (MAPK) and Phosphatidylinositol-3-kinase (PI3K)/AKT pathways. Journal of Cellular Physiology 227, 1709–1720.

Hatzivassiliou, G., Zhao, F., Bauer, D.E., Andreadis, C., Shaw, A.N., et al., 2005. ATP citrate lyase inhibition can suppress tumor cell growth. Cancer Cell 8, 311–321.

Kridel, S.J., Axelrod, F., Rozenkrantz, N., Smith, J.W., 2004. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. Cancer Research 64, 2070–2075.

Kuhajda, F.P., 2006. Fatty acid synthase and cancer: New application of an old pathway. Cancer Research 66, 5977–5980.

Kuhajda, F.P., Pizer, E.S., Li, J.N., Mani, N.S., Frehywot, G.L., et al., 2000. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. Proceedings of the National Academy of Sciences of the United States of America 97, 3450–3454.

Kusakabe, T., Maeda, M., Hoshi, N., Sugino, T., Watanabe, K., et al., 2000. Fatty acid synthase is expressed mainly in adult hormone-sensitive cells or cells with high lipid metabolism and in proliferating fetal cells. The Journal of Histochemistry and Cytochemistry 48, 613–622.

Liu, Y., Zuckier, L.S., Ghesani, N.V., 2010. Dominant uptake of fatty acid over glucose by prostate cells: A potential new diagnostic and therapeutic approach. Anticancer Research 30, 369–374.

Mashima, T., Seimiya, H., Tsuruo, T., 2009. *De novo* fatty-acid synthesis and related pathways as molecular targets for cancer therapy. British Journal of Cancer 100, 1369–1372.

Mason, P., Liang, B., Li, L., Fremgen, T., Murphy, E., et al., 2012. SCD1 inhibition causes cancer cell death by depleting mono-unsaturated fatty acids. PLoS One 7, e33823.

Menendez, J.A., Lupu, R., 2007. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nature Reviews Cancer 7, 763–777.

Mullen, P.J., Yu, R., Longo, J., Archer, M.C., Penn, L.Z., 2016. The interplay between cell signaling and the mevalonate pathway in cancer. Nature Reviews Cancer 16 (11), 718–731.

Orita, H., Coulter, J., Tully, E., Kuhajda, F.P., Gabrielson, E., 2008. Inhibiting fatty acid synthase for chemoprevention of chemically induced lung tumors. Clinical Cancer Research 14, 2458–2464.

Pavlova, N.N., Thompson, C.B., 2016. The emerging hallmarks of cancer metabolism. Cell Metabolism 23 (1), 27–47.

Price, D.T., Coleman, R.E., Liao, R.P., Robertson, C.N., Polascik, T.J., et al., 2002. Comparison of [18F] fluorocholine and [18F]fluorodeoxyglucose for positron emission tomography of androgen dependent and androgen independent prostate cancer. The Journal of Urology 168, 273–280.

Rohrig, F., Schulze, A., 2016. The multifaceted roles of fatty acid synthesis in cancer. Nature Reviews Cancer 16 (11), 732–749.

Rysman, E., Brusselmans, K., Scheys, K., Timmermans, L., Derua, R., et al., 2010. *De novo* lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. Cancer Research 70, 8117–8126.

Samudio, I., Harmancey, R., Fiegl, M., Kantarjian, H., Konopleva, M., et al., 2010. Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. The Journal of Clinical Investigation 120, 142–156.

Stylli, S.S., Kaye, A.H., Lock, P., 2008. Invadopodia: At the cutting edge of tumor invasion. Journal of Clinical Neurosciences 15, 725–737.

Further Reading

Swinnen, J.V., Brusselmans, K., Verhoeven, G., 2006. Increased lipogenesis in cancer cells: New players, novel targets. Current Opinion in Clinical Nutrition & Metabolic Care 9 (4), 358–365.

Wagle, S., Bui, A., Ballard, P.L., Shuman, H., Gonzales, J., et al., 1999. Hormonal regulation and cellular localization of fatty acid synthase in human fetal lung. The American Journal of Physiology 277, L381–390.

Willemarck, N., Rysman, E., Brusselmans, K., Van Imschoot, G., Vanderhoydonc, F., et al., 2010. Aberrant activation of fatty acid synthesis suppresses primary cilium formation and distorts tissue development. Cancer Research 70, 9453–9462.

Zaidi, N., Royaux, I., Swinnen, J.V., Smans, K., 2012a. ATP citrate lyase knockdown induces growth arrest and apoptosis through different cell- and environment-dependent mechanisms. Molecular Cancer Therapeutics 11, 1925–1935.

Zaidi, N., Swinnen, J.V., Smans, K., 2012b. ATP-citrate lyase: A key player in cancer metabolism. Cancer Research 72, 3709–3714.

Zaidi, N., Lupien, L., Kuemmerle, N.B., Kinlaw, W.B., Swinnen, J.V., Smans, K., 2013. Lipogenesis and lipolysis: The pathways exploited by the cancer cells to acquire fatty acids. Progress in lipid research 52 (4), 585–589.

Zha, S., Ferdinandusse, S., Hicks, J.L., Denis, S., Dunn, T.A., et al., 2005. Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer. Prostate 63, 316–323.

Zhan, Y., Ginanni, N., Tota, M.R., Wu, M., Bays, N.W., et al., 2008. Control of cell growth and survival by enzymes of the fatty acid synthesis pathway in HCT-116 colon cancer cells. Clinical Cancer Research 14, 5735–5742.
Zhang, F., Du, G., 2012. Dysregulated lipid metabolism in cancer. World Journal of Biological Chemistry 3 (8), 167–174.
Zhou, W., Han, W.F., Landree, L.E., Thupari, J.N., Pinn, M.L., et al., 2007. Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. Cancer Research 67, 2964–2971.

Lynch Syndrome

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Glossary

Epigenetic change Heritable alteration in the function (expression) of a gene without a change in DNA sequence. Epigenetic mechanisms include DNA methylation, histone modification, and noncoding RNA (e.g., micro-RNA)-based gene regulation.

Gene penetrance The proportion of individuals with a particular variant (genotype) that express an associated trait (phenotype).

Haploinsufficiency Half of the normal level of the protein product of a gene (as a result of a heterozygous mutation, for example) is insufficient for normal function.

Loss of heterozygosity (LOH) The absence of one of the two parental alleles in tumor tissue from an individual who is constitutionally heterozygous for the marker used to measure LOH. LOH can expose a mutant tumor suppressor gene in the remaining allele.

Microsatellite instability (MSI) The number of repeat units of a microsatellite repeat is different in tumor DNA compared to normal DNA from the same individual, resulting in a difference in the microsatellite length between normal and tumor tissue. During replication, DNA polymerase may slip from the DNA strand at the repeat region, causing deletion or addition of repeat units, and the errors remain if postreplicative mismatch repair is defective.

Abbreviations

CIMP CpG island methylator phenotype
CMMRD Constitutional mismatch repair deficiency
EMAST Elevated microsatellite alterations at selected tetranucleotide repeats
FCCTX Familial colorectal cancer, type X
LLS Lynch-like syndrome
LS Lynch syndrome
MMR DNA mismatch repair
MSI Microsatellite instability
MSI-H High-degree MSI
MTS Muir–Torre syndrome
TCGA The Cancer Genome Atlas
TS Turcot syndrome

Lynch Syndrome: Definitions and Phenotypes

Lynch syndrome (LS) is an autosomal dominant disorder caused by a defect in one of the DNA mismatch repair (MMR) genes. The syndrome is characterized by the development of colorectal carcinoma, endometrial carcinoma, and other cancers. Three main sets of clinical criteria have been formulated to select families suspected of having LS for molecular and other studies (Table 1). These include the stringent Amsterdam criteria I and II and the less stringent Bethesda criteria.

Absent MMR protein(s) and DNA microsatellite instability (MSI) serve as tumor markers for LS. These are not, however, specific for LS since similar abnormalities occur in 15% of sporadic cancers as well. A panel of five microsatellite markers, the so-called Bethesda panel (BAT25, BAT26, D2S123, D5S346, and D17S250) has been recommended for screening purposes. Instability at two or more microsatellite loci indicates high-degree MSI (MSI-H) (see section "MMR Gene Dysfunction and MSI").

Table 2 summarizes the clinical and genetic features of LS and its variants. The identification of a pathogenic germline mutation in one of the DNA MMR genes *MLH1* (MutL Homolog 1), *MSH2* (MutS Homolog 2), *MSH6* (MutS Homolog 6), or *PMS2* (post-meiotic segregation increased 2 (*S. cerevisiae*)) is required for LS diagnosis. The genetic definition of LS covers the Muir–Torre syndrome, where sebaceous gland tumors co-occur with LS-type internal malignancy. Rare individuals who inherit defective alleles from both parents in a recessive manner resulting in homozygosity or compound heterozygosity for predisposing mutation have constitutional mismatch repair deficiency (CMMRD). Clinical presentation is severe and includes childhood cancers such as hematological malignancies and brain tumors, and early-onset colorectal cancer. Germline mutations in MMR genes may also be responsible for Turcot syndrome (TS), in which primary brain tumors, usually glioblastomas, are accompanied by multiple colorectal

Table 1 The Amsterdam I and II and revised Bethesda criteria for the clinical diagnosis of LS

Amsterdam criteria I
There should be at least three relatives with colorectal cancer (CRC); all the following criteria should be present:
(1) One should be a first-degree relative of the other two
(2) At least two successive generations should be affected
(3) At least one CRC should be diagnosed before age 50
(4) Familial adenomatous polyposis should be excluded
(5) Tumors should be verified by pathological examination
Amsterdam criteria II
There should be at least three relatives with a Lynch syndrome-associated cancer (colorectal cancer (CRC), cancer of the endometrium, small bowel, ureter, or renal pelvis): all of the following criteria should be present:
(1) One should be a first-degree relative of the other two
(2) At least two successive generations should be affected
(3) At least one should be diagnosed before age 50
(4) Familial adenomatous polyposis should be excluded in the CRC case (s) if any
(5) Tumors should be verified by pathological examination
Revised Bethesda criteria
The criteria can be used to select individuals for MSI analysis. Individuals that meet one of these criteria are suspected of having Lynch syndrome.
(1) Colorectal cancer diagnosed in a patient $<$50 years of age
(2) Presence of synchronous, metachronous colorectal, or other Lynch syndrome-related tumors[a], regardless of age
(3) Colorectal cancer with MSI-H phenotype diagnosed in a patient $<$60 years of age
(4) Patient with colorectal cancer and a first-degree relative with a Lynch syndrome-related tumor, with one of the cancers diagnosed under age 50 years
(5) Patient with colorectal cancer with two or more first-degree or second-degree relatives with a Lynch syndrome-related tumor, regardless of age

[a]Lynch syndrome-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter, renal pelvis, biliary tract, and brain tumors, sebaceous gland adenomas, keratoacanthomas, and carcinoma of the small bowel.

Table 2 Lynch syndrome and related phenotypes

Syndrome	*MIM no.*[a]	*Predisposing genes and their relative shares of all mutations*[b]	*Mode of inheritance*	*Clinical features*
Lynch syndrome (LS)	120436 120435	*MLH1* (40%) *MSH2* (34%) *MSH6* (18%) *PMS2* (8%)	AD	Colonic and extracolonic cancers of a defined spectrum (Amsterdam criteria II), occurring earlier than in the average population (at ~40–60 years of age)
Muir–Torre syndrome (MTS)	158320	*MSH2* > *MLH1* > *MSH6*	AD	Multiple sebaceous gland adenomas co-occurring with visceral malignancies, such as colorectal carcinoma
Constitutional mismatch repair deficiency (CMMRD)	276300	*PMS2* > *MSH6* > *MSH2, MLH1*	AR	Childhood cancers, mainly hematological malignancies and/or brain tumors, combined with early-onset colorectal cancers
Turcot syndrome (TS)	276300	See CMMRD	AR (AD)[c]	Primary brain tumors co-occurring with multiple colorectal adenomas

AD, autosomal dominant; *AR*, autosomal recessive.
[a]Mendelian Inheritance in Man (www.omim.org/).
[b]The designation *A* > *B* indicates that mutations in gene *A* are more frequent than in gene *B*.
[c]TS arising as a variant of CMMRD shows recessive transmission. TS may also be associated with germline mutation of the Adenomatous Polyposis Coli (*APC*) gene (MIM no. 175100), in which case it is dominantly inherited.

adenomas. TS is a variant of LS if associated with a heterozygous MMR gene mutation, CMMRD if associated with a homozygous or compound heterozygous MMR gene mutation, and familial adenomatous polyposis if associated with a heterozygous mutation of the gene for adenomatous polyposis coli (*APC*).

LS is among the most prevalent hereditary cancer syndromes in man. It accounts for 1%–3% of unselected colorectal or endometrial carcinomas and some 15% of those with MSI or absent MMR protein. The population incidence of LS is around 1:400.

LS Genes and Their Functions

Postreplicative DNA Mismatch Repair

LS genes are conserved in evolution (Table 3). The postreplicative MMR system in *E. coli* includes three main proteins: MutS (recognizes the mismatch), MutH (marks the nascent strand by making a nick), and MutL (couples mismatch recognition to the

Table 3 Lynch syndrome genes in evolution and phenotypes resulting from germline mutations

E. coli	S. cerevisiae	H. sapiens	*Phenotype resulting from germline mutation*
MutS	Msh2	*MSH2*	LS, MTS, CMMRD[a]
	Msh6	*MSH6*	LS, CMMRD[a]
	Msh3	*MSH3*	Attenuated adenomatous polyposis/CMMRD[a]
	Msh1	Not identified	N/A
	Msh4	*MSH4*	No Lynch syndrome-associated mutations known
	Msh5	*MSH5*	No Lynch syndrome-associated mutations known
MutL	Mlh1	*MLH1*	LS, MTS, CMMRD[a]
	Pms1	*PMS2*	LS with reduced penetrance, TS/CMMRD[a]
	Mlh2	*PMS1*	No Lynch syndrome-associated mutations known
	Mlh3	*MLH3*	LS?
MutH	Not identified	Not identified	N/A

LS, Lynch syndrome; *MTS*, Muir–Torre syndrome; *CMMRD*, constitutional mismatch repair deficiency; *TS*, Turcot syndrome; *N/A*, not applicable.
[a]Biallelic germline mutations.

downstream events). MMR acts on the newly synthesized strand; therefore, a mechanism of strand discrimination is necessary. In *E. coli*, a methyl group on the template strand directs MutH to make a nick on the opposite strand. Thus, hemimethylated DNA is corrected on the unmethylated strand and the methylated strand serves as a template.

Multiple homologues of MutS and MutL exist in eukaryotes. In humans, five MutS homologues (MSH2, MSH6, MSH3, MSH4, and MSH5) and four MutL homologues (MLH1, PMS2, PMS1, and MLH3) have been identified and at least six of them play a direct role in MMR (**Table 3**, **Fig. 1**). MSH4 and MSH5 are necessary for meiotic (and possibly mitotic) recombination but do not participate in MMR. The role of human PMS1 in MMR is unclear (see later).

The MMR proteins form heterodimers in different combinations and have different substrate specificities (**Table 4**). The main mismatch-binding factor is hMutSα, consisting of MSH2 and MSH6, which recognizes single-base mispairs and insertion–deletion loops (IDLs). Another mismatch-binding heterodimer is hMutSβ, formed by MSH2 and MSH3, acting mainly on IDLs. According to

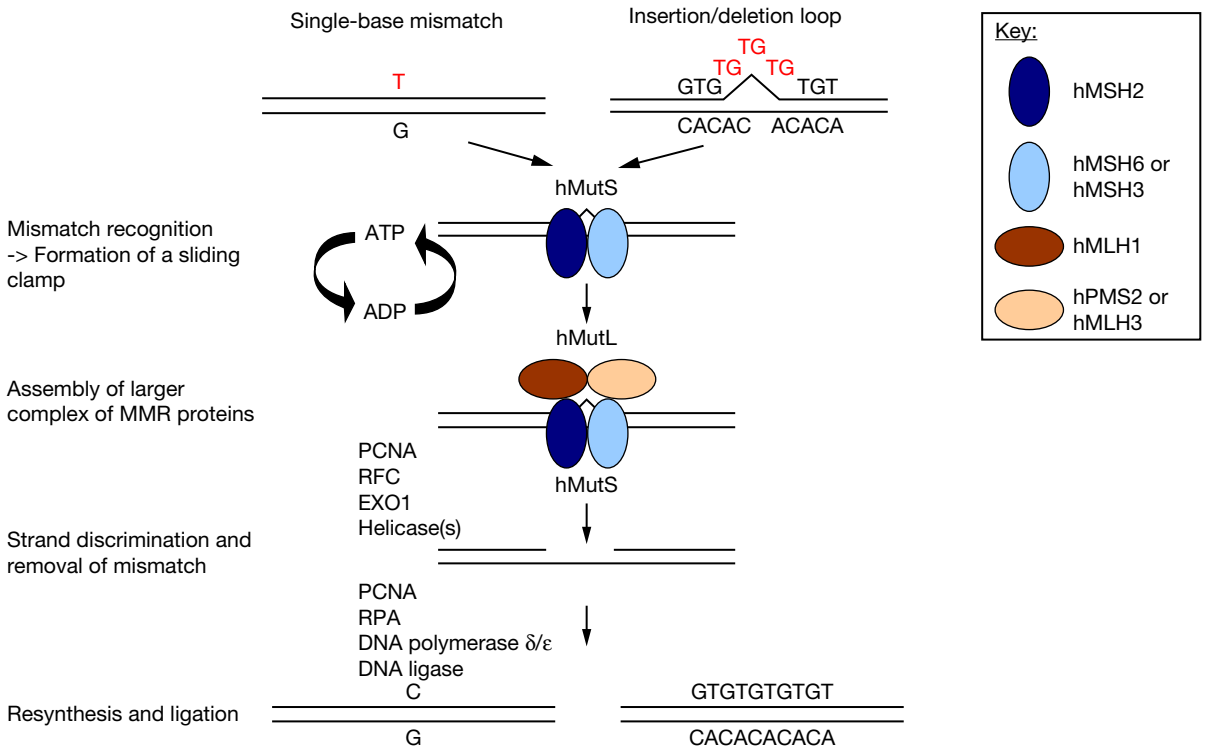


Fig. 1 DNA mismatch repair. The main biochemical steps necessary for the correction of replication errors, single-base mismatches, and insertion/deletion loops in the newly synthesized strand (*red*), are shown. *PCNA*, proliferating cell nuclear antigen; *RFC*, replication factor C; *EXO1*, exonuclease 1; *RPA*, replication protein A.

Table 4 Complexes of human MMR proteins and their substrate specificities

Heterodimer	*Components*	*Type of mismatches repaired*	*Predominant type of MSI (defective gene)*
hMutSα	MSH2 + MSH6	Single-base mismatches, ins/del loops	MSI-H (*MSH2* or *MSH6*)
hMutSβ	MSH2 + MSH3	Ins/del loops	MSI-L/EMAST (*MSH3*)
hMutLα	MLH1 + PMS2	Single-base mismatches, ins/del loops	MSI-H (*MLH1* or *PMS2*)
hMutLβ	MLH1 + PMS1	?	?
hMutLγ	MLH1 + MLH3	Single-base mismatches, small loops	MSI-L/EMAST or MSI-H (*MLH3*)

the "molecular switch" or "sliding clamp" model, the hMutS heterodimer binds to mismatched DNA in an ADP-bound form. Mismatch binding induces an ADP to ATP exchange that allows the hMutS complex to convert to a sliding clamp. The purpose of the sliding clamp that diffuses away from the mismatch is thought to be to empty the mismatch site for the loading of additional hMutS clamps and to recruit the hMutL heterodimer and the necessary downstream factors to the site of repair. The hMutL heterodimer coordinates the interplay between the mismatch recognition complex and other proteins necessary for MMR. The main hMutL complex is hMutLα, consisting of MLH1 and PMS2 and responsible for single-base mismatch and IDL repair. Alternative hMutL heterodimers are hMutLγ (MLH1 and MLH3), which may predominantly contribute to IDL repair, and hMutLβ (MLH1 and PMS1), which does not appear to participate in MMR.

When one of the component proteins (e.g., MSH2) is absent, its pair in the heterodimer (MSH6 in hMutSα) becomes unstable as well, whereas the expression of components belonging to other heterodimers (MLH1 and PMS2) is unaffected. Defined patterns of absent MMR proteins in immunohistochemical evaluations of tumor tissues can help prioritize individual MMR gene(s) for germline mutation screens in suspected LS.

No MutH homolog has been identified in yeast or mammalian cells, and two main alternative mechanisms for strand discrimination have been proposed. First, nicks that separate Okazaki fragments on the lagging strand may mark the nascent strand during replication. Second, MutLα has a proliferating cell nuclear antigen-dependent endonuclease activity that allows strand-specific cleavage.

The predominant role of *MLH1* and *MSH2* as LS predisposition genes (**Table 2**) is easy to understand given that their protein products are obligatory components in all types of heterodimers (**Table 4**), followed by *MSH6* and *PMS2*. *MLH3* mutations are rare (the protein is functionally redundant with PMS2). No LS-predisposing heterozygous germline mutations are known for *MSH3* (functionally redundant with *MSH6*); however, biallelic (homozygous) mutations may cause susceptibility to attenuated adenomatous polyposis. No convincing LS-associated germline mutations are known for *PMS1*, *MSH4*, or *MSH5*.

MMR Gene Dysfunction and MSI

Substrate specificities of the individual MMR proteins are reflected in different MSI phenotypes in tumors (**Table 4**). MSI-H is detected with the Bethesda panel of two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250); at least two unstable markers indicate MSI-H. (If only one marker or none is unstable, a tumor is said to have low-degree MSI (MSI-L) or be microsatellite-stable (MSS), respectively.) It has been suggested that the mononucleotide repeat markers BAT25 or BAT26 alone are sensitive and specific indicators of MSI-H. *MSH2* and *MLH1* mutations are associated with MSI-H involving mononucleotide and dinucleotide (and other short tandem) repeats. The same is true for *PMS2* mutations. In the case of *MSH6* mutations mononucleotide repeats are predominantly affected. In tumors from *MLH3* mutation carriers, mononucleotide repeats may be less informative than dinucleotide and tetranucleotide repeats and MSI phenotypes may range from MSI-high to the absence of MSI.

Loss of *MSH3* function may underlie a distinct form of MSI called EMAST (elevated microsatellite alterations at selected tetranucleotide repeats). EMAST and MSI-L are likely to be the same. EMAST can be frequent as an acquired defect in colorectal cancers or result from rare biallelic germline mutations in *MSH3* associated with polyposis predisposition. While no consensus definition for EMAST exists to date, a commonly used definition is 1–2 tetranucleotide repeat markers mutated out of 5 or more markers examined.

Other Functions of MMR Genes

Genomic instability is an "enabling characteristic" that, by generating genetic diversity, expedites the acquisition of the fundamental hallmarks of cancer that are prerequisites for tumor growth and metastasis. Apart from the correction of replication errors, MMR proteins have replication-independent (so-called noncanonical) functions whose failure may play important roles in conferring selective advantage on subclones of mutant cells. MMR proteins recognize diverse types of endogenous and exogenous damage, such as heterocyclic amine (e.g., PhIP) DNA adducts and damage induced by oxidation or alkylation. If possible, the lesions are corrected; otherwise, MMR proteins signal DNA damage to cell cycle arrest or apoptosis. In these processes, MMR proteins interact with other players of the DNA damage response pathway, including ATM and ATR, protein kinases involved in apoptosis and cell cycle regulation.

Recombination between DNA sequences that are related but nonidentical (homeologous) generates mismatches that act as substrates for the MMR pathway. MMR proteins correct mismatches occurring in recombination and suppress recombination between homeologous sequences. If recombination-related mismatches are not corrected, gene conversion is reduced in mitotic cells and "postmeiotic segregation" (PMS) is increased in meiotic cells (*S. cerevisiae*).

The MMR system can also promote mutations when appropriate. For example, MSH4 and MSH5 facilitate meiotic crossover between homologous chromosomes. MMR proteins also promote somatic hypermutation and class switch of antibody genes.

LS Genes and Knudson's Two-Hit Hypothesis

Generally, both gene copies of a given MMR gene need to be inactivated for a phenotype in agreement with Knudson's two-hit hypothesis for tumor suppressor genes. As a consequence, protein expression of the affected MMR gene is absent in tumor tissue and microsatellite repeats become unstable (Fig. 2, upper panel). The frequencies of immunohistochemical aberrations and especially MSI display some organ-specific variation (Fig. 2, lower panel), which is discussed in greater detail in the section "**LS Tumor Spectrum and Its Molecular Basis**."

In LS, the first hit inactivating one allele of the responsible MMR gene is inherited, whereas the second hit inactivating the remaining wild-type allele occurs in, and is restricted to, the somatic cancer progenitor cell in a target tissue. Either one of the two hits, or both of them, may be genetic or epigenetic. Both hits are usually genetic in LS-associated cancer and consist of a point mutation or a large rearrangement for the first hit and loss of the wild-type allele (loss of heterozygosity, LOH), gene conversion, or point mutation for the second hit (Fig. 3). Occasionally, promoter methylation of a MMR gene (*MLH1* or *MSH2*) provides the first hit (see section "**Constitutional Epimutations Predisposing to LS**"). Promoter methylation can also provide the second hit, although this is relatively rare.

In sporadic tumors with MSI, two inactivating hits, one in each allele of a MMR gene, occur somatically prior to tumor initiation. In the most common situation, the two hits are epigenetic and consist of biallelic methylation of *MLH1*. In approximately half of MMR-deficient tumors without germline mutations or promoter methylation, two somatic mutations of a MMR gene, or a somatic mutation and LOH, explain the MMR deficiency (Lynch-like syndrome (LLS), see section "**Differential Diagnosis of LS**").

Although MMR genes are typically recessive on cellular level, a single allele is sometimes sufficient to initiate tumorigenesis (haploinsufficiency). MMR gene dosage makes a clear difference regarding phenotypic consequences. Heterozygosity versus homozygosity for the predisposing mutation causes drastically different constitutional phenotypes (LS vs. CMMRD, Table 2). In somatic target tissues, the amount of available MMR protein may regulate tumorigenesis in a function-specific manner. For example, it has been shown that DNA damage signaling requires higher dosage of the MLH1 protein than DNA MMR. Furthermore, MLH1 haploinsufficiency in sporadic cancers and cell lines (such as pancreatic and renal cell cancers) prone to *MLH1*-LOH has been reported to cause a distinctive form of genomic instability involving increased rates of insertion/deletion mutations without

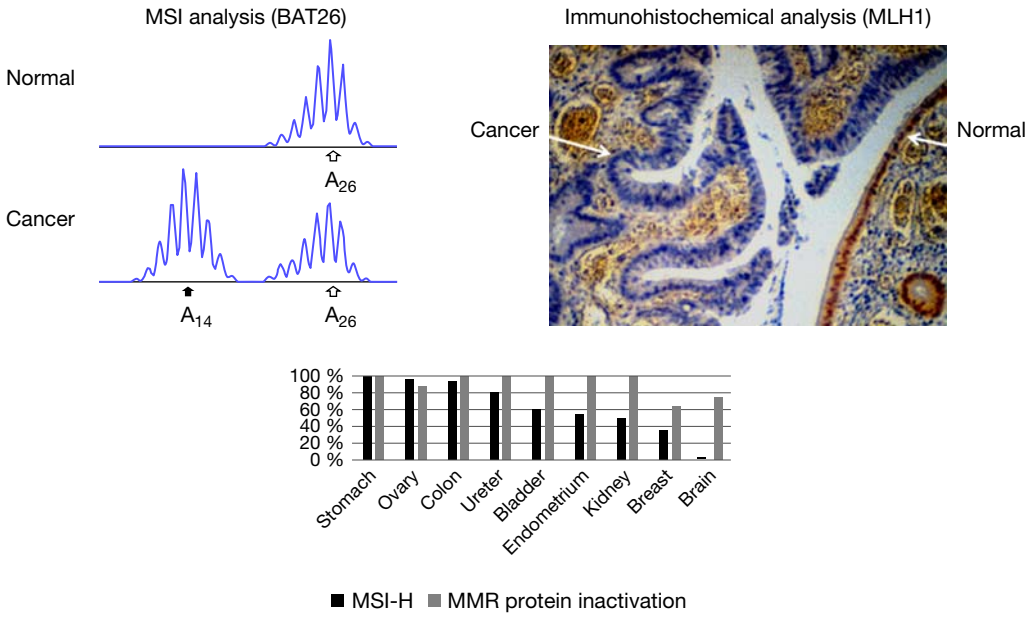


Fig. 2 Microsatellite instability and absent MMR protein as hallmarks of LS tumors. *Top left*: BAT26 includes a stretch of 26 consecutive adenine nucleotides (A_{26}); shortened alleles (A_{14} in this example) indicate MSI. *Top right*: Staining with an MLH1 antibody reveals absent MLH1 expression (*blue color*) in colorectal carcinoma cells from an *MLH1* mutation carrier, whereas MLH1 is normally expressed (*brown color*) in the patient's non-neoplastic mucosa. *Bottom*: The frequencies of LS tumors with MSI and MMR protein inactivation show organ-specific variation.

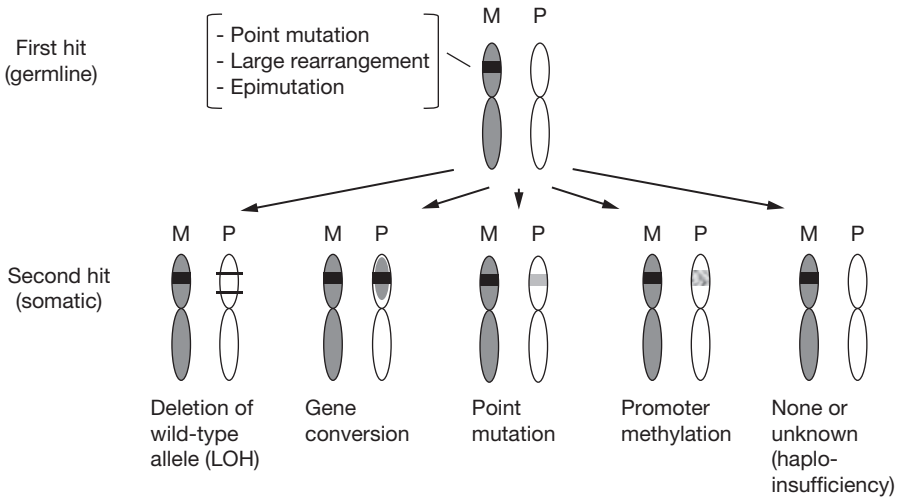


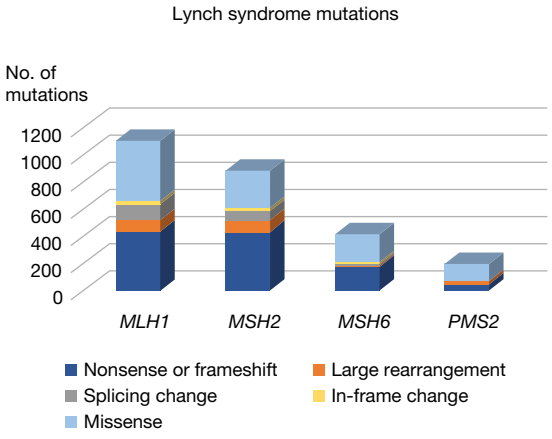
Fig. 3 Mechanisms of two-hit inactivation of MMR genes in LS (see text). M refers to the maternal and P to the paternal allele (the maternal allele was randomly chosen as the target of the first hit).

conventional MSI. The truncating insertion/deletion mutations can inactivate tumor suppressor genes and thereby drive sporadic tumor development.

Germline Mutations Predisposing to LS

The International Society for Gastrointestinal Hereditary Tumors (InSiGHT) has collected information of LS-associated MMR gene mutations in a public database established in 1994. *MLH1*, *MSH2*, *MSH6*, and *PMS2* account for 40%, 34%, 18%, and 8%, respectively, of the over 3000 unique germline sequence variants of MMR genes deposited to the database to date (Fig. 4, left, and www.insight-group.org). Mutations are scattered throughout the genes. Most *MLH1* and *MSH2* mutations and an important proportion of *MSH6* mutations are truncating nonsense or frameshift mutations. Missense changes, which lead to single amino acid substitutions without altering the reading frame, also account for a significant share (30%–60%) of the known mutations in all four genes.

It was precisely the abundance of missense-type changes that prompted a recent large-scale effort by InSiGHT to classify each MMR gene variant according to pathogenicity (Fig. 4, right). The classification is based on variant, family, and tumor characteristics



Classification for pathogenicity

InSiGHT class	Examples of variants	Predictive testing	Surveillance of variant carriers
5 (Pathogenic)	Nonsense, frameshift, large rearrangements, missense with proven pathogenicity	Yes	Full high-risk guidelines
4 (Likely pathogenic)	Splicing aberrations (untested)	Yes	Full high-risk guidelines
3 (Uncertain)	Missense, small in-frame changes	No	Tailored for each family
2 (Likely non pathogenic)	Intronic and synonymous variants	No	Treat as no mutation detected
1 (Non pathogenic)	Polymorphisms	No	Treat as no mutation detected

Fig. 4 Mutations predisposing to LS and translation of mutation information to clinical recommendations. *Left*: Based on information deposited in the InSiGHT database, the number of unique mutations discovered for each main LS susceptibility gene is shown, along with a breakdown into five mutation types within each gene. *Right*: An individual variant can be assigned a pathogenicity class from 1 (non-pathogenic) to 5 (pathogenic) on the basis of evaluation criteria formulated by the InSiGHT. Typical examples of changes representing each category are given. Possibilities for clinical use in predictive testing of at-risk relatives or regular surveillance of variant carriers vary according to the class of pathogenicity.

on the one hand and a variety of functional assays on the other hand. It is linked to clinical recommendations as follows. Classes 5 and 4 indicate a "pathogenic" and "likely pathogenic" variant, respectively, implying that a causative mutation was detected. Surveillance according to full high-risk guidelines is warranted and predictive testing of at-risk relatives should be made available. Nonsense and frameshift mutations constitute a majority of class 5 and 4 variants. Class 3 comprises variants of unknown significance (VUS), predominantly missense changes. Clinical management has to be tailored case by case. Classes 2 and 1 indicate a "likely nonpathogenic" and "nonpathogenic" variant, respectively, suggesting that no causative mutation was found. Carriers of class 2 and 1 variants should be clinically treated as "no mutation detected." Intronic variants and synonymous (silent) variants with no associated RNA aberration are typical representatives of category 2 and variants with frequency $\geq 1\%$ in the general population (polymorphisms) of category 1.

Most MMR gene mutations are inherited from either parent and de novo mutations are rare. A majority of MMR gene mutations are unique (specific to a single family). Nevertheless, some prevalent recurrent mutations are known and may arise de novo or represent founder mutations. Founder mutations originate from a single ancestor, revealed in present-day carriers by shared haplotypes of various sizes depending on the age of the mutation. Founder mutations are enriched in isolated populations, such as the Finns, Newfoundland population, or Ashkenazi Jews.

A genetic point mutation is the main type of germline mutation in MMR genes. Analysis of LS cohorts from different geographic locations indicates a frequency of 15% for large genomic rearrangements. Mutations typically abrogate one or several capabilities necessary for a functional MMR system, such as MMR protein expression, transport to the nucleus, heterodimer formation, DNA mismatch binding, and ATP–ADP cycling (Fig. 1). In a small but significant percentage of LS-suspected families with MMR-deficient tumors and negative for point mutations and large rearrangements, the predisposing defect is regulatory and consists of a constitutional epimutation in an MMR gene. The latter mechanism is discussed in more detail in the next section.

Constitutional Epimutations Predisposing to LS

Constitutional epimutation can be defined as constitutional hypermethylation at the promoter of one allele of a given (nonimprinted) gene leading to silencing of expression from that allele in all main somatic tissues. If induced by a genetic alteration (usually located *in cis*), epimutation is secondary and otherwise primary. Primary and secondary epimutations of *MLH1* have been described (Fig. 5A and B). The only known type of constitutional epimutation of *MSH2* is a secondary epimutation caused by deletions of the 3′ end of the *EPCAM* gene (Fig. 5C). After removal of stop codon, transcription of *EPCAM* reads into the

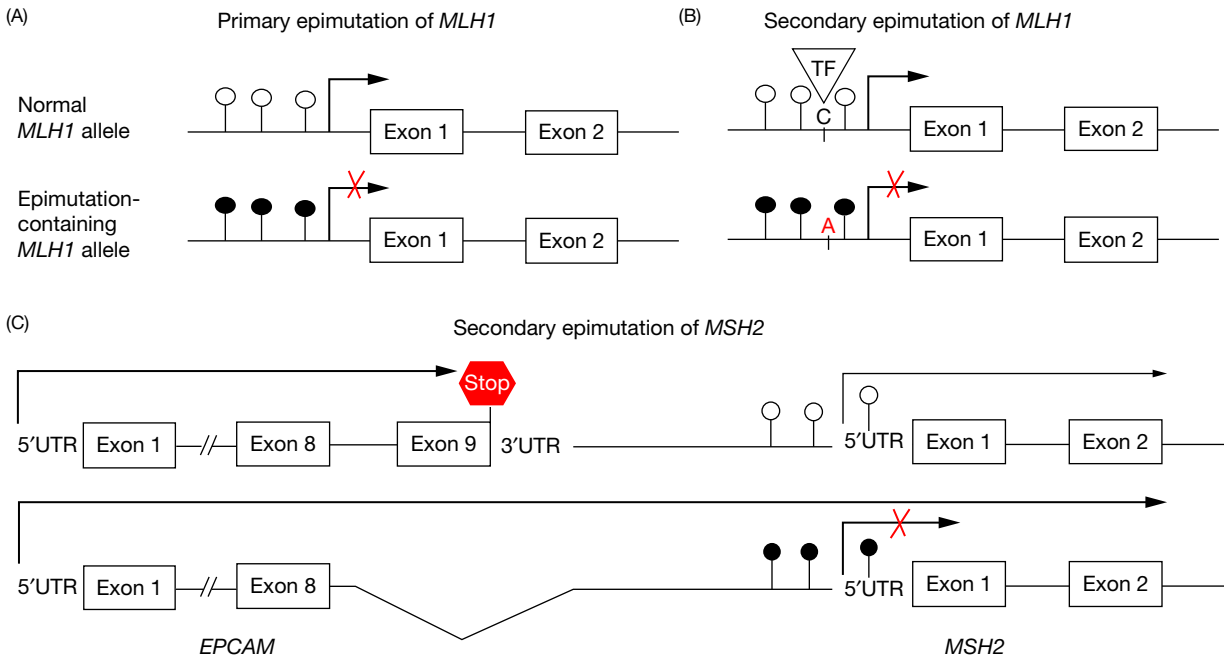


Fig. 5 Mechanisms of constitutional epimutations in MMR genes. In *A* to *C*, the upper allele is normal (open lollipops denote unmethylated CpG sites and an arrow depicts active transcription) whereas the lower allele contains an epimutation (methylated CpG sites and transcriptionally inactive). (A) CpG sites at the promoter of an epimutation-containing *MLH1* allele are methylated (*black lollipops*) blocking transcription from that allele (*crossed arrow*). No apparent reason (e.g., a genetic change) for methylation can be identified, which is why the epimutation is designated primary. (B) A single nucleotide polymorphism (C to A) abolishes transcription factor (TF) binding and leads to secondary methylation at the *MLH1* promoter. (C) Owing to a genomic deletion, the stop codon of the *EPCAM* gene is lost and transcription continues into the adjacent *MSH2* gene, causing secondary methylation of the structurally normal *MSH2* promoter.

adjacent, structurally normal *MSH2* gene inducing promoter methylation of *MSH2*. In tumor tissues of constitutional epimutation carriers, the remaining wild-type allele is inactivated by a genetic or epigenetic mechanism according to Knudson's two-hit hypothesis (Fig. 3).

Carriers of constitutional epimutations of *MLH1* or *MSH2* are clinically indistinguishable from carriers of conventional (structural) MMR gene mutations (Table 5). However, family features and segregation patterns make a distinction. Epigenetic changes are erased on passage through the germline, preventing regular transmission from parent to child. Therefore, primary constitutional epimutations segregate in a non-Mendelian manner and are seldom associated with any remarkable family history of cancer (the revised Bethesda criteria may be fulfilled, Table 1). Examination of the families of primary epimutation carriers has revealed variable patterns of transmission, including apparent heritability, mosaic epigenetic inheritance, and reversion of the methylated allele to normal active state. Importantly, it has been shown that asymptomatic low-level somatic mosaicism for a *MLH1* epimutation in a parent can produce a nonmosaic constitutional epimutation in the offspring, resulting in early-onset colorectal cancer. Such observations make genetic counseling of *MLH1* epimutation carriers and their families quite challenging. In contrast, secondary epimutations of *MLH1* or *MSH2* give rise to classical LS families and regularly cosegregate with their *cis*'acting genetic changes in a dominant Mendelian fashion. Nevertheless, the mechanism of transmission may display distinct features compared to that of a genetic mutation. Secondary epimutation of *MLH1* has been shown to undergo a complete but transient reversion in the germline, being erased in spermatozoa but reinstated in somatic cells of the next generation.

To date, more than 50 index cases with primary or secondary constitutional epimutation of *MLH1* are known. Constitutional epimutations may explain 1%–10% of mutation-negative Lynch-suspected families with silenced MLH1 expression in tumors (Table 5). A comparable number of index cases with *EPCAM* deletion-induced *MSH2* epimutation have been described. Their share of Lynch suspected families without conventional germline mutations and with absent MSH2 protein in tumor tissues ranges between 0% and 40% depending on possible founder effects.

Tumorigenesis in LS

Precursor Lesions

Colorectal tumorigenesis in LS is traditionally considered to follow the adenoma–carcinoma sequence, which forms the rationale of using colonoscopy screening and removal of adenomas as an important means of cancer prevention in MMR gene mutation carriers. In sporadic colorectal tumorigenesis, the complete adenoma–carcinoma sequence can take 15 years or more. The presence of one defective copy of the predisposing MMR gene in every cell of LS individuals may shorten the process to only a few years or less. MMR genes are "caretaker" genes which when mutated, may accelerate tumor progression in particular, while the incidence of polyps is not markedly different from the average population. This is in contrast to "gatekeeper" genes such as *APC* that accelerate tumor initiation and underlie polyposis syndromes. Compatible with the underlying MMR defect, adenomas in LS often show aggressive features such as villosity and poor differentiation (high grade). Additionally, tumor-infiltrating lymphocytes (TILs) are common because of neoantigens resulting from mutant proteins.

Recent evidence suggests that adenomas in LS may not always be easily detectable or may not exist prior to colorectal carcinoma development. For example, a report from a prospective LS database based on 10 centers from Europe and Australia

Table 5 Constitutional epimutations of *MLH1* and *MSH2* in LS predisposition

	MLH1	MSH2
Type of epimutation	Primary or secondary	Secondary
Gene defects associated with secondary epimutation reported in the literature	• Genomic deletion of *MLH1* exons 1–2 • Genomic deletion of *MLH1* exon 1 • Duplication of *MLH1* and flanking regions • c.-27C > A in the *MLH1* promoter	• Deletions of *EPCAM* removing stop codon
Tumor phenotype	MSI Lack of MLH1 (and PMS2) protein	MSI Lack of MSH2 (and MSH6) protein
Clinical phenotype	May present as a sporadic case (primary epimutation) or as a classical Lynch syndrome family (secondary epimutation). Age at onset and tumor spectrum are similar to Lynch syndrome. Atypical phenotypes (e.g., increased number of polyps) are possible	Presents as a classical Lynch syndrome family. Colorectal cancer risk is high. Endometrial cancer risk is increased with deletions extending close to the *MSH2* promoter
Pattern of transmission	Variable, from apparent lack of heritability (primary epimutation) to autosomal dominant (secondary epimutation)	Autosomal dominant
Share of LS[a]	1%–10%	10%–40%

[a]Estimated from Lynch-suspected cases with MMR-deficient tumors and negative screens for conventional germline mutations in MMR genes.

(http://LScarisk.org) shows that colorectal cancer is surprisingly frequent despite colonoscopic surveillance with 1–3-year intervals. Furthermore, a long-term (mean 56 months) follow-up analysis of close to 900 MMR gene mutation carriers enrolled in the Colorectal Adenoma/Carcinoma Prevention Program 2 (CAPP2) surprisingly reveals that aspirin exhibits delayed protection against colorectal cancer without reducing adenoma incidence. These observations imply that colorectal carcinomas in LS may arise from lesions other than adenomas. Such possible alternative lesions remain to be identified and defined histologically and molecularly.

Endometrial cancer, the most common extracolonic cancer in LS, is thought to develop via endometrial hyperplasias as precursor lesions. Accordingly, endometrial cancer prevention in LS is based on screening for endometrial hyperplasias by regular endometrial biopsies. Epidemiological studies suggest that atypical and complex hyperplasias are associated with increased risks of malignant transformation. According to the current knowledge, ovarian carcinomas of endometrioid and clear cell types, the predominant histological types of ovarian carcinoma in LS, also arise from endometrial epithelium, with atypical endometriosis as a recognized precursor. The fact that the abovementioned precursor lesions of endometrial and ovarian carcinoma share many genetic and epigenetic characteristics of the corresponding malignancies, such as MMR defects, tumor suppressor promoter methylation, and mutations in *ARID1A*, *PIK3CA*, or *PTEN* strongly supports their malignant potential.

Somatic Mutations in LS Tumors

Owing to defective MMR, LS tumors belong to the "hypermutated" category defined by The Cancer Genome Atlas (TCGA) for unselected colorectal, endometrial, and other cancers. Whole-exome sequencing of MSI colorectal tumors by the TCGA network has revealed somatic nonsynonymous mutation rates above 12 per 10^6 bases, with a median number of ~700 mutations per tumor. This is 10–100-fold compared to MMR-proficient tumors (median 58 mutations per tumor). The profiles of mutant genes differ in hypermutated and nonhypermutated tumors. For example, frameshift mutations in coding repeats within *ACVR2A*, *TGFBR2*, and other genes are characteristics of MSI tumors. The karyotypes are usually diploid and copy number changes are relatively rare. Molecular features typical of MSI tumors broadly apply to LS tumors as well. Additionally, certain universal signatures of tumor types, such as mutations in the adenoma–carcinoma progression sequence genes *APC*, *KRAS*, and *TP53* in colorectal tumors and *ARID1A*, *PIK3CA*, and *PTEN* genes in endometrial and nonserous ovarian cancers, are largely preserved in LS tumors, although mutation frequencies of individual genes may vary.

The *BRAF* oncogene is an important exception to the generally similar patterns of mutant genes between sporadic and LS tumors with MSI. Analysis of large cohorts of colorectal cancers indicates that *BRAF* V600E mutation occurs with a frequency of 1.4% in tumors from MMR gene mutation carriers (LS), compared with 5% in MSS cases and 63% in sporadic MSI colorectal cancers. *BRAF* V600E mutation coincides with *MLH1* promoter "C region" methylation and strongly argues against LS. *BRAF* V600E mutation can therefore be utilized as a biomarker for predicting MMR-negative mutation status in MSI colorectal cancers.

Epigenetic Alterations in LS Tumors

The MMR system can influence the epigenetic machinery and vice versa. For example, deficient MMR can induce mutations in genes with key roles in epigenetic regulation (such as *ARID1A* and other chromatin regulator genes), therefore having the potential to alter epigenetic modifications. Coding repeats, common targets of frameshift mutations in MMR-deficient tumors, are part of several epigenetic regulator genes and can be one reason why epigenetic regulation is commonly affected in LS and sporadic cancers with MSI. Conversely, MMR genes can be epigenetically regulated by promoter methylation (*MLH1*) and micro-RNAs (*MSH2*, *MSH6*, and *MLH1*). Moreover, the histone mark H3K36me3 is required to recruit hMutSα to chromatin in the process of MMR. In light of this interplay, it is not unexpected that apart from somatic mutations, coordinated methylation of CpG islands that are normally unmethylated (CpG island methylator phenotype, CIMP) and global hypomethylation (with the long interspersed element 1, LINE-1, as a surrogate marker) are integral parts of LS tumorigenesis. CIMP has the potential to silence hundreds of tumor suppressor genes per cancer cell. DNA hypomethylation in turn can activate oncogenes and increase chromosomal instability via LINE-1 retrotransposition.

Sporadic MSI colorectal cancers with *MLH1* promoter methylation typically display high CIMP phenotypes. More or less the same loci are involved in LS-associated colorectal cancers, but the frequencies of tumors with methylation are lower. Promoter methylation appears early and increases along with tumor progression. Promoter methylation of *SFRP1* and *SLC5A8*, established tumor suppressor genes of colorectal carcinogenesis, has been shown to be significantly elevated in normal mucosa from MMR gene mutation carriers previously diagnosed with colorectal carcinoma, as compared to mutation carriers of a comparable age with no prior colorectal carcinoma diagnosis, suggesting that methylation changes may form cancer-prone "fields" in histologically normal mucosa. Moreover, methylation increases with histological dysplasia, with 0% of normal mucosae, 15% of adenomas with low-degree dysplasia, 23% of adenomas with high-degree dysplasia, and 50% of carcinomas from LS individuals reported to display CIMP-high with established marker genes.

Analogous observations are available from endometrial tumorigenesis, except that different tumor suppressor genes (e.g., *RASSF1* and *CHD13*) are preferentially affected given the tissue-specific nature of epigenetic regulation. Retrospective examination of serial endometrial biopsy samples from a long-term follow-up of LS mutation carriers has revealed MMR defects and elevated promoter methylation at tumor suppressor gene loci up to 12 years before endometrial carcinoma development. Furthermore, promoter methylation of CpG islands associated with tumor suppressor genes and microRNA genes stratifies endometrial lesions into a low-methylator group (normal endometrium and simple hyperplasia) and a high-methylator group (complex hyperplasia

with and without atypia and endometrial carcinoma) suggesting that increased methylation accompanies endometrial tumorigenesis. The findings apply to both LS-associated and sporadic endometrial lesions emphasizing their universal nature.

The highest levels of global hypomethylation occur in sporadic MSS colorectal cancers. Extensive hypomethylation is not a feature of LS-associated colon cancers; however, when present, LINE-1 hypomethylation has been reported to be associated with a worse prognosis compared with LS patients without significant hypomethylation. In line with observations from LS colorectal cancers, LS-ovarian carcinoma shows a significantly higher average number of methylated tumor suppressor genes and lower degree of LINE-1 hypomethylation compared with the corresponding sporadic disease, which may contribute to the favorable prognosis of LS-ovarian cancer (see section "**Molecular Profiles in Tumors Versus Disease Outcome**").

Temporal Relationship Between MMR Deficiency and Other Tumorigenic Events

MMR defects are among the earliest detectable alterations in cancer-prone target tissues of LS individuals. Intestinal resections for small or large bowel cancer from LS mutation carriers display lesions termed MMR-deficient crypt foci with a frequency of one focus per 1 cm^2 of nontumorous mucosa, as compared to none in non-LS control patients. MMR protein is absent and MSI is present in such lesions, indicating biallelic MMR gene inactivation. The abundance of MMR-deficient crypt foci contrasts with the low number of adenomas or carcinomas observed in LS and suggests that most lesions do not progress to malignancy. In colorectal polyps from MMR gene mutation carriers, the prevalence of MMR deficiency increases with the size and dysplasia of adenomatous polyps, from 67% in adenomas with low-degree dysplasia to 100% in adenomas with high-degree dysplasia; the latter frequency is similar to colorectal carcinomas. Hyperplastic polyps from LS individuals rarely ($<5\%$) display MMR defects and their malignant potential is considered low. In analogy to colorectal tumorigenesis, the prevalence of MMR defects increases with endometrial tumor progression in LS. Decreased MMR protein expression has been reported to occur in 7% in normal endometrium, 40% in simple hyperplasia, and $\sim 100\%$ in complex hyperplasia with or without atypia and likewise in endometrial carcinoma; the frequencies of MSI are somewhat lower.

The observations of abundant MMR-deficient crypt foci without progression to visible tumors on the one hand and adenoma development without biallelic MMR gene inactivation on the other hand imply the need of other oncogenic events besides MMR deficiency. As mentioned earlier, DNA methylation changes can be early events in tumorigenesis. Promoter methylation of *SFRP1* and *SLC5A8* may form field defects in histologically normal colonic mucosa from LS individuals. Studies on sporadic cases has revealed frequent hypermethylation of *SFRP1* and *SLC5A8* promoters in aberrant crypt foci, the earliest detectable morphological lesions of colorectal tumorigenesis that usually lack *APC* mutations, and methylation is accompanied by decreased expression of the corresponding proteins. The repair gene *MGMT* encoding O^6-methylguanine DNA methyltransferase is a further gene whose loss of expression, usually by promoter methylation, can form field defects in normal mucosa from LS and sporadic cases. The resulting failure to process mutagenic methyl adducts may trigger cellular transformation by inducing mutations in cancer-related genes such as *KRAS* or by inactivating the MMR genes by mutations or promoter methylation. It has been postulated that methylation tolerance due to *MGMT* field defects may initiate sporadic or LS-associated MSI-colorectal cancer prior to MMR deficiency. As, however, no such studies are available that would have examined the earliest lesions (aberrant crypts) for both MMR and methylation abnormalities, it remains unsettled which aberrations come first.

Mutations of *APC*, an important gatekeeper of colon tumorigenesis, are known to occur early in colon tumorigenesis; therefore, this gene has been used in studies addressing the temporal relationship between MMR defects and other molecular events. Investigations on mice heterozygous for the *Apc* Min mutation and knockouts for selected MMR genes (*Min*/+, *Msh2* −/− and *Min*/+, *Mlh1* −/− mice) demonstrate that MMR deficiency changes the spectrum of somatic *Apc* alterations from LOH, the usual second hit, to point mutations, suggesting that deficient MMR exerts its effect before *Apc*. On the other hand, comparison of the *APC* mutation spectra in sporadic MSI and MSS colon cancers has failed to identify in the former tumors any clear excess of changes characteristic of MMR defects, such as preferential involvement of repeat sequences, suggesting that MMR deficiency occurs after *APC* mutations. Taken together, studies conducted to date have remained conflicting or inconclusive in an attempt to determine the chronological order of MMR defects relative to other molecular events in multistep tumorigenesis.

Molecular Profiles in Tumors Versus Disease Outcome

Evidence from a prospective LS database (http://LScarisk.org) on MMR gene mutation carriers without previous cancer and under regular surveillance shows that the 10-year survival after the first cancer is excellent: 91% for colorectal cancer, 98% for endometrial cancer, 89% for ovarian cancer, and 87% for any cancer. Although early detection probably plays a role, LS cancers are additionally likely to have intrinsic factors that contribute to the favorable outcome compared to cancers arising in the general population.

Early studies already found that despite aggressive histological features (poor differentiation) of LS-associated colorectal carcinoma, LS patients experience a definite survival advantage over average colorectal carcinoma patients. Since the same applies to unselected colorectal carcinoma with MSI, it is logical to reason that MMR deficiency offers a plausible explanation. Increased mutational load triggering apoptosis of tumor cells can be one possible mechanism. The favorable effects of deficient MMR may also be related to the generation of neoantigens that can elicit strong antitumor immune responses. Multiple genes encompass coding microsatellite repeats prone to insertion/deletion mutations, resulting in neopeptides with altered carboxy-terminal sequences that are recognized as foreign by the immune system. Some peptides derived from mutant genes (*TGFBR2*, *AIM2*, *HT001*, and

TAF1B in one study and *ASTE1*, *HNF1A*, and *TCF7L2* in another study) have been identified as especially immunogenic and may help develop vaccination-based immunotherapy in LS. Pronounced immune responses provoked by various neopeptides may eliminate MMR-deficient crypt foci present in nontumorous mucosa of LS individuals. Progression and metastasis of tumors that have already developed may be prevented by similar mechanisms. Furthermore, the active immune microenvironment is counterbalanced by immune inhibitory signals, resulting in overexpression of several immune checkpoint molecules, such as PD-1 and PD-L1. This may make metastatic MMR-deficient cancers particularly susceptible to pembrolizumab and other immune checkpoint inhibitors.

Ovarian carcinomas arising in LS individuals are mainly of nonserous histology (endometrioid and clear cell type) and well or moderately differentiated early-stage tumors at diagnosis, as opposed to sporadic ovarian carcinomas, a majority of which are serous and advanced at presentation. Although the abovementioned clinicopathological differences are likely to contribute to differential disease outcomes, the 5-year survival of LS patients with even stage III or IV ovarian cancer is 59% compared to 28% in sporadic cases. A novel molecular profile for LS-associated ovarian carcinoma has been described, consisting of normal p53 expression, lack of *KRAS* mutation, MMR deficiency, significantly higher rate of methylated tumor suppressor genes and lower degree of LINE-1 hypomethylation compared to sporadic endometrioid and clear cell ovarian cancers. This markedly different overall molecular profile compared to sporadic ovarian cancers may in part explain the surprisingly high survival rates of LS-associated ovarian carcinoma.

Genotype-Phenotype Correlations in LS

Cancer Risks Associated With Germline Mutations in MMR Genes

The life-time risks of cancer vary according to the mutant gene, which may reflect the degree of functional redundancy of the corresponding proteins in MMR (**Table 4**). *MLH1* and *MSH2* mutations have high penetrance, with 60%–80% of mutation carriers developing some form of cancer during their lifetime. The mean age of onset is ~45 years for colorectal cancer and ~45–55 years for other cancers. *MSH6* and especially *PMS2* mutations have lower penetrance, 25%–54% for *MSH6* and ~20%–30% for *PMS2*. The mean age at onset is 55–60 years for colorectal cancer and 54 years for endometrial cancer in *MSH6* mutation carriers. Heterozygous carriers of *PMS2* mutation have a mean age at onset of around 50 years for colorectal and other cancers. The type (truncating vs. missense) or location (relative to different functional domains) of MMR gene mutations is poorly correlated with clinical phenotype.

LS Tumor Spectrum and Its Molecular Basis

The Amsterdam II criteria (**Table 1**) acknowledge cancers of the colon and rectum, endometrium, small bowel, ureter, and renal pelvis as LS-associated cancers, because these are significantly more frequent in LS compared to the average population. Since the Amsterdam II criteria were formulated, significantly increased standardized incidence ratios have repeatedly been reported for cancers of the stomach, ovaries, and pancreas as well as for certain other cancers (footnote in **Table 1**), which combined with molecular profiles characteristic of LS, such as MMR protein loss and MSI, justifies their inclusion in the LS spectrum. Recently, breast cancer and prostate cancer have also been suggested to belong to the LS tumor spectrum, based on MMR deficiency in tumors from MMR gene mutation carriers, in addition to some published evidence of increased risk in LS compared to the average population. While confirmation from additional study series is still warranted for reliable conclusions, a recent investigation compared the rate of MMR deficiency in breast carcinomas from proven mutation carriers versus noncarriers from the same families, resulting in frequencies of 65% versus 0% ($P < 0.001$). Moreover, the age at onset of breast carcinoma in mutation carriers was earlier if the tumor was MMR-deficient, suggesting that MMR deficiency does play a role in breast cancer development in LS.

Why MMR gene mutation carriers are predisposed to a narrow spectrum of cancers, considering that they carry one defective copy of a given MMR gene in all their cells, is not well understood. Some possible mechanisms of organ selectivity are depicted in **Fig. 6**. First, the individual MMR genes may be associated with different tumor spectra. For example, the risk of extracolonic cancers has been suggested to be higher in *MSH2* than *MLH1* mutation carriers. As another example, female carriers of *MSH6* mutations are at a higher risk of endometrial than colorectal cancer.

Second, the dosage of the MMR gene or protein is important for phenotype. As discussed earlier (**Table 2**), homozygosity or compound heterozygosity for germline mutation results in a separate syndrome, CMMRD. At present, ~150 cases with CMMRD are known. Childhood cancers of the hematological system and brain, signs of neurofibromatosis type 1 (café-au-lait spots), and coexistence of colorectal and brain tumors (TS) are common manifestations of CMMRD. The distinct tumor spectrum may reflect the sensitivity of neural and hematological progenitor cells to MMR deficiency via specific somatic target genes, such as *NF1*. In contrast to LS with heterozygous MMR gene mutations (**Fig. 2**, upper right), CMMRD patients lack expression of the MMR protein(s) in question not only in cancer tissue but in normal tissue as well. Despite biallelic MMR gene inactivation, MSI is not usually detectable by standard methods in peripheral blood lymphocytes because of clonal heterogeneity; however, immortalized lymphoblastoid cells, which are oligoclonal, can reveal MSI. Another line of evidence to support the significance of MMR gene or protein dosage for phenotypic consequences comes from observations of haploinsufficiency in somatic cells (see section "**LS Genes and Knudson's Two-Hit Hypothesis**"). Different organs may have different requirements for MMR gene dosage or

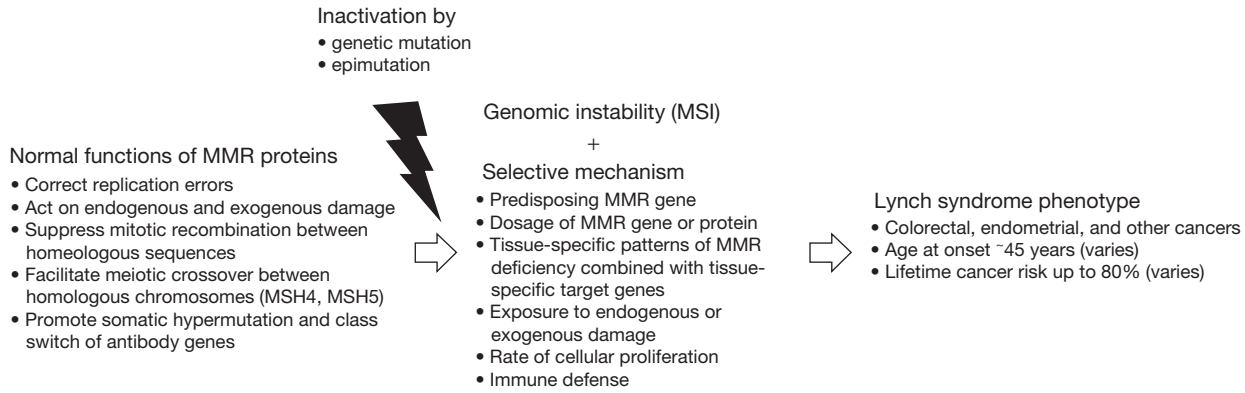


Fig. 6 Possible mechanisms that may lead to organ selectivity in LS. MMR gene inactivation by genetic or epigenetic mechanisms (see **Fig. 3**) abrogates normal functions of MMR proteins and results in genomic instability (see **Fig. 2**), which together with various selective mechanisms can target specific organs for tumor development and contribute to the overall LS phenotype (see text).

protein, which may affect their susceptibility to tumor development. For example, analysis of second hits in *MLH1* mutation carriers shows that deletion of the wild-type *MLH1* allele is more common in colorectal than endometrial cancer.

Third, tissue specificity for MMR deficiency and genes targeted by failing MMR may be a factor behind organ selectivity. In LS, immunohistochemical analysis of cancers regularly demonstrates absent MMR protein corresponding to the gene mutant in the germline, but the frequency of MSI-high varies from 80% to 100% (stomach, ovary, colon, and ureter) to ~50% (bladder, endometrium, and kidney) and even less (35% for breast and 0% for brain tumors) (**Fig. 2**, lower panel). Clonal heterogeneity is a feature of LS and sporadic MMR-deficient tumors and can partly explain the different frequencies of MSI between tumor types. Observations of *BAT* markers showing shorter allelic shifts in endometrial than colorectal cancers from LS patients offer another indication of tissue-specific patterns of clonal growth. Genes with coding repeats are preferential targets in MMR-deficient cancers (see earlier) and different genes confer selective advantage for different cancers, such as the *TGFβ* superfamily for gastrointestinal cancers and *PTEN* for endometrial cancers. Organ-specific differences in the manifestation of MMR deficiency can therefore contribute to LS tumor spectrum via tissue-specific target genes with mutation-prone sequences.

Fourth, exposure to endogenous and exogenous damages can have serious consequences when the ability of the MMR system to repair such damage is compromised due to inherited mutations. Such damage can be exogenous (many heterocyclic amines are of dietary origin) or endogenous (such as inflammation-induced oxidation). The accumulation of damage could facilitate cancer development in exposed organs, such as the gastrointestinal tract and endometrial epithelium. Western-style diet has been shown to increase colorectal adenomas in LS mutation carriers and colon adenomas and carcinomas in *Mlh1*+/− mice. Likewise, tobacco smoking appears to increase colorectal adenoma risk in LS mutation carriers. Such observations imply a reduced capacity of MMR gene mutation carriers to repair dietary and tobacco-associated damage.

Fifth, tumors initiated by unrepaired mutations or carcinogen-derived adducts may show differential progression rates in different tissues depending on cellular proliferation. Colon and many other epithelial cells have fast turnovers, and short cell cycles may allow less time to repair errors. Stem cell divisions in the epithelium continue throughout life. Hematopoietic tissue, which is likewise highly proliferative, may be less cancer-prone because of fewer stem cell divisions during lifetime. Proliferation rates may explain why a majority of LS-associated tumors are epithelial and why MMR deficiency can occasionally (in CMMRD) cause predisposition to hematological malignancies.

Finally, immunological processes may play a role in organ-specific tumor susceptibility. MMR deficiency induces frameshift mutations that can have differential effects on the immune system. Cell surface proteins responsible for antigen processing and presentation may lose expression as a consequence of frameshift mutations of genes encoding them and this may make it possible for tumor cells to escape from immune surveillance. For example, mutations in the *B2M* gene coding for beta 2 microglobulin can abrogate HLA class I antigen expression on the surface of tumor cells, allowing evasion from the attacks by cytotoxic T cells. Mutations in other types of genes such as *ACVR2* or *TGFBR2* may result in the formation of neoantigens and induce a strong immune response against cancer cells. High levels of TILs in colon, gynecological, and other tumors from LS patients are indicators of active immune surveillance. Differential manifestation of MMR defects (see earlier) combined with possible variations in the inherent efficacy of immune surveillance in different organs may contribute to organ selection in LS.

Differential Diagnosis of LS

A suspicion of LS typically arises because of a family history of LS-spectrum cancers or because of MSI and/or immunohistochemical loss of MMR protein in tumor tissue. Starting from family or tumor studies, a number of alternative diagnoses are possible and need to be considered in differential diagnosis (**Fig. 7**, **Table 6**).

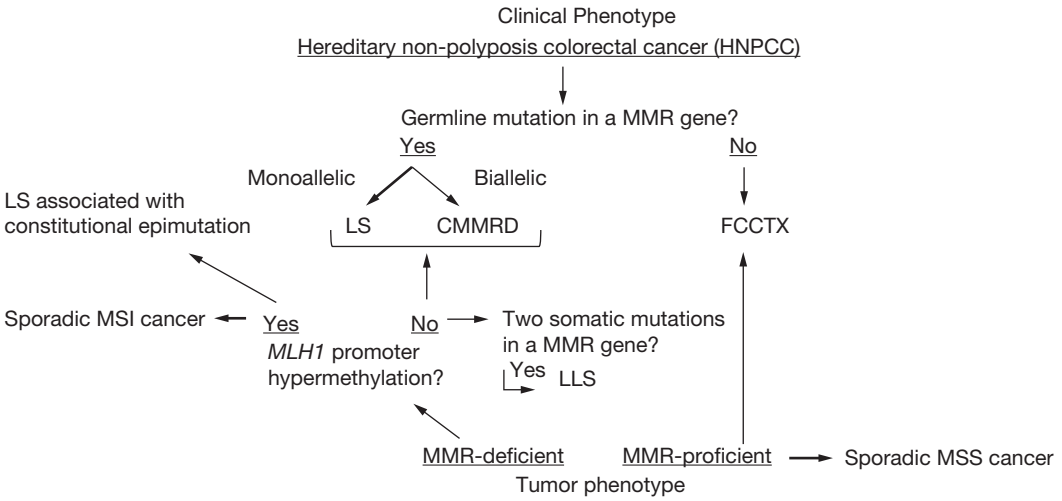


Fig. 7 Differential diagnosis of LS. Clinical and tumor phenotypes are common starting points for molecular evaluation to differentiate LS from overlapping conditions (see **Table 6**). When two options exist, a *thick arrow* denotes the most likely alternative.

Table 6 Syndromes that overlap with or mimic LS

Syndrome	*Share among unselected CRCs (%)*	*Responsible gene*	*Family features*	*Tumor characteristics*
Familial colorectal cancer type X (FCCTX)	~2	Mostly unknown	Hereditary/familial	No MMR defects in a family that fulfills the Amsterdam criteria
Sporadic MSI	~15	*MLH1*	Sporadic	MSI-H or absent MLH1 protein due to *MLH1* methylation
Lynch-like syndrome (LLS)	2.5	*MLH1*, *MSH2*, *MSH6*, or *PMS2*	Sporadic (some familial clustering reported)	MSI-H or absent MMR protein not explained by methylation of *MLH1* or germline mutation of MMR genes. Attributable to two inactivating somatic events of MMR genes
POLE/*POLD1*-associated hypermutability	1–2	*POLE* or *POLD1*	Sporadic or hereditary	Rate of nonsynonymous mutations comparable to that caused by MMR defects, but MSI-H is absent. Somatic mutations result in "ultramutated" tumors, whereas germline mutations predispose to polymerase proofreading-associated polyposis (PPAP)

CRC, colorectal cancer; *MSI-H*, high-degree of microsatellite instability.

Familial Colorectal Cancer Type X

Before the discovery of the LS genes in the 1990s, the Amsterdam Criteria (**Table 1**) were used to identify families with a presumably inherited form of colorectal carcinoma. Families that meet these clinical criteria were called hereditary non-polyposis colorectal cancer (HNPCC). Molecular analyses have revealed that in about half of such families, MSI or absent MMR protein in tumors or a germline MMR gene defect cannot be identified. Such families are referred to as familial colorectal cancer "type X" (FCCTX), because the genetic basis of this disease is largely unknown. FCCTX forms the MMR-proficient subset and LS with pathogenic germline mutations in MMR genes, the MMR-deficient subset of HNPCC. FCCTX families show site-specific (mainly distal) colorectal cancer, as opposed to colonic (mainly proximal) and extracolonic cancers seen in LS; furthermore, the mean age at cancer onset is 10 years higher (55 years in FCCTX vs. 45 years in LS). A number of novel candidates for FCCTX susceptibility genes have recently been identified, including *GALNT12*, *BMPR1A*, *RPS20*, *SEMA4*, and *FAN1*. The genes function in different biological pathways and each seems to be restricted to a small number of families.

Colorectal carcinomas from FCCTX families display a remarkably low LINE-1 methylation (i.e., strong LINE-1 hypomethylation) as a common denominator. As low LINE-1 methylation is also a feature of normal mucosae from FCCTX patients, it may represent a field defect or a constitutional alteration. The mechanistic basis of FCCTX-associated hypomethylation remains to be uncovered by future studies.

Sporadic MSI-H Colorectal Cancer Associated With MLH1 Promoter Methylation

If a colorectal cancer is MMR-deficient, the main subgroup (~15% of all colorectal cancers) to be considered is sporadic colorectal cancer with acquired biallelic methylation of the *MLH1* promoter. Like LS-associated colorectal cancer, sporadic colorectal cancer with *MLH1* methylation preferentially affects the proximal colon; however, the age at onset is higher (~70 years) compared to LS and, for reasons that remain to be clarified, there is a clear female predominance. The presence of *BRAF* V600E mutation combined with *MLH1* promoter methylation identifies the sporadic MSI-H subgroup of colorectal cancers. A comparable (or even higher, up to 30%) share of unselected endometrial carcinomas may be MMR-deficient because of biallelic *MLH1* methylation. While *BRAF* V600E mutation is absent in LS-associated endometrial carcinoma in analogy to LS-colorectal cancer, *BRAF* mutations are so infrequent in endometrial carcinomas in general that *BRAF* V600E testing has no role in differentiating sporadic MMR-deficient endometrial carcinoma from LS-associated disease.

Lynch-Like Syndrome

Approximately 2.5% of all colorectal cancers are MMR-deficient in the absence of promoter methylation or causal germline mutations, which is referred to as LLS. In half of the cases, two somatic events that inactivate *MLH1*, *MSH2*, *MSH6*, or *PMS2* are detectable. These events can consist of two point mutations or a point mutation and LOH. The mean age at onset of colorectal cancer and predominant location in the proximal colon resemble LS. Unlike LS, LLS is an acquired disease, although some family clustering by unknown mechanisms has been reported.

The profiles of molecular alterations in MMR-deficient tumors may depend on the underlying defect. While hypermutability with repeat sequences as preferential targets is a feature common to all tumors with MMR deficiency, the CIMP phenotype combined with *BRAF* mutation is a particular characteristic of sporadic MSI colorectal cancers with *MLH1* methylation, as discussed earlier. Preliminary evidence suggests that *BRAF* mutations are absent LLS colorectal carcinomas, thus resembling LS tumors. More frequent mutations in *PIK3CA* may distinguish LLS colorectal and endometrial tumors from LS tumors.

POLE or POLD1-Associated Hypermutability

Hypermutability without MSI-H in tumors can result from proofreading mutations of the replicative DNA polymerases Pol ε and Pol δ (the polymerases are depicted in MMR in **Fig. 1**). Somatic *POLE* proofreading domain mutations occur in 1%–2% of colorectal cancers and 7%–12% of endometrial cancers (somatic *POLD1* mutations are rare). Germline mutations affecting the *POLE* and *POLD1* proofreading domains predispose to polyposis that is often relatively mild (polymerase proofreading-associated polyposis). Interestingly, faulty proofreading owing to somatic or germline mutations in *POLE* or *POLD1* can induce mutations in MMR genes and thereby explain some LLS cases with MSI-H.

Prospective Vision

The fact that LS cannot be diagnosed on the basis of clinical or tumor phenotype alone emphasizes two important aspects, first, that the syndrome serves as a general model for common cancers and second, that LS and related phenotypes are breaking down to separate subsets by modern molecular methods.

At present, germline mutations in MMR genes are detectable in up to 88% of LS families fulfilling the Amsterdam criteria and showing MSI in tumors. Smaller or atypical families and families not prescreened by MSI or immunohistochemical analyses of tumor tissues show mutation frequencies of 10%–40% depending on the method of ascertainment. Deep sequencing by targeted gene panels and whole exomes or genomes, which will be replacing current single gene-based approaches, is likely to increase the fraction of LS with detectable predisposing mutations and to unravel the genetic basis of colon cancer families unrelated to MMR defects.

The complex phenotype of LS is associated with multiple puzzles that remain to be solved by future investigations. Why does the penetrance of MMR gene mutations vary so much even between individuals with identical predisposing mutations? Related to the former question: what is the relative contribution of genetic versus nongenetic factors to LS phenotype? What is the clinical and functional significance of MMR gene variants of the VUS type? Which tumors are unequivocal components of the LS tumor spectrum and by which mechanisms? With a better understanding of the pathogenesis of LS, will cancer eventually become preventable in a large fraction of mutation carriers?

Apart from defining the spectrum of genes and mutations predisposing to LS, genome-wide screens (as opposed to previous targeted approaches) can identify low-penetrance alterations that may modify the LS phenotype. Comprehensive genetic, epigenetic, and expressional profiling of tumor tissues may define tumorigenic mechanisms in LS and identify clinically actionable somatic alterations in analogy to efforts on sporadic cancers (TCGA). One in ten carriers of MMR gene mutations may never develop cancer during their lifetime and identification of possible protective factors, inherited or acquired, would be of particular interest. Clinical and molecular studies need to be combined with epidemiological investigations to explore the impact of environmental and lifestyle factors on LS phenotype—an area that is a largely uncharted in LS compared to sporadic cancer. By integrating clinical, molecular, and epidemiological information, a complete picture of LS can be built, which is likely to advance cancer research and facilitate the management of LS individuals.

Acknowledgments

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See also: Colorectal Cancer: Pathology and Genetics. DNA Mismatch Repair: Mechanisms and Cancer Genetics. Genetic Instability.

Further Reading

Bak, S.T., Sakellariou, D., Pena-Diaz, J., 2014. The dual nature of mismatch repair as antimutator and mutator: for better or for worse. Frontiers in Genetics 5, 287.
Burn, J., Mathers, J.C., Bishop, D.T., 2013. Chemoprevention in Lynch syndrome. Familial Cancer 12, 707–718.
Carethers, J.M., 2017. Microsatellite instability pathway and EMAST in colorectal cancer. Current Colorectal Cancer Reports 13, 73–80.
Hampel, H., de la Chapelle, A., 2013. How do we approach the goal of identifying everybody with Lynch syndrome? Familial Cancer 12, 313–317.
Kloor, M., Huth, C., Voigt, A.Y., Brenner, A., Schirmacher, P., von Knebel Doeberitz, M., Bläker, H., 2012. Prevalence of mismatch repair-deficient crypt foci in Lynch syndrome: a pathological study. Lancet Oncology 13, 598–606.
Lynch, H.T., Snyder, C.L., Shaw, T.G., Heinen, C.D., Hitchins, M.P., 2015. Milestones of Lynch syndrome: 1895–2015. Nature Reviews Cancer 15, 181–194.
Peltomäki, P., 2014. Epigenetic mechanisms in the pathogenesis of Lynch syndrome. Clinical Genetics 85, 403–412.
Sijmons, R.H., Hofstra, R.M., 2016. Review: Clinical aspects of hereditary DNA mismatch repair gene mutations. DNA Repair (Amst) 38, 155–162.
Sloane, M.A., Nunez, A.C., Packham, D., Kwok, C.T., Suthers, G., Hesson, L.B., Ward, R.L., 2015. Mosaic epigenetic inheritance as a cause of early-onset colorectal cancer. JAMA Oncology 1, 953–957.
Thompson, B.A., Spurdle, A.B., Plazzer, J.-P., Greenblatt, M.S., Akagi, K., Al-Mulla, F., Bapat, B., Bernstein, I., Capellá, G., Den Dunnen, J., du Sart, D., Fabre, A., Farrell, M.P., Farrington, S.M., Frayling, I.M., Frebourg, T., Goldgar, D.E., Heinen, C.D., Holinski-Feder, E., Kohonen-Corish, M., Lagerstedt-Robinson, K., Leung, S.Y., Martins, A., Moller, P., Morak, M., Nystrom, M., Peltomaki, P., Pineda, M., Qi, M., Ramesar, R., Rasmussen, L.J., Royer-Pokora, B., Scott, R.J., Sijmons, R., Tavtigian, S.V., Tops, C.M., Weber, T., Wijnen, J., Woods, M.O., on behalf of InSiGHT, Macrae, F., Genuardi, M., 2014. Application of a five-tiered scheme for standardized classification of 2,360 unique mismatch repair gene variants lodged on the InSiGHT locus-specific database. Nature Genetics 46, 107–115.
van Duijnhoven, F.J., Botma, A., Winkels, R., Nagengast, F.M., Kampman, E., 2013. Do lifestyle factors influence colorectal cancer risk in Lynch syndrome? Familial Cancer 12, 285–293.
Vasen, H.F., Tomlinson, I., Castells, A., 2015. Clinical management of hereditary colorectal cancer syndromes. Nature Reviews Gastroenterology and Hepatology 12, 88–97.

Relevant Websites

www.insight-group.org—International Society for Gastrointestinal Hereditary Tumours.
www.LScarisk.org—Prospective Lynch syndrome database.
www.omim.org/—Online Mendelian Inheritance in Man.

Malignant Skin Adnexal Tumors: Pathology and Genetics

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Abbreviations

EMPD Extramammary Paget's disease
EMPSGC Endocrine mucin-producing sweat gland carcinoma
MAC Microcystic adnexal carcinoma
MTS Muir-Torre-Syndrome
NOS Not otherwise specified
PCACC Primary cutaneous adenoid cystic carcinoma
PPT Proliferating pilar tumor

Malignant Sweat Gland Tumors

Malignant sweat gland tumors comprise the largest group of skin adnexal carcinomas. They are classically subdividing according to their behavior into low- and high-grade carcinomas. Low-grade tumors may show locally destructive growth with risk for local recurrences and rare metastasis to loco-regional lymph nodes while disseminated metastasis and disease-related mortality is a complication of high-grade neoplasms. Morphologically, sweat gland carcinomas frequently mimic adenocarcinomas of visceral primary sites, especially of the breast. They should not be mistaken for cutaneous metastasis but reliable separation is often difficult requiring careful clinical correlation and work-up.

Low-Grade Sweat Gland Carcinomas

Primary Cutaneous Cribriform Carcinoma

Primary cutaneous cribriform carcinoma is a rare tumor of indolent behavior. No local recurrences or metastases have been described to date and it is unclear whether this tumor should be regarded as a true carcinoma. It presents as slowly growing nodules of few centimeters with a preference for the lower extremities of middle-aged adults. Females are twice as commonly affected as males. Complete removal is curative.

Histological features: The tumors are well circumscribed and situated in the dermis without connection with the overlying epidermis (Fig. 1A). They are composed of interconnecting strands of medium-sized cuboidal tumor cells with florid duct

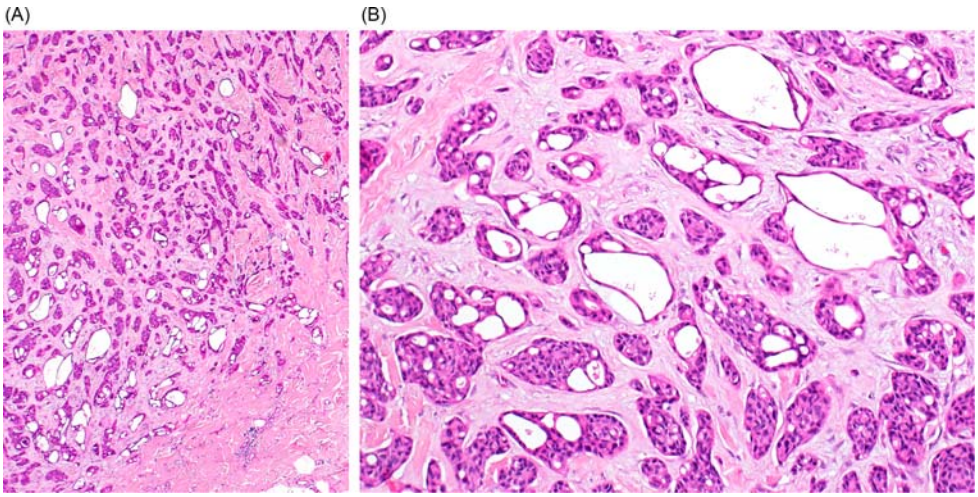

Fig. 1 Primary cutaneous cribriform carcinoma. This well circumscribed but unencapsulated nodular tumor is located within dermis and composed of interconnecting epithelial nests and strands separated by a loose fibrous stroma (A). Extensive ductal differentiation leads to its cribriform appearance. The tumor cells are cuboidal without significant cytological atypia (B).

formation, resulting in a cribriform architecture (Fig. 1B). The mitotic activity is low and there is no nuclear pleomorphism or tumor necrosis. Focal decapitation secretion may be found.

Immunohistochemistry: Expression of cytokeratins AE1/3, MNF116, Cam5.2, and CK7 is present. EMA and CEA staining illustrates luminal differentiation. No myoepithelial cell layer is present.

Differential diagnosis: Primary cutaneous cribriform carcinoma may resemble tubular adenoma. These benign tumors show a tubular growth pattern with intervening stroma. They lack the cribriform architecture and show a preserved myoepithelial layer. Separation from the more aggressive adenoid cystic carcinoma is of greater importance. Cutaneous adenoid cystic carcinoma is characterized by a diffusely infiltrative growth, and the cribriform architecture is due to mucinous pseudocysts rather than true duct formation.

Endocrine Mucin-Producing Sweat Gland Carcinoma

Endocrine mucin-producing sweat gland carcinoma (EMPSGC) is a rare disease, closely related to solid papillary carcinoma of the breast or endocrine ductal carcinoma in situ. The tumors may arise in association with and may represent a precursor of mucinous carcinoma. EMPSGC presents as a solitary, slowly growing lesion in the periorbital region or cheek, with a strong predilection for the eyelids. The patients are elderly adults (median: 70 years) with a female predilection (F:M = 2:1). Local recurrence following complete excision is a rare event and no metastases or disease related mortality have been described.

Histological features: EMPSGC is a well-circumscribed and lobulated dermal based tumor with solid, cystic and papillary differentiation in varying proportions (Fig. 2A). The solid tumor lobules are composed of monomorphous, medium-sized round and oval cells with a pale eosinophilic cytoplasm that may contain mucin (Fig. 2B). The nuclei are placed centrally and show stippled chromatin. Cytological atypia is limited and nuclear pleomorphism is rare. Mitotic activity is noted. The presence of extracellular mucin with the formation of mucinous pseudocysts is a characteristic finding (Fig. 2B). True cystic structures are present in varying amounts and may show decapitation secretion. A subset of tumors shows transition to invasive mucinous carcinoma with small tumor islands suspended in large mucin lakes. Tumor necrosis is usually not a feature.

Immunohistochemistry: The tumor cells express neuroendocrine markers (synaptophysin, chromogranin, neuron-specific enolase, CD57) and low molecular cytokeratins (Cam5.2) and CK7. Epithelial membrane antigen (EMA) highlights luminal differentiation. EMPSGC also expresses estrogen and progesterone receptors. The tumor cells are negative for CK20 and S100. No myoepithelial differentiation is found.

Differential diagnosis: Mucinous carcinoma is less circumscribed with invasion of deeper tissues. Mucin filled lakes containing scattered epithelial islands dominate the histological pattern. Cutaneous metastases from a visceral primary, particularly of breast origin, are an important differential diagnosis, which requires clinico-pathological correlation. The adenoid-cystic variant of basal cell carcinoma contains more pronounced cytological atypia, shows peripheral palisading and lacks duct differentiation. Merkel cell carcinoma is characterized by a sheet-like or trabecular architecture and clearly infiltrative growth. It lacks mucin and is cytokeratin 20 positive.

Primary Cutaneous Mucinous Carcinoma

Mucinous carcinoma arising primarily in the skin is a rare sweat gland tumor that affects the scalp and face, especially the eyelids of elderly adults (median: 76 years) with a predilection for females. The nodular tumors grow slowly and often show a violaceous discoloration. They are of low-grade malignant potential with local recurrence rates of 20%–26% but low risk for metastases, mainly

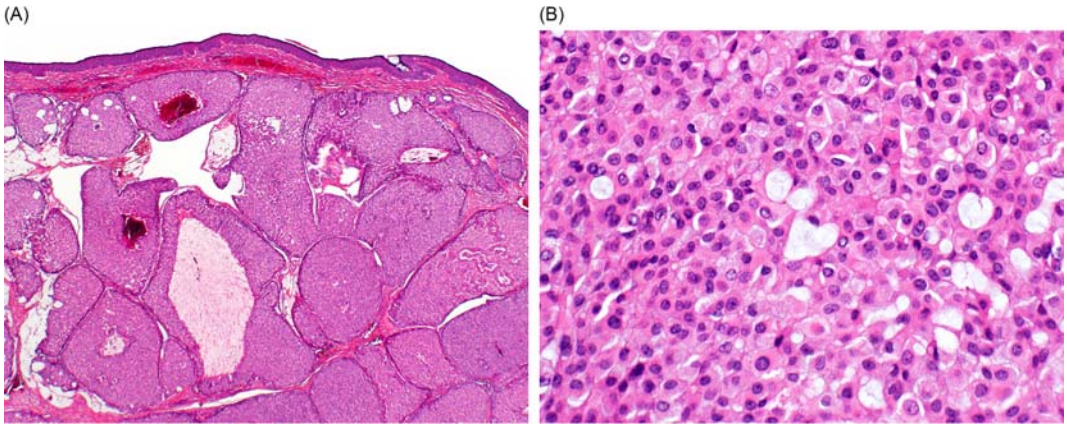

Fig. 2 Endocrine mucin-producing sweat gland carcinoma. This multilobular dermal based tumor shows a basophilic appearance (A). It is composed of medium sized round to polygonal cells containing eosinophilic cytoplasm and centrally located nuclei with indistinct nucleoli. Intracytoplasmic mucin droplets and extracellular mucin pools are also present (B).

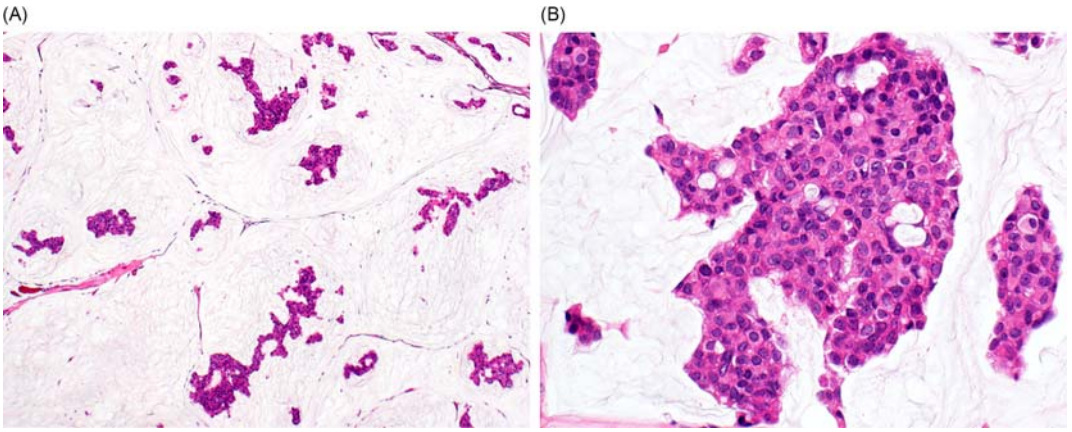

Fig. 3 Primary cutaneous mucinous carcinoma. The tumor is composed of nests and strands in an abundant mucinous matrix separated by thin fibrous septa (A). The tumor cells are polygonal and contain eosinophilic cytoplasm and irregular vesicular to hyperchromatic nuclei (B).

to loco-regional lymph nodes. Disseminated spread and disease related mortality is exceptional. Importantly, the diagnosis requires an investigation for a possible underlying mucinous adenocarcinoma of visceral sites, especially the breast, ovary, gastrointestinal and genitourinary tract.

Histological features: The tumors show an invasive growth in dermis and subcutaneous fat. They are lobulated showing fine, delicate fibrous septa and are composed of irregularly shaped epithelial islands and strands embedded in pools of mucin (Fig. 3A). The tumor cells are cuboidal containing eosinophilic cytoplasm occasionally with mucin droplets (Fig. 3B). Nuclear pleomorphism is variable. Areas of cribriform as well as solid growth are present in some tumors. Glandular structures with papillary projections and decapitation secretion may also be seen.

Immunohistochemistry: The tumor cells are positive for cytokeratins AE1/AE3, Cam 5.2., CK7, and estrogen (ER) and progesterone receptors (PR). Duct differentiation is highlighted by EMA and CEA staining. They are negative for CK20.

Differential diagnosis: Primary cutaneous mucinous carcinoma needs to be distinguished from cutaneous metastases from visceral primaries. Tumors of colonic origin can be distinguished by the presence of so-called "dirty" necrosis and expression of CK20. Visceral primaries of other sites, especially breast, require careful clinic-pathological correlation and work-up.

Microcystic Adnexal Carcinoma

Microcystic adnexal carcinoma (MAC, syringomatous carcinoma, malignant syringoma, sclerosing sweat duct carcinoma) is a rare carcinoma that usually affects adults in their 5th and 6th decade without gender bias. It typically involves the face, especially the nasolabial and periorbital regions. Other sites are rarely affected but it may be related to infiltrating syringomatous adenoma of the nipple. The tumors present as slowly growing, poorly circumscribed plaques measuring several centimeters. They are locally destructive and show high local recurrence rates of around 30%–40%. Metastasis to regional lymph nodes and systemic disease are exceptionally rare. Wide local excision or Mohs surgery are the treatment of choice to ensure complete removal and prevent against local recurrence.

Histological features: The tumor is located in the dermis and is characterized by a diffusely infiltrative architecture with invasion of subcutaneous fat, skeletal muscle, fascia and rarely bone (Fig. 4A). Morphologically it shows both follicular and sweat duct differentiation. The tumor consists of solid cords and strands of small to medium sized basaloid cells in a dense fibrotic stroma (Fig. 4B). Cytological atypia is minimal and mitoses are rare. Superficially, keratocysts and dystrophic calcifications are present. Ductal differentiation is most evident in the deeper parts of the tumor (Fig. 4C). Perineural infiltration is commonly present.

Immunohistochemistry: The tumor cells express pan-cytokeratins. EMA and CEA highlight duct differentiation.

Differential diagnosis: MAC may resemble syringoma, desmoplastic trichoepithelioma, infiltrative basal cell carcinoma and desmoplastic squamous cell carcinoma. Separation from syringoma and desmoplastic trichoepithelioma is important as these are benign skin adnexal tumors. Both tumors are confined to the superficial and mid dermis and lack the diffusely infiltrative and deep growth of MAC. Reliable separation is often challenging and may be impossible on superficial biopsies. In these cases, a deeper repeat biopsy or complete excision is recommended for a definitive diagnosis. Sclerosing basal cell carcinoma and squamous cell carcinoma lack ductal differentiation and show more pronounced cytological atypia.

Primary Cutaneous Adenoid Cystic Carcinoma

Primary cutaneous adenoid cystic carcinoma (PCACC) is rare. It affects middle-aged to elderly adults (median: 62 years) without sex predilection. It presents as slowly growing nodules and plaques, often measuring multiple centimeters. There is a predilection for the head and neck area, especially the scalp, but the anatomic distribution is wide, also including the genital area, in particular the

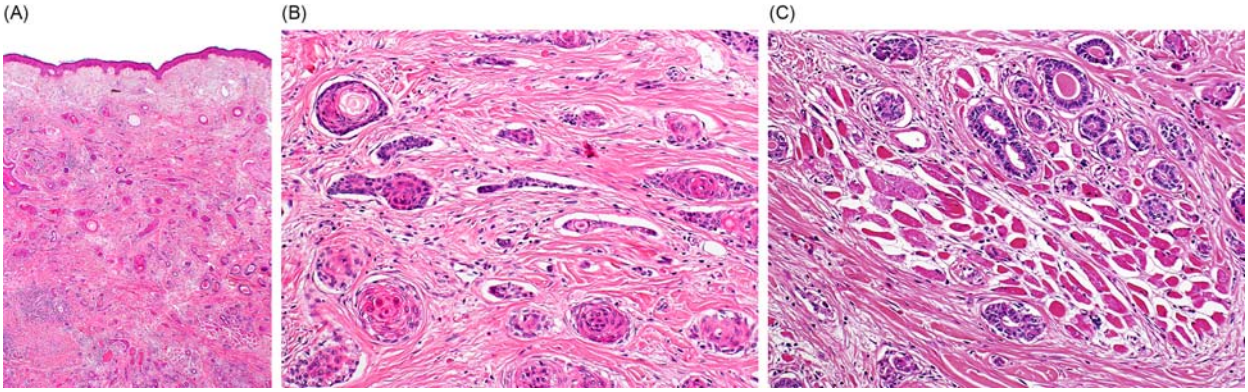

Fig. 4 Microcystic adnexal carcinoma. The tumor is characterized by a diffusely infiltrative growth within dermis (A). It consists of cords and strands showing hair follicular differentiation in a sclerotic stroma. Small keratocysts are also present (B). In the deeper aspects, ductal differentiation is evident. The tumor invades skeletal muscle (C).

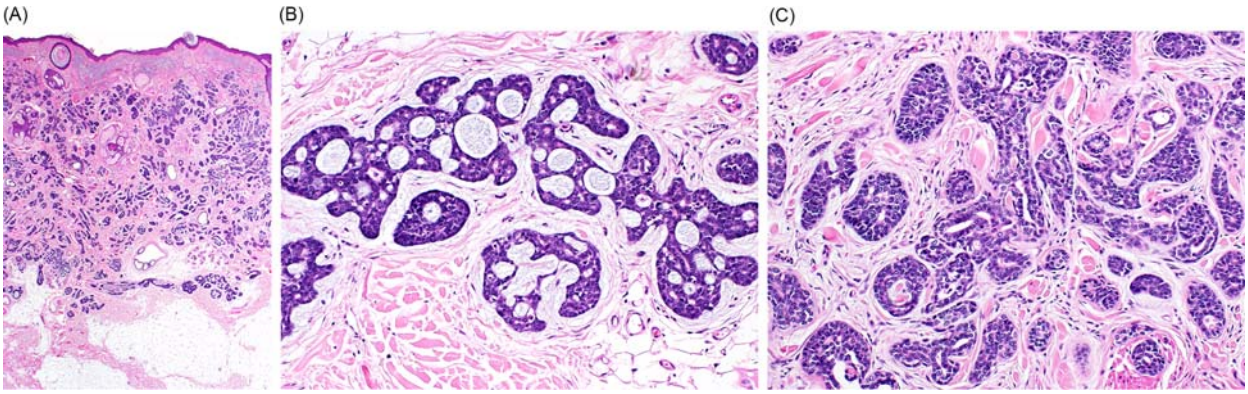

Fig. 5 Primary cutaneous adenoid cystic carcinoma. This basophilic tumor is poorly circumscribed and shows an infiltrative growth within dermis with invasion of subcutaneous adipose tissue (A). The tumor cells are medium sized without marked nuclear pleomorphism. Mucin filled pseudocyst give rise to the typical cribriform architecture (B). Ductal differentiation is a focal finding (C).

vulva. In contrast to its visceral counterparts the behavior of primary cutaneous tumors is less aggressive, characterized by locally destructive growth and risk for local recurrence. The risk for metastatic spread and mortality is however low.

Histological features: The tumor is poorly demarcated with an infiltrative growth in dermis and subcutaneous fat (Fig. 5A). It is composed of variably sized and shaped nests, tumor lobules and cords and strands of basaloid epithelioid cells with little cytoplasm and hyperchromatic nuclei. Cytological atypia is mild to moderate but mitotic activity is readily identified. The individual tumor islands are surrounded by stromal mucin leading to the formation of mucinous pseudocysts and the characteristic cribriform appearance (Fig. 5B). Ductal differentiation is focally present and perineural infiltration is usually seen (Fig. 5C).

Immunohistochemistry and genetics: The tumor expresses cytokeratins AE1/3 and Cam5.2. Myoepithelial differentiation is present and highlighted by SMA, SOX10 and S100 staining. Most tumors show positivity for CD117. Ductal structures stain with CEA and EMA.

The t(6;9)(q22-23;p23-24) translocation leading to the MYB-NFIB fusion gene and resulting in MYB overexpression characteristic of visceral adenoid cystic carcinoma has also been found in primary cutaneous tumors.

Differential diagnosis: Primary cutaneous tumors need to be distinguished from cutaneous extension or metastasis from visceral adenoid cystic carcinoma. This requires careful clinico-pathological correlation and work-up as the histological, immunohistochemical and genetic features are identical. Adenoid cystic basal cell carcinoma can be differentiated from PCACC by the presence of palisading of tumor cells, the stromal cleft artifact and lack of duct differentiation.

Squamoid Eccrine Ductal Carcinoma

Squamoid eccrine ductal carcinoma is a rare and likely under-recognized entity, closely related if not identical to adenosquamous carcinoma of the skin. The tumor mainly affects the head and neck area of elderly males. It presents as solitary and often ulcerated nodules and plaques. The tumors are characterized by local recurrence rates of 25% and occasional metastasis to regional lymph nodes. Systemic disease and mortality are rarely reported events.

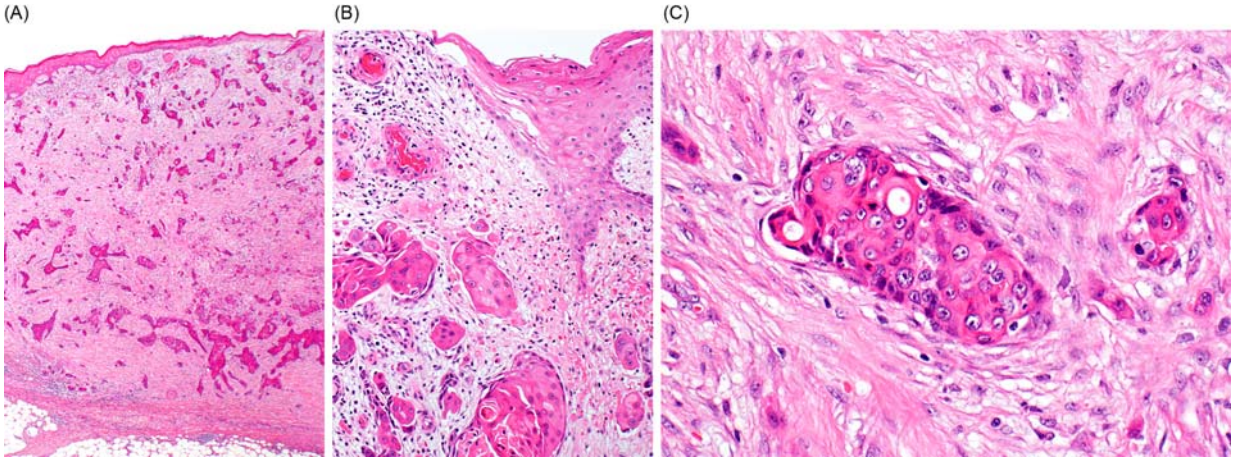

Fig. 6 Squamoid eccrine ductal carcinoma. The tumor is situated in the dermis, is poorly circumscribed and shows a diffusely infiltrative growth invading subcutaneous fat (A). In the superficial aspects of the tumor squamous cell differentiation is typical and may be morphologically identical to squamous cell carcinoma. A connection with the overlying epidermis is often present and there may be surface ulceration (B). Eccrine ductal differentiation is found in the deeper reaches of the tumor consisting of irregular cords and strands of pleomorphic cuboidal cells in a desmoplastic stroma. Mitotic activity is high (C).

Histological features: The tumors are situated in the dermis with a diffusely infiltrative growth, frequently invading subcutaneous fat, skeletal muscle and fascia (Fig. 6A). They are characterized by a dual differentiation towards squamous cell carcinoma in the superficial aspects and eccrine ductal carcinoma in the deeper reaches. A connection with the overlying epidermis is often present and there may be surface ulceration. The tumors are indistinguishable from squamous cell carcinoma superficially (Fig. 6B). In the deeper areas, they are composed of irregular cords and strands of pleomorphic cuboidal cells in a desmoplastic stroma (Fig. 6C). Mitotic activity is brisk and ductal differentiation is present. Tumor necrosis, perineural infiltration and lymphovascular invasion may be present.

Immunohistochemistry: Duct differentiation is highlighted by immunohistochemistry for CEA and EMA.

Differential diagnosis: Squamoid eccrine ductal carcinoma may be mistaken for squamous cell carcinoma on superficial biopsies. Eccrine porocarcinoma may show focal squamous differentiation but lacks the zonation. Microcystic adnexal carcinoma also shows duct and squamoid differentiation but lacks the pronounced cytological atypia.

High-Grade Sweat Gland Carcinomas

Porocarcinoma

Porocarcinoma (malignant eccrine poroma) affects elderly people in their 7th and 8th decade without gender bias. It is typically found on the lower limbs but can also be seen on the trunk, head and neck area and upper extremities. Risk factors include trauma, burning, radiotherapy and potentially also immunosuppression. Porocarcinoma presents as long-standing solitary verrucous tumors, polypoid nodules or plaques measuring several centimeters. Ulceration is common. The behavior is similar to squamous cell carcinoma if adjusted for tumor thickness. The risk for local recurrences is 17% with potential for lymph nodes metastasis. Although rare, the presence of distant metastatic spread is an adverse prognostic factor associated with high mortality rates.

Histopathological features: Porocarcinoma is a poorly delineated dermal based tumor showing multifocal epidermal connection and epidermal ulceration (Fig. 7A). Its borders may be pushing or diffusely infiltrative and invasion of subcutis and deeper structures may be present. A preexisting benign poroma is noted in 11% of cases. The tumors are composed of irregularly shaped and interconnecting strands and islands of polygonal epithelioid cells with varying degrees of cytological atypia and nuclear pleomorphism (Fig. 7B). The identification of duct differentiation is a prerequisite for the diagnosis (Fig. 7C). Tumor necrosis, perineural infiltration and lymphovascular invasion may also be present. Other rare features include squamous or clear cell change and sarcomatoid differentiation. The tumors can be pigmented by melanocytic colonization.

Immunohistochemistry: The tumor cells express pancytokeratins. P16 is overexpressed in most porocarcinomas as is p53. Duct differentiation is highlighted by EMA and CEA staining.

Differential diagnoses: Squamous cell carcinoma can be separated by the absence of duct differentiation. Squamoid eccrine ductal carcinoma shows a zonation with areas indistinguishable from squamous carcinoma and eccrine ductal carcinoma. When adjusted for tumor thickness the behavior of porocarcinoma, squamous cell carcinoma and squamoid eccrine ductal carcinoma is similar.

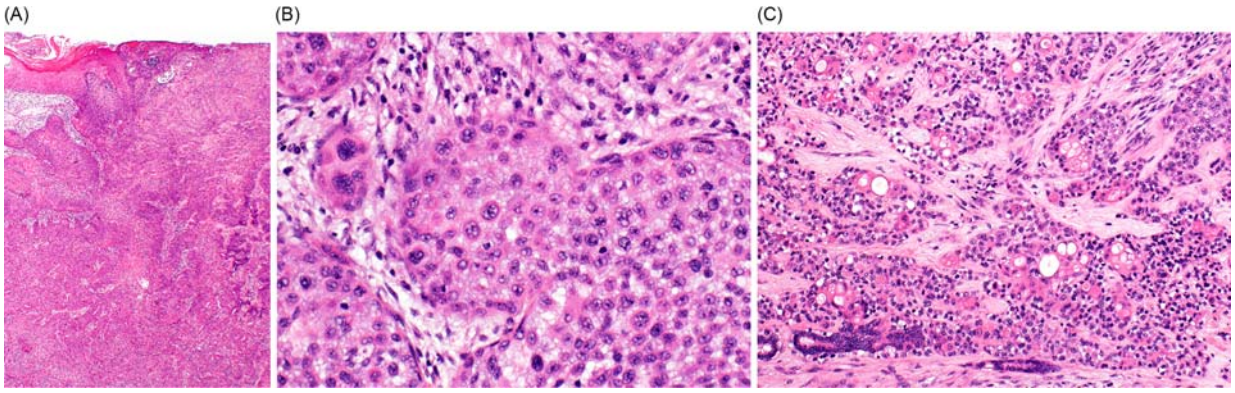

Fig. 7 Porocarcinoma. This large dermal based tumor shows ulceration and connection with the overlying epidermis (A). It consists of solid nests and islands of poroid polygonal cells with varying degrees of cytologic atypia (B). Ductal differentiation is invariably present and its identification is necessary for the diagnosis (C).

Hidradenocarcinoma

Hidradenocarcinoma (clear cell hidradenocarcinoma, malignant hidradenoma) is a rare tumor with a wide anatomic distribution and a predilection for adults. The face and the extremities appear to be most frequently affected. The tumors have potential for aggressive behavior with high local recurrence rates (50%–75%) and a high potential to metastasize to lymph nodes, lung and bone. Wide local excision is the treatment of choice. No clear guidelines exist regarding sentinel lymph node biopsy. The possibility should be considered and discussed on an individual basis.

Histopathological features: Hidradenocarcinoma is located in the dermis as a multinodular tumor with an infiltrative growth and invasion of deeper structures (Fig. 8A). The tumor lacks an epidermal connection and is composed of polygonal epithelioid cells with eosinophilic cytoplasm and variable nuclear pleomorphism (Fig. 8B). Clear cell change and squamoid features may also be present. Duct differentiation or cystic elements are invariably seen and their recognition is necessary for the diagnosis (Fig. 8C). In addition, mitotic activity, tumor necrosis, perineural infiltration and lymphovascular invasion may be seen. A preexisting benign hidradenoma is observed in a small subset of tumors.

Immunohistochemistry and genetics: The tumor cells express cytokeratin and frequently Her-2/neu. Ductal differentiation is demonstrated by EMA and CEA stains. Immunohistochemical overexpression of P53 is seen in the majority of tumors despite low frequency of mutations in the TP53 gene. The translocation t (11;19) can be detected in some hidradenocarcinomas analogous to hidradenoma.

Differential diagnoses: Morphologically low-grade tumors are a particular diagnostic challenge and need to be separated from hidradenoma. Tumors with infiltrative margins and cytological atypia need to be regarded with care and require complete excision. In this context, it is also important to be aware that morphologically and biologically benign hidradenomas may rarely show lymphovascular invasion and even lymph node deposits. Morphologically high-grade tumors require separation from cutaneous metastases from visceral primaries by careful clinical correlation, work-up and imaging techniques.

Apocrine Carcinoma

Apocrine carcinoma is a rare adenocarcinoma mainly affecting the axilla of adults with a mean age of 60 years and no significant gender predilection. It presents as solitary, slowly growing erythematous or violaceous nodules. The disease course can be aggressive

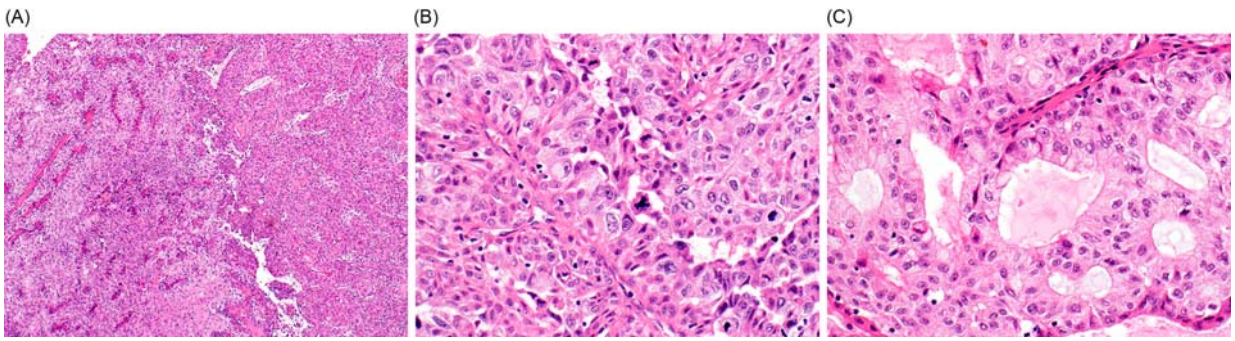

Fig. 8 Hidradenocarcinoma. The tumor shows a solid growth and arises from a preexisting hidradenoma seen on the right-hand side (A). Marked cytological atypia, nuclear pleomorphism and mitotic figures are present (B). In areas duct differentiation is appreciated (C).

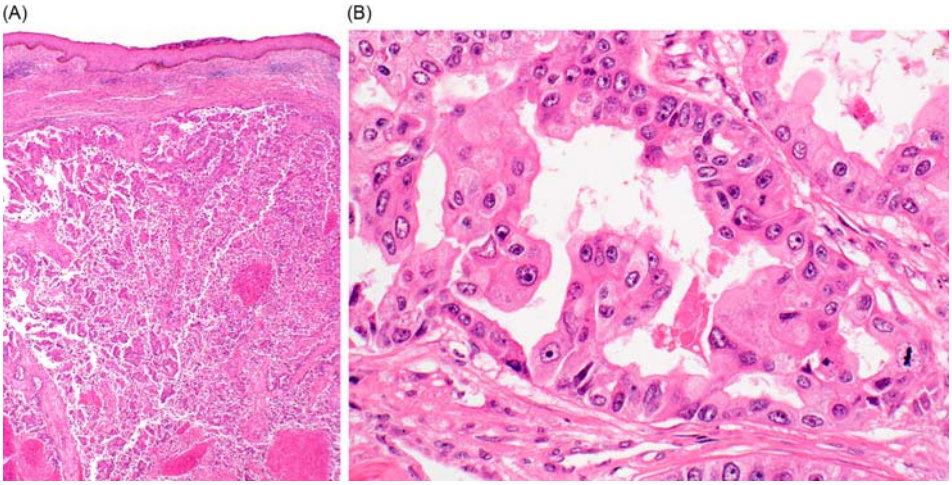

Fig. 9 Apocrine carcinoma. This dermal-based tumor shows a mixture of cystic and papillary elements (A). It is composed of large epithelioid cells with abundant eosinophilic cytoplasm containing vesicular nuclei and prominent eosinophilic nucleoli. Mitotic activity is identified (B).

with metastatic disease to lymph nodes and distant organs (lungs, liver, bone) and associated mortality. Histological grading appears to be a good predictor of outcome. The treatment consists of wide local excision.

Histopathological features: Apocrine carcinoma is lobulated and located in the dermis, frequently also invading subcutaneous fat (Fig. 9A). It is poorly circumscribed with infiltrative borders. The tumor cells are polygonal containing abundant eosinophilic cytoplasm and vesicular nuclei with prominent nucleoli. Cytological atypia and mitotic activity are variable and tumor necrosis may be seen (Fig. 9B). The growth pattern may show solid, tubular and cystic structures in a dense fibrotic stroma. Papillary structures are also frequently seen and decapitation secretion is consistently present. Adjacent apocrine glands are usually found close to the tumor. The tumors can be graded according to the modified Bloom Richardson grading system, which correlates with outcome.

Immunohistochemistry and genetics: The tumor cells express CAM5.2, AE1/AE3, EMA, CEA, GCDFP-15, mammaglobin, ER and PR. They are negative for HER2/neu and adipophilin.

Differential diagnosis: Apocrine carcinoma needs to be differentiated from metastatic adenocarcinoma, especially of breast origin. This differential diagnosis is challenging due to the morphologic overlap and presentation in the axilla. It requires careful clinical correlation. In addition, an immunohistochemical panel of ER and PR, HER2/neu, adipophilin, CK5/6, mammaglobin may be helpful: cutaneous apocrine carcinoma is likely to be adipophilin and Her2/neu negative, ER and PR positive and also shows a strong positive staining with CK5/6 and mammaglobin.

Digital Papillary Adenocarcinoma

Digital papillary adenocarcinoma is a rare but distinctive sweat gland carcinoma with a narrow anatomic distribution and a marked male predominance. The tumor shows a strong predilection for the distal extremities, particularly the digits. It presents as small erythematous or brown nodules, ranging from few millimeters to multiple centimeters. A wide age range is affected, including children and adolescents. The tumors have potential for aggressive behavior independent of the histological degree of differentiation. The local recurrence rates are high (20%–40%) and metastatic disease is seen in approximately 15%, mainly to lymph nodes and lung. Wide local excision or amputation is the recommended treatment. It may positively influence the disease course with reduced local recurrence and distant metastatic rates.

Histological features: Digital papillary adenocarcinoma shows a wide histological spectrum ranging from bland and innocuous appearing tumors to poorly differentiated examples with high-grade morphology. The majority of tumors are well-circumscribed and located in the deep dermis and superficial subcutaneous adipose tissue (Fig. 10A). They are solid and cystic with additional papillary, micropapillary and tubular growth patterns (Fig. 10B and C). The tumor cells are cuboidal basaloid cells with varying degrees of cellular atypia and nuclear pleomorphism, ranging from mild to severe. Mitotic figures are usually present. A second myoepithelial cell layer is present. Tumor necrosis and a more infiltrative growth are rare additional findings (Fig. 10C).

Immunohistochemistry and genetics: Tumor cells express cytokeratins. The luminal differentiation can be highlighted by EMA and CEA staining. S100, p63, podoplanin, SMA and calponin staining demonstrates a second myoepithelial cell layer.

Differential diagnosis: In view of the bland histological features and the preserved myoepithelial cell layer digital papillary adenocarcinoma may easily be mistaken for benign skin adnexal tumors, especially apocrine cystadenoma, hidradenoma and tubular adenoma. Apocrine cystadenoma is exceptionally rare on acral sites and this diagnosis should be made only after careful consideration. In reality, the vast majority of cystic and papillary tumors thought to be apocrine cystadenomas are in fact digital papillary adenocarcinomas. Hidradenoma shares many of the histological features with digital papillary adenocarcinoma. Papillary differentiation is usually not a prominent finding and the presence of any cytological atypia should be a warning sign. Tubular adenoma is

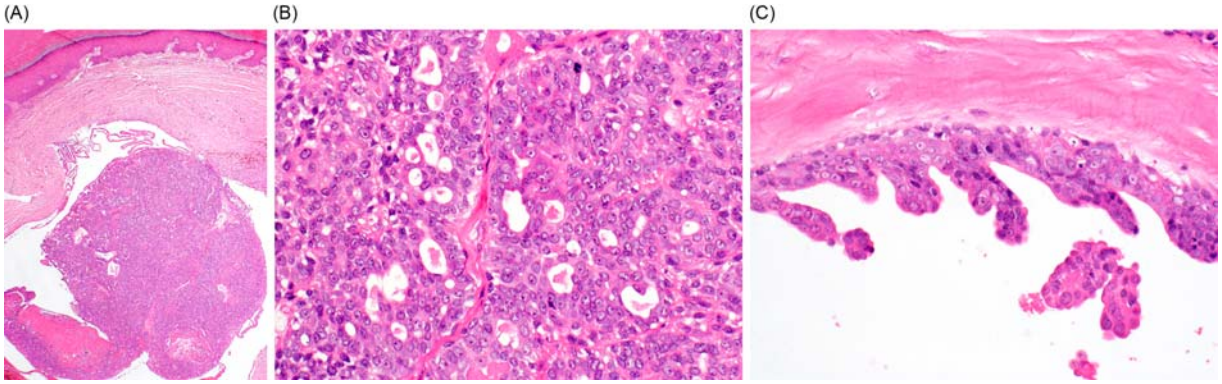

Fig. 10 Digital papillary adenocarcinoma. The tumor is well-circumscribed and situated in the dermis with a solid and cystic architecture (A). In areas duct formation and a tubular growth are noted (B). The cystic structures show micropapillae and a second myoepithelial cell layer can be seen (C).

characterized by a striking tubular growth with intervening stroma. A solid growth component is not observed. Metastases from papillary adenocarcinomas of visceral origin can be excluded by the demonstration of the myoepithelial differentiation.

Malignant Neoplasms Arising from Preexisting Spiradenoma, Cylindroma or Spiradenocylindroma

These rare tumors are defined by the presence of a malignant component arising in the background of a preexisting spiradenoma (spiradenocarcinoma), cylindroma (cylindrocarcinoma) of a hybrid spiradenoma-cylindroma. They present as solitary nodules with a preference for the head and neck and the extremities of elderly adults without gender bias. A long-standing history with recent enlargement may be given. The tumors may develop in association with the autosomal dominant Brooke Spiegler syndrome. The syndrome is characterized by the development of multiple adnexal neoplasms, especially cylindroma, spiradenoma, spiradenocylindroma and trichoepithelioma. The Brooke Spiegler syndrome is linked to mutations resulting in the inactivation of the tumor suppressor gene CYLD.

Morphologically the tumors arising from preexisting spiradenoma and cylindroma are divided into low- and high-grade, which correlates with outcome. Low-grade carcinomas have a 20% risk of local recurrence but distant metastasis and disease-related mortality is seen almost exclusively in high-grade carcinomas.

Histological features: These multinodular tumors are located in the deep dermis and subcutis and often show pushing rather than diffusely infiltrative borders. Recognition of a preexisting spiradenoma, cylindroma or hybrid tumor is essential for the diagnosis. Morphologically low-grade tumors show a similar architecture to spiradenoma on low power examination (Fig. 11A). They are composed of a monotonous population of uniform basaloid cells with mild to moderate cytological atypia and increased mitotic activity (Fig. 11B). A hallmark feature is the lack of the dual cell population, typical of spiradenoma and cylindroma. Additional findings include clear cell change and the formation of squamous eddies. Tumor necrosis and ulceration may also be observed. Morphologically high-grade tumors show a wide range of histological features. They are characterized by high-grade cytological atypia and nuclear pleomorphism and atypical mitoses, resembling poorly differentiated carcinoma or adenocarcinoma, NOS (Fig. 11C). Necrosis is a frequent finding.

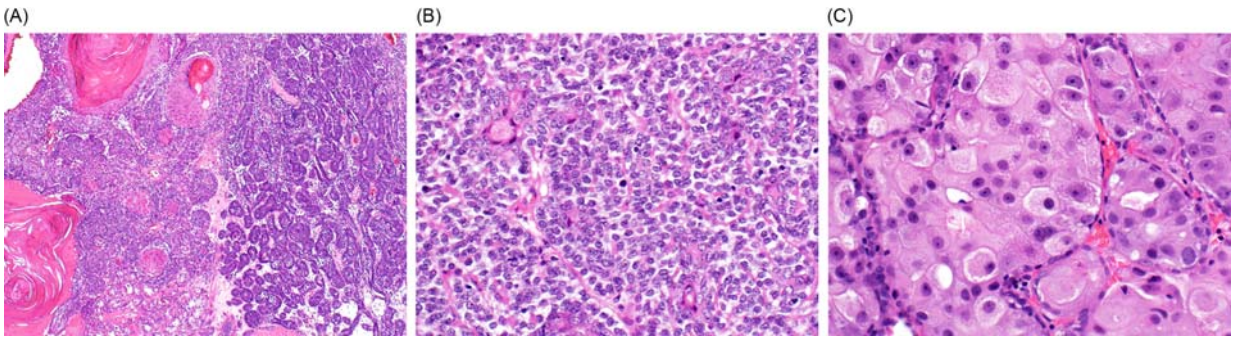

Fig. 11 Spiradenocarcinoma. The malignant nodular tumor (on the left-hand side) arises in association with a preexisting benign spiradenoma (on the right-hand side) (A). Morphologically low-grade tumors show a monotonous proliferation of uniform basaloid cells with mild to moderate degrees of cytological atypia and increased mitotic activity. The dual cell population characteristic of benign spiradenoma is lost (B). Morphologically high-grade spiradenocarcinoma is characterized by marked cytological atypia and resembles poorly differentiated adenocarcinoma (C).

Immunohistochemistry and genetics: The tumor cells express cytokeratins, duct differentiation is highlighted by EMA or CEA staining. Immunohistochemical overexpression of MYB is used as surrogate marker for the t(6;9) translocation seen in benign spiradenomas and cylindromas. MYB expression is lost, at least in the low-grade malignant tumors and serves as a helpful diagnostic marker to distinguish these tumors from their benign counterparts. Adnexal neoplasms arising in patients with Brooke Spiegler syndrome are likely to show mutations in the CYLD gene mapped on chromosome locus 16q12–13.

Differential diagnosis: The separation of morphologically low-grade spiradenocarcinoma from benign spiradenoma is particularly challenging. It requires careful examination with recognition of loss of the dual cell population, the presence of cytological atypia and mitotic activity. Immunohistochemical loss of MYB expression is helpful in this setting. High-grade tumors resemble metastases from visceral primaries. The recognition of a benign precursor is essential for the correct diagnosis.

Extramammary Paget's Disease

Extramammary Paget's disease (EMPD) is a rare adenocarcinoma. The majority of tumors develop primary to the skin which is subject of this article. Less commonly, EMPD presents secondary to the skin as cutaneous spread of an underlying visceral carcinoma, especially of the rectum, the urogenital tract, endocervix and stomach. Primary cutaneous EMPD develops in areas rich in apocrine sweat glands, especially in the vulva and perianally. Other sites include the penis, scrotum, axillae, umbilicus, and very rarely the face, scalp, trunk and extremities. It shows a predilection for females in their sixth to eighth decade of life. EMPD presents as erythematous, pigmented or erosive plaques accompanied by pruritus or pain. Early lesions can resemble eczema. In late stages nodules and ulceration are noted.

Primary cutaneous EMPD is usually diagnosed in the intraepidermal state (carcinoma in-situ). It shows a high risk for local recurrences but the overall prognosis is excellent. In contrast, invasion is seen in approximately 10% of EMPD and is associated with potential for aggressive behavior with regional and distant metastasis resulting in disease related mortality. Treatment for localized disease includes radical excision and radiotherapy. In-situ disease may also respond to photodynamic therapy and local immunomodulators (imiquimod).

Histological features: Primary cutaneous EMPD presents as an intraepidermal carcinoma in most cases. It is composed of medium-sized to large round to epithelioid cells arranged singly and in clusters (**Fig. 12**A). The tumor cells are scattered throughout the epidermis and may also involve hair follicular epithelium and sweat ducts. They contain abundant cytoplasm and vesicular nuclei showing variable pleomorphism. Intracytoplasmic mucin may be seen. Signet ring cell and glandular differentiation have been described. The involved epidermis may be hyperplastic and ulceration may be a feature. An invasive component is present in approximately 10% of cases. The invasive component shows a diffusely infiltrative growth involving dermis and deeper structures. It may be solid or nested and can show single cell infiltration. Signet ring or acinar differentiation may also be evident (**Fig. 12**B).

Immunohistochemistry: The tumor cells are PAS-positive and express low molecular weight cytokeratins (CAM5.2) and cytokeratin 7. Tumor cells are also positive for EMA, CEA, GCDFP-15 and mucicarmine 1. Androgen receptors and Her-2/neu are variably positive.

Differential Diagnoses: Most importantly, an epidermotropic metastasis of an internal adenocarcinoma needs to be excluded. Metastasis from colonic carcinoma is usually positive for both CK20 and CDX-2 in contrast to EMPD. Melanoma in situ and superficial spreading melanoma may show morphological similarities but can be distinguished by staining for melanocytic markers (S100, MelanA, HMB45). Clear cell squamous cell carcinoma is negative for GCDFP-15 and CEA but positive for p63.

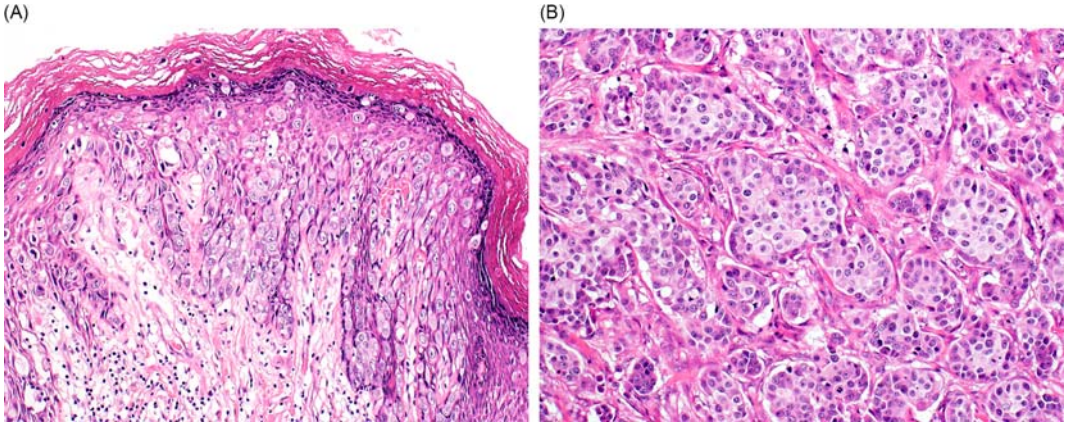

Fig. 12 Extramammary Paget's disease. The epidermis is diffusely infiltrated by medium sized tumor cells with abundant eosinophilic to clear cytoplasm and vesicular nuclei containing prominent eosinophilic nucleoli. The tumor cells are arranged singly and in small clusters, there is surrounding epidermal acanthosis (A). The invasive component consists of irregular nests of tumor cells. The invasive tumor cells are morphologically identical to the intraepidermal cells. Mitoses are readily spotted (B).

Eccrine Ductal Carcinoma NOS

Eccrine ductal carcinoma (eccrine adenocarcinoma) NOS is a diagnosis of exclusion. These rare tumors do not fit with any of the entities described above, and there are no systematic studies in the literature. Histologically they may be identical to metastases from visceral primaries, particularly of breast origin. Their diagnosis is only possible after a visceral primary has been excluded by clinical work-up and imaging studies. The tumors present as firm erythematous nodules involving a wide range of anatomical locations, especially of middle aged and elderly adults. The disease course is aggressive with high recurrence, metastasis and mortality rates.

Histological features: Eccrine ductal carcinoma is located in the deep dermis and may invade subcutaneous fat and deeper structures (Fig. 13A). The tumors are poorly circumscribed and consist of nests and strands of pleomorphic cuboidal cells with prominent ductal differentiation in a sclerotic stroma (Fig. 13B). Mitotic activity is brisk and tumor necrosis may be present. Perineural infiltration and lymphovascular invasion are common features.

Immunohistochemistry: The tumor cells express cytokeratins and CEA. Hormone receptors, GCDFP-15 and S100 are variably positive.

Differential diagnosis: Eccrine ductal carcinoma is morphologically identical to cutaneous metastasis from adenocarcinomas of visceral sites. The diagnosis of eccrine ductal carcinoma is one of exclusion requiring a careful clinical work-up and imaging of the patient.

Malignant Tumors with Follicular Differentiation

Pilomatrix Carcinoma

Pilomatrix carcinoma (malignant pilomatricoma, matrical carcinoma) is a rare neoplasm of low-grade malignancy and a predilection for male adults. The tumor presents as nodules of a few to multiple centimeters with a wide anatomical distribution. The head and neck area and the back are most commonly involved. Local recurrences are common but loco-regional and distant metastases are rare.

Histological features: Pilomatrix carcinoma is a multinodular dermal based tumor and may show invasion of subcutis, skeletal muscle and fascial tissues (Fig. 14A). The irregularly shaped and sized tumor lobules are composed of basophilic tumor cells with varying degrees of cytological atypia and a brisk and atypical mitotic activity. In addition, the tumor shows the distinctive hair matrix keratinization with ghost or shadow cell differentiation (Fig. 14B). Confluent necrosis, perineural infiltration and lymphovascular invasion may be present. Occasionally, colonization by melanocytes is seen.

Immunohistochemistry and genetics: Pilomatrix carcinoma is associated with mutations in beta-catenin that have also been identified in pilomatricoma, its benign counterpart. By immunohistochemistry, the tumor cells show nuclear expression of beta-catenin and cyclin D.

Differential diagnosis: Differentiation from the benign pilomatricoma is challenging. An infiltrative growth pattern, the presence of cytological atypia and tumor necrosis are the most reliable differentiating features. Basal cell carcinoma with matrical differentiation shows a peripheral palisade and a stromal cleft artifact.

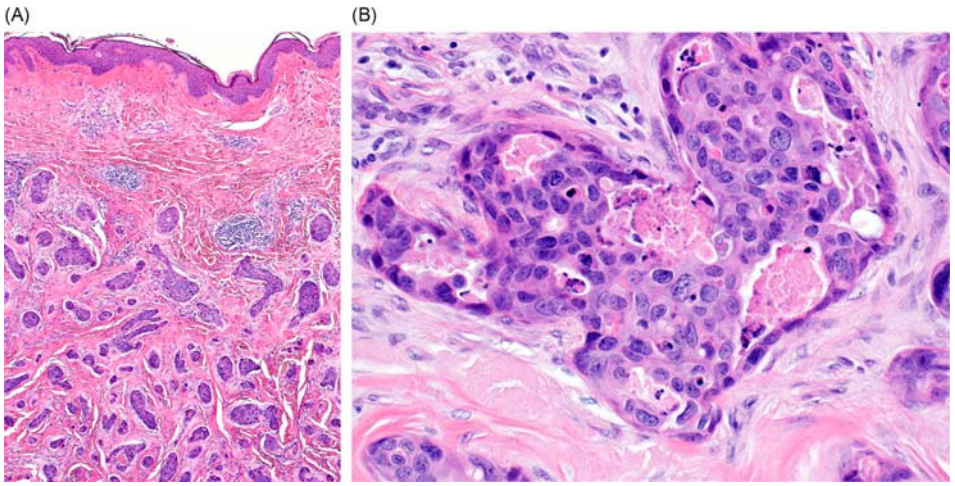

Fig. 13 Eccrine ductal carcinoma, NOS. The dermal based tumor is composed of irregularly shaped basaloid nests and lobules (A). There is marked nuclear pleomorphism and prominent duct differentiation. A desmoplastic stroma surrounds the tumor nests (B).

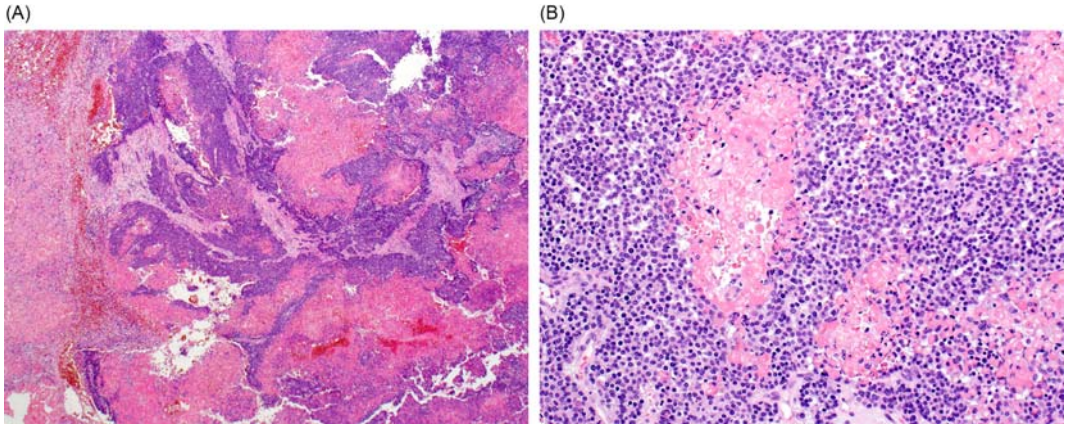

Fig. 14 Pilomatrix carcinoma. This large tumor is composed of irregular lobules containing sheets of basophilic cells and keratin (A). The tumor cells show a monotonous growth pattern and keratinization with ghost- or shadow-cell differentiation (B).

Trichilemmal Carcinoma

Trichilemmal carcinoma is a rare carcinoma of low-grade malignancy. It affects sun-exposed skin, particularly of the face, scalp, neck and hands of the elderly with a mean age of 71 years. The tumors are solitary erythematous nodules and plaques that may ulcerate. After complete excision trichilemmal carcinoma recurs very rarely. Regional lymph node metastases and systemic disease are rare events, but have been described, especially in immunocompromised patients.

Histopathological features: Trichilemmal carcinoma is a dermal based tumor with pushing borders composed of irregular tumor lobules, nests and strands (Fig. 15A). A diffusely infiltrative architecture is rarely observed. The tumor may extend into deep dermis and invades subcutaneous fat. A multifocal connection with the overlying epidermis or with hair follicles is typically present and there may be epidermal ulceration. The neoplastic cells show trichilemmal characteristics with clear to palely eosinophilic cytoplasm. Trichilemmal keratinization is invariably present either in the form of intracytoplasmic hyaline droplets, in form of small horn cysts or as large confluent areas (Fig. 15B). Cytological atypia and nuclear pleomorphism are variable and range from mild to severe. Nuclear palisading may be seen in the periphery of tumor lobules with surrounding hyaline basement membrane material. Mitoses are often numerous including atypical forms. Tumor necrosis may be an additional finding.

Immunohistochemistry: The tumor cells express cytokeratins and the cytoplasm is PAS-diastase positive. With rare exception, there is no expression of EMA and CEA.

Differential diagnoses: Differentiation from clear cell squamous cell carcinoma is difficult. The trichilemmal keratinization and hyaline basement membrane material as well as EMA negativity are diagnostic clues. Basal cell carcinoma with outer root sheath differentiation shows palisading of peripheral cells and retraction artifacts between tumor lobules and tumor stroma. Porocarcinoma and hidradenocarcinoma are separated by the presence of ductal differentiation.

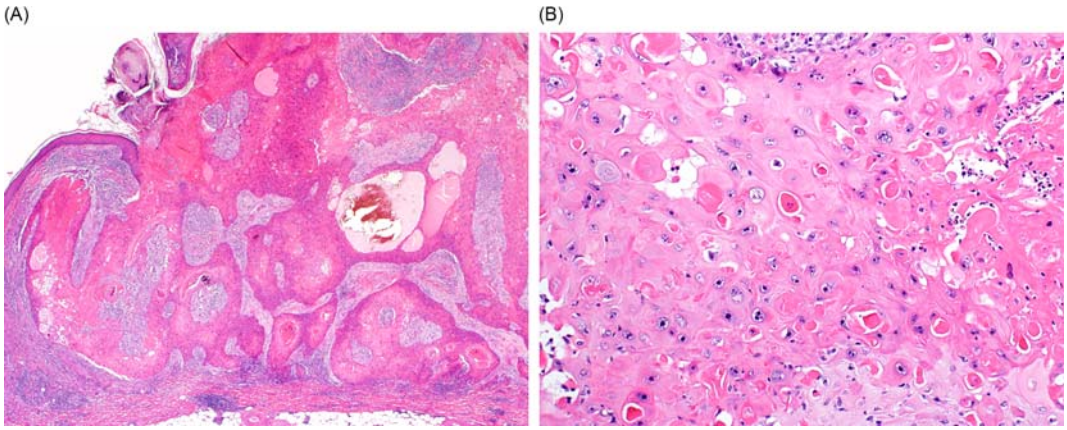

Fig. 15 Trichilemmal carcinoma. The tumor is connected with the surface epithelium and shows a multinodular growth pattern with pushing borders (A). It consists of large epithelial cells with abundant palely eosinophilic cytoplasm and trichilemmal keratinization. Nuclear pleomorphism is marked (B).

Proliferating Pilar Tumor

Proliferating pilar tumor (PPT) is a rare neoplasm that shows a predilection for elderly women. The tumors are usually located on the scalp. Other locations include eyelids, face, neck, vulva, trunk and extremities. PTT presents as slowly growing subcutaneous nodules measuring several centimeters. The majority of proliferating pilar tumors is benign. Local destructive growth and metastases to regional lymph nodes and distant organs is rare and seen in tumors with adverse histological features as listed below.

Histopathological features: The tumor has a multinodular growth pattern in deep dermis and may extend into the subcutaneous fat, skeletal muscle and fascia (**Fig. 16**A). It shows solid and cystic differentiation composed of large epithelioid cells with palely eosinophilic cytoplasm, vesicular nuclei and prominent trichilemmal keratinization (**Fig. 16**B). The periphery of tumor lobules is composed of smaller, more basophilic cells that may show palisading and an outer hyaline basement membrane. The presence of marked cytological atypia, atypical mitotic activity, an infiltrative growth pattern, tumor necrosis, perineural infiltration and lymphovascular invasion confers risk for more aggressive behavior.

Immunohistochemistry: Immunohistochemistry plays no role in the diagnosis of these tumors.

Differential diagnosis: Squamous cell carcinoma and trichilemmal carcinoma are characterized by an epidermal connection. They both lack the classical solid and cystic multilobular growth pattern characteristic of proliferating pilar tumor.

Sebaceous Carcinoma

Sebaceous carcinoma is a rare neoplasm that is classically divided into periocular (sebaceous carcinoma of the eyelids) and extraocular sebaceous carcinoma.

Periocular sebaceous carcinoma affects the elderly and appears to be more common in Asia. It has potential for aggressive behavior with local recurrence rates of 30%–40% and metastases to regional lymph nodes and distant organs in up to 25%. Metastatic disease is associated with high mortality and poor 5-year survival.

Extraocular sebaceous carcinoma is rare. It is mainly found in the head and neck area of elderly adults and presents with erythematous and often ulcerated nodules and plaques. The presentation outside the head and neck area, young age at presentation and multiple sebaceous neoplasms raise suspicion for the Muir-Torre-Syndrome (MTS), a variant of hereditary nonpolyposis colorectal cancer syndrome (Lynch syndrome). The behavior of extraocular sebaceous carcinoma is thought to be similar to that of periocular tumors. Treatment of choice is complete excision and long-term follow-up.

Histological features: Sebaceous carcinomas are circumscribed and multinodular or diffusely infiltrative dermal based tumors, often invading underlying subcutaneous tissues (**Fig. 17**A). They may show multifocal epidermal connection and ulceration (**Fig. 17**B). The tumor cells are epithelioid with basophilic cytoplasm and vesicular nuclei. Admixed in varying proportions are pale staining tumor cells with a vacuolated cytoplasm and indented nuclei (**Fig. 17**C). Poorly differentiated tumors show a predominance of the basaloid cells and sebaceous differentiation is difficult to appreciate on histology (**Fig. 17**D). There may be marked cytological atypia and nuclear pleomorphism. Mitoses are numerous and tumor necrosis is frequently seen. Lymphovascular or perineural invasion may be present. An intraepidermal growth with florid pagetoid spread is a feature seen particularly in the periocular tumors.

Immunohistochemistry: Tumor cells are positive for cytokeratin 7, p63 and p40. Sebaceous differentiation is highlighted by staining for EMA and adipophilin. The immunohistochemical demonstration of sebaceous differentiation may be difficult in poorly differentiated tumors.

Differential diagnosis: Sebaceous carcinoma needs to be distinguished from basal cell carcinoma, basaloid squamous cell carcinoma and Merkel cell carcinoma. The demonstration of sebaceous differentiation on morphology and immunohistochemistry is the clue to the correct diagnosis. In contrast to basal cell carcinoma sebaceous carcinoma does not show palisading of peripheral

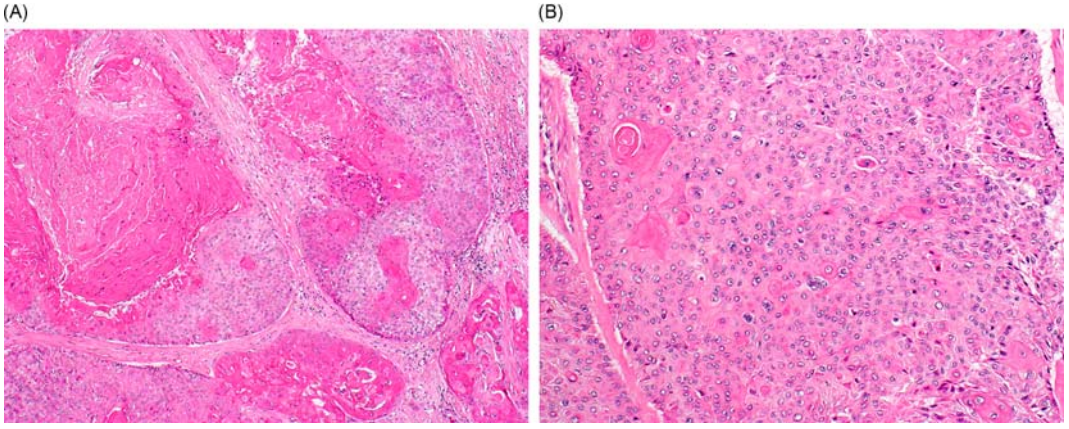

Fig. 16 Proliferating pilar tumor. The lobulated tumor is composed of solid epithelial elements and abundant trichilemmal keratinization (A). There is cytological atypia and numerous mitotic figures are present (B).

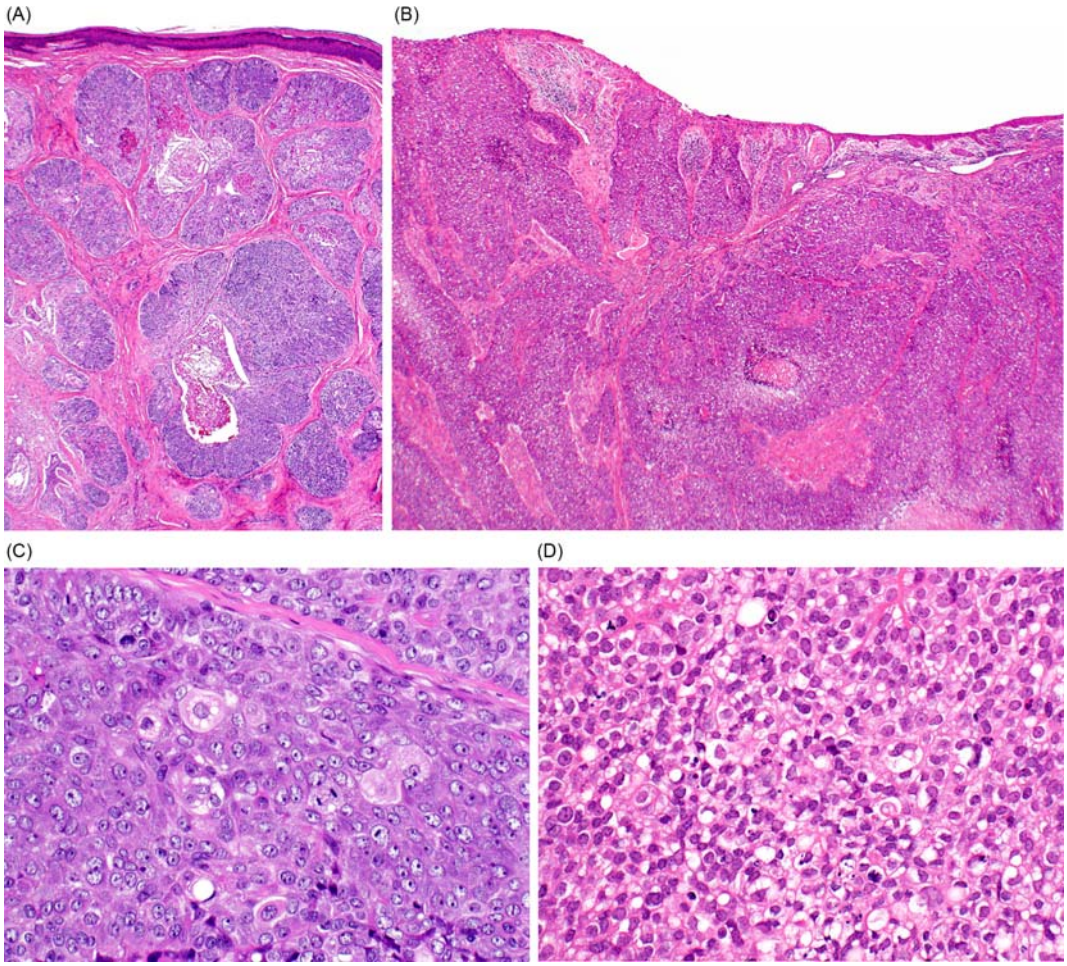

Fig. 17 Sebaceous carcinoma. This basaloid tumor is characterized by a multinodular growth within dermis (A). Multifocal epidermal connection and ulceration are present (B). The tumor is composed of basaloid cells with cellular atypia and admixed pale staining cells with a vacuolated cytoplasm (C). Sebaceous differentiation is difficult to appreciate in poorly differentiated examples (D).

cells, no retraction artifacts and is usually BerEp4 negative. Merkel cell carcinoma expresses CK20 which is consistently negative in sebaceous carcinoma.

Prospective Vision

Insight into the clinical behavior and underlying molecular events of skin adnexal carcinomas is still limited. Larger and more comprehensive studies with genetic analysis are necessary to better define these tumors and develop novel targeted therapeutic options, especially for disseminated disease.

See also: Squamous Cell and Basal Cell Carcinoma of the Skin: Diagnosis and Treatment.

Further Reading

Brenn, T., 2015. Malignant sweat gland tumors: An update. Advances in Anatomic Pathology 22, 242–253.
Calonje, E., Brenn, T., Lazar, A., McKee, P.H., 2012. McKee's pathology of the skin, fourth ed. Vol. 2. Elsevier, Edinburgh.
Flux, K., 2017. Sebaceous neoplasms. Surgical Pathology Clinics 10, 367–382.
Flux, K., Brenn, T., 2017. Cutaneous sweat gland carcinomas with basaloid differentiation: An update with emphasis on differential diagnoses. Clinics in Laboratory Medicine 37, 587–601.
Kazakov, D., Michal, M., Kacerovska, D., McKee, P.H., 2012. Cutaneous adnexal tumors. Wolters Kluwer Health, Philadelphia.
Patterson, J., 2015. Weedon's skin pathology, 4th ed. Elsevier, Edinburgh.
Van der Horst, M.P.J., Brenn, T., 2017. Update on malignant sweat gland tumors. Surgical Pathology Clinics 10, 383–397.

Malignant Tumors of the Eye, Conjunctiva, and Orbit: Diagnosis and Therapy

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Primary Intraocular Malignant Tumors

Uveal Melanoma

Diagnosis

Uveal melanoma is the most frequent primary intraocular tumors of adults. The age at diagnosis is around 60 years. For most patients, conservative management by proton beam therapy, plaque brachytherapy or transcleral resection is possible with equal survival than enucleation. Metastasis occurs in 50% of the patients mostly in the liver and survival with liver metastasis is short. For many years, there has been little progress in the management of metastatic disease but there is now increasing research and interest in many centers.

Blue and green eyes are more frequently involved and uveal melanoma is very rare in melanodermic patients. Usual visual symptoms include scotomas, phosphenes, loss of vision if the tumor is involving the macula or if the central retina is detached. Large peripheral tumors can cause visual field defects. Diagnosis relies mainly on fundus examination. Location of the tumor is very variable from ciliary body to posterior pole. Small ciliary body masses are often asymptomatic at first. They can invade the iris angle but are only visible with dilated pupil. Most tumor appears as a choroidal or ciliochoroidal mass with variable amount of pigmentation (sometimes very dark, sometimes amelanotic). The typical shape is the mushroom shape due to rupture of Bruch's membrane. Orange pigment can be present on the surface representing lipofuscin deposits. Serous retinal detachment is often associated sometimes visible only on OCT, sometimes clinically visible and associated to inferior exudative retinal detachment. Useful ancillary test are ultrasonography of the eye and fluorescein angiography. MRI can be useful when there is invasion of optic disk or suspected extra scleral extension. Ultrasonography usually shows typical attenuation of ultrasounds due to the very dense tumor tissue. The tumors appear hypoechogenic at the base with frequent choroidal excavation. On fluorescein angiography, it is possible to see a typical double circulation in achromic tumors in the early phase. Later tumors all show hyperfluorescence with surface pin points.

In the absence of appropriate treatment, uveal melanoma can invade the sclera and grow in the orbit. Extrascleral extension can be detected by ultrasonography or MRI. MRI is also helpful to verify the absence of optic nerve invasion for tumors invading the optic disk. If extrascleral extension is detected it can be treated by brachytherapy or proton beam but large extrascleral extension requires enucleation followed by external beam radiation.

Differential diagnosis includes choroidal naevi and other benign pigmented lesions such as melanocytomas and congenital hypertrophy of the retinal pigment epithelium, choroidal metastasis, choroidal hematomas, choroidal hemangiomas and choroidal osteomas. The most difficult and frequent situation is to differentiate between suspicious choroidal naevi and melanoma. The risk factors for a nevus to grow into a melanoma have been extensively described by JA Shields and CL Shields. They are diameter of >7 mm, thickness of >2 mm, orange pigment, subretinal fluid, visual symptoms, absence of drusen and location close to the optic disk. In studies performed with fluorescein angiography it has been shown that the presence of pin points is a significant risk factor. Risk for growth increases when the number of risk factors increases. The decision to treat depends on the number of risk factors, the location of the tumor and age of the patients. Decision should always be made with informed consent of the patient.

Unique metastasis can sometimes be difficult to differentiate from achromic melanoma. Choroidal metastasis can be seen in all cancer but more frequently in breast cancer and lung cancer. In lung cancer, metastasis is the first symptom of the disease in many cases. It is recommended to perform thoracoabdominal CT in all patients with achromic fundus mass.

Prognosis

Clinical risk factors for metastatic disease are well known: largest tumor diameter and anterior tumor location are important. Histological risk factors are the presence of epithelioid cells, mitotic count, vascular loops and presence of intravascular tumor cells. Largest tumor diameter should be correlated with histological and cytogenetic studies.

There has been a lot of progress in the understanding of genetics of uveal melanoma during the past 10 years. Since the first papers on genetic abnormalities based on karyotype, chromosome comparative genomic hybridization (CGH) and fluorescent in situ hybridization (FISH) analyses monosomy 3 has been recognized as bad prognostic factor. Other chromosomal imbalances, especially gain of 8q, have been reported as important events for metastasis. Molecular techniques such as CGH- and single nucleotide polymorphism (SNP)-arrays, and multiplex-ligation-dependent probe arrays (MLPA), has made it possible to reliably assess the status of genomic markers of prognostic value. These studies have highlighted genetic heterogeneity with isodisomy on chromosome 3. For others gene expression profile is the most precise way to predict the prognosis of uveal melanoma. All these advances were made possible thanks to the collaboration of ophthalmologist oncologists and geneticists in multidisciplinary teams.

Local treatment

The treatment of uveal melanoma by radiotherapy was popularized by Stallard who initiated the use of cobalt plaque brachytherapy in the 1950s with encouraging results. In the 1970s Zimmerman and colleagues showed that enucleation of the eye does not prevent metastatic dissemination compared to conservative management of uveal melanoma. Since then treatment by brachytherapy or

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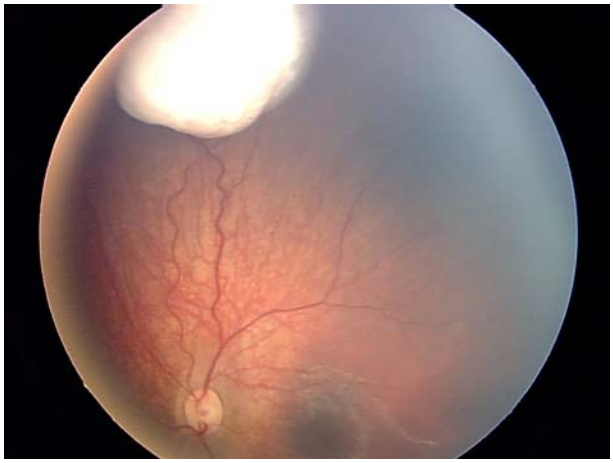

Fig. 1 Uveal melanoma (fundus).

accelerated heavy particles has been used extensively. Compared to brachytherapy and to fractionated stereotactic radiotherapy, proton beam therapy has the advantage of delivering a homogenous dose of radiation to the entire tumor. This is due to the physical properties of accelerated proton beam which deliver the dose precisely with sharp decrease after the delivery point called the Bragg peak (Fig. 1).

The first step to treat uveal melanoma with proton beam is radiological spotting of the tumor by clip positioning. During a surgical procedure, tantalium rings of 2.5 mm in diameter with two holes in the middle are sutured to the sclera around the tumor base that has been visualized by transillumination of the globe. Proton beam therapy is planned and performed 2 weeks after surgery. Head immobilization during radiotherapy is ensured by a custom-made thermoplastic mask associated with a bite block. All the data collected during surgery (axial length, clip measurements, tumor diameter, tumor thickness) are sent to the physicist in charge of the planning of therapy. It is mandatory to have good pictures of the fundus, ultrasonography of the eye and biometry. EYEPLAN software is used for dosimetry based on three-dimensional (3D) reconstruction of the eye, including the tumor. The irradiated volume is the tumor volume plus a safety margin of 2.5 mm around the tumor. The standard dose delivered is 60 Grays in four fractions. During treatment, patients are seated on a robot chair facing the beam and are asked to aim at small target light placed at a known angle adopted during the treatment planning process.

Local control is excellent. The local recurrence rate at 10 years is usually around 5%. Secondary enucleation is performed in 10% to 15% of patients either for complications or for local recurrence. Complications include retinal detachment, maculopathy, papillopathy, cataract, glaucoma, vitreous hemorrhage, and dryness can occur. The most severe complication that usually leads to secondary enucleation is neovascular glaucoma and it is encountered after irradiation of large to extra-large tumors. The toxic tumor syndrome was recently described. It is supposed to result from the residual tumor scar producing proinflammatory cytokines and VEGF, leading to intraocular inflammation and neovascular glaucoma. Additional treatments after proton beam such as TTT (trans pupillary thermotherapy), endoresection of the tumor scar or intravitreal injections of anti-VEGF antibody may reduce the rate of these complications.

Plaque radiotherapy is the best option when proton beam therapy is not available, when the patient's general health precludes proton beam, for small anterior tumors or for tumors situated anteriorly in the superior and external quadrant (where the dose at the entrance of proton beam can cause lacrimal gland atrophy). Iodine 125 seeds can be used. This is a low gamma energy radiation emitting isotope and this radiation can be stopped by heavy metals such as gold or lead. Gold or lead plaques (12–20 mm diameter) are prepared with iodine 125 seeds and sutured on the sclera at the location of the tumor. The usual calculated dose is 90 Grays at the apex of the tumor. The time duration is calculated by the physicist and the plaque is then removed surgically. Ruthenium is a Beta emitting isotope with less penetration and less diffusion. Plaques are already prepared and can be used for multiples applications. It is recommended to avoid the use of ruthenium for tumor with a thickness of >6 mm.

Role of biopsy at the time of local treatment

Ocular oncologists have recently changed their approach to uveal melanoma in most centers. During many years, it has been considered that biopsy was not necessary in the management of uveal melanoma. Diagnostic techniques were reliable enough to allow performing conservative treatment by radiotherapy without histopathological confirmation of diagnosis. More recently it has become evident intraocular biopsies at the time of local treatment are needed to better characterize the disease at cell and molecular level. The techniques of transcleral and transvitreal fine needle aspiration biopsies were published on large series of patients, with very few side effects. Today most centers are able to offer fine needle aspiration biopsies for cytogenetic studies despite in principle risk of tumor dissemination, vitreous hemorrhage and retinal detachment; On the other hand, the benefits for the patient are still

limited as there is still a lack of efficient chemotherapy to prevent metastasis in high-risk patients. Others benefits of genetic studies are the possibility of entering a closer follow-up protocol to detect early liver metastasis and to participate to adjuvant therapy protocols.

Management and follow-up

Besides genetic studies of the primary tumor, the presence of circulating tumor cells could be an indicator for metastatic risk and is currently under evaluation. A few centers have started to use adjuvant therapy protocols. In the past, there has been three published studies on adjuvant therapies in uveal melanoma, one randomized with DTIC, one nonrandomized with interferon and one with intraarterial fotemustine. None of these trials gave statistically significant results. There have been a lot of controversies on how to follow patients that have been treated for uveal melanoma. Early detection of metastasis by repeated liver ultrasonography can allow the surgical resection of localized metastasis and therefore increase survival. Some of the new targeted therapies are active in 10% of metastatic patients. Ultrasonography of the liver every 6 months is usually recommended. Liver MRI is useful, in particular to confirm liver metastasis but TEP scan are sometimes not detecting metastasis from uveal melanoma though they are very sensitive in the case of cutaneous melanoma.

Oncologists have become recently more interested in this disease and many new drugs or protocols are already under trial for metastatic disease. Chemoembolization is employed for the liver but is not useful when the patient has other localizations of the metastatic disease. Vaccine protocols are available in HLA2 patients, with limited success but there is still ongoing work. Current research is investigating the prognosis and predictive significance of transcriptomic analysis. Proteins involved in the migration of tumor cells from the eye to the liver and adhesion and proliferation of the metastasis are being studied.

Other primary intraocular malignancies in adults are very rare. Some rare cases of adenocarcinoma of the pigmented epithelium have been published. Oculocerebral lymphoma is a rare disease due to large cells lymphomas. The vitreoretinal lymphoma is associated generally with central nervous system lymphoma. A typical retinal infiltrate is associated with the presence of abnormal cells in the vitreous. Diagnosis can usually be made by vitreous cytology or more rarely by biopsy of the cerebral lesions. Treatment must be performed in reference centers.

Retinoblastoma

Diagnosis

Retinoblastoma is the most common malignant intraocular tumor in children with an estimated incidence of 1/20,000 live births. Retinoblastoma is the first tumor for which a genetic origin was demonstrated. According to the Knudson hypothesis, retinoblastoma carcinogenesis is dependent upon inactivating mutation on the two alleles of the *RB1* tumor suppressor gene located on chromosome 13q14.2.2. The tumor can arise in a child carrying a germline mutation on one of the two alleles. A second hit mutation of the other allele in a precursor cell is responsible for the appearance of a retinal tumor. This predisposition can be transmitted by a parent carrying the mutation (familial form) or can occur as a de novo event. In this setting, retinoblastoma is usually bilateral or unilateral multifocal and the child is at risk of developing other extra ocular tumors (soft tissue sarcoma, osteosarcoma, carcinoma, high-grade glioma, malignant melanoma). Treatment must take into account the risk of developing second tumors, which can be increased by treatment with mutagenic agents (chemotherapy), X-rays, and radiotherapy. Retinoblastoma occurs almost exclusively in children under the age of 15 years and the median age at diagnosis is lower for the bilateral form (<12 months) than for the unilateral unifocal form (24 months). Early diagnosis is a priority, as it allows the child to be cured with preservation of almost normal vision in at least one of the two eyes. However, despite well-known clinical signs, retinoblastoma is still often diagnosed at an advanced stage, requiring aggressive therapy with a high-risk of enucleation of the affected eye in unilateral forms and of the two eyes in bilateral forms.

The clinical presentation of retinoblastoma is well known. Leukocoria is the presenting sign in 60% of cases, but is often a late sign. At the beginning, leukocoria can be intermittent, only visible in specific direction of gaze or with specific light. If no diagnosis is made, leukocoria become more and more visible and permanent. It can easily be detected on photos with flash especially if the anti-red eye system is disconnected. The second most common early sign of retinoblastoma is strabismus, generally related to a macular tumor. Provided that strabismus is not confused with accommodative strabismus, treatment can be curative and allow preservation of the eye. Less common clinical presentations may also be encountered, generally indicating advanced forms (buphthalmia, neovascular glaucoma, orbital inflammation, phthisis bulbi, etc.), which generally do not allow globe-conserving treatment. The clinical presentation varies according to the stage of the disease and the presence of one or several tumors in either or both eyes, with symmetrical or asymmetrical lesions. The tumor is visible as a white retinal mass with a variable blood supply depending on the size of the tumor and may present calcifications. The tumor (or tumors) may present as an endophytic form, in which the tumor extends into the vitreous, an exophytic form, associated with retinal detachment and subretinal infiltration; or a mixed form, comprising vitreous involvement and retinal detachment. Diffuse infiltrating retinoblastoma is a rare form of retinoblastoma with infiltration of the retina without tumor mass. Examination shows vitreous infiltration and sometimes hypopyon (**Fig. 2**).

Retinoma (or retinocytoma) is a spontaneously arrested or spontaneously regressive form that resembles treated retinoblastoma with a grayer color, intense calcification, and a zone of atrophy around the lesion. These lesions can be detected on ocular fundus examination of a parent of a child with retinoblastoma or in a child consulting for leukocoria or strabismus. These lesions must be carefully monitored in young children, as they can progress to active retinoblastoma (**Fig. 3**).

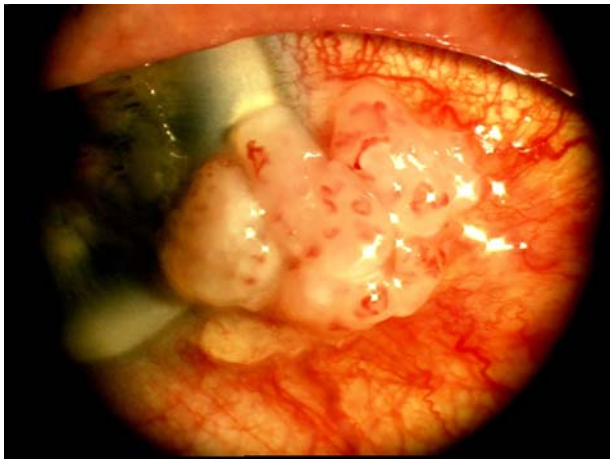

Fig. 2 Leukocoria.

Fig. 3 Retinoblastoma (fundus).

Prognosis

There are also several well-known differential diagnoses (Coats disease, persistent hyperplastic primary vitreous, medulloepithelioma, uveal melanoma in children, retinal detachment due to other causes, *Toxocara canis* infection, etc.). Examination in a reference center and modern imaging comprising duplex Doppler ocular ultrasound and magnetic resonance imaging (MRI) allow confirmation of the diagnosis in the vast majority of cases.

Several classifications have been proposed to address various issues, but none of them are fully satisfactory. The first classification proposed by Reese-Ellsworth was developed in the age of external beam radiotherapy to evaluate the chances of globe-conserving radiotherapy. The second classification, the International Intraocular Retinoblastoma Classification (ABC classification), was developed by Murphree in 2005 in the age of systemic chemotherapy. This classification, ranging from A to E, can be used to estimate the possibility of preserving the eye by systemic chemotherapy in combination with local therapy. This classification remains widely used. The new TNM classification (eighth American Joint Committee on Cancer TNM classification) estimates survival and the chances of preserving the eye by taking into account clinical risk factors (cT), histological risk factors (pT), the presence or absence of a germline mutation (H), and of course the presence of lymph node (N) or distant metastasis (M). Modern MRI imaging is also used for intraorbital and central nervous system staging.

The treatment of retinoblastoma was very much improved during the past 30 years. The cure rate in developed countries is closed to 100%. Radiotherapy has been the treatment of choice until early 1990s but has now been phased out because of the risk of second cancer in the field or radiation that can be as much as 30% in patients carrying RB1 mutation. Chemotherapy is now the treatment of choice. The combination of intravenous carboplatin, vincristine and etoposide found to be active on metastasis is also very efficient on intraocular retinoblastoma. Depending on the severity, patients with bilateral retinoblastoma can be treated with a combination of two to three drugs. More recently the use of intraarterial chemotherapy with melphalan, topotecan, and carboplatin has allowed very impressive regression of the tumors. Chemotherapy can also be injected directly in the vitreous cavity in selected cases. Disseminated metastatic disease or cerebral involvement (pinealoma or metastases via the optic tract) requires intensive chemotherapy with autologous bone marrow transplantation. Cerebral involvement is associated with a poorer prognosis.

The second factor that has improved survival and eye preservation rates is the use of local therapy. Most teams specialized in the treatment of retinoblastoma agree that chemotherapy alone cannot ensure perfect control of intraocular disease but must be combined with local therapy (diode laser transpupillary thermotherapy, laser, cryoapplication, brachytherapy). Early diagnosis, as a result of information campaigns directed to the general population, healthcare professionals, and ophthalmologists, allows ablation of small tumors exclusively by local therapy or a combination of carboplatin and transpupillary thermotherapy. Local therapies can also be also used as second-line treatment for local relapse.

Another important factor for improved patient survival is the establishment of specialized centers comprising multidisciplinary teams composed of ophthalmologists, oncologists, pediatric oncologists, radiation physicians, radiologists, geneticists, anesthetists, and so on. This high level of multidisciplinary competence allows tailored treatments based on the findings of the initial ophthalmological examination, which must be performed under general anesthesia. In centers treating retinoblastomas, genetic information concerning the parents of an affected child and patients treated for retinoblastoma during childhood is essential and germline mutation screening must be systematically proposed, with due consideration to mosaicism.

Chemotherapy

Various drugs are used for intravenous, intraarterial, subconjunctival, or intravitreal chemotherapy depending on the team (most commonly carboplatin, vincristine, etoposide, topotecan, melphalan). Intravenous chemotherapy alone cannot cure retinoblastoma but chemoreduction with intravenous chemotherapy followed or combined with local therapy is still widely used by many teams with recognized efficacy and acceptable systemic toxicity. The intraarterial route of administration developed by Kaneko, using melphalan or topotecan or carboplatin alone or in combination, allows drug administration directly into the ophthalmic artery, thereby limiting systemic distribution of chemotherapy and its adverse effects. Consequently, local administration cannot be used to treat micrometastases in advanced forms of the disease. This route of administration is not devoid of local toxicity on the choroidal and retinal blood supply and must be performed by an experienced team of neuroradiologists. In a large meta-analysis based on 208 publications on the subject, only 28 articles with sufficient follow-up could be analyzed. Most of these studies were retrospective and noncomparative. Enucleation was avoided in 66% of cases and 20 cases of metastases were reported. These cases of metastases probably occurred in the context of treatment of advanced retinoblastoma. Nevertheless, this treatment is very effective in unilateral forms with visual potential (*c*T2 or group C or D). Some authors also use intraarterial chemotherapy to treat bilateral forms. The place of this treatment remains controversial in advanced unilateral forms (*c*T3 or V) regardless of the subgroup in the absence of visual potential. No clear consensus has been reached, as the indications for intraarterial chemotherapy vary from one team to another. No multicenter prospective studies comparing intraarterial chemotherapy and systemic chemotherapy are available and the published studies are all too often retrospective and present excessive bias to allow reliable scientific results.

There is also insufficient follow-up concerning the sensitivity of bone and soft tissues to repeated exposure to low doses of X-rays necessary for catheter positioning by radiologists: these doses are not negligible in the context of constitutional alteration of the *RB1* gene, associated with an increased risk of second tumor (essentially sarcoma) in the irradiated field.

Intravitreal administration of melphalan or topotecan is particularly effective treatment for vitreous seeding. A rigorous technique is essential due to the risk of tumor cell dissemination along the needle track. Some cases of extraocular tumor spread have been described. This phenomenon is probably rare, but several cases that have been reported to the author and have never been published. Compliance with the most commonly accepted doses (20–30 μg of melphalan) is important to avoid major complications, including phthisis bulbi for doses higher than 50 μg.

Radiotherapy and other local therapies

External beam radiotherapy (EBRT) is now rarely used due to the risk of local complications and the major risk of sarcoma in the irradiated field. The incidence of second cancer in the irradiated field, but also outside of the irradiated field, was estimated to be 51% at 50 years by a team in New York on the basis of a very large series. Brachytherapy is generally used for peripheral tumors, usually as second-line treatment. Orbital brachytherapy using rows of iodine 125 plus a plaque can be used instead of EBRT after enucleation in the presence of extraocular tumor spread or positive optic nerve resection margins (pT4), in combination with systemic chemotherapy. Other focal Treatments include triple freeze-thaw cryotherapy with freezing as far as the tip of the tumor for peripheral tumors inaccessible to laser ablation not exceeding a thickness of several millimeters. Laser thermotherapy is used as an adjuvant to chemotherapy or alone for small tumors or localized relapses. We use a diode laser (810 nm) with continuous wavelength delivered via an operating microscope or via the indirect ophtalmoscope. The laser is absorbed at the level of the pigment epithelium and produces energy and hyperthermia of which is synergistic with carboplatin (chemothermotherapy). Duration and intensity is adapted to the size of the tumor and degree of pigmentation (5–20 min, 400–800 mW). A complete ophthalmological assessment, including bilateral dilated ocular fundus examination under general anesthesia, MRI, and a pediatric assessment, is essential before any treatment decisions and should be done in emergency.

Genetic information is essential in familial forms. Prenatal diagnosis is possible if the mutation is identified. Embryo selection and in vitro fertilization of embryos not carrying a constitutional alteration of the *RB1* gene is possible in some countries. Parents must be informed about this possibility. A child born to a parent with a history of retinoblastoma must be examined during the first week of life and, in the absence of visible tumor, must be examined at least monthly under general anesthesia. Small *c*T1a tumors detected on clinical examination or optical coherence tomography can be treated by transpupillary thermotherapy alone.

Retinoblastoma survival is around 95% in high-resource countries but is much less in low-resource countries where diagnosis is often made at late stage and access to care constrained. Programs to improve retinoblastoma care are ongoing in many countries. In low-resource countries the rate of ocular conservation is improving but careful evaluation is needed for treatment, balance of benefits and risk, side effects and long term results and metastatic rate.

Medulloepithelioma

Medulloepitheliomas are very rare, almost always unilateral congenital tumors arising from the ciliary epithelium usually becoming symptomatic between 2 and 4 years of age. They are most often localized in the ciliary body. They often are slow growing but some of them can be very aggressive with possible orbital recurrence or metastasis.

Malignant Conjunctival Tumors

The conjunctiva is the outermost layer of the ocular surface, above the sclera, and also forms a continuous membrane with the innermost layer of the eyelids behind the tarsal plane. Anatomically these regions are described as bulbar and tarsal conjunctiva, and they merge in the conjunctival fornices. Histologically, the conjunctiva is composed of an epithelium, overlying a basal membrane that separates it from the conjunctival stroma, that is densely vascularized, innervated, and contains immune elements. This structure has clinical consequences since most primary conjunctival neoplasia develop initially within the epithelium, and may become invasive when they progress into the stroma, where there is a risk of local spread and distant metastases.

Conjunctival invasive squamous cell carcinoma

Invasive squamous cell carcinoma is a continuation of the spectrum of conjunctival intraepithelial neoplasia (CIN), a minimally aggressive lesion localized to the conjunctival epithelium. Severe CIN, also termed in situ squamous cell carcinoma, can progress through the basement membrane, invade adjacent tissues and become malignant. Invasive squamous cell carcinoma can also metastasize (in $<2\%$ of cases), usually to the regional and cervical lymph nodes. Therefore, CIN is a major risk factors for invasive squamous cell carcinoma. Other identified factors include UV-light exposure, human papillomavirus infection (especially HPV-16, that may be associated to a better prognosis), immunosuppression, especially by human immunodeficiency virus (HIV), associated to more aggressive tumors, and conditions that predispose to epithelial malignancies, such as atopic eczema and xeroderma pigmentosum, a rare genetic skin disorder characterized by an extreme sensitivity to UV light. The reported incidence of invasive squamous cell carcinoma ranges from 0.02 to 3.5 per 100,000, and the disease seems more frequent in men (estimated male–female ratio, 3:1) and in elderly subjects (Fig. 4).

The main diagnostic challenge is the similar appearance of CIN and invasive squamous cell carcinoma, which are sometimes difficult to differentiate clinically. Both lesions develop in the interpalpebral region of the conjunctiva, often adjacent to the limbus, with part of the lesion overlapping the cornea, and can more rarely extend to the conjunctival fornices. Although most frequently affecting elderly Caucasian males, they can be diagnosed at all ages, in both genders, and in patients of all ethnicities. While CIN

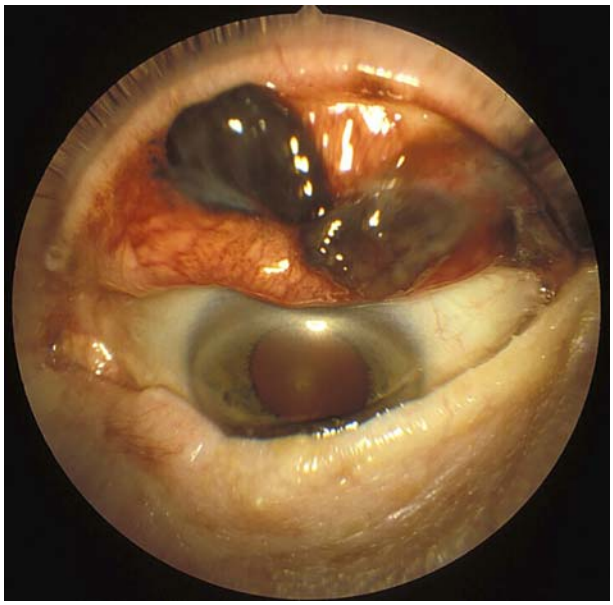

Fig. 4 Conjunctival invasive carcinome.

presents as a fleshy, with moderate elevation and a sessile base, invasive squamous cell carcinoma can have multiple presentations, from a localized gelatinous mass to a large elevated, papillomatous lesion. It is often associated with dilated conjunctival feeder vessels. A frequent feature is the multilobular aspect of the lesion.

Variants of squamous cell carcinoma need to be recognized. The diffuse form, flat and poorly demarcated, may be underdiagnosed. Two aggressive forms, the mucoepidermoid and spindle cell variants (< 5% of conjunctival squamous cell carcinoma) carry a more severe prognosis. The mucoepidermoid form may progress intraocularly with the development of large supra-uveal mucinous cysts. Spindle cell carcinoma is more aggressive locally with a higher risk of distant organ metastases.

Diagnosis should rely on pathological examination after primary complete surgical excision. The characteristic histological features include abnormal keratin-producing epithelial cells with mitotic activity. A small proportion of cases are poorly differentiated with atypical pleomorphic giant cells and dyskeratosis. The main characteristic is that the tumor extends beyond the basal conjunctival membrane into the subepithelial connective tissue. The mucoepidermoid form harbors an epidermoid component with vacuolated cytoplasms and variable mucin accumulation. The spindle cell variant presents with pleomorphic spindle cells of epithelial origin. Differential diagnoses, besides CIN, include all nonpigmented conjunctival malignancies may resemble invasive conjunctival squamous cell carcinoma. Notable entities include amelanotic conjunctival melanoma; mucoepidermoid and spindle cell carcinoma, detailed above; conjunctival basocellular carcinoma, a very rare entity; and diffuse conjunctival extension of eyelid sebaceous carcinoma, that carries a higher metastasis and mortality rates than invasive squamous cell carcinoma and should be recognized.

The local prognosis is related to the risk of local recurrence, which is globally quite high (40%), in the absence of neoadjuvant treatments, such as irradiation, as detailed below. The systemic prognosis is generally favorable and related to the risk of developing metastases. Although rare in invasive squamous cell carcinoma, metastatic disease is conditioned by tumor size, the depth of local invasion, and presence of immunosuppression (especially HIV infection). According to these factors, locoregional extension should be explored at regular intervals by ultrasound exploration of head and neck lymph nodes (for lower risk cases) or Positron Emission Tomography (for higher risk cases).

Regarding treatment, a schematic drawing or preferably a color photograph of the lesion at diagnosis, before any therapeutic intervention is mandatory. Initial management consists in a surgically complete excision, that should be performed with a "no touch" approach similar to pigmented lesions (avoiding direct contact of instruments with the lesion, and replacing instruments for conjunctival suture/reconstruction), given the possibility of presumed epithelial malignancy to be eventually identified as amelanotic melanoma upon pathological analysis. For the same reason, surgery is preferably performed under general anesthesia to avoid orbital seeding. Cryotherapy of tumor margins combined to excision contributes to reduce the risk of local recurrence. If pathological examination confirms the diagnosis of squamous cell carcinoma (or other malignant epithelial tumor), neoadjuvant radiotherapy of the tumoral site by either conventional external radiotherapy, plaque brachytherapy or proton beam irradiation should be performed, according to the initial tumor topography. These treatments allow to reduce dramatically the recurrence rate from 40% to 2%, and their major adverse effect is the acceleration of cataract formation. Topical chemotherapy by mitomycin C eyedrops is indicated as neoadjuvant therapy for CIN. The initial recommended dose is 0.02%, four times daily, two 2-week cycles. In case of relapse, the same treatment at the higher dose of 0.04% is indicated. Interferon eyedrops, administered four times daily for 6 months, are an alternative to mitomycin C, in patients with severe corneal alterations or in those with limited disease.

With adjuvant, topical chemotherapy having widely improved local control and recurrence rates, future developments rely in the higher integration of treatment approaches. In a near future, advances in diagnosis techniques, for instance by impression cytology, and by tumoral genome detection, may improve management of conjunctival epithelial malignancies by allowing their detection and referral to tertiary centers at earlier stages.

Conjunctival melanoma

Conjunctival melanoma is a rare, malignant invasive melanocytic tumor, affecting the bulbar conjunctiva, and more rarely the conjunctival fornices or the palpebral conjunctiva. Its incidence increased with age, and is higher in individuals in their 50–60s or older. It only rarely occurs in subjects younger than 30 years. There seem to be a recent increase in the incidence of conjunctival melanoma, according to data from the United States and Northern Europe. Conjunctival melanoma has a variable, but usually aggressive course, characterized by a tendency to recur often after simple surgical excision. It therefore requires adjuvant treatments to optimize local control. Moreover, conjunctival melanomas frequently metastasize. This tumor is composed by variably pigmented malignant melanocytes (**Fig. 5**).

Commonly recognized risk factors for conjunctival melanoma include lighter skin (although it has been scarcely studied in black-skinned subjects), exposure to UV light (this explains the preferential localization to exposed parts of the conjunctiva, nasal and temporal to the limbus, but does not account for the possibility of melanoma localized in the conjunctival fornices), primary acquired melanocytosis (75% of conjunctival melanoma arise from primary acquired melanocytosis) and conjunctival nevi (25% of conjunctival melanoma arise from conjunctival nevi).

At clinical presentation, conjunctival melanoma usually consists in a nodular pigmented elevation, most often located close or adjacent to the limbus, with possible extension over the corneal epithelium, and usually surrounded by a fine, planar, granular pigmentation of the conjunctiva. Usually sharply demarcated, the borders of the pigmented tumor may be ill-defined, taking the appearance of diffuse of multiple nodules, which often occurs in cases arising over areas of primary acquired melanocytosis. It can occasionally develop in the conjunctival fornices or palpebral conjunctiva, in which case it may remain undetected until the diagnosis is made when the tumor is advanced. Conjunctival melanoma can also be nonpigmented (amelanotic), making it

Fig. 5 Conjunctival melanoma.

difficult to distinguish clinically from malignancies of the conjunctival epithelium. In addition, seeding of the palpebral conjunctiva by continuous contact with a bulbar lesion may occur.

Diagnosis relies on histopathological evaluation after surgical excision, identifying the morphology of malignant melanocytes, that may vary from low-grade spindle cells to epithelioid cells. It affects the basal conjunctival epithelium at early stages, and secondarily invades the stroma. Morphological evaluation may be challenging, for instance in purely fusiform or amelanotic presentations. Immunostaining can be used to identify S-100 protein (sensitive but not specific), HMB-45 or melan-A (more specific), SOX10 (very sensitive and specific), and other proteins expressed by melanoma cells. Ki-67 labeling evaluates the depth of the proliferative activity and help differentiate malignant from naevic pigmented lesions. The pathological evaluation also recognizes preexisting naevi or primary acquired melanocytosis.

Differential diagnoses include conjunctival nevus, primary acquired melanocytosis (with are also risk factors and may coexist with melanoma), ethnic conjunctival melanocytosis in dark-skin subjects, squamous cell carcinoma (difficult to differentiate from completely amelanotic melanomas), ciliary or very peripheral choroidal melanoma with extrascleral extension, conjunctival foreign body (especially long-standing metallic elements progressively surrounded by a brown halo due to perilesional oxidation), and most other benign or malign conjunctival tumors that may harbor variable pigmentation.

Prognosis is hampered by the difficulty to locally control the tumor due to frequent recurrences and the tendency to spread by simple contact, and by the high rate of metastasis (up to 40% 10 years after diagnosis). Preferred sites of metastasis are regional lymph nodes, and follow-up by serial ultrasonography of cervical lymph nodes is mandatory. The particularity of conjunctival melanoma is the serious risk of aggravating the prognosis in cases of mismanagement, especially by improper steps during surgery. Contact of surgical instruments with the tumor itself must be avoided, and new forceps and scissors must be used after excision for repair of the conjunctival defect by either direct suture, or amniotic membrane graft for wider defects. To date, intraorbital disease is treated by orbital exenteration, illustrating the catastrophic course that may result from improper surgical management. Although difficult to estimate, the overall mortality related to conjunctival melanoma is close to 25% at 10 years.

Regarding treatment, as for epithelial malignancies, a color photograph of the lesion at diagnosis, before any therapeutic intervention, is mandatory. The treatment of conjunctival melanoma consists in removing surgically the malignant tissue, and is associated to additional multidisciplinary treatment modalities to limit the risk of recurrence. Surgical excision of the whole tumor with safety margins is the first step. Due to the tendency of melanoma cells to disseminate and develop in seeded tissues, the excision of any pigmented or nonpigmented conjunctival tumors (due to the possibility of amelanotic melanoma resembling spindle cell

carcinoma) should be performed using "no touch" technique under general anesthesia to avoid orbital seeding by local anesthesia infiltration. The tumor should be totally removed, with 2-mm security margins macroscopically devoid of any tumor. In order to limit tumor spread, biopsies must also be avoided, except for diagnostic confirmation of advanced cases requiring orbital exenteration. Additional cryotherapy can be performed on tumor margins to destroy microscopic lesions or invisible primary acquired melanosis. A double freeze-thaw process is recommended, but overtreatment should be avoided due to the destructive effect on underlying ocular structures, and the inflammatory conjunctival reaction. Adjuvant irradiation by plaque brachytherapy or proton beam therapy is performed for invasive forms of conjunctival melanoma. Adjuvant topical chemotherapy by 0.04% mitomycin C (four times daily, two 2-week cycles) aims at treating primary acquired melanosis lesions.

The recent advent of neoadjuvant therapy after surgical excision seems to dramatically reduce recurrences of conjunctival melanoma. Radiotherapy by either proton beam irradiation or plaque brachytherapy has provided ocular oncologists with an unprecedented tool to treat conjunctival melanoma and reduce the rate of recurrences. With now a few years' follow-up, topical adjuvant chemotherapy by mitomycin seems efficient in controlling primary acquired melanocytosis, a major predisposing risk factor that also favors recurrences. Finally, progress remains to be made in the search for efficient treatment strategies for metastatic disease, a frequent course of this aggressive conjunctival neoplasia.

Caruncular malignant tumors

Due to the particular histological origin of the caruncle, an evolutionary remnant of the third eyelid still present in certain species of birds or reptiles, it harbors cutaneous elements such as hair follicle and sebaceous glands, and can be affected by tumors types usually found on eyelids or conjunctiva.

Sebaceous gland carcinoma is a rare malignant tumor arising from sebaceous glands of the caruncle, similarly to eyelid sebaceous gland carcinoma that arises from sebaceous glands of the eyelids. A purely conjunctival variant also exists that develops insidiously as a flat diffuse "pagetoid" lesion at the surface of the conjunctiva. Both forms may pose diagnostic dilemmas. The caroncular/palpebral form may be initially mistaken and managed as a chalazion (a benign meibomian gland cyst), and the diffuse conjunctival form as an ocular surface inflammation (such as chronic conjunctivitis or episcleritis). Histologically, the lesions are composed of pleomorphic cells with prominent nucleoli. A characteristic but not systematical aspect consists in disseminated malignant cells within the conjunctival epithelium, termed "pagetoid infiltrate." If advanced, this infiltrate can replace completely the normal epithelium (in situ carcinoma). Sebaceous gland carcinoma is aggressive and can metastasize, with an estimated mortality of 25% at 5 years. Local treatment relies on surgical excision, that must be complete, and additional cryotherapy or mitomycin C topical chemotherapy.

Caruncular melanoma presents as variably pigmented, noncystic solid lesions. They are diagnosed and managed similarly to conjunctival melanoma. Due to the high risk of seeding into the lacrimal drainage system, because of the proximity of the caruncle to the lacrimal ducts, lacrimal plugs may be employed.

Malignant Orbital Tumors

The orbit is a circumscribed anatomical region where skeletal, muscular, vascular, and neural elements coexist. A wide array of lesions can produce orbital masses, including inflammatory syndromes, thyroid-related extraocular muscle dilation, and benign or malignant tumors of muscular, skeletal, vascular or neural origin. Although rare, malignant orbital tumors should be recognized and appropriately managed. Three of the most important malignancies localizing to the orbit will be detailed in this section.

Rhabdomyosarcoma represents the most frequent orbital malignancy in childhood and teenage. Median age at diagnosis is 8 years but the tumor can develop during the first two decades of life. This tumor derives from skeletal muscle. It is favored by local irradiation, which is of particular importance for the follow-up of children previously irradiated for retinoblastoma, a common treatment modality until the early 1990s. These patients are at higher risk of developing secondary tumors, including rhabdomyosarcoma, with devastating consequences.

Clinical manifestations are nonspecific and result from the orbital volume occupation by the developing tumor. They include proptosis, lateral or vertical globe displacement in primary gaze position, variable eye movement limitations, ptosis of the superior eyelid, eyelid or conjunctival swelling, pain and detection of a palpable protruding mass in most advanced cases. Most often, the mass is localized in the superonasal part of the orbit and the globe is displaced inferiorly and temporally.

Diagnosis relies on radiological suspicion and pathological confirmation. Imaging by computed tomography (CT) scan shows an irregular, moderately demarcated mass generally not involving the extraocular musculature. Magnetic resonance (MR) imaging reveals on T1 an hypointense lesion, as compared to extraocular muscles, and a hyperintense T2 images, enhanced after gadolinium injection. If accessible to biopsy, rhabdomyosarcoma may harbor different cell morphologies, the most frequent being the embryonal type, and the more aggressive the alveolar type. Embryonal type displays skeletal muscle cells at different stages of embryogenesis. The alveolar type displays separated cells with septae resembling pulmonary alveolar structures. Differential diagnoses include anterior or posterior orbital cellulitis, nonspecific orbital inflammation, capillary hemangioma, lymphangioma, rupture dermoid cyst, and any orbital may present as an orbital space-occupying process and should be differentiated from rhabdomyosarcoma.

Prognosis depends on the presence of metastases in distant organs, such as lung or lymph nodes. The local long-term prognosis may also be influenced by the treatment strategy, with patients receiving radiotherapy to the facial and orbital region in their childhood or teenage years at higher risk of developing secondary neoplasias of skin, bone, or soft tissue.

Regarding treatment, initial management is surgical and consists in a biopsy of the orbital mass for diagnosis conformation. When possible, the entire tumor should be removed. Once the diagnostic is ascertained by histopathological evaluation, the treatment relies on radiotherapy and chemotherapy, alone or combined, according to guidelines.

The optimization of irradiation treatment strategies (total dose, dose fractioning, irradiation modality) will hopefully reduce the burden of secondary tumors arising years or even decades after successful management. Progresses in chemotherapeutic approaches may contribute to reduce partially or totally the amount of radiation administered.

Malignant tumors of the lacrimal gland

Approximately 10% of orbital lesions involve the lacrimal gland. Malignant lesions of several histological type can affect the lacrimal gland, associated with different prognosis. Primary epithelial tumors arise from epithelial structures of the lacrimal gland. Nonepithelial neoplasia include inflammatory, infiltrative, lymphoid lesions. Adenoid cystic carcinoma, pleomorphic adenocarcinoma and primary ductal carcinoma are the most frequent epithelial malignant tumors affecting the lacrimal gland, among several histological diagnoses, and will be detailed in this section.

Lacrimal gland malignancies do not have any gender or ethnic predilection. They occur more frequently in middle-aged individuals, with the exception of adenoid cystic carcinoma that showing incidence peak in childhood. The clinical presentation of all tumor types is very similar, reflecting the space-occupying orbital process located superiorly and temporally. The main sign is a progressive, unilateral proptosis, with medial and inferior displacement of the eyeball, possibly associated with pain in the more aggressive types due to local sensitive nerve invasion. Frequently, patients experience progressive diplopia. Posterior neural invasion induces cutaneous hypoesthesia in the territory of the 5th cranial nerve (upper cheek, periocular area).

Diagnosis relies on imaging by CT scan and MRI to visualize a round or oval-shaped mass, located in the superior temporal orbit, possibly displaying irregular contours. Bone erosion is visible on CT scan in case of large, aggressive tumors. These imaging modalities provide certain features suggestive of malignancy, like intralesional calcifications, but cannot distinguish between pathological types. MRI show lesions with low T1 signal, high T2 signal, and moderate enhancement by contrast agents. Diagnosis also requires pathological examination after ultrasound-guided or surgical biopsy, or preferably whole lesion removal. Each histological type harbors distinctive features. Adenoid cystic carcinoma can be cribriform (a pattern described as "Swiss cheese"), sclerosing, basaloid (associated to a worse prognosis), tubular or comedocarcinoma. Pleomorphic adenocarcinoma harbors a proportion of malignant epithelial cells within a benign pleomorphic adenoma. Ductal carcinoma presents proliferative epithelial cells within lacrimal ducts, resulting in cystic dilation. Differential diagnoses include symptoms and signs related to malignant tumors tend to progress more rapidly than in benign lesions. Pain is also more frequently associated to malignant than benign lesions, due to neural invasion. Benign lacrimal gland lesions include pleomorphic adenoma, also termed "benign mixed tumor," and ductal epithelial cysts, also termed "dacryops."

Epithelial malignant neoplasia of the lacrimal gland are aggressive tumors. Prognosis depends mostly on histological subtype, the main factor influencing local invasiveness and the tendency for distant metastases. Treatment includes Irradiation, chemotherapy, and in most advanced cases exenteration.

Improvements in the care of rhabdomyosarcoma patients rely on two major axes. First, better screening of at risk individuals, namely those previously treated for another malignancy in childhood, is needed. Prolonged and sustained follow-up in tertiary centers with standardized imaging protocols will improve the detection of new tumors, and the understanding of risk factors and progression patterns of these secondary tumors. Second, current improvements in therapeutic modalities by mini-invasive or endoscopic orbitofacial surgery, brachytherapy, optimized external irradiation, and pharmaceutical approaches may hopefully improve the local and systemic prognosis of these aggressive tumors.

Orbital metastases

The tumors that have the higher propensity for seeding metastases to the orbit are carcinomas of the breast, lung, kidney, prostate, and gastrointestinal system. Recognizing orbital metastases is of importance since in 20% of cases patients have no history of cancer and orbital metastasis is the revealing manifestation of the primary neoplasia. Orbital metastases follow the age distribution of solid organ tumors and are more common in elderly subjects. They are very rare in children. They manifest with the classical signs of orbital space-occupying lesions, with a very rapid onset and progression, and frequent pain. Paradoxically, certain tumors induce endophthalmos rather than proptosis, like breast or gastric neoplasia, that tend to induce tissue contraction.

Regarding diagnosis, some primary cancer types have preferential localizations within the orbit, visible on imaging. For instance, breast cancer localizes to soft orbital tissue and prostate cancer tends to localize to bone. Several cancer types generate demarcated, oval-shaped orbital metastases that resemble benign lesions. A biopsy is mandatory to ascertain the nature of an orbital mass and its benign or malign characteristics. The final diagnosis is made by the pathologist (or cytologist, when only needle aspiration is feasible instead of surgical biopsy), who identifies the histological nature of the primary neoplasia. Differential diagnoses include all intraorbital lesions and should be explored by cytological, or preferably histological analysis. Ultrasound-guided biopsy for cytological analysis is a diagnostic option for lesions not accessible surgically, or for patients unfit for invasive orbital surgery. Certain metastases from epithelial tumors in distant organs pose a diagnostic challenge because they may lack specific histological features to distinguish them from orbital, conjunctival or eyelid epithelial neoplasia.

Orbital metastases indicate advanced metastatic disease and are classically associated to a poor prognosis. They are frequently associated to brain metastases. Nonetheless, advances in chemotherapy, especially drugs modulating the immune system, seems to

improve the survival of metastatic patients. Treatment involves irradiation, chemotherapy, or hormone treatment are possible management strategies for orbital metastases, depending on the primary tumor type, stage and general status of the patient.

Progress in fine-needle, ultrasound-guided biopsy techniques should improve diagnostic procedures. Novel chemotherapeutic agents may bring new possible treatment lines for advanced metastatic disease in many different primary neoplasia, variably effective on metastatic disease. However, despite of the rich vascularization of the orbit supplying the eye and extraocular musculature, intraorbital spaces are moderately accessible to oral or intravenous drug administration. Progress in drug delivery to the orbit will improve the management and prognosis of orbital metastases.

See also: Eye and Orbit Cancer: Pathology and Genetics.

Further Readings

Abramson, D.H., Shields, C.L., Munier, F.L., Chantada, G.L., 2015. Treatment of retinoblastoma in 2015: Agreement and disagreement. JAMA Ophthalmology 133 (11), 1341–1347.

Aerts, I., Sastre-Garau, X., Savignoni, A., Lumbroso-Le Rouic, L., Thebaud-Leculee, E., Frappaz, D., et al., 2013. Results of a multicenter prospective study on the postoperative treatment of unilateral retinoblastoma after primary enucleation. Journal of Clinical Oncology 31 (11), 1458–1463.

Ahmed, S., Goel, S., Khandwala, M., Agrawal, A., Chang, B., Simmons, I.G., 2006. Neuroblastoma with orbital metastasis: Ophthalmic presentation and role of ophthalmologists. Eye (London, England) 20 (4), 466–470.

Al-Jamal, R.T., Cassoux, N., Desjardins, L., Damato, B., Konstantinidis, L., Coupland, S.E., et al., 2015. The pediatric choroidal and ciliary body melanoma study: A survey by the European ophthalmic oncology group. Ophthalmology 123 (4), 898–907.

American Brachytherapy Society—Ophthalmic Oncology Task Force, 2014AmericanBrachytherapySociety—OphthalmicOncologyTaskFor. American Brachytherapy Society consensus guidelines for plaque brachytherapy of uveal melanoma and retinoblastoma. Brachytherapy 13 (1), 1–14.

Bakhshi, S., Singh, P., Chawla, N., 2008. Malignant childhood proptosis: Study of 104 cases. Journal of Pediatric Hematology/Oncology 30 (1), 73–76. https://doi.org/10.1097/MPH.0b013e31815a0599.

Bellaton, E., Bertozzi, A.I., Behar, C., Chastagner, P., Brisse, H., Sainte-Rose, C., et al., 2003. Neoadjuvant chemotherapy for extensive unilateral retinoblastoma. The British Journal of Ophthalmology 87 (3), 327–329.

Boutroux, H., Levy, C., Mosseri, V., Desjardins, L., Plancher, C., Helfre, S., et al., 2015. Long-term evaluation of orbital rhabdomyosarcoma in children. Clinical & Experimental Ophthalmology 43 (1), 12–19.

Brisse, H.J., de Graaf, P., Galluzzi, P., Cosker, K., Maeder, P., Goricke, S., et al., 2015. Assessment of early-stage optic nerve invasion in retinoblastoma using high-resolution 1.5 Tesla MRI with surface coils: A multicentre, prospective accuracy study with histopathological correlation. European Radiology 25 (5), 1443–1452.

Brouwer, N.J., Marinkovic, M., van Duinen, S.G., Bleeker, J.C., Jager, M.J., Luyten, G.P.M., 2017. Treatment of conjunctival melanoma in a Dutch Referral Centre. The British Journal of Ophthalmology. https://doi.org/10.1136/bjophthalmol-2017-311082.

Cassoux, N., Charlotte, F., Sastre, X., Orbach, D., Lehoang, P., Desjardins, L., 2009. Conservative surgical treatment of medulloepithelioma of the ciliary body. Archives of Ophthalmology 128 (3), 380–381.

Cassoux, N., Cayette, S., Plancher, C., Lumbroso-Le Rouic, L., Levy-Gabriel, C., Asselain, B., et al., 2013. Choroidal melanoma: Does endoresection prevent neovascular glaucoma in patient treated with proton beam irradiation? Retina 33 (7), 1441–1447.

Cassoux, N., Rodrigues, M.J., Plancher, C., Asselain, B., Levy-Gabriel, C., Lumbroso-Le Rouic, L., et al., 2014. Genome-wide profiling is a clinically relevant and affordable prognostic test in posterior uveal melanoma. The British Journal of Ophthalmology 98 (6), 769–774.

Desjardins, L., Lumbroso-Le Rouic, L., Levy-Gabriel, C., Cassoux, N., Dendale, R., Mazal, A., et al., 2012. Treatment of uveal melanoma by accelerated proton beam. Developments in Ophthalmology 49, 41–57.

Finger, P.T., 2009. The 7th edition AJCC staging system for eye cancer: An international language for ophthalmic oncology. Archives of Pathology & Laboratory Medicine 133 (8), 1197–1198.

Gombos, D.S., Kelly, A., Coen, P.G., Kingston, J.E., Hungerford, J.L., 2002. Retinoblastoma treated with primary chemotherapy alone: The significance of tumour size, location, and age. The British Journal of Ophthalmology 86 (1), 80–83.

Harbour, J.W., 2009. Molecular prognostic testing and individualized patient care in uveal melanoma. American Journal of Ophthalmology 148 (6), 823–829.e1.

Hirst, L.W., 2007. Randomized controlled trial of topical mitomycin C for ocular surface squamous neoplasia: Early resolution. Ophthalmology 114 (5), 976–982.

Kheir, W.J., Tetzlaff, M.T., Pfeiffer, M.L., Mulay, K., Ozgur, O., Morrell, G., et al., 2016. Epithelial, non-melanocytic and melanocytic proliferations of the ocular surface. Seminars in Diagnostic Pathology 33 (3), 122–132.

Kim, I.K., Lane, A.M., Jain, P., Awh, C., Gragoudas, E.S., 2016. Ranibizumab for the prevention of radiation complications in patients treated with proton beam irradiation for choroidal melanoma. Transactions of the American Ophthalmological Society 114, T2.

Kivela, T., Kujala, E., 2013. Prognostication in eye cancer: The latest tumor, node, metastasis classification and beyond. Eye (London, England) 27 (2), 243–252.

Kivela, T.T., Piperno-Neumann, S., Desjardins, L., Schmittel, A., Bechrakis, N., Midena, E., et al., 2016. Validation of a prognostic staging for metastatic uveal melanoma: A collaborative study of the European ophthalmic oncology group. American Journal of Ophthalmology 168, 217–226.

Lane, A.M., Egan, K.M., Harmon, D., Holbrook, A., Munzenrider, J.E., Gragoudas, E.S., 2009. Adjuvant interferon therapy for patients with uveal melanoma at high risk of metastasis. Ophthalmology 116 (11), 2206–2212.

Lumbroso-Le Rouic, L., Savignoni, A., Levy-Gabriel, C., Aerts, I., Cassoux, N., Salviat, F., et al., 2015. Treatment of retinoblastoma: The Institut Curie experience on a series of 730 patients (1995 to 2009). Journal Français d'Ophtalmologie 38 (6), 535–541.

Munier, F.L., Gaillard, M.C., Balmer, A., Soliman, S., Podilsky, G., Moulin, A.P., et al., 2012. Intravitreal chemotherapy for vitreous disease in retinoblastoma revisited: From prohibition to conditional indications. The British Journal of Ophthalmology 96 (8), 1078–1083. https://doi.org/10.1136/bjophthalmol-2011-301450.

Nguyen, D.T., Houillier, C., Choquet, S., Cassoux, N., Soussain, C., Le Cossec, C., et al., 2016. Primary Oculocerebral lymphoma: MTX Polychemotherapy alone on intraocular disease control. Ophthalmology 123 (9), 2047–2050.

Pandey, K., Singh, P., Singh, A., Pandey, H., 2011. Primary sebaceous gland carcinoma of the bulbar conjunctiva without involvement of eyelid: A clinical dilemma. Oman Journal of Ophthalmology 4 (2), 97–99. https://doi.org/10.4103/0974-620X.83665.

Papakostas, T.D., Lane, A.M., Morrison, M., Gragoudas, E.S., Kim, I.K., 2017. Long-term outcomes after proton beam irradiation in patients with large choroidal melanomas. JAMA Ophthalmology 135 (11), 1191–1196.

Sant, D.W., Tao, W., Field, M.G., Pelaez, D., Jin, K., Capobianco, A., et al., 2017. Whole exome sequencing of lacrimal gland adenoid cystic carcinoma. Investigative Ophthalmology & Visual Science 58 (6), BIO240–BIO246.

Sellam, A., Desjardins, L., Barnhill, R., Plancher, C., Asselain, B., Savignoni, A., et al., 2014. Fine needle aspiration biopsy in uveal melanoma: Technique, complications, and outcomes. American Journal of Ophthalmology 162, 28–34.e1.

Shields, C.L., Shields, J.A., Kiratli, H., De Potter, P., Cater, J.R., 1995. Risk factors for growth and metastasis of small choroidal melanocytic lesions. Ophthalmology 102 (9), 1351–1361.

Shields, C.L., Shields, J.A., Cater, J., Gunduz, K., Miyamoto, C., Micaily, B., et al., 2000. Plaque radiotherapy for uveal melanoma: Long-term visual outcome in 1106 consecutive patients. Archives of Ophthalmology 118 (9), 1219–1228.

Shields, C.L., Jorge, R., Say, E.A., Magrath, G., Alset, A., Caywood, E., et al., 2016. Unilateral retinoblastoma managed with intravenous chemotherapy versus intra-arterial chemotherapy. Outcomes based on the International Classification of Retinoblastoma. Asia-Pacific Journal of Ophthalmology 5 (2), 97–103.

Shields, C.L., Chien, J.L., Surakiatchanukul, T., Sioufi, K., Lally, S.E., Shields, J.A., 2017. Conjunctival tumors: Review of clinical features, risks, biomarkers, and outcomes—The 2017 J. Donald M. Gass Lecture. Asia-Pacific Journal of Ophthalmology 6 (2), 109–120.

Yousef, Y.A., Soliman, S.E., Astudillo, P.P., Durairaj, P., Dimaras, H., Chan, H.S., et al., 2016. Intra-arterial chemotherapy for retinoblastoma: A Systematic Review. JAMA Ophthalmology. https://doi.org/10.1001/jamaophthalmol.2016.0244.

Melanoma: Pathology and Genetics

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Glossary

Radial growth phase (RGP) An atypical melanocytic proliferation that enlarges along the radii of an imperfect circle and is limited to epidermis and superficial dermis. Clinically, the lesion tends to be relatively flat with oval or circular borders. Clark used the term RGP to describe the clinical peripheral enlargement of melanoma, but not the growth direction of melanocytic cells, as they may grow upward in the epidermis, downward into the papillary dermis, or peripherally, determining enlargement of the margins. Melanoma in situ and invasive melanoma that has not yet metastasized, are in the radial growth phase.

Vertical growth phase (VGP) An atypical melanocytic proliferation that expands as a spheroidal nodule in the dermis and is associated with the risk of subsequent metastatic disease. Clark used the term VGP to describe the focal appearance within the radial growth phase of a new clone of melanocytic cells, which is characterized by a distinct expansive growth preference, similar to that of a metastasis.

Nomenclature

AKT AKT serine/threonine kinase
ALK ALK receptor tyrosine kinase
ARAF A-Raf proto-oncogene, serine/threonine kinase
ARID2 AT-rich interaction domain 2
ATM ATM serine/threonine kinase
BAP1 BRCA1 associated protein 1
BRAF B-Raf proto-oncogene, serine/threonine kinase
BRCA1 BRCA1, DNA repair associated
BRCA2 BRCA2, DNA repair associated
CCND1 Cyclin D1
CDH1 Cadherin 1
CDH11 Cadherin 11
CDK4 Cyclin-dependent kinase 4
CDKN2A Cyclin-dependent kinase inhibitor 2A
CHEK2 Checkpoint kinase 2
CIMP CpG island methylator phenotype
CM Cutaneous melanoma
CN Congenital nevus
CRAF Raf-1 proto-oncogene, serine/threonine kinase
CSD Cumulative solar damage
CTNNB1 Catenin beta 1
CYSLTR2 Cysteinyl leukotriene receptor 2
DDX3X DEAD-box helicase 3, X-linked
EGFR Epidermal growth factor receptor
EIF1AX Eukaryotic translation initiation factor 1A, X-linked
ERBB1 Erb-B2 receptor tyrosine kinase 1
ERBB2 Erb-B2 receptor tyrosine kinase 2
ERK Extracellular signal-regulated kinase
EZH2 Enhancer of zeste 2 polycomb repressive complex 2 subunit
FBXW7 F-box and WD repeat domain containing 7

 https://doi.org/10.1016/B978-0-12-801238-3.65095-X

FISH Fluorescent in situ hybridization
FoxP3 Forkhead box P3
GAB2 GRB2 associated binding protein 2
GDP Guanosine diphosphate
GNA11 G protein subunit alpha 11
GNAQ G protein subunit alpha q
GTP Guanosine triphosphate
HER2 Human epidermal growth factor receptor 2
HRAS HRas proto-oncogene, GTPase
IDH1 Isocitrate dehydrogenase (NADP+) 1, cytosolic
IDO Indoleamine-2,3-dioxygenase
IκB Inhibitor of kappa B
KDR Kinase insert domain receptor
KIT KIT proto-oncogene receptor tyrosine kinase
KRAS KRAS proto-oncogene, GTPase
LM Lentigo maligna
LMM Lentigo maligna melanoma
MAP2K1 Mitogen-activated protein kinase kinase 1
MAPK Mitogen-activated protein kinase
MBN Melanoma arising in blue nevus
MC1R Melanocortin-1 receptor
MCN Melanoma arising in congenital nevus
MEK MAPK/ERK kinase
MET MET proto-oncogene, receptor tyrosine kinase
MITF Microphthalmia-associated transcription factor
mTOR Mechanistic target of rapamycin kinase
NF1 Neurofibromin 1
NFKBIE NFKB inhibitor epsilon
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS Next-generation sequencing
NM Nodular melanoma
NMSC Nonmelanoma skin cancer
NRAS NRAS proto-oncogene, GTPase
NTRK1 Neurotrophic receptor tyrosine kinase 1
NTRK3 Neurotrophic receptor tyrosine kinase 3
PALB2 Partner and localizer of BRCA2
PDGFRA Platelet derived growth factor receptor alpha
PD-L1 Programmed death ligand 1
PI3K Phosphatidylinositol 3-kinase
PIK3CA Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha
PIP2 Phosphatidylinositol 4,5-bisphosphate
PIP3 Phosphatidylinositol 3,4,5-trisphosphate
PLCB4 Phospholipase C beta 4
POT1 Protection of telomeres 1
PPP6C Protein phosphatase 6 catalytic subunit
PREX2 Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 2
PTEN Phosphatase and tensin homolog
PTPN11 Protein tyrosine phosphatase, nonreceptor type 11
RAC1 Rac family small GTPase 1
RAD50 RAD50 double strand break repair protein
RAF1 Raf-1 proto-oncogene, serine/threonine kinase
RB1 RB transcriptional corepressor 1
RET Ret proto-oncogene
RGP Radial growth phase

RICTOR RPTOR independent companion of MTOR complex 2
ROS Reactive oxygen species
ROS1 ROS proto-oncogene 1, receptor tyrosine kinase
RTK Receptor tyrosine kinase
SF3B1 Splicing factor 3b subunit 1
SNX31 Sorting nexin 31
SOS1 SOS Ras/Rac guanine nucleotide exchange factor 1
SPRED1 Sprouty related EVH1 domain containing 1
SSM Superficial spreading melanoma
STK19 Serine/threonine kinase 19
TACC1 Transforming acidic coiled-coil containing protein 1
TCGA The Cancer Genome Atlas
TERT Telomerase reverse transcriptase
TET Tet methylcytosine dioxygenase
TME Tumor microenvironment
TP53 Tumor protein p53
UM Uveal melanoma
UV Ultraviolet
VEGFR2 Vascular endothelial growth factor receptor 2
VGP Vertical growth phase
WT Wild-type
WT1 Wilms tumor 1

Introduction

The approach to the classification of melanoma has evolved from the traditional clinicopathologic to a novel molecular classification. Based on recent knowledge, it is generally agreed that melanoma definition cannot be exclusively confined to the cytoarchitectural morphology observed under light microscope, since melanoma molecular features are much more intricate, and genetics has a remarkable clinical impact for effective systemic therapy. Cutaneous melanoma is the malignant tumor with the highest mutation load and a prevailing cytosine to thymine nucleotide transition signature, which is attributable to UV radiation. While such a signature has been found to be a characteristic of melanoma arising on sun-exposed skin, acral and mucosal melanomas have been shown to have structural changes and mutational signatures not related to UV radiation damage. The most significantly mutated genes in cutaneous melanoma are BRAF, NRAS, CDKN2A and TP53, in acral melanoma NRAS and NF1, in mucosal melanoma SF3B1, and in 80%–90% of uveal melanomas GNAQ or GNA11, whose mutations are mutually exclusive and happen early in the pathogenesis.

Overall, the most frequent mutations are those in noncoding DNA sequences and involve the TERT promoter. The TERT gene encodes the catalytic subunit of telomerase, and its transcriptional regulation is usually the limiting step in telomerase activity. Telomerase activity is usually silenced in normal tissues, causing telomeres to shorten with each successive round of cell division. The expression of telomerase is considered a hallmark of tumorigenesis, as over 90% of human cancers express the enzyme, including melanoma. TERT mutation results in cell immortalization by extension of the replicative lifespan of cells, allowing them to avoid replicative senescence in response to critical telomere shortening.

In addition, whole-genome sequencing of melanoma has unveiled how different mutational pathways are implicated in melanoma pathogenesis contributing to the development of targeted therapies.

The molecular perspective has developed as a reinterpretation of the clinicopathological classification on the basis of genotype–phenotype correlation. As proposed by Bastian, the taxonomy of melanocytic neoplasia is structured according to a dichotomous approach that differentiates lesions that originate from intraepithelial melanocytes, and are variably associated with UV radiation damage, from those lesions arising from nonintraepithelial melanocytes that can be uveal or nonuveal (brain, visceral organs, and proliferation in the dermis). While the first type of lesion is correlated with patient's age, the second type does not show strict correlation with age distribution, with the exception of melanoma on congenital nevus, which occurs primarily in children.

Melanomas can be considered as a group of biologically distinct categories showing differences in age and ethnic distribution, clinical and histological features, pattern of metastasization, etiopathogenetic role of UV radiation, primary oncogenic mutations, somatic alterations, and pathways of mutational mechanisms. Furthermore, analysis of The Cancer Genome Atlas (TCGA), mainly in metastatic melanoma dataset, has indicated that, independently of the oncogenic mutations (BRAF, NRAS, NF1), melanomas with immune signature have a better survival than those without immune signature. According to presence or not of an immune

signature, two major subsets of melanomas characterized by the presence or absence of a gene expression profile indicative of a pre-existing T cell-inflamed tumor microenvironment (TME) have been described. The T cell-inflamed subset of tumors is characterized by a type I Interferon transcriptional profile, the presence of T cell transcripts, T cell recruitment through chemokines and macrophage activation markers. Accordingly, immunohistochemistry confirms the positivity for CD8+ T cells, macrophages, and some B cells and plasma cells. While a subset of T cells specific for tumor antigens is present in tumor microenvironment, functional analysis indicates various degrees of dysfunction of these T cells due to immune suppressive mechanisms acting at the level of the TME. Transcripts encoding IDO, PD-L1, and FoxP3 and positivity for PD-L1 and IDO protein expression, and also nuclear FoxP3 + CD4+ cells by immunohistochemistry, are present within T cell-inflamed melanomas in the same region as CD8+ T cells. This immune adaptive resistance feature represented a clinical challenge recently addressed by the adoption of immune checkpoint inhibitors, which unleash the immune system against melanoma.

Genetic Features

Cutaneous melanoma originates in melanocytes, whose biology and role in melanin production have been well studied and characterized. Melanin plays a role in the dispersion of UV radiation and in the absorption of reactive oxygen species (ROS); physiologically, the protein product of the TP53 gene plays a crucial role in melanocytes for the control of cell response to DNA damage related to increased UV exposure and ROS levels. In particular, the damage to DNA induced by UV rays determines specific mutational signatures, many of which are driven by TP53, which therefore define the frequent events of melanomagenesis in the skin. Considering data from TCGA and the results of the main initial studies with screening approaches based on mutational analysis of the entire exome/genome by next-generation sequencing approaches, cutaneous melanoma has been shown to possess a very high prevalence of somatic mutations, both in primary lesions and—at an even higher level—in metastatic lesions (Fig. 1). The mean mutation rate of cutaneous melanoma, calculated by TCGA from whole-exome sequencing of 318 primary and metastatic melanomas originating from nonglabrous (hair-bearing) skin, has been estimated ranging from 18 to 21 mutations per megabase, which is among the highest rates reported for any type of cancer, with the exception of colorectal cancer carrying deficient mismatch repair (MMR) mechanisms. Approximately three-fourths (range, 69%–82%) of these genomic variations are represented by C>T substitutions, which is associated with another small fraction (about 4%–5%) of CC > TT transitions; all these variants were strictly dependent on a mutagenic effect of UV rays and typically cause the so-called *UV signature*. Overall, the mutation rate in melanomas

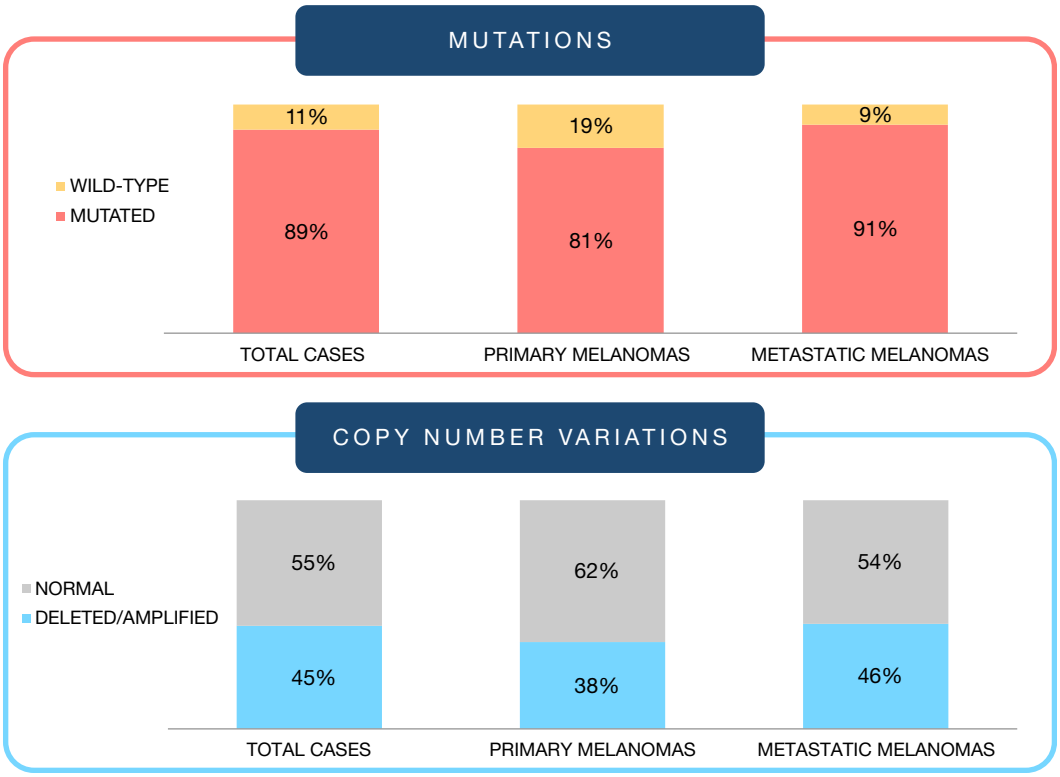

Fig. 1 Mutations and copy number variations in primary and metastatic melanomas. Nine out of ten melanomas are genetically mutated. Copy number alterations are present in 45% of cases. The frequency of mutations and copy number variations increases significantly from primary to metastatic lesions; indeed, metastatic melanomas have a higher mutation burden.

arising on chronically sun-exposed skin was approximately five times higher compared to those on skin not subject to chronic damage by the sun. Finally, UV signature appears to be slightly more prevalent in metastatic lesions (about 85% of the total amount of detected mutations) than primary tumors (about 75%).

About 10% of melanomas occur in families with increased recurrence of the disease, sometimes associated with the presence of other neoplastic diseases (such as pancreatic carcinoma or brain tumors). Approximately, 40% of these familial melanomas are due to hereditary transmission of mutations in high penetrance susceptibility genes, including *cyclin-dependent kinase inhibitor* 2A (CDKN2A; the most frequently mutated gene for predisposition to melanoma, located on chromosome 9p21), *cyclin-dependent kinase 4* (CDK4; 12q14), *breast cancer gene 1 (BRCA1)-associated protein 1* (BAP1; 3p21), *telomerase reverse transcriptase* (TERT; 5p15), *protection of telomeres 1* (POT1; 7q31), and *microphthalmia-associated transcription factor* (MITF; 3p13—susceptibility is quite exclusively associated with the intermediate risk variant pE318K). Carriers of BAP1 mutations show an intermediate risk for cutaneous melanoma (CM), which becomes much higher in families with coexistence of CM cases and uveal melanoma (UM): 3% in UM cases (population based), 15% in CM families, and 28% in CM–UM families. Overall, carriers of germline mutations in CDKN2A/CDK4 and BAP1 genes may present an increased predisposition to either cutaneous melanoma (for BAP1, also to other melanoma subtypes such as UM; for CDKN2A/CDK4, also to higher prevalence of atypical melanocytic nevi), either to an increased incidence—though with a penetrance markedly lower than melanoma—of other types of cancer (particularly, pancreatic and renal carcinoma, brain tumor and mesothelioma). In about 60% of families with melanoma recurrence, the predisposition to the disease is probably due to the combination of variants—both mutations and functional polymorphisms (the latter often associated with specific environmental exposure conditions)—in susceptibility genes with a lower penetrance. In other words, the increased predisposition to melanoma would seem to be linked to the inheritance of specific patterns of genetic alterations rather than mutations in single, stronger susceptibility genes (hence, the difficulty encountered so far in identifying additional melanoma-predisposing genes).

In addition to the model of dominant inheritance described above, melanoma may represent a subordinate malignancy in the context of other tumor syndromes. Members of families with rare hereditary syndromes—such as Cowden syndrome (due to mutations in PTEN), Li Fraumeni syndrome (TP53 mutations), Lynch syndrome (inactivation of genes controlling DNA mismatch repair mechanisms) and xeroderma pigmentosum (mutations in multiple predisposing genes)—or relatively more common genetic conditions, such as hereditary breast and ovary cancers (mainly, mutations in BRCA1–2, but also mutations in ATM, CDH1, CHEK2, PALB2, PTEN, RAD50, TP53), present a poorly defined but higher risk of melanoma.

All these observations strongly support the hypothesis that the pathogenesis of melanoma is complex and involves both genetic and nonhereditary or extrinsic factors.

Molecular Pathways

Summarizing the data generated by the main NGS analyses on the whole-exome/genome, the molecular pathways involved in the melanoma pathogenesis depend on the following genes: BRAF (able to activate the pathway downstream, which includes two effector proteins MEK and ERK), NRAS (whose activation is able to promote the activity of downstream pathways: BRAF-MAPK and PI3K-AKT), NF1 (able to induce the activation of NRAS), CDKN2A (able to activate the pathways dependent on the two proteins encoded by this tumor suppressor gene: $p16^{CDKN2A}$-CDK4-RB and $p14^{CDKN2A}$-MDM2-p53 pathways), and PTEN (activating the PI3K-AKT-mTOR pathway). From a therapeutic point of view, besides BRAFV600, there are no alterations currently considered relevant in clinical decision making. Testing for NRAS or NF1 mutations is largely a matter of identifying patients who are potential candidates for clinical trials. Preliminary evidence supports the importance of CDKN2A and/or PTEN inactivation as mediators of acquired resistance to BRAF inhibitor-based therapy, but these alterations cannot be currently considered routinely in clinical decision making.

BRAF

The RAF kinase family consists of three proteins (ARAF, BRAF, and CRAF), all of which are part of the signal transduction cascade named mitogen-activated protein kinase (MAPK) pathway. In melanoma, the BRAF gene is mutated in 45%–50% of cases; the most prevalent mutation (about 90% of cases) is represented by a substitution of a valine with glutamic acid at codon 600 ($BRAF^{V600E}$). The remaining BRAF mutations often occur at the same codon (V600-K/D/R); therefore, mutations in codons other than V600 are not very common (among them, K601 is the most prevalent). The constitutive oncogenic activation of BRAF—through a process of phosphorylation of MEK and ERK, the effectors immediately downstream of the MAPK pathway—promotes a continuous, uncontrolled stimulation of cell proliferation. There is an inverse relationship between BRAF mutation prevalence and age-decade. The majority of patients <30 years and 25% ≥ 70 years harbor a BRAF-mutant melanoma. Among BRAF-mutant melanomas, the frequency of non-V600E genotypes (including V600K) raises with increasing age. Non-V600E genotypes have been found in <20% of patients <50 years and >40% in those ≥ 70 years. A higher degree of cumulative sun-induced damage correlates with V600K but not V600E melanoma. Melanomas that carry BRAF mutation are characterized by an enrichment of alterations in PTEN, corroborating the frequent coexistence of BRAF and PTEN mutations in melanoma. At the same time, a large fraction of melanomas with mutated BRAF exhibits a reduced or absent expression of $p16^{CDKN2A}$ or p53 proteins, due to the coexistence of mutations inactivating the corresponding genes (CDKN2A and TP53, respectively). The demonstration that BRAF is mutated

in a large fraction (65%–80%) of common nevi suggests that its oncogenic activation is a necessary but not sufficient condition for the development of melanoma (in fact, it is considered an initiation event in melanocyte transformation). Therefore, the onset of alterations in other genes able to cooperate with BRAF seems to act as the main mechanism underlying the transformation and neoplastic progression in the melanocytic lineage. It has finally been demonstrated that some genetic variants of the *melanocortin-1 receptor membrane receptor* (MC1R) are not able to adequately stimulate the production of melanin protecting the skin from the damage of UV rays. The combination of the presence of such variants of MC1R and exposure to UV rays (especially, when intermittent) was also considered responsible for the oncogenic activation of BRAF in this specific subgroup of melanomas.

RAS

The products of the RAS family are composed of three tissue-specific isoforms: HRAS, KRAS and NRAS. NRAS mutations are found in 20%–25% of melanoma cases; again, they occur almost exclusively in a single gene codon (Q61, about 90% of cases); in the remaining 10% of cases, the mutated codon is G12 or G13. The oncogenic stimulation of RAS is able to activate specific cytoplasmic downstream proteins with kinase function: RAF and *phosphatidylinositol 3-kinase* (PI3K).

RAS mutations have been demonstrated to be mutually exclusive with BRAF mutations in nearly all cases (coexistence of the two genes mutated in a constitutive manner are reported in a fraction of cutaneous melanomas, $<2\%$–3% of cases). It has been observed that, in the presence of NRAS activation (both for the acquisition of mutations or functional oncogenic induction), the translation of the mitogenic signal in the MAPK pathway can be switched to CRAF, which therefore acquires a key role in maintaining cell proliferation stimulation in this subset of melanomas. Interestingly, an increased activation of CRAF, as well as the presence of NRAS mutation, have been described as responsible for the acquired resistance to BRAF inhibitors.

In addition to BRAF, genetic alterations (mutations and gene copy number changes) in other important components of pathways downstream of RAS that control cell proliferation and survival—including PIK3CA (altered in 5% of cases) and AKT3 (8%)—occur at lower frequency. In fact, a vast majority of the PI3K-PTEN/AKT genes downstream of RAS are functionally silenced in melanoma. In this pathway, an exception is represented by a markedly higher frequency of mutations (about one fifth of melanomas in NGS studies) in the PREX2 gene regulating PTEN.

A limited fraction of melanomas that are not mutated in BRAF and RAS may carry activating mutations in KIT, a tyrosine kinase receptor of the cell membrane, resulting in a continuous induction of cell proliferation, through functional stimulation, mainly of the MAPK pathway. In particular, KIT mutations have been described in acral melanomas (about 10% of cases) and in those from chronically sun-exposed skin areas (5% of cases) among cutaneous melanomas, showing, however, the highest prevalence in mucosal melanomas, particularly in anal melanomas (15%–20% of cases).

NF1

Germline mutations in NF1 cause an inherited multisystem genetic disorder, neurofibromatosis type 1, which is characterized by changes in skin coloring pigmentation, as well as the growth of both benign and malignant tumors. In preclinical models, NF1 mutation suppresses BRAF-induced senescence in melanocytes, promoting melanocyte proliferation and enhancing melanoma development. The Cancer Genome Atlas has also identified a subset of melanomas (approximately 15% of cases) in which activation of the MAPK pathway is due to inactivating mutations of the NF1 gene. Physiologically, NF1 encodes for neurofibromin, a RAS-GTPase-activating protein, which negatively regulates *RAS* signaling by facilitating hydrolysis of RAS-GTP to the RAS-GDP-inactive form; therefore, mutations functionally silencing NF1 result in RAS activation. In this perspective, the activation of several RASopathy genes—such as SOS1, PTPN11, RAF1, and SPRED1—appears to occur more frequently in subtypes of melanomas with NF1 mutations. At the same time, these data are a clear demonstration that NF1 cooperates with other RASopathy genes in melanomagenesis. Although the mutations of NF1 are the most prevalent alterations in the group of BRAF and RAS wild-type melanomas (about two-fifths of these cases, which, however, represent 4%–5% of the total cutaneous melanomas), they are present at almost the same frequency in BRAF- and RAS-mutated melanomas (3%–4% of cases). NF1 is a key driver of melanoma, and this is suggested by three biological features: (i) a high frequency of nonsilent exonic mutations, (ii) a low frequency of synonymous or intronic mutations, (iii) it occurs in a considerable portion of studied melanoma specimens.

Melanomas with NF1 mutations generally occur on chronically sun-exposed skin or in elder individuals and show a high mutation burden. Additionally, NF1 mutations characterize certain clinicopathologic melanoma subtypes, specifically desmoplastic melanoma. Unlike BRAF-mutated melanomas, those with the NF1 subtype have a stronger correlation with the UV-induced mutagenesis. Overall, occurrence of mutations in NF1, alone or associated with BRAF or RAS mutations, induces a higher mutational load and, consequently, a greater probability of generating neoantigens. Due to this increased antigen capacity, tumors with mutations in NF1 and/or constitutive activation of RASopathy genes are thought to be addressed to immunotherapeutic treatment with immune checkpoint inhibitors.

In several melanoma cell lines, there is evidence that NF1 ablation decreases the sensitivity of melanoma to BRAF inhibitors. NF1 mutations are present in BRAF-mutant tumors intrinsically resistant to BRAF inhibitors, as well as in melanomas of patients showing resistance to BRAF inhibitors. Correspondingly, NF1/BRAF-mutant murine tumors are resistant to BRAF inhibitors but sensitive to combined inhibition of MAPK/ERK and mTOR pathways.

Triple Wild-Type Melanomas

According to TCGA classification, cutaneous melanoma can be categorized into four genetic subgroups on the basis of MAPK driver mutations: BRAF, RAS (N-H-K), NF1 (lacking a BRAF p.V600 or RAS p.G12, G13 and Q61 hotspot mutation) and *Triple wild-type* (*WT*) melanomas.

Triple wild-type melanomas represent a genetic distinct entity for several reasons: (i) only ~30% of *Triple WT* melanomas harbor a UV signature compared with over 90% considering the other three molecular subtypes; (ii) somatic copy number analysis shows that *Triple WT* melanomas comprise a significant number of cases with copy number amplifications; amplicons include the 4q12 minimal common region containing KIT, PDGFRA and KDR (also known as VEGFR2), as well as amplifications in loci encompassing TERT, CDK4 and CCND1; (iii) *Triple WT* melanomas have more complex structural rearrangements and candidate fusion drivers, although few recurrent fusions have been described so far.

CDKN2A

The CDKN2A gene encodes two proteins: $p16^{CDKN2A}$ and $p14^{CDKN2A}$. It acts as tumor suppressor gene in a recessive manner; inactivation of both alleles is necessary for the development of melanoma. In patients with melanoma familiarity, 20%–40% of probands may carry germline mutations in CDKN2A. At the somatic level, about two-thirds of melanoma patients instead present a CDKN2A gene inactivation; these melanomas seem to be resistant to BRAF inhibitors, or to BRAF and MEK inhibitors. In particular, activation of the downstream CDK4-RB effectors through inactivation of $p16^{CDKN2A}$ seems to be correlated with disease progression; indeed, its prevalence significantly increases during transition from primary tumors to metastatic melanomas and melanoma cell lines. Similarly, the inactivation of $p14^{CDKN2A}$ causes the reduction of the p53 protein levels, with consequent impairment of the cell-cycle progression control and inhibition of the apoptosis, contributing to increase the aggressiveness of tumor cells and their refractoriness to therapy. Activation of the CDKN2A-dependent pathway may also be associated with the amplification of the CyclinD1 (CCND1) gene, which is generally found in melanomas negative for BRAF and NRAS mutations. However, in a fraction (about 15%) of mutated BRAF melanomas, the coexistence of CCND1 amplification seems to confer resistance to BRAF inhibitors.

PI3K-PTEN

In addition to MAPK, the second cell regulating pathway—mainly dependent on RAS activation—consists of the PI3K protein pathway which is characterized by signal transduction through the PTEN, PI3K, and AKT (in particular, the AKT3 isoform in melanoma) protein cascade. Oncogenic activation of the PI3K pathway can occur through several mechanisms, including: mutation and/or amplification of genes encoding RTKs (e.g., EGFR (*ERBB1*) and HER2 (*ERBB2*)), subunits of PI3K (e.g., p110α, p110β, p85α, and p85β), AKT (*AKT1*), or activating isoforms of RAS. Within the PI3K/AKT/mTOR pathway, AKT plays a major role, being at the crossroad and implicated in the oncogenic mutation, the immunotolerance, and the developing of de novo and acquired treatment resistance in various tumor types treated with targeted therapies. Furthermore PTEN (encoding phosphatase and tensin homolog, the major PIP3 phosphatase) is frequently mutated.

In physiological conditions, the phosphatase activity of the PTEN protein reduces the intracellular level of the PIP2 and PIP3 phosphoinositoles, which are produced by the activation of PI3K, in order to regulate the activation level of downstream AKT and its mTOR substrate, thus modulating the synthesis of proteins involved in apoptosis and cell survival. In melanoma, the PTEN gene is deleted in about a third of cases, with complete loss of expression of the corresponding protein in 10%–20% of primary melanomas; the level of this loss increases during neoplastic progression, up to 40%–50% in melanoma cell lines. The combined effect of the PTEN loss and the PI3K-AKT pathway activation results in aberrant growth of melanoma cells and increased survival capacity with the acquisition of apoptosis resistance.

All of these findings clearly indicate that there are distinct molecular subtypes of melanoma; the well-characterized ones are summarized in **Table 1**. From a practical point of view, the characterization of these subtypes becomes extremely important for an increasingly correct therapeutic approach and an appropriate definition of clinical–biological behavior in patients with melanoma.

Epigenetic Events

Alongside the genetic alterations, data are emerging that increasingly support a role of epigenetic events in the development and progression of melanoma. The latter are defined as inheritable alterations in gene expression without a change in the DNA sequence. Main epigenetic modifications include:

- posttranslational modifications of histones and remodeling of nucleosomal complexes;
- methylation of cytosine–guanine (CpG) dinucleotides from accessible DNA;
- gene silencing by noncoding RNAs, either short or long, whose expression can in turn be directly regulated through epigenetic modifications of the corresponding genes.

Table 1 Main molecular subtypes of melanoma: frequent mutations and rearrangements

Subtype	*Most frequently mutated genes (≥ 10%)*	*Less frequently mutated genes (< 10%)*	*Rearranged genes*
BRAF mutant	TP53, CDKN2A, PTEN, ARID2	PPP6C, NF1, MAP2K1, RAC1, IDH1, DDX3X, SNX31, TACC1, CTNNB1, PREX2, PIK3CA, STK19, EZH2, FBXW7, RB1, WT1	CDKN2A-del, CCND1-ampl, PTEN-del, MITF-ampl, TERT-ampl
RAS mutant	CDKN2A, TP53, ARID2, NF1, PPP6C	DDX3X, RAC1, IDH1, PTEN, MAP2K1, RB1, TACC1, PREX2, CTNNB1, FBXW7, PIK3CA, STK19, WT1	CDKN2A-del, PTEN-del, CCND1-ampl, TERT-ampl, MITF-ampl, KIT-ampl, CDK4-ampl
$BRAF^{neg}/RAS^{neg}$ mutant	NF1, TP53, ARID2, RAC1	KIT, CDKN2A, PTEN, IDH1, MAP2K1, RB1, SNX31, PPP6C, PIK3CA, STK19, EZH2, WT1, PREX2	CDKN2A-del, CCND1-ampl, PTEN-del, TERT-ampl, CDK4-ampl, KIT-ampl, MITF-ampl

del, gene deletion; ampl, gene amplification.

A particularly interesting result is given by the fact that the CpG island methylator phenotype (CIMP) is able to stratify patients with melanoma. The CIMP defines tumors that have a higher level of overall DNA methylation associated with a state of hyper-methylation of the CpG islands. The CIMP, initially observed in colorectal carcinoma, has been described in various other types of cancer, including melanoma. The TCGA highlighted the occurrence of a hyper-methylation status in both primary and metastatic melanomas (in some cases, as for CDH11, gene transcription is active only in the lymph nodes and not in the primary tumor). Hyper-methylation seems to be associated with mutations in most of the genes mainly involved in melanomagenesis (the RAS-mutated subtype is associated with CIMP to a greater extent than the BRAF-mutated subtype). Somatic mutations of the ARID2 and IDH1 genes, both involved in chromatin remodeling, appear to be significantly associated with the CIMP cluster. In particular, IDH1 (located on chromosome 2q33) encodes an enzyme that converts isocitrate into α-ketoglutarate (also known as 2-oxoglutarate). The loss of the IDH1 function results in a reduction in the production of α-ketoglutarate, which is a necessary co-substrate for the TET family enzymes—composed of TET1, TET2 and TET3—essential for the DNA de-methylation pathway. TET2 catalyzes the oxidation steps on the methyl group of 5-methylcytosine to produce 5-hydroxy-methylcytosine, 5-carboxyloxosine and 5-formylcytosine, and ultimately cytosine. This newly discovered pathway has a role in controlling methylation levels and maintenance of the DNA epigenetic fidelity; this led to the definition of the TET genes as the "guardians of the epigenome."

Overall, melanoma is even more heterogeneous than expected from the molecular point of view. Complexity of the disease is thus given by a combination of driver somatic mutations (both genetic and cytogenetic) and epigenetic modifications able to negatively and/or positively induce expression aberrations and even genomic changes.

Classification of Melanoma

Considering the molecular approach, once a melanoma has been diagnosed, it is relevant to know which primary mutation that melanoma harbors. Primarily, BRAF, NRAS, HRAS, KIT, GNAQ and GNA11 mutations should be tested and, hence, the lesion classified. As mentioned above, these mutations are typically mutually exclusive, and each is more or less frequently associated with a different UV-related clinical scenario. The diagnosis of melanoma still relies on histopathological examination, however, the clinicopathological classification proposed by Clark and McGovern can be integrated in a molecular multidimensional reclassification where nine main melanoma classes with distinct mutational pathways have been identified:

Pathway I. Low-CSD Melanoma/Superficial Spreading Melanoma (SSM)
Pathway II. High-CSD Melanoma/Lentigo Maligna Melanoma (LMM)
Pathway III. Desmoplastic Melanoma
Pathway IV. Malignant Spitz Tumor
Pathway V. Acral Melanoma
Pathway VI. Mucosal Melanoma
Pathway VII. Melanoma arising in Congenital Nevus
Pathway VIII. Melanoma arising in Blue Nevus
Pathway IX. Uveal Melanoma

Considering the causative role of UV radiation, a distinction can be made between melanomas originating on sun-exposed skin in susceptible individuals and associated with cumulative solar damage varying from low (Low-CSD Melanoma/SSM) to high (High-CSD Melanoma/LLM and Desmoplastic Melanoma), as observed on clinical–histological ground, and melanomas arising on sun-shielded skin or sites without consistent association with UV exposure (Malignant Spitz Tumor, Acral Melanoma, Melanoma arising in Congenital Nevus, Melanoma arising in Blue Nevus, Mucosal Melanoma and Uveal Melanoma) (**Fig. 2**). In cutaneous biopsies, signs of sun damage are denoted by solar elastosis which consists of ribbon-like basophilic fibers produced by injured fibroblasts.

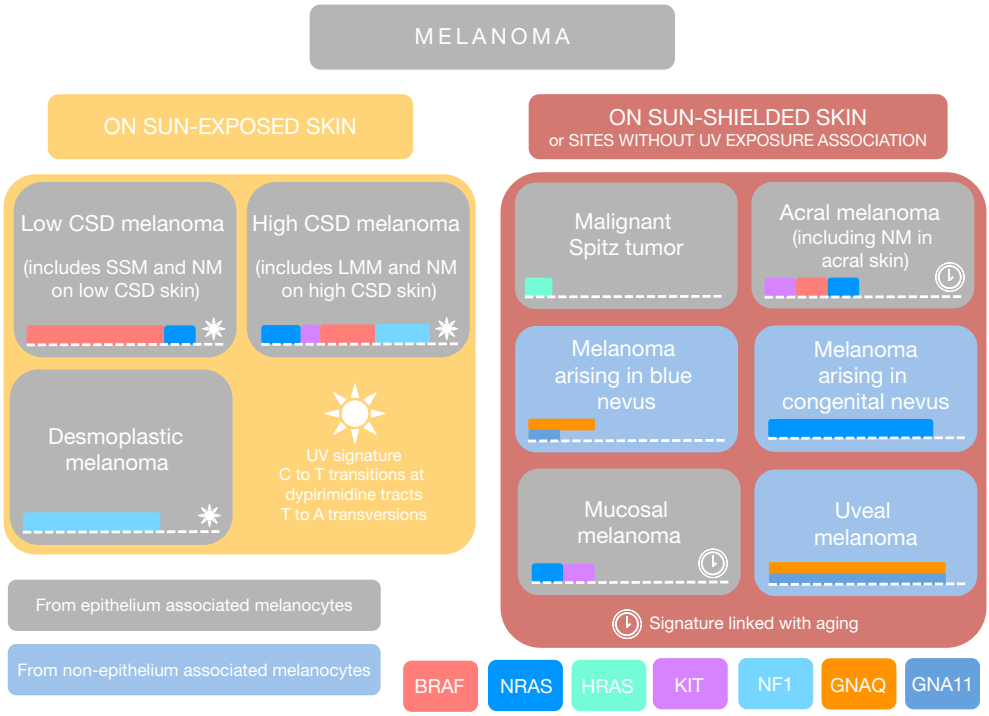

Fig. 2 Classification of melanoma integrating genotype–phenotype correlation. The heterogeneity of melanoma has been postulated for years, until a taxonomy based on molecular pathways emerged.

Such detail is referred to as a measure of cumulative solar damage (CSD); unfortunately, this criterion has limitations in diagnostics of doubtful cases, as most melanomas arise on low-CSD skin that has been intermittently exposed to sun, rather than chronically and severely sun-damaged skin.

Low Cumulative Solar Damage Melanoma

Low-CSD melanoma arises on intermittently sun-exposed skin of trunk and extremities (except palms, soles and nails) in young individuals (third to sixth decade) with a high number of nevi and fewer NMSC. Increased risk is determined by recurrent sunburns in childhood and the use of tanning beds. It is typically a SSM that shows radial growth phase (RGP) and often intense pigmentation, in the absence of marked solar elastosis. Compared to other melanoma mutational pathways, this category is most frequently affected by BRAF mutation (predominantly V600E) in 50%–70% of cases.

Such peculiarity is relevant for the possibility of treating patients showing BRAF-mutated metastatic melanoma with BRAF and MEK inhibitors. Among the primary oncogenic mutations, NRAS is also frequently mutated (15%–20%), while KIT mutation is rarely described (1%). Loss-of-function mutations include CDKN2A, TP53 and PTEN. Chromosomal aberrations are common and involve gains of chromosome 6p, 7 (copy number increases that favor mutant BRAF allele), 8q, 1q, 20q and 17q, and losses at chromosome 9, 10 (driven by PTEN), 6q and 8p. TERT promoter mutations play a role in melanoma pathogenesis via alteration in the regulation of telomere protection. Remarkably, Hayward and colleagues demonstrated that TERT promoter mutations in melanoma determine shorter telomere length. Low-CSD melanoma class includes a subset of nodular melanoma (NM) that might originate as vertical growth phase (VGP) on RGP, the latter being displaced by the rapid nodular growth, and rare variants that recognize similar molecular pathways: Melanoma in BAP1 Inactivated Naevus, Melanoma in Deep Penetrating Nevus, Melanoma in Pigmented Epithelioid Melanocytoma.

High Cumulative Solar Damage Melanoma

High-CSD melanoma develops on chronically sun-exposed skin of the head and neck region, dorsal surface of the forearm, and lower legs in elderly people, and is frequently associated with NMSC. Long-standing occupational solar UV exposure is a risk factor. This category includes LMM and NM in high-CSD skin. In contrast to low-CSD melanoma that can originate from nevus, high-CSD melanoma can develop from melanoma in situ (LM). In up to 30% of cases, loss-of-function mutation of the tumor suppressor NF1 can be found, without, at the present, any therapeutic implication in clinical decision making. It is also characterized by mutually exclusive activating mutations of NRAS (20%–25%), BRAF (more frequently V600K) or KIT (1%–10%). Loss of CDKN2A is typical of the VGP of these tumors, while TERT promoter mutations emerge earlier in the progression, in the intraepithelial lesion.

Desmoplastic Melanoma

Desmoplastic melanoma is a rare tumor that typically arises on the head and neck region. The lesion is characterized by intradermal proliferation of atypical spindled melanocytes that are generally poorly pigmented, often making the diagnosis difficult, and by marked tropism for neural structures, which determines increase of recurrence risk. A lentiginous-pigmented component is often associated. Indeed, desmoplastic melanoma more commonly arises as the VGP of a melanoma in situ LM type on CSD skin. It may also present de novo, or more rarely as the progression of another lentiginous type melanoma that can be acral or mucosal melanoma. Desmoplastic melanoma shows a high mutation load (median of 62 mutations per megabase) that ranks it, like high-CSD melanoma, among the most highly mutated cancers. NRAS and KIT are rarely mutated, while in 55%–93% of cases there are inactivating mutations of NF1, mainly represented by truncating mutation or homozygous deletion. Such genetic (predominantly NF1 mutated) and clinical (primarily head and neck localized) features define the uniqueness of desmoplastic melanoma. In addition, TP53 is mutated in 48% of cases. Gain-of-function mutation of NFKBIE have been identified in 14.5%. Interestingly, as NFKBIE mutated desmoplastic melanomas, NFKBIE mutated nondesmoplastic melanomas show neither BRAF nor NRAS mutations. NFKBIE encodes IκBε, one of the three traditional IκB proteins, whose function is that of inhibiting NF-κB signaling pathway by promoting cytosolic sequestration of NF-κB dimers in unstimulated cells, thus preventing transcription.

Malignant Spitz Tumor

Malignant Spitz Tumor or Spitz melanoma is a cancer morphologically composed of large spindled and/or epithelioid melanocytes. It may be indistinguishable from Atypical Spitz Tumor. CSD is typically absent or minimal. Compared to other melanomas, the Malignant Spitz Tumor tends to be less aggressive and present at a younger age. This melanoma subtype is defined by characteristic genetic alterations that comprise mutation of HRAS on chromosome 11, BRAF mutation with loss of BAP1 and, above all, mutually exclusive tyrosine kinase fusions in ALK, ROS1, RET, NTRK1, NTRK3, BRAF and MET genes that have been found in 39% of cases. The identification of chromosomal translocations with expression of fusion proteins, although having no role in distinguishing Spitz nevus from Spitz melanoma, is relevant to the understanding of the molecular pathogenesis of melanocytic lesions and broadens the treatment of these neoplasms to targeted therapies.

Acral Melanoma

Acral melanoma, once referred to as "acral lentiginous melanoma," is localized on glabrous acral skin, that is, the palms, soles and nail apparatus. It shows a peculiar ethnic distribution, being the most common melanoma subtype among Asian, Hispanic and African populations. Trauma may represent a risk factor. Delayed diagnosis correlates with poor prognosis. Earlier lesions in the RGP typically show lentiginous proliferation that progresses in the VGP, characterized by spindled and desmoplastic atypical melanocytes. Compared to other melanoma subtypes, acral melanoma has a low mutational load, characterized mostly by genomic rearrangements and multiple high level amplifications of CCND1, CDK4, GAB2, RICTOR and TERT loci. KIT mutations occur in 15%–40% of cases, NRAS in 15%–20% and BRAF in 15%. Loss-of-function mutations occur in NF1 and CDKN2A.

Mucosal Melanoma

Mucosal melanomas are rare tumors that arise in mucosal membranes. They can be found in the nasal cavity and sinuses, along the gastrointestinal tract (from the oral cavity to the anorectum), along the genitourinary tract (especially vulvar and vaginal region) or in the tarsal conjunctiva. As they originate on mucosa, the causative role of solar UV exposure is absent. Genomically, they show similarities to acral melanoma. Compared to cutaneous melanomas on sun-exposed skin, mucosal melanoma has a considerably lower mutational load, characterized by the presence of frequent copy number variations and structural changes. BRAF mutation is generally absent. 20%–40% of cases are KIT mutated, 15%–20% NRAS mutated. It is interesting to note that there are mutations that occur more frequently in some mucosal localizations than others, suggesting that mucosal melanomas are a group of distinct molecular lesions: up to 43% of vaginal melanomas are NRAS mutated, 25% of anorectal melanomas are KIT mutated. For this reason, there have been attempts to subdivide melanomas arising on mucosa, but they are rare and, therefore, it is difficult to make statistically significant studies.

Melanoma Arising in Congenital Nevus

Congenital nevi are benign melanocytic proliferations that are already present at birth as they arise in the fetus. They are classified according to their dimension in small (<1.5 cm), medium (1.5–20 cm), large (20–40 cm) and giant (>40 cm). The size corresponds to the largest diameter that can be expected in adulthood (predicted adult size or PAS) because congenital nevi extend in proportion to the child's growth. Risk factor for the development of melanoma is the lesion size: in giant congenital nevus, the risk increases up to 10%–15%. In pre-pubertal patients with large congenital nevus, the risk for the development of melanoma is 1%–2%, which is ten-thousand times more frequent than same-aged general population. Large congenital nevi have a 2%–42% risk of malignant transformation and a 6%–14% lifetime risk of developing melanoma. MCN presents as a nodular growth in the context of congenital nevus, though rapidly growing nodules are more often mimickers of melanoma, including atypical

proliferative nodules, as they have a benign behavior. MCN arises within the cutaneous lesion intradermally or subcutaneously before puberty, at the dermo-epidermal junction in adulthood. Gain-of-function somatic mutation in NRAS at codon 61 with the substitution of glutamine by lysine (Q61K) or by arginine (Q61R) have been found in >90% of large and giant CN, and 70% of small and medium CN. In contrast to large-giant CN, small-medium ones harbor in 30% of cases $BRAF^{V600E}$ mutation.

Melanoma Arising in Blue Nevus

Blue nevus is a frequent dermal melanocytosis. The name derives from the color blue that results from the Tyndall effect. The two most important variants are common blue nevus and cellular blue nevus. The common blue nevus consists of an exclusively dermal proliferation and it most frequently arises on the dorsal surface of the hands, albeit ubiquitous. The cellular blue nevus is a proliferation of spindled melanocytes arranged in bundles involving reticular dermis and hypodermis. MBN usually arises on the cellular blue nevus variant and appears as a dermal proliferation of large atypical spindled and/or epithelioid melanocytes with moderate to severe cytologic atypia and necrosis. MBN has conceptual affinity with uveal melanoma, as both originate from melanocytes that are not intraepithelial, and it is not a coincidence that their genetic mutations extensively overlap. Indeed, melanomas arising on blue nevus show GNAQ and GNA11 gain-of-function mutations (Q209 in Exon 5).

Additional mutations have been described in SF3B1 and BAP1. Chromosomal aberrations, such as gains in chromosome 6p and/or 8q, monosomy of 3, and loss in 1p, are similar to those described in uveal melanoma mutational pathway.

Uveal Melanoma

Uveal melanoma, the most common intraocular tumor in adults, arise from melanocytes in the uvea, the middle vascular layer of the eye consisting of three parts: iris, ciliary body, and choroid. Uveal melanomas are not related to sun exposure, as demonstrated by the absence of UV signature. The mutation load is lower than CSD cutaneous melanoma. In 80%–90% of uveal melanomas, there are GNAQ or GNA11 mutations which are mutually exclusive and happen early in the pathogenesis. Other mutations involving the Gαq pathway include PLCB4 and CYSLTR2. BAP1 mutation, which affects up to 50% of cases, SF3B1 (18%), and EIF1AX occur while the tumor progresses, and correlate with different risks of metastasis. Chromosome 8 and 3 can be studied via FISH for additional information on prognostication, since monosomy of chromosome 3 and isochromosome 8q correlate with poor prognosis with higher risk of liver metastasis.

Prospective Vision

Melanoma research is now moving toward a holistic vision, defining melanoma with specific histopathologic and genetic features in a unique and complex individual, aimed at achieving the most effective treatment and the best outcome. Genotype–phenotype correlation is a key lever in that sense. The efforts in cataloging the genomic architecture of different subtypes of melanoma have led to the study of a distinct set of genetic alterations, even in noncoding regions, and such developments open new frontiers in our understanding of melanoma pathogenesis. High-throughput technologies have revolutionized melanoma research, and will continue to do so, especially in defining the genetic progression of melanoma subtypes from their precursors, as well as characterizing metastatic melanoma polyclonality.

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Further Reading

Bastian, B.C., 2014journal. The molecular pathology of melanoma: An integrated taxonomy of melanocytic neoplasia. Annual Review of Pathology: Mechanisms of Disease 9, 239–271.

Clark Jr., W.H., Ainsworth, A.M., Bernardino, E.A., et al., 1975journal. The developmental biology of primary human malignant melanomas. Seminars in Oncology 2 (2), 83–103.

Clark Jr., W.H., Elder, D.E., Van Horn, M., 1986journal. The biologic forms of malignant melanoma. Human Pathology 17 (5), 443–450.

Curtin, J.A., Fridlyand, J., Kageshita, T., et al., 2005journal. Distinct sets of genetic alterations in melanoma. New England Journal of Medicine 353, 2135–2147.

Girotti, M.R., Gremel, G., Lee, R., et al., 2015journal. Application of sequencing, liquid biopsies, and patient-derived xenografts for personalized medicine in melanoma. Cancer Discovery 6 (3), 286–299.

Hayward, N.K., Wilmott, J.S., Waddell, N., et al., 2017journal. Whole-genome landscapes of major melanoma subtypes. Nature 545 (7653), 175–180.

Hodis, E., Watson, I.R., Kryukov, G.V., et al., 2012journal. A landscape of driver mutations in melanoma. Cell 150 (2), 251–263.

Leachman, S.A., Lucero, O.M., Sampson, J.E., et al., 2017journal. Identification, genetic testing, and management of hereditary melanoma. Cancer Metastasis Reviews 36 (1), 77–90.

Liu, F., Wang, L., Perna, F., Nimer, S.D., 2016journal. Beyond transcription factors: How oncogenic signaling reshapes the epigenetic landscape. Nature Reviews. Cancer 16 (6), 359–372.

Palmieri, G., Ombra, M.N., Colombino, M., et al., 2015journal. Multiple molecular pathways in melanomagenesis: Characterization of therapeutic targets. Frontiers in Oncology 5, 183.
Shain, A.H., Yeh, I., Kovalyshyn, I., et al., 2015journal. The genetic evolution of melanoma from precursor lesions. New England Journal of Medicine 373, 1926–1936.
The Cancer Genome Atlas Network, 2015journal. Genomic classification of cutaneous melanoma. Cell 161 (7), 1681–1696.
Whiteman, D.C., Pavan, W.J., Bastian, B.C., 2011journal. The melanomas: A synthesis of epidemiological, clinical, histopathological, genetic, and biological aspects, supporting distinct subtypes, causal pathways, and cells of origin. Pigment Cell & Melanoma Research 24 (5), 879–897.
Woodman, S.E., Lazar, A.J., Aldape, K.D., Davies, M.A., 2012journal. New strategies in melanoma: Molecular testing in advanced disease. Clinical Cancer Research 18 (5), 1195–1200.
Zhang, T., Dutton-Regester, K., Brown, K.M., Hayward, N.K., 2016journal. The genomic landscape of cutaneous melanoma. Pigment Cell & Melanoma Research 29 (3), 266–283.

Metastatic Signatures—The Tell-Tale Signs of Metastasis

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Introduction

Metastasis is defined as the spread of a disease from one organ, or "primary site", to another non-connected "secondary site". In the context of cancer, metastasis refers to the process of a single cell leaving the original "primary" tumor, traveling through the circulatory or lymphatic system, and invading into a distant organ to create a second tumor or metastasis. For a cancer cell, this is an incredibly challenging multi-step feat that most cannot achieve. Indeed, while millions of cells are shed from a tumor every day, very few clinically detectable metastatic colonies are usually formed. Briefly, the steps of metastasis are as follows:

1. Cells separate from the primary tumor
2. Tumor cells invade through the basal membrane into a blood or lymphatic vessel.
3. Tumor cells intravasate into the circulatory or lymphatic system
4. Tumor cells travel throughout the body as circulating tumor cells (CTCs) until halting at a distant organ, at which point they are referred to as disseminated tumor cells (DTCs)
5. Tumor cells extravasate from the vessels into the secondary organ and re-initiate proliferation

Early Work Defining the Metastatic Process

Metastases have been observed in mummies estimated to be around 2400 years old, and have been described in manuscripts as far back as 200 CE. However, the term metastasis was not coined until 1829 by French surgeon Joseph Récamier. It was taken from the Greek meaning removal, migration, change or revolution. At the time, very little was understood about cancer, but Récamier was considered an expert on breast malignancy. In his book "Sur le traitment de cancer," one of the earliest medical texts on oncology, he describes the suffering of cancer patients of the day and in particular, several breast cancer patients with metastasis to the brain. In his text, Récamier was the 1st to propose that metastasis was due to the translocation of cells by local infiltration at the primary site, invasion of the veins, and distant growth in the brain.

The theory of metastasis continued to take shape through the 1800s with the work of Rudolf Virchow, who in 1858 suggested that metastatic tumor dissemination to secondary sites was determined largely by mechanical factors. More specifically Virchow hypothesized that circulating tumor cells somewhat randomly became arrested at a distant organ because they were stuck in the vasculature. However, in 1889 Stephen Paget proposed a slightly different theory in his Lancet paper "Distribution of secondary growths in cancer of the breast". Paget, who is now referred to as the father of metastatic theory, analyzed 735 fatal cases of breast cancer and argued the distribution of metastases could not be due to chance alone. The patterns he observed did not simply follow blood flow distribution as suggested by Virchow. Instead Paget's theory referred to tumor cells as "seeds", and stated that their distribution throughout the body must be understood by studying the properties of the "soil" of distant organs. Paget drew parallels with botany, stating that seeds can only live and grow if they fall on congenial soil. While this theory is currently widely accepted, it was challenged as recently as 1928 by James Ewing who theorized that location of metastases was primarily determined by the anatomy of the vascular and lymphatic channels that drain the primary tumor.

These theories were ultimately merged and supplemented by several metastasis studies published by Isaiah Fidler. His groundbreaking studies demonstrated that metastasis occurred in a series of sequential steps that involve cancer cells with different metastatic capabilities. Dr. Fidler suggested that different "seeds" interact with their microenvironment, leave the primary tumor, and ultimately either land in a "congenial soil" or perish. Dr. Fidler's work also showed that tumor cells could leave the primary tumor by entering the bloodstream or the lymphatics. This theory of the metastatic process took into account Virchow, Paget and Ewing's work and suggested that successful cancer clones metastasized in a stochastic manner. It also hinted at the notion that while many cancer cells leave the primary site, very few are successful at creating a metastasis, as cells with differing characteristics are selected for or lost along the way. Today's current understanding of the metastatic process is built upon the observations and studies of clinicians and researchers going back centuries, and while considerable strides have been made in the last few decades, metastatic disease remains an incredible challenge to treat.

Current Clinical Observations and Challenges in Treating Metastasis

Metastasis is the primary clinical challenge in the treatment of cancer as it is unpredictable in onset and it exponentially increases the clinical impact to the patient. It is a key element in cancer staging systems such as the tumor/node/metastasis (TNM) staging system, where metastasis places a cancer in Stage IV. The possibilities of curative treatment are greatly reduced, or often entirely removed, when a cancer has metastasized. For example, in breast cancer approximately 20–50% of patients diagnosed at an early stage are expected to develop metastatic disease, and because the treatment of primary tumors is quite good, around 90% of breast cancer deaths in the U.S. are instead due to untreatable metastases. It remains a major challenge to eliminate, identify, or remove metastases, and there are several factors that have led to this disparity in curative treatments between localized and metastatic tumors.

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Metastatic cancer is multifaceted and therefore complex to study and treat. Firstly, not all cancers metastasize, but those that most commonly do - lung, breast, skin (melanoma), colon, kidney, prostate, pancreas, and liver – don't all metastasize to the same distant organs. For example, breast cancer usually spreads to the brain, bone, or lungs, yet colorectal cancer will primarily spread to the liver. Furthermore, the sensitivity of clinical imaging techniques cannot always expose small metastatic lesions. Another key characteristic that confounds treatment is the drug resistance often observed in metastatic lesions. The current treatments available target the biology of the primary tumor, for which ample patient tissue is available for study. Metastatic tissue is often not resected from patients and therefore not widely available to study; because of this the current therapies are often unable to eliminate the metastatic cells which can be significantly altered during the metastatic process.

Finally, another major challenge in the treatment of metastatic cancer is cancer cell dormancy. Once shed from the primary tumor, dormant cancer cells cease dividing but survive in a quiescent state, often in the bone or other organs, waiting for appropriate environmental conditions to trigger proliferation again. This period of quiescence can last from months to many years. Dormancy creates a large hurdle for treatment as individual dormant cells cannot be detected in the clinic and most therapies target proliferating cells. Furthermore, the creation of drugs to target cells in this state is incredibly difficult as the outcome of effectively targeting dormant cells may not be measurable for upwards of 10 years and may only be recognized as the absence of further metastasis.

Ultimately, in order to improve patient outcomes, clinicians must be able to identify the spread of cancer or a patient's potential for metastasis early in their disease. The identification and use of such "metastatic signatures" will be described in this chapter.

Tumor-Intrinsic Signatures of Metastasis

Gene Expression Profiling of the Primary Tumor

Molecular profiling of tumors following patient surgery has enabled oncologists to categorize tumor types beyond the organ of origin to several subtypes, ultimately guiding physicians' prognoses and treatments. A major factor in this subcategorization has been the assessment of somatic gene mutations, amplifications, and tumor-specific gene expression patterns. Interestingly, epidemiological studies combining the genetic analysis of patient tumors with patient outcomes has also revealed which subtypes, and which genetic alterations in the primary tumor, are likely to result in metastatic disease.

The categorization of breast cancers into subtypes based on gene expression is a prime example of using primary tumor characteristics to predict patient outcomes. Breast cancer subtypes have been extensively categorized by the expression levels of the estrogen and progesterone hormone receptors, and cell surface marker HER2. The 4 major subtypes of breast cancer are Luminal A which expresses both hormone receptors but is negative for HER2, Luminal B which expresses the hormone receptors and either HER2 or Ki-67, Triple negative/Basal-like which is hormone-receptor negative and HER2 negative, and finally HER2-enriched tumors which are hormone-receptor negative but HER2 positive. Analysis published by the American Society of Clinical Oncology has revealed that the Luminal A type has the lowest rate of metastasis, while Luminal B HER2 positive has the highest. When metastasis does occur, subtypes can also be used to predict patterns of metastatic spread. For example, high rates of brain metastases are seen among HER2-enriched, basal-like, and Triple negative types, whereas brain metastases are less frequently seen in the luminal/HER2 groups. Interestingly, bone is the predominant site of metastasis for the luminal/HER2 groups but the least common site for the basal subtype.

In addition to using the expression of specific markers, primary tumor metastatic propensity can be evaluated by different gene expression programs. With recent advances in technology, it has become possible to compare gene expression patterns between primary tumors and metastases. Over the last decade microarray and RNA sequencing studies of patient tissue, mouse metastasis models, and cell lines have informed numerous biomarker panels that can be used to predict metastasis and patient outcome. Several cancer gene signature tests are used clinically to guide personalized treatment decisions for breast, colon, or prostate cancer. Such panels can typically assess expression of 10–70 genes that span cellular pathways critical for cell adhesion, migration, cell-cell signaling, differentiation, and metabolism. As well as creating robust biomarker panels that can direct treatment for individual cancer types, there is also an ongoing effort to create multi-cancer gene expression biomarker panels for cancer metastasis.

Many studies have demonstrated that microRNAs, and the altered expression of specific microRNAs, may play essential roles as either promoters or suppressors of metastatic progression. By altering the expression of networks of genes, microRNAs can be powerful determinants of pathway activation and cellular phenotype. For example, by down regulating a specific network of target transcripts, miR-155 promotes the dissolution of tight junctions via TGFβ-induced cell plasticity and RhoA suppression, ultimately increasing cell migration and metastasis. In addition, sustained expression of miR-155 through positive feed-back of TGF-β signaling has been associated with dysregulation of cell polarity in solid tumors. Conversely, miR-29b has previously been reported as a metastasis suppressor. miR-29b targets transcripts encoding prometastatic effector molecules involved not only in angiogenesis and collagen remodeling such as VEGFA, ANGPTL4, PDGF, and MMP9, but also in differentiation and plasticity such as ITGA6, ITGB1, and TGF-b. miR-29b is enriched in luminal breast cancers and loss of miR-29b increases metastasis and promotes a mesenchymal phenotype. As well as contributing to each tumor cell's metastatic capacity, microRNAs can also function to induce tumor cell non-autonomous alterations. In section "**Systemic Signatures of Metastasis**" of this chapter we will discuss, microRNA involvement in the preparation of the distant pre-metastatic niche.

Somatic Mutations of the Primary Tumor

Several high-quality genomic studies have explored the genetic relationship between primary and metastatic lesions in order to understand metastatic evolution. It is now well accepted that metastases arise from a clone of unique cells within the primary tumor. DNA copy number analysis of metastatic prostate cancers has indicated a common clonal origin in most cases, although sub-clonal alterations have also been observed in metastases. Whole-genome sequences of a basal-like primary breast tumor and corresponding brain metastases have consistently shown that while copy number alterations and overall mutational spectra within the genome are not significantly different, specific mutations revealed a subset of cancer cells from the primary tumor were preferentially enriched in metastatic lesions. Genomic sequencing analyses of pancreatic cancer lesions also suggested that metastatic lesions are clonal in nature, but also likely require additional driver mutations that are not found in the primary tumor.

Phylogenetic analysis of metastatic progression in breast cancer using somatic mutations and copy number aberrations (CNAs) has shed yet further light on the timing of the evolution of metastases. In agreement with earlier studies, descent of metastases from a common origin was observed in patients diagnosed with early stage breast cancer, however multiple seeding events from the primary tumor were observed in patients diagnosed with advanced stage disease. Interestingly, the number of late single nucleotide variants and CNAs increased as distant metastases evolved and can therefore give an indication of the time elapsed since they diverged from their common ancestor. Not surprisingly, there is a correlation between overall patient survival and the phylogenetic branch lengths of the metastases. This phylogenetic analysis ultimately revealed that metastases from patients with longer cancer histories are genetically more distant from their primary tumor tissue of origin than those of patients with a shorter cancer history.

Despite a large number of studies into the evolution of metastatic tumor cells, no specific driving mutations of metastasis have been identified. However, there are several tumor progression-promoting genetic alterations that often result in more aggressive and metastatic disease. In the case of metastatic colorectal cancer, associations have been made between mutational activation of the KRAS, BRAF, PIK3CA, and NRAS oncogenes and metastatic patterns in patients. The presence of a KRAS mutation is associated with decreased liver metastases and increased lung, brain, and bone metastases. BRAF mutation, a poor prognostic factor, is associated with decreased liver-limited metastasis and increased peritoneal and distant lymph node metastases. Conversely, PIK3CA and NRAS mutations do not clearly affect outcomes in the metastatic setting, although PIK3CA is associated with concurrent KRAS mutations. Our expanding understanding of how somatic mutations correlate with metastatic patterns contributes to the clinical care of patients by focusing treatment and guiding surveillance strategies for metastasis.

Inherited Predisposition to Metastasis

The inherited basis for many cancers has been thoroughly studied. For example, inherited predisposition to breast and ovarian cancer with a BRCA1 mutation is commonly assessed in the clinic. However, the inherent diversity in the host genetic background has also been shown to influence metastatic risk. In 1998, a pioneering study showed that an identical oncogenic event in the mammary tissue of mice with different genetic backgrounds led to the development of mammary tumors with similar primary tumor properties but with distinct propensity for metastasis. Linkage analysis has further mapped several genomic loci that modify metastatic potential in mice. With recent advances in global transcript analysis, several studies have now demonstrated the influence of germline polymorphisms and gene networks on the susceptibility to metastatic disease.

Inherited predisposition to metastasis often impacts metastasis in a manner specific to cancer type and subtype. However, recent work suggests that inherited variants can function to modify metastasis by tumor cell-autonomous and non-autonomous mechanisms. Consider that while a patient may have inherent specific expression of genes that benefit tumor cell proliferation or survival, they may also harbor variants in immune surveillance or metabolism pathways that alter the tumor cell's road to the secondary site. In a series of genetics studies in mice, several inherited tumor-intrinsic factors have been identified as metastasis modifiers. One such factor is human circadian rhythm gene Arntl2, for which polymorphisms associated with its expression correlate with metastatic prognosis of ER- breast cancer patients by a tumor intrinsic mechanism. Ultimately, future and ongoing studies will extend this area of research to human patients with the application of genome-wide association study (GWAS) methods. The goal of such studies is to enable early screening of patients for inherited metastatic modifying genes, and develop targeted therapies for specific groups.

Epigenetic Signatures of Metastasis

The study of genetic lesions alone has been unable to explain the complexity of the aberrations that arise in a cancer cell. It is now recognized that cancer is both a genetic and an epigenetic disease. Epigenetics is defined as the inheritance of changes in gene activity that are independent of DNA sequence. The two main epigenetic events involved in gene regulation, development, and carcinogenesis are histone modifications and DNA methylation. In cancer cells, the fine control of epigenetic mechanisms is lost and the disruption of epigenetic patterns promotes the expression of the tumoral phenotype. The alteration of epigenetic marks on the genome is a key tool in successful metastasis, as these marks are reversible and allow the cell to adapt as it travels to the metastatic site. A noteworthy example of such a phenomenon is the *E*-Cadherin gene, which is silenced in some human cancers due to DNA hypermethylation. It has been shown that in some primary tumors displaying E-cadherin hypermethylation, the corresponding metastases are unmethylated at the E-cadherin gene and regain gene expression at this locus. Demethylation of E-cadherin and re-expression are thought to be indispensable for the incorporation of metastatic cells into their new cellular environment.

Recent work in metastatic pancreatic cancer has provided tremendous insight into the epigenetic changes that arise in metastases that seed different sites. Using matched primary and metastatic samples, researchers revealed differences in epigenetic reprogramming within subclones of the primary tumor that seeded local (peritoneal) and distant (liver and lung) metastases. Differences in the epigenetic reprogramming between local and distant metastatic lesions were also observed. Interestingly, no significant changes were seen in the tumors of patients whose cancer spread only locally. However, tumors from patients with distant metastases showed substantial epigenetic changes both in the distant metastases and in the subclone of the primary tumors from which they arose. The genes impacted by these epigenetic alterations in lung metastases were associated with oxidoreductase activity as well as DNA damage, rendering corresponding cell cultures resistant to oxidative stress and chemotherapy. Also, genes encoding epithelial and mesenchymal identity were reciprocally expressed between the local and distant metastases, such that peritoneal metastases were epithelial and lung metastases were poorly differentiated. This work suggests that epigenetic regulation by metastatic cells is paramount to reaching and growing secondary tumors in specific organs. It also highlights the complexity of metastatic cancers and the multifaceted treatment strategy that must be implemented to eradicate it.

Metabolic Changes in Metastatic Cells

It has long been appreciated that metabolism is altered in cancer cells. In 1955, Otto Warburg presented his work showing the nuanced manner in which cancer cells utilize glucose and oxygen. Since this discovery, metabolism in cancer has been thoroughly studied and the conditions to which tumor cells can adapt and thrive by metabolic modifications is wide and varied. In recent years, further specialization of metabolic changes has been observed during tumor cell dissemination into the circulation and establishment of metastatic lesions.

The dissemination of malignant cells is an integral characteristic of advanced stages in transformation. While contributions of peroxide signaling to metastasis may be context-dependent, elevated levels have been found to mediate metabolic changes that facilitate anchorage-independent survival and consequent metastatic spread. The mitochondria generate reactive oxygen species (ROS), which are required for KRAS-induced anchorage-independent growth through regulation of the ERK/MAPK signaling pathway, resulting in the transduction of a survival signal. The peroxide signal underlying anchorage-independent expansion has now been tied to ATP generation, which is increased to satisfy energy requirements for cells that lose contact with the substratum. The elevation of peroxide levels must be tightly regulated however, as intracellular ROS can be limiting to cell survival. This is just one example of the specific metabolic changes wielded by tumor cells to aid in the process of dissemination from the primary tumor.

Interestingly it has also been revealed that the tropism of metastatic cells may in part be dependent on their ability to adapt to the metabolic environment of the secondary organ. For example, the liver is largely hypoxic with uneven glucose levels due to competing hepatocytes. Both glucose and oxygen are key substrates in both glycolytic and oxidative metabolism and therefore metastatic cells in the liver are forced to adapt. It has been observed in patients with colon cancer that metastatic cells are able to escape this potential metabolic stress by capitalizing on the higher levels of creatine in the liver, which is synthesized and secreted by hepatocytes. Through the modulation of miRNAs these cells are able to upregulate and secrete Creatine Kinase B (CKB) into the extracellular space. Once secreted, CKB catalyzes phosphocreatine from ATP and creatine. Phosphocreatine can then be imported back into the tumor cells through specific upregulated transporters, and used to regenerate ATP. Through this mechanism the cells bypass glycolytic and oxidative metabolism pathways and essentially scavenge energy from the secondary organ's extracellular environment. Pharmacological inhibition of CKB has been shown to substantially decrease the level of liver metastasis in mouse models and may be a useful therapeutic strategy in the clinic.

Signatures of Metastasis in the Tumor Microenvironment

Tumor Vasculature

Not only is tumor vasculature important for tumor growth, it is also key in tumor cell dissemination in the initial steps of metastasis. It is well established that tumor vasculature is distinct to that of healthy tissue, and these distinctive qualities can influence the spread of tumor cells into the circulation. Normal vasculature is maintained through a careful balance of pro- and anti-angiogenic factors and is composed of a hierarchical distribution of arterioles, capillaries, and venules. In contrast, tumor vasculature lacks this hierarchical structure and is mainly composed of immature differentiated and undifferentiated vessels with high permeability. Undifferentiated tumor vessels frequently present with either collapsed or an absent lumen, and consequently tumor vasculature is inefficient in carrying blood flow. Due to this phenomenon, and despite increased intra-tumoral microvessel density, the tumor microenvironment is often hypoxic when compared to healthy tissue. This hypoxia can promote the generation of new vessels and also induce metastasis. Tumor blood vessels can form by the common processes of neovascularization, but often also through alternative mechanisms. "Vascular mimicry" or VM is one such alternate process and involves the creation of tumor cell-lined channels for fluid transport independent of typical endothelial cell-driven angiogenesis. It is this process of VM that has most recently become the focus of attention as a signature of metastatic cancer.

The initial morphological, clinical, and molecular characterization of VM was performed using human melanoma as a model. These tumor cells were shown to express endothelial, embryonic/stem cell, and tumor markers; they were also shown to form channels, networks, and tubular structures rich in laminin, collagens, and heparin sulfate proteoglycans and contain plasma as well as

red blood cells. Since these initial characterizations in the late 1990s VMs have been hypothesized to function as "escape routes" for metastatic cells, and indeed several studies have demonstrated that VM is implicated in poor patient clinical prognosis. In a 2015 study, Wagenblast and colleagues showed VM can indeed pave a way for breast cancer metastasis. Using a polyclonal mouse model of breast tumor heterogeneity and a systems genetics approach, they identified discrete clones within the primary tumor that displayed phenotypic specializations and their corresponding gene expression profiles. Clones that efficiently entered the vasculature expressed two secreted proteins, Serpine2 and Slpi, which are necessary and sufficient in programing tumor cells for vascular mimicry. This study suggests that vascular mimicry drives the ability of breast tumor cells to contribute to distant metastases. Furthermore, this study also revealed that in the clinic SERPINE2 and SLPI are overexpressed preferentially in patients that have lung-metastatic relapse. Thus, these two secreted proteins, and the VM phenotype they promote, may be a relevant signature of metastatic progression in human cancer.

Tumor-Associated Immune Cells

A substantial amount of clinical data has indicated that tumor infiltration of certain immune cell types correlates with poor prognosis of cancer patients. Inflammation in the tumor microenvironment can contribute to the initiation, promotion, and progression of malignant disease by fostering a nurturing environment for tumor progression and metastasis. Specifically, chronic inflammation modifies the transformed tissue by providing an abundant source of growth factors, cytokines, chemokines, prostaglandins, and reactive oxygen species, which promote angiogenesis and metastasis. As a result, the tumor microenvironment is frequently rich with immune cells including innate immune cells, such as mast cells, tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs), as well as adaptive immune cells including T and B lymphocytes and dendritic cells that can have tumor and metastasis-promoting properties. Accumulating evidence has indicated that myeloid cells, such as TAMs and MDSCs, contribute to early steps of metastasis by promoting tumor cell migration and invasion into blood vessels.

Macrophages are considered to be highly plastic cells and have distinct functions in response to environmental signals. Accumulating data suggest that the tumor microenvironment polarizes recruited macrophages from a potentially tumor-reactive state to a tumor-promoting state. These 'tumor-educated' macrophages influence the early steps of the metastatic cascade by promoting tumor cell invasion of the surrounding tissue and intravasation into the circulation. Research has shown that in breast cancer mouse models the ablation of macrophages inhibits tumor angiogenesis, suggesting that TAMs promote metastasis by increasing the density of leaky blood vessels that provide pro-tumorigenic and pro-invasive factors, as well as a route of escape for tumor cells. TAMs have also been found to secrete factors such as epidermal growth factor (EGF), which activate tumor cell receptors and enhance invasion and motility by increasing invadopodium formation and matrix degradation. Along with this, TAMs also secrete many cytokines that recruit tumor cells to the blood vessels and remodel the extracellular matrix. Interestingly, intravital imaging has also revealed that perivascular TAMs aid in tumor cell invasion of surrounding tissues by directly associating with tumor cells as they intravasate. In the clinic, breast cancer patients are given a 'tumor microenvironment for metastasis' (TMEM) score, which is an indication of the number of TAM, endothelial cell, and tumor cell interactions observed within a tumor. A high TMEM score, and thus high number of interactions, is a signature associated with an increased risk of metastasis.

Tissue Rigidity and Metastasis

Mechanical properties of the extracellular matrix (ECM) have become increasingly recognized as functionally important during tumor progression. In particular, tissue rigidity has emerged as an important factor during metastasis. Briefly, recent work shows that mesenchymal-like tumor cells enhance ECM remodeling through deposition of additional ECM molecules, which alters the local ECM, increases the pool of available growth factors, and potentiates the mesenchymal phenotype. Tumor cells have also been shown to hijack the normal functions of stromal cells to facilitate ECM remodeling. Take for example cancer-associated fibroblasts, which can drive matrix stiffening through collagen crosslinking. Force mapping of human and mouse breast tumors revealed invasive tumors have heterogeneous mechanical properties. More specifically, when compared to the tumor core, the periphery is relatively stiffer which is consistent with the observation that cells from the invasive front of tumors are prone to be more mesenchymal before dissemination. While research continues into this phenomenon, such a finding does suggest that local mechanical properties of a niche may control the movement of tumor cells. Indeed, studies in 3D culture systems and mouse tumor models have shown that increased tissue rigidity has a functional role in driving tumor invasion and malignancy. More notably, in breast cancer patients, increased tissue rigidity due to dense clusters of collagen fibers and fibroblast at the tumor site correlates with metastatic recurrence and poor patient survival.

In 2011, Conklin et al. performed a thorough study of the relationship between the long-term survival rate of human patients and the tumor-associated collagen signature 3 (TACS-3). TACS-3 is defined as bundles of straightened and aligned collagen fibers that are oriented perpendicular to the tumor boundary which specifically increase matrix stiffness through increased collagen deposition and matrix alignment. In this study, the presence of TACS-3 was evaluated in tissue biopsies from 196 breast cancer patients. Remarkably, the presence and extent of TACS-3 observed in primary tumor samples was confirmed as a prognostic indicator regardless of tumor grade or subtype. In this study, even the presence of small TACS-3 sites, and therefore only niche regions of stiffness, carried an increased risk of long-term relapse. This study produced strong statistical evidence for poor survival in patients with TACS-3, and showed that routine TACS assessment can be performed with clinical histopathological samples. Ultimately, while much is

left to ascertain about the role of ECM and tissue stiffness in metastasis, it is clear that TACS-3 is a strong biomarker for the prediction for breast cancer survival.

Systemic Signatures of Metastasis

Tumor-Secreted Factors

Cancer is able to grow and spread by implementing unique survival and proliferative pathways. This subsequently results in the production and release of specific metabolic, hormonal, and exosomal signatures. With recent advances in blood and liquid biopsies, many different tumor-secreted factors have been identified within the peripheral blood of cancer patients. By using liquid biopsy, many of the unique signatures produced by tumors can now be observed serially – a current limitation – and without invasive tissue biopsies. While tumors produce specific factors distinct from other organs, they also have signatures unique to their type and even molecular subtype. Not surprisingly, tumors undergoing metastatic spread have also been shown to produce measurable factors indicative of unique metastatic characteristics such as preferred metastatic site or "organotropism", and therapeutic sensitivity or response. The specific ways in which tumor-secreted factors are being used as signatures of metastatic spread are discussed in the following sections.

Hormones and metabolites

For some cancer types, the use of circulating metabolites and hormones as biomarkers for diagnosis has become common in the clinic. One example of a cancer prognosis marker is Prostate-specific antigen (PSA), for which serum levels are used to screen for prostate cancer in older male patients. Another serum test in early clinical use is a three protein signature indicative of glioblastoma for which the levels of CRP, LYAM1, and BHE40 are assessed. Recent work has also revealed that some metastatic tumors can be identified by changes in circulating metabolites and hormones. Currently this is a young field, however with the increasing use of liquid biopsy in the clinic, the number of metabolite and hormone-based serum screens for metastasis will likely continue to grow.

In several cancer types, including breast, lung, and prostate cancers, Parathyroid hormone-related protein (PTHrP) has been identified as a bone metastasis-specific serum biomarker. In normal physiology PTHrP is widely expressed in embryos and is critical to organ development. PTHrP is also critical during lactation, where it is secreted from the mammary tissue into the circulation to mobilize skeletal calcium for milk production. PTHrP also contributes to the pathophysiology of several cancers, and its production by disseminated tumor cells in the bone microenvironment has been shown to promote osteoclastic activity and contribute to osteolytic bone metastases. PTHrP appears to also play a central role in cancer-associated hypercalcemia. Thus the serum levels of PTHrP and calcium are together very important biomarkers for bone metastasis of several cancers. As well as a biomarker, PTHrP inhibitors have attractive prospects for clinical treatment. Two small nucleotide analogs have been reported to inhibit production of PTHrP by tumor cells and reduce bone lesions with higher survival rates in animal models, but are not yet in use for human patients.

Cell-free nucleic acids

Due to advances in nucleotide sequencing, along with a better understanding of tumor and metastasis mutational signatures, the assessment of circulating DNA and RNA has recently become an exciting new tool for the diagnosis of cancer and metastasis. Circulating, cell-free DNA (cfDNA) are fragments of DNA found in the blood. cfDNA was first described in 1948, but cfDNA fragments originating from tumor cells (ctDNA) were not characterized until the 1980s. The origin of ctDNA has not been well defined yet, but is thought to result from cell death. Nucleosomes play essential roles in the fragmentation of DNA during programmed cell death and genome-wide nucleosome mapping has shown that ctDNA fragments can be distinguished from non-tumor derived cfDNA by distinct patterns of nucleosome spacing.

ctDNA as a prognostic biomarker is currently used for several different types of cancers, including melanoma, colorectal cancer, cervical cancer, and pancreatic cancer. Through the assessment of ctDNA, somatic oncogenic Ras, p53 and other metastasis-related gene mutations, plus epigenetic signatures can be detected. As well as providing insight into metastasis promoting genetic changes occurring in tumors, the relative abundance of ctDNA can also be used as a prognostic marker. It was recently shown that ctDNA is found at a relatively high concentration in the peripheral circulation in patients with metastatic cancer compared with localized disease: ctDNA detection in patients with no radiographic evidence of metastasis varies between 49 and 78%, compared with 86–100% in metastatic disease.

Circulating RNAs (cf-RNA) in human cancer patients were first described in the 1990s in patients with different types of cancers. As mRNA is critical in intracellular protein translation, extracellular mRNAs provide a rare glimpse into the status of the intracellular processes. Non-coding RNAs which largely function to regulate gene expression also make up a large percentage of cf-RNAs. While the majority of research to date has focused on circulating microRNAs and long non-coding RNAs, the number of studies of other RNA classes has recently grown. Interestingly, despite the unstable nature of RNA and the presence of RNA degrading factors in the blood, cf-RNA is stable enough for use as a biomarker. This is likely because tumor derived cf-RNA is often released in vesicles from tumor and apoptotic cells.

cf-RNA is used as a biomarker in several ways. In serum obtained pre-surgically from patients with early stage colorectal cancers, a panel of 6 circulating miRNAs can predict cancer recurrence. Similarly, changes in cf-miRNA patterns within the same patients can be monitored over time to assess response to therapy. In addition, several studies have indicated the usefulness of cf-RNA as a marker of host response to treatment. In terms of metastatic cancer, cf-RNA is currently evaluated to reflect the gene expression within the

primary tumor, and as such its use in the prognosis of metastatic disease is limited by the current understanding of cancer spread. Essentially, cf-RNA is screened for distinct primary tumor gene expression signatures indicative of metastatic spread. As the use and study of cf-RNA increases, it is likely that the nuances of metastatic disease will be further revealed in circulating nucleotides, increasing their use in the clinic for metastatic prognosis.

Tumor-secreted exosomes

Exosomes are spherical or cup-shaped nanovesicles 40–100 nm in diameter that are secreted by many cell types and can be found in most body fluids such as urine and blood. They are end-products of the recycling endosomal pathway which involves endocytosis, formation of early and late endosomes, multivesicular bodies, and secretion with the help of specific cellular pathways. Exosomes are distinct from other secreted lipid entities such as microvesicles and ectosomes, which are microvesicles derived from neutrophils or monocytes and apoptotic bodies. The basic makeup of exosomes consists of a lipid bilayer containing transmembrane and non-membrane bound proteins and nucleic acids. Each of these components give insight into the state of the cell of origin, and, not surprisingly, exosomes released from tumor cells have been found to contain proteins involved in cancer pathogenesis such as mutant KRAS. Interestingly, exosomes can transfer their constituents and cargo to neighboring or distant cells, and it is in this way that tumor exosomes are thought to aid in tumor cell invasion of local stroma, immune cell suppression, and preparation of the metastatic site for colonization.

As a signature of metastasis, exosomes can be evaluated to determine organotropism of tumor cells. In a seminal study published in 2015, David Lyden's group showed that exosomes and their contents play a large role in determining the secondary site of metastasis. In their study, exosomes from lung-, liver- and brain-tropic tumor cells were shown to fuse preferentially with resident fibroblasts and epithelial cells at their predicted destinations. In addition, by taking up tumor-derived exosomes, the organ-specific cells were altered to create a pre-metastatic niche (section "**The Premetastatic Niche**"). This was revealed when treatment of mice with exosomes from lung-tropic models redirected the metastasis of bone-tropic tumor cells to the lung. Assessment of the protein signatures within exosomes from different organotropic tumors revealed distinct integrin expression patterns. Specifically, exosomal integrins $\alpha6\beta4$ and $\alpha6\beta1$ were associated with lung metastasis, while exosomal integrin $\alpha v\beta5$ was linked to liver metastasis. Ultimately this study reveals a new tool for predicting and monitoring the site of cancer recurrence, as exosomal integrin signatures can be used to predict organ-specific metastasis.

Circulating Tumor Cells

The hypothesis that circulating tumor cells (CTCs) are an essential prerequisite to metastasis was first proposed by the Australian pathologist Thomas Ashworth in the mid-19th century. Today, the identification and molecular characterization of CTCs in cancer patients remains key to further understanding the metastatic process and developing new therapeutic targets for treatment. Fortunately, recent advances in technology have provided a means to reliably identify and isolate tumor cells in the peripheral blood stream of cancer patients, opening up new avenues of research into their biological significance.

Release of CTCs into the circulation is frequently termed shedding. A study using a single rat model reported that CTCs are shed from solid tumors at a daily rate of 3.2 to 4.1×10^6 per gram of tissue. However, this cell loss via blood comprised about 10% of the tumor weight and resulted in a CTC count of approximately 20,000 CTCs/mL blood, a rate much higher than that observed in human cancer patients. Clinical data published in 2016 suggests a much lower rate of tumor cell shedding in humans at around 3–5 CTCs/7.5 mL blood for breast, prostate cancer, and colon cancer patients. To date, the majority of clinical studies have focused on CTC enumeration in guiding prognosis in metastatic cancer patients. This work aims to use CTC number as a signature to discriminate which patients are more likely to benefit from adjuvant treatment, as well as a method to monitor the efficacy of therapy.

CTC number surveillance, as a marker of metastatic potential, is also predicted to detect relapse at a much earlier stage than current clinical or radiological tests. Pioneering studies in breast, prostate, and colorectal cancer have shown that higher CTC number predicts for worse prognosis, and a change in number following initiation of therapy can be predictive of survival outcome. Essentially, the characterization of CTCs from a 'simple' blood test could serve as a non-invasive 'real-time tumor biopsy' permitting an up-to-date snapshot of a patient's tumor biology. In this case, molecular characterization of CTCs will also be essential for understanding the status of treatment.

The characterization of CTCs on a molecular level is also useful is determining the course of treatment for recurrent and metastatic disease. For example, HER2 is a targetable factor in breast cancer and the expression of HER2 has been detected in CTCs of metastatic breast cancer patients in cases where primary tumors were negative at the original diagnosis. Subsequent clinical studies have shown that despite a HER2-negative status of the original tumor biopsy, patients with HER2-positive CTCs do benefit if treated with HER2-specific therapy to target CTCs and consequent metastatic lesions. Similarly, acquired genetic mutations can be detected in CTCs, and as next generation sequencing and whole cell proteomics become more accessible, the signatures associated with metastasis will likely become more obvious. The recent advances in isolating and evaluating CTCs are exciting and have greatly accelerated progress in understanding, treating, and monitoring metastasis.

Disseminated Tumor Cells

As described in the previous section, tumors are able to shed cells into the circulation in large numbers. However, simply entering the blood stream alone is not enough to initiate metastasis. A fraction of CTCs are also capable of entering distant sites and

persisting as DTCs. Interestingly, research has revealed that the presence of disseminated tumor cells can be observed before the detection of a primary tumor, suggesting that shedding and colonization of distant tissues can occur at very early stages of the disease. DTCs are currently of great interest to the metastasis research community, as they can remain dormant for many years before initiating growth of a metastasis. However, the exact "trigger" that pushes DTCs from dormancy to metastatic outgrowth is still unclear.

The use of DTC characteristics as a prognostic indicator, or even a sign of likely metastasis, is also in its infancy for several reasons. Firstly, the study of DTCs is difficult because their isolation in the clinic invariably requires invasive procedures. Current strategies are limited to isolating DTCs from tissue that is convenient to collect and lacks common epithelial tumor markers, such as lymph nodes or bone marrow. Markers such as cytokeratins and proliferation-associated Ki67 are then used to detect epithelial cells foreign to that tissue. As new markers are identified and verified, they are increasingly being applied in a multi-marker fashion. Detection of DTCs in lymph nodes and bone marrow is primarily used before and after therapy, however their use as an indicator for relapse remains weak. Despite DTCs predicting adverse outcomes, the majority of DTC-positive patients do not develop metastases. This suggests that the ability to take up residence in bone marrow or lymph node is not sufficient to initiate the growth of a metastatic lesion. The presence of DTCs in these tissues also does not indicate which other organs may also be occupied. Rather, it is thought that a change in the microenvironment of these cells must also occur to initiate the switch from dormant to proliferative state. Furthermore, as well as the technical caveats in analyzing DTCs, the timing of dissemination remains a confounding factor in their use as biomarkers for relapse or sensitivity to therapy. Regardless, the study of such cells with more refined detection, isolation, and characterization technologies has the potential to make DTCs clinically useful in metastatic disease management and also as therapeutic targets.

The Premetastatic Niche

As described in section "**Current Clinical Observations and Challenges in Treating Metastasis**" of this chapter, the "seed and soil" hypothesis of metastasis was proposed by Paget in the late 1800s. Modern research continues to expand on this concept, with a rapidly growing emphasis on the "soil". Today, the "soil" of metastasis describes not only the distant organ but also a suitable microenvironment for metastatic growth, often referred to as the premetastatic niche. The microenvironment within distant organs is critical in the initiation of metastatic growth, as the dissemination of tumor cells alone is not sufficient for metastasis (section "**Disseminated Tumor Cells**"). First described by Kaplan and colleagues in 2005, the premetastatic niche is conceived to form before tumor cells arrive in the tissue. It is populated with bone marrow-derived cells (BMDCs) that promote the chemoattraction and attachment of disseminated cancer cells, as well as numerous other cell types that together impact cancer cell extravasation, survival, colonization, and aggressive growth. Revolutionary work by the Lyden group has revealed that tumor-derived exosomes, which are described in section "**Tumor-secreted exosomes**", initiate the formation of the premetastatic niche by reprograming stromal cells and determining organ specificity. Further work has found that a premetastatic niche can also be further enhanced in a tumor-independent manner by tissue damage or systemic physiological changes such as pregnancy. The unique features of the premetastatic niche can change surrounding tissue remarkably, revealing several signatures of possible metastatic development.

There are various key alterations that have been shown to prime tissue for the growth of metastatic lesions. One of the first observed phenomena is the recruitment of BMDCs to the premetastatic niche by a myriad of tumor secreted factors that can both mobilize the BMDCs from the bone marrow and attract them to specific sites. One example is primary tumor STAT3 signaling which induces secretion of factors such as IL6 and IL10 from tumor cells. This secretion results in widespread STAT3 activation in distant organs. In premetastatic lungs, STAT3 activation stimulates fibroblasts to produce fibronectin (FN) and induces the clustering of a specific BMDC type, the myeloid-derived suppressor cells (MDSCs), which together create a favorable site for metastatic growth. Interestingly, MDSCs are not the only immune cell found in the premetastatic niche, however there is widespread heterogeneity among research model systems. Neutrophils, macrophages, T-Cells, and MDSCs are all reported to infiltrate premetastatic tissues, and contribute to the favorable conditions for growth.

As well as the recruitment of several foreign cell types, the premetastatic niche is also prepared by the reprogramming of resident stromal cells. In bone tissue, senescence is induced in osteoblasts which results in their elevated secretion of IL6 and other factors that promote NFATc1-driven osteoclastogenesis. Osteoclasts function to remodel bone, a process that creates osteolytic lesions and provides a suitable environment for metastatic growth. Yet another example can be found in the premetastatic lung. Normal lung fibroblasts express the miR-30 family of microRNAs to limit MMPs and stabilize vasculature. Cancer cells, however, secrete exosomes whose contents can reprogram miR-30 family expression in lung fibroblasts, resulting in elevated MMPs and ultimately increased vascular permeability and metastasis.

Vascular permeability is key to the establishment of metastasis (section "**Tumor Vasculature**") and is targeted by exosomes for tumor-derived adaptation in preparation of the premetastatic niche. Specifically, tumor-secreted exosomes can promote dissemination of CTCs by transporting regulators of the vascular endothelial barriers to distant tissues. This modulation of the blood vessels in the premetastatic niche is key, as they directly control the arrest and extravasation of CTCs into the tissue. For example, brain metastasis by breast cancer cells is partially facilitated through the secretion of exosomes carrying miR-181c, which modulates actin to promote systemic vascular leakiness and the destruction of the blood-brain barrier.

Metabolic manipulation within target tissues of metastasis is another specific form of tumor-induced stromal cell reprogramming. Upon dissemination into a distant site, cancer cells must compete with the surrounding tissue for nutrients to establish a metastatic colony. Breast cancer cells have been shown to secrete exosomes containing miR-122, which reprograms lung

fibroblasts to decrease their glucose consumption by suppressing pyruvate kinase. By decreasing glucose consumption of resident cells, the cancer cells have increased glucose availability, which facilitates their proliferation and survival.

Finally, alterations in ECM are observed early in the formation of the premetastatic niche. One such change is the accumulation of FN, which can be induced by primary tumor-secreted factors. Hypoxia in the primary tumor has been shown to induce the secretion of FN and lysyl oxidase (LOX), which colocalize in the lung premetastatic niche to recruit myeloid cells in the lung. LOX can also mediate collagen cross-linking, which in turn increases MMP2 activity to also recruit myeloid cells. This phenomenon functions as a positive feedback loop because myeloid cells break down collagen IV, releasing it into the blood to function as a chemoattractant to draw yet more myeloid cells to the niche. Activated fibroblasts are another cell within the premetastatic niche that also excretes LOX, which results in collagen deposition and ECM stiffening to promote metastatic cell survival and engraftment.

The majority of premetastatic niche characteristics have been observed in the laboratory and are not routinely assessed clinically as they would require potentially unnecessary invasive procedures. However, several biomarkers of premetastatic niche formation can be identified by non-invasive blood biopsy. Current focus is being placed on circulating exosome contents, as tumor- secreted exosomes play a large role in adapting the distant stromal cells at the site of metastasis. By analyzing tumor-secreted factors clinicians may be able to determine the likelihood of metastatic spread, the organ of metastasis, or target mediators of premetastatic niche development.

Prospective Vision

Early detection and the interruption of metastasis pathways holds promise for the treatment of cancer patients with, or at risk of, metastatic disease. Target discovery efforts are currently focused on blocking metastasis at several steps, as the degree of metastatic progression in patients often cannot be determined. The obstacles that must be overcome for a tumor cell to achieve metastasis provide several vulnerabilities to exploit. The ability to enter the circulatory system, evade immune surveillance, extravasate into distant tissues, switch from dormancy to growth phase once disseminated, and establish a premetastatic niche are all essential steps of interest to target therapeutically. Detection and screening for metastatic pre-disposition are also important aspects of the current efforts in treating patients. The use of liquid biopsies continues to improve physicians' abilities to predict secondary site location and measure treatment response. In addition, studies of inherited predisposition have potential to begin early screening of cancer patients to determine their likelihood of developing secondary tumors. Finally, as new drugs are tested pre-clinically and in patients, researchers have begun to consider a specific shift in the end points of clinical trials for metastasis therapeutics. Preclinical metastasis therapies often provide a significant delay in the development of metastases rather than reducing the size of established lesions. It is thought that "time to new lesion" end points will be more beneficial in the prevention of an initial metastasis, or additional metastases. In summary, with sights focused squarely on improving detection and treatment, creative minds and the advancement of technology continues to accelerate our grasp of the nuanced biology of metastasis.

See also: Cell Adhesion During Tumorigenesis and Metastasis.

Further Reading

Hendrix, M.J.C., Seftor, E.A., Hess, A.R., Seftor, R.E.B., 2003. Angiogenesis: vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nature Reviews. Cancer 3, 411–421.

Hunter, K., 2006. Host genetics influence tumour metastasis. Nature Reviews. Cancer 6, 141–146.

Karagiannis, G.S., Goswami, S., Jones, J.G., Oktay, M.H., Condeelis, J.S., 2016. Signatures of breast cancer metastasis at a glance. Journal of Cell Science 129, 1751–1758.

Liu, Y., Cao, X., 2016. Characteristics and significance of the pre-metastatic niche. Cancer Cell 30 (5), 668–681.

Paget, S., 1889. The distribution of secondary growths in cancer of the breast. Lancet 1, 99–101.

Siravegna, G., Marsoni, S., Siena, S., Bardelli, A., 2017. Integrating liquid biopsies into the management of cancer. Nature Reviews. Clinical Oncology. Published online 02 march 2017.

Steeg, P.S., 2016. Targeting metastasis. Nature Reviews. Cancer 16, 201–218.

Smith, H.A., Kang, Y., 2013. The metastasis-promoting roles of tumor-associated immune cells. Journal of Molecular Medicine (Berlin, Germany) 91 (4), 411–429.

Valastyan, S., Weinberg, R.A., 2011. Tumor metastasis: molecular insights and evolving paradigms. Cell 147 (2), 275–292.

Metformin

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Glossary

AMPK 5′ AMP-activated protein kinase or AMPK is an enzyme that plays a role in cellular energy homeostasis. It is expressed in a number of tissues, including the liver, brain, and skeletal muscle. The net effect of AMPK activation is stimulation of hepatic fatty acid oxidation, ketogenesis, stimulation of skeletal muscle fatty acid oxidation and glucose uptake, inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, and modulation of insulin secretion by pancreatic beta-cells. Recently AMPK is emerging as a possible metabolic tumor suppressor and target for cancer prevention and treatment. Activation of AMPK has been found to oppose tumor progression in several cancer types and offers a promising cancer therapy. AMPK activity opposes tumor development and progression in part by regulating inflammation and metabolism.

IGFs Insulin-like growth factors I (IGFs) are important mediators of growth, development, and survival. They are synthesized by almost any tissue in the body, and their action is modulated by a complex network of molecules, including binding proteins, proteases, and receptors, which all comprise the IGF system. IGFs may promote cell cycle progression and inhibition of apoptosis either by directly associating with other growth factors or indirectly by interacting with other molecular systems which have an established role in carcinogenesis and cancer promotion. In addition, increased serum levels of IGFs and/or altered levels of their binding proteins are associated with increased risk for developing several malignancies.

MAPK A mitogen-activated protein kinase (MAPK or MAP kinase) is a type of protein kinase that is specific to the amino acids serine, threonine, and tyrosine. MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock, and proinflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.

mTOR The mammalian target of rapamycin is a kinase that in humans is encoded by the MTOR gene. mTOR is a member of the phosphatidylinositol 3-kinase-related kinase family of protein kinases. mTOR functions as a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, and transcription. Furthermore, mTOR also promotes the activation of insulin receptors and insulin-like growth factor 1 receptors.

REDD1 The protein regulated in development and DNA damage response 1 (REDD1) is a protein that in humans is encoded by the DDIT4 gene. REDD1 acts as a negative regulator of mTOR, and its clinical interest is based primarily on its effect on mTOR, which has been associated with aging and linked with diseases such as diabetes and cancer.

Introduction

Metformin is an orally administrated drug commonly used to lower blood glucose concentrations in patients with type II diabetes mellitus (T2DM). There is intense interest in the cancer prevention research community regarding the potential use of metformin (1,1-dimethylbiguanide) to decrease cancer incidence or cancer-related mortality. A series of recent metaanalyses confirmed the association of diabetes or prediabetes (impaired fasting glucose and/or impaired glucose tolerance) with an increased risk of cancer. Such metaanalyses have estimated a relative risks (RR) of 1.1–2.5 for cancer risk at various organ sites in patients with T2DM, as shown in **Table 1**.

Insulin-resistance and hyperinsulinemia are the principal conditions associated with T2DM. Various mechanisms have been reported to explain the relationship of T2DM with cancer incidence. In particular, insulin can exert its oncogenic effect through the stimulation of growth factors, such as insulin-like growth factor-I (IGF-I), and the concomitant reduction of the binding proteins (BPs) for these growth factors.

Metformin reduces gluconeogenesis and hepatic glucose production and improves insulin sensitivity by increasing peripheral glucose uptake. Thus, it does not increase the circulating insulin, and it is a relative safe drug with a good pharmacokinetic profile and mild toxicity that is usually well tolerated. The most common side effects are nausea and diarrhea, usually self-limited and transient. The only potential major adverse event during therapy is lactic acidosis, but a direct association between this condition and metformin use has not yet been clearly demonstrated. Moreover, this disease is rare and primarily confined to patients with concomitant renal and hepatic disorders.

Despite many epidemiological studies and metaanalyses showing an association between metformin use and reduced cancer incidence and mortality (fully discussed later), only a minority of these associations have a robust supporting evidence without suggestion of bias. In particular, time-related biases have been recently recognized as a major potential source of error in some epidemiologic studies, imposing a reevaluation of the literature linking metformin use to reduced cancer incidence, and indicating the need to conduct properly designed randomized clinical trial. The chapter summarizes the scientific evidence of metformin use to

Table 1 Metaanalyses on cancer incidence in patients with diabetes

Cancer	*First author (PY)*	*No. of studies*	*SRR*	*95% CI*
Any cancer	Noto et al. (2011)	12	1.10	1.04–1.17
Breast	Boyle et al. (2012)	39	1.27	1.16–1.39
	Liao et al. (2011)	10	1.23	1.18–1.27
	Larsson et al. (2007)	20	1.20	1.12–1.28
	Hardefeldt et al. (2012)	43	1.20	1.13–1.29
Bladder	Larsson et al. (2006)	16	1.24	1.08–1.42
	Zhu et al. (2013)	36	1.35	1.17–1.56
Biliary duct	Jing et al. (2012)	5	1.60	1.38–1.87
Colorectum	Deng et al. (2012)	24	1.26	1.20–1.31
	Jiang et al. (2011)	41	1.27	1.21–1.34
Endometrium	Friberg et al. (2007)	16	2.10	1.75–2.53
Esophagus	Huang et al. (2012)	17	1.30	1.12–1.50
Gallbladder	Ren et al. (2011)	21	1.43	1.18–1.72
Gastric	Tian et al. (2012)	25	1.11	1.00–1.24
	Ge et al. (2011)	21	1.09	0.98–1.22
Kidney	Larsson and Wolk (2011)	9	1.42	1.06–1.91
	Bao et al. (2013)	24	1.40	1.16–1.69
Liver	Gao et al. (2010)	14	3.33	1.82–6.10
Liver (HCC)	Wang et al. (2012)	18	2.01	1.61–2.51
Leukemia	Castillo et al. (2012)	26	1.22	1.03–1.44
Lung	Lee et al. (2013)	34	1.11	1.02–1.20
Multiple Mieloma	Castillo et al. (2012)	34	1.22	0.98–1.53
Non-Hodgkin lymphoma	Mitri et al. (2008)	15	1.19	1.04–1.35
	Castillo et al. (2012)	26	1.22	1.07–1.39
Ovary	Lee JY	19	1.17	1.02–1.33
	Wang et al. (2017)	13	1.19	1.06–1.34
Pancreas	Ben et al. (2011)	35	1.94	1.66–2.27
Prostate	Kasper and Giovannucci (2006)	19	0.84	0.76–0.93
	Bansal et al. (2013)	45	0.86	0.80–0.92
Thyroid	Schmid et al. (2013)	6	1.17	0.99–1.39

PY, Publication year; *SRR*, Summary relative risk; 95% CI, 95% confidence interval.

reduce cancer incidence and mortality, from putative mechanisms of action through critical evaluation of observational studies and the limited data on clinical trials.

Molecular Mechanisms of Metformin in Cancer and Diabetes

Despite being introduced clinically in the 1950s, the exact mechanism of action of metformin has not been fully elucidated. This applies not only to its antihyperglycemic and antihyperinsulinemic effects but also to its anticancer properties. Two major mechanistic categories are relevant to the anticancer action of metformin: an indirect and a direct mechanism, not mutually exclusive and converging on the same molecular pathways.

Indirect Mechanisms

This category involves "endocrine-type effects" related to its insulin-lowering activity. Metformin acts on the liver to inhibit hepatic production of glucose, antagonizing glucagon-mediated effects that lead to hyperglycemia in diabetics. To a lesser extent metformin increases insulin-mediated uptake of glucose in skeletal muscle, though not by increasing insulin levels, as the drug actually decreases the hyperinsulinemia that is seen in diabetes. Since high insulin levels are known to stimulate proliferation of some common cancers, this decrease in insulin levels may slow tumor proliferation in hyperinsulinemic patients.

Direct Mechanisms

In this category metformin exerts its effects directly on the target cells by an insulin-independent mechanism that leads to suppression of ATP production via metformin's inhibition of mitochondrial complex I. NADH-ubiquinone oxidoreductase (Complex I) plays an essential role in biosynthesis and redox control during proliferation, resistance to cell death, and metastasis of cancer cells. Both routes of metformin activity converge on the mTOR (mammalian target of rapamycin) pathway resulting in inhibition of its proproliferative activity.

Table 2 Possible pathways involved in the anti-cancer effects of metformin

Mechanism	*Function*
AMPK-dependent	Inhibition of cell mitosis and proliferation Upregulation of the p53 axis DNA synthesis Growth inhibition Cell apoptosis
AMPK-independent	REDD1 mediates cell cycle arrest Induced apoptosis
Suppression of mTOR	Inhibition of cell growth Induction of apoptosis Induction of autophagy Inhibition of tumor promotion Suppression of HER2 overexpression
Suppression of IGF signaling	Prevention of IGF-1R upregulation Reduction of cell proliferation and invasion Reduction of circulating insulin and IGF-1 Arrest of cell growth and proliferation
The MAPK signaling pathway	Arrest of tumor cells migration and invasion Inhibition of cell growth, colony formation Induction of cell cycle arrest Survival signals blockade

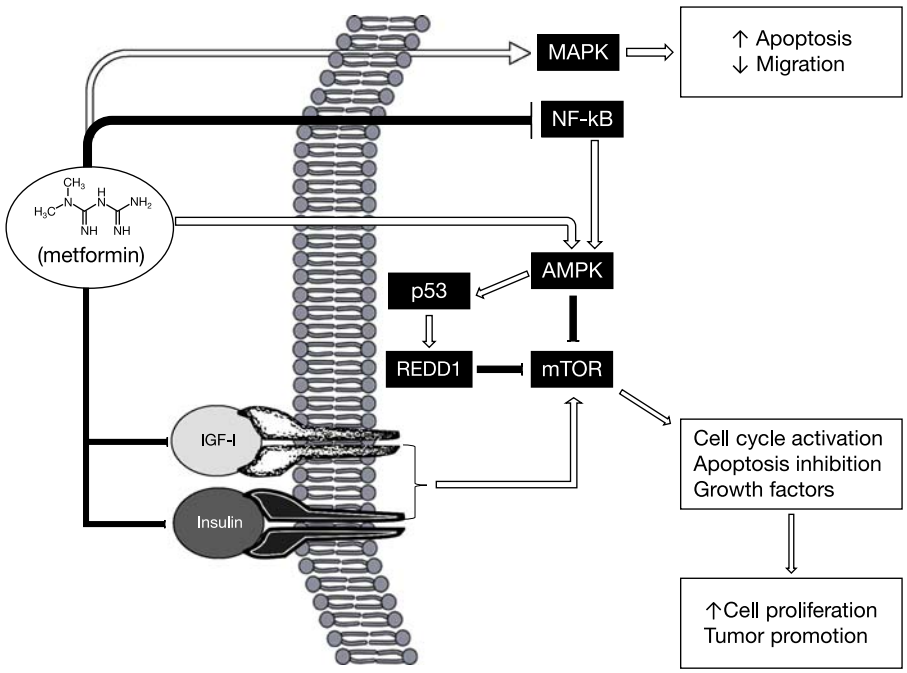

Fig. 1 Main signaling pathways involved in the anti-cancer effects of metformin.

Table 2 and Fig. 1 show some possible signaling pathways involved in the anticancer effects of metformin. Some effects are mediated by the activation of adenosine monophosphate protein kinase (AMPK), an intracellular energy sensor whose activation inhibits cell mitosis and proliferation by directly influencing the dynamics of cell division. Metformin stimulates AMPK through upregulation of the p53 axis. However, certain antitumor effects of metformin are independent of the AMPK signaling pathway. Metformin directly influences mTOR in a p53-dependent manner through an AMPK-independent mechanism increasing the level of REDD1, a negative regulator of mTOR, thus contributing to the cell circle arrest. mTOR regulates cellular energy homeostasis by modulating protein synthesis and autophagy, and exerting significant positive effect on cell proliferation and tumorigenesis. An imbalanced activation of mTOR is associated with malignant tumor progression, drug resistance, and a worse prognosis. mTOR metformin-induced inhibition of the mTOR pathway has been demonstrated in different types of cancer and through AMPK-dependent and -independent pathways.

The suppression of IGF signaling is another possible mechanism of action: insulin and IGFs are key regulators of metabolism and cancer progression by activating signaling pathways associated with cell growth and proliferation. There are two subtypes of IGF, IGF-1 and IGF-2, which are both mitogenic and antiapoptotic. IGF-1 receptor (IGF-1R) binds to the ligand IGF-1, IGF-2, or insulin to promote autophosphorylation of tyrosine at its kinase domain and with a consequent activation of signaling through the phosphatidylinositol-3-kinase PI3K/Akt/mTOR and RAS/RAF/mitogen-activated protein kinase (MAPK) pathways.

Emerging evidence suggests that metformin decreases IGF-1 by indirectly downregulating insulin and insulin-binding proteins to reverse hyperinsulinemia.

Metaanalyses of Observational Studies and Randomized Controlled Trials Examining the Effect of Metformin on Cancer Incidence and Mortality

Multiple metaanalyses of observational studies have reported the use of metformin is associated with a decrease in cancer incidence ranging from 14% to 40%, along with a decrease in mortality in the same range (Table 3). Organ sites include the breast, colon, liver, pancreas, prostate, endometrium, and lung, among others. However, these studies have a multitude of study designs, from cohort and case-control studies through randomized controlled trials. As the level of scientific evidence increases with the study design, the effect of metformin on cancer incidence appears to decrease. Thakkar et al. showed that while cancer incidence was decreased by 30% in cohort studies and 10% in case-control studies, there was no clear effect in randomized controlled trials. Gandini et al. performed a metaanalysis with particular attention to biases and confounders and also found that the risk reduction for cancer incidence decreased from 31% for all studies to 29% for prospective studies to 5% for randomized clinical trials.

Several authors suggested that the existing metformin literature might be subject to important sources of time-related bias, which potentially overestimate cancer-reducing effects. Time-related bias is a form of differential misclassification bias that can be avoided by appropriate accounting of follow-up time and exposure status in the design and analysis of studies. There are different types of time-related biases: (i) immortal time bias, when unexposed time is misclassified as drug-exposed time; (ii) time-window bias, when there are differential exposure opportunity time windows between exposed and unexposed subjects; (iii) time-lag bias, when there's a comparison of treatment given during different stages of the disease. Immortal time bias refers to a period of cohort follow-up time during which a cancer event (that determines end of follow-up) cannot occur. Immortal time bias can arise when the period between cohort entry and date of first exposure to metformin, during which cancer has not occurred, is either misclassified or excluded and not accounted for in the analysis. In particular this is frequently found in studies that compare "ever users" against "nonusers" and do not take into account, in the analysis and in the study design, of time of use (when patients' start and stop metformin). Caution should be used also when analyzing cohort studies where a first-line therapy with metformin is compared with second- or third-line therapies. Patients are unlikely to be at the same stage of diabetes, which can induce confounding of the association with an outcome (e.g., cancer incidence) by disease duration. An outcome related to the first-line therapy may also be attributed to the second-line therapy if it occurs after a long period of exposure. Such a situation requires matching on disease duration and consideration of latency time windows in the analysis.

Table 3 Metaanalyses of observational studies and randomized controlled trials examining the effect of metformin on cancer incidence and mortality

Author (PY)	*No. of studies included*	*Cancer incidence SRR (95%CI)*	*Cancer mortality SRR (95% CI)*
DeCensi et al. (2010)	11	0.68 (0.52–0.88)	0.70 (95% CI, 0.51–0.96)
Noto et al. (2012)	6	0.67 (0.53–0.85)	0.66 (95% CI, 0.49–0.88)
Soranna et al. (2012)	17	0.61 (0.54–0.70)	NR
Thakkar et al. (2013)	24	0.70 (0.67–0.73) Co 0.90 (0.84–0.98) CC 1.01 (0.81–1.26) RCT	NR
Franciosi et al. (2013)	53	0.73 (0.61–0.88) 0.98 (0.81–1.19) RCT	0.65 (95% CI, 0.53–0.80)
Gandini et al. (2014)	47	0.69 (0.52–0.90) 0.95 (0.69–1.30) RCT	0.66 (95% CI, 0.54–0.81)
Wu et al. (2015)	265	0.86 (0.83–0.90) 0.88 (0.83–0.92) Co 0.71 (0.63–0.80) CC 1.05 (0.94–1.18) RCT	0.70 (95% CI, 0.53–0.94) 0.66 (95% CI, 0.49–0.89) Co 0.91 (95% CI, 0.37–2.23) RCT
Ma et al. (2017)	23	0.52 (0.40–0.68) 0.64 (0.48–0.86) Co 0.50 (0.36–0.70) CC 0.84 (0.10–6.83) RCT	NR

PY, Publication year; *SRR*, Summary relative risk; *95% CI*, 95% confidence interval; *NR*, Not reported; *Co*, cohort study; *CC*, case-control study; *RCT*, randomized control trial.

In the metaanalysis performed by Gandini et al., time-related biases were taken into account, resulting in a diminution of the protective effect on cancer incidence. The risk reduction in cancer incidence at all sites decreased from 30% to 10%, albeit still statistically significant. When focusing on specific organ sites, metformin use was significantly associated with decreased breast cancer incidence, but the magnitude of the effect was not clinically relevant. For colon cancer, 8% decrease in incidence was calculated. For prostate and pancreatic cancers, metformin use was not associated with a statistically significant protective effect. For lung cancer, examination of all studies showed a 18% decrease in cancer incidence. Adjustment for time-related biases decreased this protective effect to a statistically significant 12%, but adjustment for smoking, which is by far the most relevant lung cancer risk factor, resulted in no association between metformin use and cancer incidence. Similarly, the strong protective effect of metformin use against liver cancer became nonsignificant when only unbiased studies were examined. It should be emphasized, however, that the total number of studies and patients was rather small for the liver cancer associations, and thus these results should be interpreted with caution. Notably, a very recent metaanalysis of studies using metformin as a reducer for liver cancer risk in diabetic patients reported that metformin use reduced the ratio of liver cancer by 48% compared with nonusers. The protective effect was validated in all the exploratory subgroup analyses, except that pooled result of post hoc analyses of two randomized controlled trials found no significant difference between subjects with metformin and those without. After adjusting for hepatitis B/C virus infection, cirrhosis, obesity, behavioral factors, and time-related bias, the association was stable, pooled OR ranged from 0.42 to 0.75.

Another potential source of confounding that is often not adequately explored in the existing literature is the effect of obesity and its surrogate, body mass index (BMI). Obesity is intimately linked to increased risk of multiple cancer types. Potential mechanisms include both direct and indirect effects of obesity on insulin, IGF-1, sex hormones, adipokines, and inflammation, many of which are directly impacted by metformin. Metformin, unlike several other antidiabetic agents, is associated with weight loss. Furthermore, a recent clinical trial showed that metformin affected breast cancer proliferation differentially according to insulin resistance status and BMI, with a trend toward inhibiting proliferation only in women with insulin resistance or high BMI.

Gandini et al. examined BMI as a potential confounder and found that adjustment for BMI decreased the protective effect of metformin for all-cancer incidence from 31% to 18%, which was still statistically significant. For breast cancer, adjustment for BMI revealed a borderline association of metformin use with cancer incidence. For colon and prostate cancer, there was no statistically significant association between metformin use and cancer incidence. Other organ sites could not be assessed due to a small number of published studies with adjusted estimates. It should be noted, however, that BMI is dynamic and therefore adjusting for a single BMI assessment might be inadequate to account for confounding by BMI dynamics over time. Furthermore, Gandini et al. were unable to assess the effect of BMI and time-related biases simultaneously due to inadequate numbers of studies that controlled for both factors.

The effect of metformin on cancer mortality is influenced by a limited literature and thus Gandini et al. were unable to determine the effect by cancer sites. However they found that metformin use was associated with a 34% decrease mortality that remained significant when the analysis was limited to prospective studies. Adjustment for BMI maintained the magnitude of the effect although adjustment for time-related biases, limited to only three studies, resulted in loss of statistical significance. Different mechanisms may be responsible for effect of metformin on cancer mortality compared with cancer incidence. Several retrospective analyses have suggested that diabetics treated with metformin during chemotherapy have longer survival than individuals treated with other antidiabetic agents. A previous mouse xenograft study showed that metformin targets breast cancer stem cells and synergizes with doxorubicin to prevent relapse. Increasing the effectiveness of chemotherapy could result in improved survival.

Taken together, these results point out the limitations of the current literature regarding the association between metformin use and cancer incidence and mortality. While all the studies, including metaanalyses, suggest that metformin use is associated with a reduced risk of cancer and death, the effect may be far smaller than previously believed.

Published Clinical Trials of Metformin in Cancer

Table 4 summarizes the main characteristics of the completed clinical trials (mainly phase II) in cancer patients with metformin. Two studies examined various doses of metformin given for 3–6 months to women after therapy for breast cancer. Goodwin et al. phase II study (2008) examined women with breast cancer who completed adjuvant therapy and whose plasma levels of insulin were at least 45 pmol/L. Metformin decreased circulating insulin levels by 22.4% significantly. Campagnoli et al. study (2012) enrolled women with breast cancer, who had completed adjuvant therapy but had elevated testosterone, and compared two different doses of metformin. The higher dose (1500 mg/day) significantly reduced serum testosterone levels and free androgen index compared to the lower dose. In 2015, Goodwin and colleagues analyzed 492 patients enrolled in the NCIC Clinical Trials Group MA.32 phase III trial (metformin vs placebo effect on invasive disease-free survival and other outcomes in early breast cancer) to explore whether metformin may improve metabolic factors (insulin, glucose, leptin, highly sensitive C-reactive protein [hs-CRP]). At 6 months, decreases in weight and blood variables were statistically significantly greater in the metformin arm (vs placebo) in univariate analyses: weight – 3.0%, glucose – 3.8%, insulin – 11.1%, homeostasis model assessment – 17.1%, leptin – 20.2%, hs-CRP – 6.7%. There was no statistically significant interaction of change in these variables with baseline BMI or insulin. Five trials examined the short-term effects (1–4 weeks) of various doses of metformin on cell proliferation (Ki-67) in women awaiting surgery for breast cancer (presurgical, window of opportunity trial). Hadad et al. (2011) found a 3.4% reduction in Ki-67 in the metformin arm, but 29% of the patients on metformin withdrew due to gastrointestinal side effects. Bonanni et al.

Table 4 Completed phase II–III clinical trials of metformin in cancer patients

First Author (year of publication)	*Phase*	*Organ*	*Number of patients*	*Patients Characteristics*	*Treatment*	*Endpoint*	*Results*
Goodwin et al. (2008)	II	Breast cancer	32	Insulin levels >45 pmol/L	MET versus no treatment	Insulin level	Significant reduction
Hosono et al. (2010)	II	Colon	23	Nondiabetic	MET versus no treatment	Number of rectal aberrant crypt foci	Significant reduction
Hadad et al. (2011)	II	Breast cancer	8 + 47	Nondiabetic	MET versus no treatment	Ki-67; transcriptome analysis; insulin level	Significant reduction
Campagnoli et al. (2012)	II	Breast cancer	125	Nondiabetic, testosterone levels ≥0.28 ng/mL	HD MET versus LD MET	Insulin; HOMA IR index; testosterone, free-androgen index	Significant reduction
Bonanni et al. (2012) Cazzaniga et al. (2013) DeCensi et al. (2014)	II	Breast cancer Presurgical	200	Nondiabetic	MET versus Placebo	Ki-67	Overall nonsignificant reduction; significant in HER2+, top hs-CRP; if HOMA > 2.8
Niraula et al. (2012)	II	Breast cancer Presurgical	39	Nondiabetic	MET versus no treatment	• TUNEL • Ki-67 • BMI, weight, HOMA • Insulin level, leptin, CRP • Symptoms/quality of life	• Significant increase • Significant reduction • Significant reduction • Nonsignificant reduction • Nonsignificant alteration
Kalinsky et al. (2014)	II	Breast cancer presurgical	35	Nondiabetic BMI ≥ 25	MET versus no treatment	• Ki-67 • BMI, cholesterol, leptin	• Nonsignificant reduction • Significant reduction
Laskov et al. (2014)	II	Endometrial cancer Presurgical	11	Nondiabetic	MET versus no treatment	Insulin level, IGF-1, IGFBP-7, Ki-67, Ps6	Significant reduction
Mitsuhashi et al. (2014)	II	Endometrial cancer Presurgical	31	Nondiabetic	MET versus no treatment	• Ki-67, Topoisomerase IIa, Ps6,ERK1/2 • AMPK, p27 • Insulin, glucose, IGF-1, leptin	• Significant reduction • Significant increase • Significant reduction
Joshua et al. (2014)	II	Prostate cancer Presurgical	24	Nondiabetic	MET (single arm)	Ki-67	Significant reduction
Goodwin et al. (2015)	III	Breast cancer	492	Nondiabetic	MET versus Placebo	Weight, glucose, insulin level, HOMA, leptin, hs-CRP	Significant reduction
Schuler et al. (2015)	II	Endometrial cancer	20	Nondiabetic BMI ≥ 30	MET (single arm)	Ki-67	Significant reduction
Chak et al. (2015)	II	Barrett's esophagus	74	Nondiabetic	MET versus Placebo	pS6K1	No reduction
Higurashi et al. (2016)	III	Colorectal adenoma	498	Nondiabetic	MET versus Placebo	Adenoma recurrence	Significant reduction in prevalence

Abbreviations: *MET*, Metformin; *AMPK*, Phospho-adenosine monophosphate-activated protein kinase; *HD*, High dose; *LD*, Low dose; *HOMA-IR*, Homeostasis model assessment-insulin resistance; *IGF-1*, Insulin-like growth factor-1; *TUNEL*, Terminal eoxynucleotidyl transferase dUTP nick end labeling; *HOMA*, Homeostasis model assessment; *hs-CRP*, Highly sensitive C-reactive protein; *IGFBP-3*, Insulin-like growth factor binding protein 3; *BMI*, Body mass index; *CRP*, C-reactive protein; *Ps6*, Phospho-ribosomal protein S6; *pS6K1*, Phosphorylated S6 kinase; *IGFBP-7*, Insulin-like growth factor binding protein 7; *ERK1/2*, Phospho-extracellular signal-regulated kinase 1/2; *ACF*, Aberrant crypt foci.

(2009) performed a larger double-blind, placebo-controlled study, where metformin effect on Ki-67 change relative to placebo was not statistically significant, with a mean proportional increase of 4.0%. Women with a HOMA index (Homeostasis Model Assessment, used to quantify insulin resistance) >2.8 had a nonsignificant decrease of 10.5% while women with a HOMA index <2.8 had a nonsignificant increase of 11.1%. The interaction between HOMA index and metformin on Ki-67 was statistically significant; a similar trend was seen with BMI although the interaction was not statistically significant. Niraula et al. (2012) in a single-arm trial reported a 3% decrease in Ki-67 ($p = 0.016$) after a median of 18 days of treatment, and a recently completed study (Kalinsky et al., 2014) showed no reduction in Ki-67. Two studies have been reported in women with early-stage endometrial cancer. Endometrial cancer prevention is of particular interest because of its relationship with obesity and the use of metformin for treatment of polycystic ovary syndrome, a condition associated with increased endometrial cancer risk. In the Laskov study (2011), 11 newly diagnosed, untreated, nondiabetic patients with endometrial cancer received metformin 500 mg tide from diagnostic biopsy to surgery. Ki-67, pAMPK, and pS6 immunohistochemistry staining were performed on the endometrial cancer before and after metformin treatment, and they were compared with a control group of 10 women with endometrial cancer who did not receive metformin. Mean plasma insulin, IGF-1, and IGFBP-7 were significantly reduced after metformin treatment. A clear reduction in Ki-67 and pS6 expression was observed by both conventional light microscope analysis and digital image analysis with a significant mean reduction in percentage of cells staining for Ki-67 and pS6. In the untreated control group expression of ki-67 was similar between the biopsy and the surgical specimens. Mitsuhashi et al. trial (2014) reported that increasing the dosage from 750 mg QD to 1500 mg or 2250 mg for 4 weeks of metformin resulted in a 44.2% decrease in Ki-67. Three additional studies in other organ systems (Barrett's esophagus, colon, and prostate) have been published. A study in Barrett's esophagus (Chack et al., 2015) showed no significant change in phosphor-serine 6 kinase (pS6K), cell proliferation, or apoptosis. Hosono et al. (2010) study examined change in the number of aberrant crypt foci (ACF, a putative precursor of colon cancer) in individuals with preexisting ACF, randomizing participants to a very low dose of metformin (250 mg/day) or no treatment for one month. A significant decrease in ACF from 8.78 ± 6.45 to 5.11 ± 4.99 was observed in the metformin arm but not in the control group. Additionally, proliferation was significantly decreased in the metformin group. Recently, Hirugashi et al. (2016) in a phase III trial reported that the administration of low-dose metformin (250 mg/daily) for 1 year to patients without diabetes after colorectal polipectomy has shown to be safe and to reduce the prevalence and number of metachronous adenomas or polyps. In the trial published by Joshua et al. (2014), men with prostate cancer were treated with metformin for 4–12 weeks prior to surgery. The primary endpoint, change in Ki-67, was statistically significant, with an absolute decrease of 1.44%. In a per patient and per tumor analyses, metformin reduced the Ki67 index by relative amounts of 29.5% and 28.6%, respectively.

Ongoing Phase III Trials With Metformin in Cancer Patients and Prevention

Table 5 summarizes the main characteristics of ongoing phase III clinical trials in cancer patients with metformin. NCT01864096 trial aims to see if metformin can delay the time to progression in men with low-risk prostate cancer when compared to a placebo. NCT01101438 is looking at whether metformin can decrease or affect the ability of breast cancer cells to grow and whether metformin will work with other therapies to keep cancer from recurring. NCT02614339 trial wants to identify the effect of adjunctive metformin on recurrence of nondiabetes mellitus stage III colorectal cancer. This trial is open-label randomized controlled study, and the primary endpoint is to compare the 3-year disease-free survival between metformin group and nonmetformin group. The secondary endpoint is to compare the 5-year overall survival and disease-specific survival between two group, to identify the safety of metformin, and to compare the recurrence rate of polyps after polypectomy between two groups. NCT01697566 study analyzes the effects of metformin and/or a program called "lifestyle intervention" on the endometrium in postmenopausal women who are also obese (both risk factors for endometrial cancer). Lifestyle intervention is made up of a series of in-person sessions with a coach to discuss strategies for losing weight and ways to increase physical activity. NCT03184493 is a prospective trial to compare the role of celebrex alone, metformin alone, and celebrex plus metformin in preventing hepatocellular carcinoma recurrence after hepatic resection. NCT02065687 is a randomized phase II/III trial to investigate how well paclitaxel, carboplatin, and metformin hydrochloride works and compares it to paclitaxel, carboplatin, and placebo in treating patients with endometrial cancer that is stage III–IV. The hypothesis is that metformin may help paclitaxel and carboplatin to work better by making cancer cells more sensitive to the drugs. NCT02040376 is a placebo-controlled, double-blind crossover trial of metformin in 30 children treated with radiation for medulloblastoma, the most common malignant brain tumor. Tests of thinking and learning and brain imaging techniques will be used to examine whether metformin can enhance cognition or promote brain repair following radiation-induced brain injury. NCT01905046 tests whether metformin is able to get rid of atypia (early cell changes that are thought to be a marker of breast cancer risk) in women at increased risk for breast cancer. A total of 300 patients with atypia (Masood Score 14–17) on random periareolar fine needle aspiration (RPFNA) at baseline are expected to be randomized between Metformin and placebo control arm. The randomization will be stratified for known BRCA mutation (BRCA1 or BRCA2 mutation vs no mutation), prior excisional biopsy (atypical epithelial hyperplasia, atypical lobular hyperplasia, flat epithelial hyperplasia vs DCIS vs LCIS), and baseline fasting insulin levels. The primary objective is to test for the presence or absence of cytological atypia in RPFNA bilateral aspirates after 12 and 24 months.

Table 5 ClinicalTrials.gov Search Results 07/14/2017

NCT number	*Recruitment*	*Conditions*	*Interventions*	*No. of patients*	*Study designs*	*Primary endpoint*
NCT01864096	Recruiting	Prostate cancer on active surveillance	Metformin versus placebo	408	Randomized; double blinded	Time to progression
NCT01101438	Active, not recruiting	Breast cancer	Metformin versus Placebo	3649	Randomized; double blinded	Invasive disease-free survival
NCT02614339	Active, not recruiting	Stage III colorectal cancer Nondiabetic	Metformin versus control	593	Randomized; open label	Disease-free survival
NCT01697566	Active, not recruiting	Healthy women; BMI $\geq$ 30	Metformin versus placebo	100	Randomized; double blinded	Ki-67
NCT03184493	Recruiting	Liver cancer	Metformin plus celebrex versus Metformin versus cerebrex	200	Nonrandomized	1-year tumor recurrence
NCT02065687	Recruiting	Endometrial cancer	Metformin plus paclitaxel and carboplatin versus paclitaxel and carboplatin	540	Randomized; double blinded	Overall survival
NCT02040376	Recruiting	Children with cranial-spinal radiation for medulloblastoma	Metformin versus placebo	30	Randomized; double blind; crossover	Fostering brain repair
NCT01905046	Recruiting	Breast ADH; BRCA1/2 mutation carrier; DCIS; LCIS	Metformin versus Placebo	400	Randomized; double blinded	Presence of atypia in RPFNA

Abbreviations: *NCT*, Number of clinical trial; *ADH*, Atypical ductal hyperplasia; *DCIS*, Ductal carcinoma in situ; *LCIS*, Lobular carcinoma in situ.

Conclusions

The emerging data from preclinical, epidemiologic, and clinical studies suggest that there is a signal for cancer-preventive potential with metformin use. There is biologic plausibility for a cancer-preventive effect given the multiple ways that metformin can interfere with cancer-promoting signaling pathways. However, both animal and epidemiologic studies have shown somewhat mixed effects. Further, the epidemiologic literature reviewed presented results that regard mainly individuals with diabetes and the effect is of lesser magnitude than previously reported once the appropriate adjustments to avoid bias and confounding are made. It remains to be determined whether a similar protective effect on cancer risk and mortality can be confirmed in nondiabetic individuals by properly designed studies. Multiple ongoing clinical trials are addressing which cancer patient cohorts are most likely to benefit from metformin.

Although the long history and clinical experience with metformin make it a very attractive candidate for drug repurposing, general recommendations about its use, particularly in nondiabetic populations, need to await the results of the ongoing studies.

See also: Chemoprevention Trials. Diabetes and Cancer. Obesity and Cancer: Epidemiologic Evidence.

References

Bansal, D., Bhansali, A., Kapil, G., Undela, K., Tiwari, P., 2013. Type 2 diabetes and risk of prostate cancer: A meta-analysis of observational studies. Prostate Cancer and Prostatic Diseases 16 (151–8), S1.

Bao, C., Yang, X., Xu, W., Luo, H., Xu, Z., Su, C., Qi, X., 2013. Diabetes mellitus and incidence and mortality of kidney cancer: A meta-analysis. The Journal of Diabetic Complications 27, 357–364.

Ben, Q., Xu, M., Ning, X., Liu, J., Hong, S., Huang, W., Zhang, H., Li, Z., 2011. Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. European Journal of Cancer 47, 1928–1937.

Bonanni, B., Puntoni, M., Cazzaniga, M., Pruneri, G., Serrano, D., Guerrieri-Gonzaga, A., Gennari, A., Stella, T.M., Galimberti, V., Veronesi, P., Johansson, H., Aristarco, V., Bassi, F., Luini, A., Lazzeroni, M., Varricchio, C., Viale, G., Bruzzi, P., Decensi, A., 2012. Dual Effect of Metformin on Breast Cancer Proliferation in a Randomized Presurgical Trial. Journal of Clinical Oncology 30, 2593–2600.

Boyle, P., Boniol, M., Koechlin, A., Robertson, C., Valentini, F., Coppens, K., Fairley, L.-L., Boniol, M., Zheng, T., Zhang, Y., Pasterk, M., Smans, M., Curado, M.P., Mullie, P., Gandini, S., Bota, M., Bolli, G., Rosenstock, J., Autier, P., 20-9-2012–. Diabetes and breast cancer risk: A meta-analysis. British Journal of Cancer 107, 1608–1617.

Campagnoli, C., Pasanisi, P., Abba, C., Ambroggio, S., Biglia, N., Brucato, T., Colombero, R., Danese, S., Donadio, M., Venturelli, E., Zito, G., Berrino, F., 2012. Effect of different doses of metformin on serum testosterone and insulin in non-diabetic women with breast cancer: A randomized study. Clinical Breast Cancer 12, 175–182.

Castillo, J.J., Mull, N., Reagan, J.L., Nemr, S., Mitri, J., 2012. Increased incidence of non-Hodgkin lymphoma, leukemia, and myeloma in patients with diabetes mellitus type 2: A meta-analysis of observational studies. Blood 119, 4845–4850.

Cazzaniga, M., Decensi, A., Pruneri, G., Puntoni, M., Bottiglieri, L., Varricchio, C., Guerrieri-Gonzaga, A., Gentilini, O.D., Pagani, G., Dell'Orto, P., Lazzeroni, M., Serrano, D., Viale, G., Bonanni, B., 2013. The effect of metformin on apoptosis in a breast cancer presurgical trial. British Journal of Cancer 109, 2792–2797.

Chak, A., Buttar, N.S., Foster, N.R., Seisler, D.K., Marcon, N.E., Schoen, R., Cruz-Correa, M.R., Falk, G.W., Sharma, P., Hur, C., Katzka, D.A., Rodriguez, L.M., Richmond, E., Sharma, A.N., Smyrk, T.C., Mandrekar, S.J., Limburg, P.J., 2015. Metformin does not reduce markers of cell proliferation in esophageal tissues of patients with Barrett's esophagus. Clinical Gastroenterology and Hepatology 13, 665–672.

DeCensi, A., Guerrieri-Gonzaga, A., Pruneri, G., Puntoni, M., Cazzaniga, M., Vingiani, A., Serrano, D., Gentilini, O., Johansson, H., Aristarco, V., Lazzeroni, M., Varicchio, C., Petri, M., Viale, G., Bonanni, B, 2014–. Metformin decreases Ki67 in HER2+ve ductal carcinoma in situ in a window of opportunity trial. San Antonio Breast Cancer Symposium.

Decensi, A., Puntoni, M., Goodwin, P., Cazzaniga, M., Gennari, A., Bonanni, B., Gandini, S., 2010. Metformin and Cancer Risk in Diabetic Patients: A Systematic Review and Meta-analysis. Cancer Prevention Research (Philadelphia, PA) 3, 1451–1461.

Deng, L., Gui, Z., Zhao, L., Wang, J., Shen, L., 2012. Diabetes mellitus and the incidence of colorectal cancer: An updated systematic review and meta-analysis. Digestive Diseases and Sciences 57, 1576–1585.

Franciosi, M., Lucisano, G., Lapice, E., Strippoli, G.F., Pellegrini, F., Nicolucci, A., 2013. Metformin therapy and risk of cancer in patients with type 2 diabetes: Systematic review. PLoS ONE 8, e71583.

Friberg, E., Orsini, N., Mantzoros, C.S., Wolk, A., 2007. Diabetes mellitus and risk of endometrial cancer: A meta-analysis. Diabetologia 50, 1365–1374.

Gandini, S., Puntoni, M., Heckman-Stoddard, B.M., Dunn, B.K., Ford, L., Decensi, A., Szabo, E., 2014. Metformin and cancer risk and mortality: A systematic review and meta-analysis taking into account biases and confounders. Cancer Prevention Research (Philadelphia, PA) 7, 867–885.

Gao, S., Yang, W.S., Gao, J., Wang, J., Xiang, Y.B., 2010. A meta-analysis of cohort studies on the association between diabetes and the risk of primary liver cancer. Zhonghua Yu Fang Yi. Xue Za Zhi 44, 711–716.

Ge, Z., Ben, Q., Qian, J., Wang, Y., Li, Y., 2011. Diabetes mellitus and risk of gastric cancer: A systematic review and meta-analysis of observational studies. European Journal of Gastroenterology & Hepatology 23, 1127–1135.

Goodwin, P.J., Parulekar, W.R., Gelmon, K.A., Shepherd, L.E., Ligibel, J.A., Hershman, D.L., Rastogi, P., Mayer, I.A., Hobday, T.J., Lemieux, J., Thompson, A.M., Pritchard, K.I., Whelan, T.J., Mukherjee, S.D., Chalchal, H.I., Oja, C.D., Tonkin, K.S., Bernstein, V., Chen, B.E., Stambolic, V., 2015. Effect of metformin vs placebo on and metabolic factors in NCIC CTG MA.32. Journal of the National Cancer Institute 107.

Goodwin, P.J., Pritchard, K.I., Ennis, M., Clemons, M., Graham, M., Fantus, I.G., 2008. Insulin-lowering effects of metformin in women with early breast cancer. Clin Breast Cancer 8, 501–505.

Hadad, S., Iwamoto, T., Jordan, L., Purdie, C., Bray, S., Baker, L., Jellema, G., Deharo, S., Hardie, D.G., Pusztai, L., Moulder-Thompson, S., Dewar, J.A., Thompson, A.M., 2011. Evidence for biological effects of metformin in operable breast cancer: A pre-operative, window-of-opportunity, randomized trial. Breast Cancer Research and Treatment 128, 783–794.

Hardefeldt, P.J., Edirimanne, S., Eslick, G.D., 2012. Diabetes increases the risk of breast cancer: A meta-analysis. Endocrine-Related Cancer 19, 793–803.

Higurashi, T., Hosono, K., Takahashi, H., Komiya, Y., Umezawa, S., Sakai, E., Uchiyama, T., Taniguchi, L., Hata, Y., Uchiyama, S., Hattori, A., Nagase, H., Kessoku, T., Arimoto, J., Matsuhashi, N., Inayama, Y., Yamanaka, S., Taguri, M., Nakajima, A., 2016. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase 3 trial. The Lancet Oncology 17, 475–483.

Hosono, K., Endo, H., Takahashi, H., Sugiyama, M., Sakai, E., Uchiyama, T., Suzuki, K., Iida, H., Sakamoto, Y., Yoneda, K., Koide, T., Tokoro, C., Abe, Y., Inamori, M., Nakagama, H., Nakajima, A., 2010. Metformin suppresses colorectal aberrant crypt foci in a short-term clinical trial. Cancer Prevention Research (Philadelphia, PA) 3, 1077–1083.

Huang, W., Ren, H., Ben, Q., Cai, Q., Zhu, W., Li, Z., 2012. Risk of esophageal cancer in diabetes mellitus: A meta-analysis of observational studies. Cancer Causes Control 23, 263–272.
Jiang, Y., Ben, Q., Shen, H., Lu, W., Zhang, Y., Zhu, J., 2011. Diabetes mellitus and incidence and mortality of colorectal cancer: A systematic review and meta-analysis of cohort studies. European Journal of Epidemiology 26, 863–876.
Jing, W., Jin, G., Zhou, X., Zhou, Y., Zhang, Y., Shao, C., Liu, R., Hu, X., 2012. Diabetes mellitus and increased risk of cholangiocarcinoma: A meta-analysis. European Journal of Cancer Prevention 21, 24–31.
Joshua, A.M., Zannella, V.E., Downes, M.R., Bowes, B., Hersey, K., Koritzinsky, M., Schwab, M., Hofmann, U., Evans, A., van der, K.T., Trachtenberg, J., Finelli, A., Fleshner, N., Sweet, J., Pollak, M., 2014. A pilot 'window of opportunity' neoadjuvant study of metformin in localised prostate cancer. Prostate Cancer and Prostatic Diseases 17, 252–258.
Kalinsky, K., Crew, K.D., Refice, S., Xiao, T., Wang, A., Feldman, S.M., Taback, B., Ahmad, A., Cremers, S., Hibshoosh, H., Maurer, M., Hershman, D.L., 2014. Presurgical trial of metformin in overweight and obese patients with newly diagnosed breast cancer. Cancer Investigation 32, 150–157.
Kasper, J.S., Giovannucci, E., 2006. A meta-analysis of diabetes mellitus and the risk of prostate cancer. Cancer Epidemiology, Biomarkers and Prevention 15, 2056–2062.
Larsson, S.C., Mantzoros, C.S., Wolk, A., 2007. Diabetes mellitus and risk of breast cancer: A meta-analysis. International Journal of Cancer 121, 856–862.
Larsson, S.C., Orsini, N., Brismar, K., Wolk, A., 2006. Diabetes mellitus and risk of bladder cancer: A meta-analysis. Diabetologia 49, 2819–2823.
Larsson, S.C., Wolk, A., 2011. Diabetes mellitus and incidence of kidney cancer: A meta-analysis of cohort studies. Diabetologia 54, 1013–1018.
Laskov, I., Drudi, L., Beauchamp, M.C., Yasmeen, A., Ferenczy, A., Pollak, M., Gotlieb, W.H., 2014. Anti-diabetic doses of metformin decrease proliferation markers in tumors of patients with endometrial cancer. Gynecologic Oncology 134, 607–614.
Lee, J.Y., Jeon, I., Lee, J.M., Yoon, J.M., Park, S.M., 2013. Diabetes mellitus as an independent risk factor for lung cancer: A meta-analysis of observational studies. European Journal of Cancer 49, 2411–2423.
Liao, S., Li, J., Wei, W., Wang, L., Zhang, Y., Li, J., Wang, C., Sun, S., 2011. Association between diabetes mellitus and breast cancer risk: A meta-analysis of the literature. Asian Pacific Journal of Cancer Prevention 12, 1061–1065.
Ma, S., Zheng, Y., Xiao, Y., Zhou, P., Tan, H., 2017. Meta-analysis of studies using metformin as a reducer for liver cancer risk in diabetic patients. Medicine (Baltimore) 96, e6888.
Mitri, J., Castillo, J., Pittas, A.G., 2008. Diabetes and risk of Non-Hodgkin's lymphoma: A meta-analysis of observational studies. Diabetes Care 31, 2391–2397.
Mitsuhashi, A., Kiyokawa, T., Sato, Y., Shozu, M., 2014. Effects of metformin on endometrial cancer cell growth in vivo: A preoperative prospective trial. Cancer 120, 2986–2995.
Niraula, S., Dowling, R.J., Ennis, M., Chang, M.C., Done, S.J., Hood, N., Escallon, J., Leong, W.L., McCready, D.R., Reedijk, M., Stambolic, V., Goodwin, P.J., 2012. Metformin in early breast cancer: A prospective window of opportunity neoadjuvant study. Breast Cancer Research and Treatment 135, 821–830.
Noto, H., Goto, A., Tsujimoto, T., Noda, M., 2012. Cancer risk in diabetic patients treated with metformin: A systematic review and meta-analysis. PLoS ONE 7, e33411.
Noto, H., Tsujimoto, T., Sasazuki, T., Noda, M., 2011. Significantly increased risk of cancer in patients with diabetes mellitus: A systematic review and meta-analysis. Endocrine Practice 17, 616–628.
Ren, H.B., Yu, T., Liu, C., Li, Y.Q., 2011. Diabetes mellitus and increased risk of biliary tract cancer: Systematic review and meta-analysis. Cancer Causes Control 22, 837–847.
Schmid, D., Behrens, G., Jochem, C., Keimling, M., Leitzmann, M., 2013. Physical activity, diabetes, and risk of thyroid cancer: A systematic review and meta-analysis. European Journal of Epidemiology 28, 945–958.
Schuler, K.M., Rambally, B.S., DiFurio, M.J., Sampey, B.P., Gehrig, P.A., Makowski, L., Bae-Jump, V.L., 2015. Antiproliferative and metabolic effects of metformin in a preoperative window clinical trial for endometrial cancer. Cancer Medicine 4, 161–173.
Soranna, D., Scotti, L., Zambon, A., Bosetti, C., Grassi, G., Catapano, A., La, V.C., Mancia, G., Corrao, G., 2012. Cancer risk associated with use of metformin and sulfonylurea in type 2 diabetes: A meta-analysis. Oncologist 17, 813–822.
Thakkar, B., Aronis, K.N., Vamvini, M.T., Shields, K., Mantzoros, C.S., 2013. Metformin and sulfonylureas in relation to cancer risk in type II diabetes patients: A meta-analysis using primary data of published studies. Metabolism 62, 922–934.
Tian, T., Zhang, L.Q., Ma, X.H., Zhou, J.N., Shen, J., 2012. Diabetes mellitus and incidence and mortality of gastric cancer: A meta-analysis. Experimental and Clinical Endocrinology and Diabetes 120, 217–223.
Wang, C., Wang, X., Gong, G., Ben, Q., Qiu, W., Chen, Y., Li, G., Wang, L., 2012. Increased risk of hepatocellular carcinoma in patients with diabetes mellitus: A systematic review and meta-analysis of cohort studies. International Journal of Cancer 130, 1639–1648.
Wang, L., Wang, L., Zhang, J., Wang, B., Liu, H., 2017. Association between diabetes mellitus and subsequent ovarian cancer in women: A systematic review and meta-analysis of cohort studies. Medicine (Baltimore) 96, e6396.
Wu, L., Zhu, J., Prokop, L.J., Murad, M.H., 2015. Pharmacologic Therapy of Diabetes and Overall Cancer Risk and Mortality: A Meta-Analysis of 265 Studies. Science Reporter 5, 10147.
Zhu, Z., Wang, X., Shen, Z., Lu, Y., Zhong, S., Xu, C., 2013. Risk of bladder cancer in patients with diabetes mellitus: an updated meta-analysis of 36 observational studies. BMC Cancer 13, 310.

Further Reading

Farmer, R.E., Ford, D., Forbes, H.J., et al., 2017. Metformin and cancer in type 2 diabetes: A systematic review and comprehensive bias evaluation. International Journal of Epidemiology 46, 745.
Heckman-Stoddard, B.M., Gandini, S., Puntoni, M., Dunn, B.K., et al., 2016. Repurposing old drugs to chemoprevention: The case of metformin. Seminars in Oncology 43, 123–133.
Lei, Y., Yi, Y., Liu, Y., et al., 2017. Metformin targets multiple signaling pathways in cancer. Chinese Journal of Cancer 36, 17.
Provinciali, N., Lazzeroni, M., Cazzaniga, M., Gorlero, F., Dunn, B.K., Decensi, A., 2015. Metformin: Risk-benefit profile with a focus on cancer. Expert Opinion on Drug Safety 14, 1573–1585.

Mevalonate Pathway

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Glossary

Aceto(acetyl)-CoA Acetyl CoA is formed from pyruvate in the final stages of glycolysis and is also a metabolite from fatty acid catabolism (beta oxidation) and a precursor of ketone bodies, which are accumulated in a status of hunger. Addition of an acetyl-residue results in aceto(acetyl)-CoA, a substrate for enzymes of the upper mevalonate pathway.

Bisphosphonate Also named diphosphonates, group of chemical compounds which contain two phosphonate groups, this group of drugs is known to inhibit osteoclasts.

GTPases A large family of hydrolase enzymes that can bind to and hydrolyze guanosine triphosphate (GTP). The GTP binding and hydrolysis takes place in the highly conserved G domain, which is common to all GTPases.

NAD(P)H Nicotinamide adenine dinucleotide phosphate, abbreviated $NADP^+$, is a cofactor on anabolic reactions, which require NAD(P)H as a reducing agent. $NADP^+$ differs from NAD^+ in the presence of an additional phosphate group on the 2′ position of the ribose ring that carries the adenine moiety.

Prenylation The addition of hydrophobic molecules to a protein or to a chemical compound. It is assumed that prenyl groups (3-methyl-but-2-en-1-yl) facilitate attachment to cell membranes.

RHOA and RHOB A family of small (~21 kDa) signaling G proteins. The members of the Rho family have been shown to regulate many aspects of intracellular actin dynamics and metabolic processes.

Squalene A 30′carbon unsaturated oily liquid hydrocarbon and an important metabolite in cholesterol biosynthesis.

Sterol regulatory element-binding protein (SREBP) SREBPs are transcription factors that bind to the sterol regulatory element DNA sequence TCACNCCAC.

Statin A class of drugs that reduce the levels of lipids in the blood by altering the enzyme activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR).

Sterol isoprenoids Basically, isoprenoids are a class of organic compounds composed of two or more units of hydrocarbons, with each unit consisting of five carbon atoms arranged in a specific pattern. These compounds include certain sterols, oxysterols, farnesol, and geranylgeraniol, as well as the diphosphate derivatives of isopentenyl, geranyl, farnesyl, geranylgeranyl, and presqualene. They regulate transcriptional and posttranscriptional events that in turn affect lipid synthesis, meiosis, apoptosis, developmental patterning, protein cleavage, and protein degradation.

Introduction

The mevalonate pathway (MP) also known as the isoprenoid pathway or 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway is an anabolic pathway providing metabolites for multiple cellular processes in eukaryotes, archaea, as well as some bacteria, thus underscoring its importance for nearly all living organisms including humans.

The mevalonate which is produced from acetoacetyl-CoA by HMGCR (Fig. 1) is further processed to sterol isoprenoids, such as cholesterol, which is an indispensable precursor of bile acids, lipoproteins, and steroid hormones, and to a number of hydrophobic molecules including nonsterol isoprenoids, such as dolichol, heme-A, isopentenyl tRNA, and ubiquinone. Intermediates of this network play important roles in the posttranslational modification of a multitude of proteins involved in inter- and intracellular signaling.

Besides its key role for cholesterol synthesis, the MP has become a challenging topic, when a large number of experimental and clinical studies suggested that inhibition of the MP might have valuable interest in human disease the management of multiple human diseases, besides cardiovascular diseases. Molecules arising from the MP are essential for cell growth and differentiation. They appear to be potential interesting therapeutic targets for many areas of ongoing research: oncology, autoimmune disorders, atherosclerosis, and Alzheimer disease. Also, considerable progress has been made in understanding the pathophysiology of two autoinflammatory disorders resulting from an inherited deficiency of mevalonate kinase (MK), the first committed enzyme of the MP.

Biochemistry of the MP

Upper MP

The mevalonate–isoprenoid pathway involves first the synthesis of 3-hydroxy-3-methylglutaryl-CoA (HMG)-CoA from acetyl-CoA through acetoacetylCoA. HMGCR, one of the best-regulated enzymes in nature, catalyzes the conversion of HMG-CoA to mevalonic acid. HMGCR is the rate-limiting enzyme of MP.

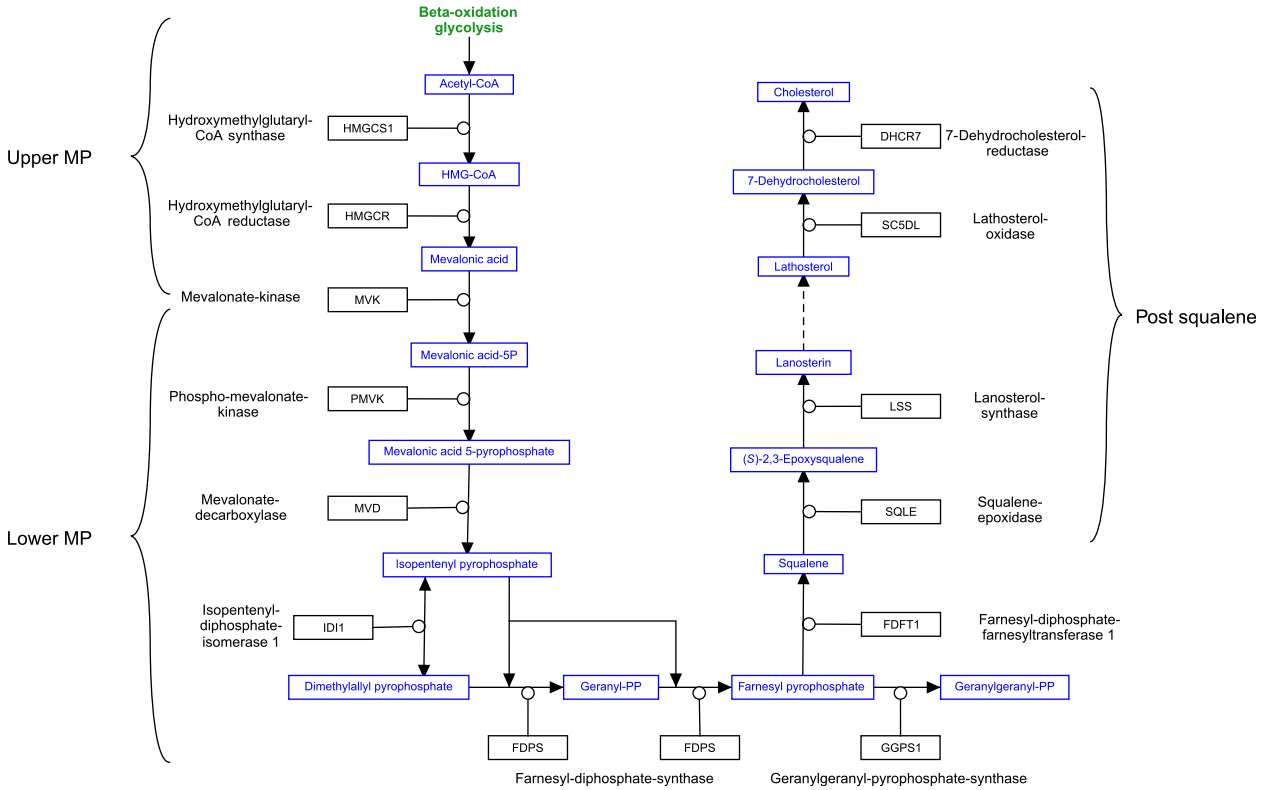

Fig. 1 Chemical reactions of the mevalonate pathway (MP). MP pathway enzymes condense three acetyl-CoA molecules in a two-step reaction to produce 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). Both reactions are reversible and in equilibria, with the intracellular concentration of acetyl-CoA being the primary driver. HMG-CoA is then reduced by HMG-CoA reductase (HMGCR) to produce mevalonic acid (MA) via an irreversible reaction. MA is then converted into isopentenyl-diphosphate through a series of enzymatic steps, which serves as a monomeric unit for the consequent synthesis of all downstream metabolites. *Dashed arrows* indicate multiple steps.

In the absence of sterol isoprenoids in the cell, a family of transcription factors, named sterol regulatory element-binding proteins (SREBPs), directly activates HMGCR gene transcription. SREBPs regulate not only HMGCR gene transcription but also every step of cholesterol synthetic pathway by increasing gene expression of all the enzymes acting in the MP. In addition, other regulatory mechanisms can influence the activity of HMGR. The degradation rate of HMGR protein is influenced by cell's requirements for isoprenoids. Cell's requirements for isoprenoids will determine the rate of translation of HMGCR mRNA.

The investigation of yeast HMGCR accounted for mevalonate production following the reaction:

$$S-HMG-CoA+2NADPH+H^{+}\rightarrow R-mevalonate+2NADP+CoASH$$

The enzyme HMGCR is found in eukaryotes, archaea, and some eubacteria. The conversion of the thioesterified HMGCoA carboxyl to an alcohol represents a two-step reduction, accounting for the stoichiometry of NADPH in the reaction. The reaction thus proceeds through the successive reduction steps to first produce bound mevaldyl-CoA, collapse of the thiohemiacetal to release CoASH and form mevaldehyde; the second reduction step then forms product mevalonate.

The eukaryotic proteins (class I HMG-CoA reductases) are associated with the endoplasmic reticulum (ER) and interact through membrane spanning helices in the N-terminal domain. Consequently, the catalytic domain follows this membrane-anchoring sequence. These class I enzymes are potently inhibited by the class of statin drugs that effectively modulate sterol synthesis and, as a result, have been heavily investigated. There is no homologous sequence for membrane association at the N-terminus in the bacterial HMG-CoA reductases (class II), and a few of these (some in recombinant form) have been isolated as soluble proteins. The *Pseudomonas mevalonii* enzyme has a degradative function, allowing this microbe to grow on mevalonate as a carbon source. In contrast, the *Staphylococcus aureus* enzyme has a biosynthetic function and is encoded by a gene within a MP gene cluster.

Despite the low overall sequence homology (<20%) and overall protein structure architecture between class I (eukaryotic) and class II (bacterial) HMGCR enzymes, there is considerable similarity between these enzymes in the positioning of active site residues important to catalytic function. Residues from two different subunits contribute to an active site. The histidine proposed to function in protonation of product Coenzyme A is appropriately positioned for this role. The aspartate implicated by mutagenesis is located within the active site and involved in a hydrogen bond network with the lysine and the glutamate that have been identified by functional studies. While both lysine and glutamate are in close proximity to the HMG-CoA thioester carbonyl that is reduced to form

mevalonate, there are different proposals concerning their precise roles in substrate carbonyl polarization and/or the proton transfers that accompany substrate reduction by NADPH.

Lower MP

The lower MP converts mevalonate into the relatively unreactive isopentenyl pyrophosphate (IPP), which further is converted to the more reactive electrophile dimethylallyl pyrophosphate. There exist (at least) three variants of the lower MP: In eukaryotes, mevalonate (MV) is phosphorylated twice in the 5-OH position and then decarboxylated to yield IPP. In some archaea such as *Haloferax volcanii*, mevalonate is phosphorylated once in the 5-OH position, decarboxylated to yield isopentenyl phosphate (IP), and finally phosphorylated again to yield IPP (Archaeal Mevalonate Pathway I). A third MP variant found in the archaeon *Thermoplasma acidophilum* phosphorylates mevalonate at the 3-OH position followed by phosphorylation at the 5-OH position. The resulting metabolite, mevalonate-3,5-bisphosphate, is decarboxylated to IP and finally phosphorylated to yield IPP (Archaeal Mevalonate Pathway II).

In eukaryotes, the above-mentioned phosphorylation is done by MK. MK also known as MVK or ATP: mevalonate 5-phosphotransferase catalyzes the transfer of ATP's c-phosphoryl to the C5 hydroxyl oxygen of mevalonic acid, resulting in formation of mevalonate 5-phosphate and ADP. The reaction was characterized in yeast; the protein is found in eukaryotes, archaea, and certain eubacteria. The enzyme was highly purified from porcine liver and was demonstrated to catalyze a sequential reaction with mevalonate substrate binding first and MgADP product released.

MK is the second essential enzyme of the isoprenoid/cholesterol biosynthesis pathway, after HMGCR, catalyzing the phosphorylation of mevalonic acid into phosphomevalonate. Although MK not has the rate-limiting properties of HMGCR, it is demonstrated that MK activity is regulated via feedback inhibition by intermediates in the isoprenoid/cholesterol pathway geranyl pyrophosphate, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP).

High-affinity feedback inhibition has also been observed using a recombinant human protein and contrasted with lower-potency inhibition (10^{-5} M) of the *Staphylococcus aureus* enzyme.

Inherited human mevalonate kinase (MVK) mutations are correlated with two diseases, accounting for mevalonic aciduria (MVA) and Hyper-IgD syndrome.

Phosphomevalonate kinase (PMK) catalyzes the next step in isoprenoid/sterol biosynthesis, converting mevalonate 5-phosphate and ATP to mevalonate 5-diphosphate and ADP.

Activity of this enzyme was demonstrated in pig liver, and the pig liver enzyme has subsequently been isolated and more extensively characterized. PMK is found in eukaryotes and some eubacteria. The amino acid sequences for animal and low-homology invertebrate PMK proteins are not orthologous to those for PMK in plants, fungi, and bacteria. Thus, the proteins that catalyze the enzymatic reaction differ widely, depending on their source. Animal and invertebrate PMK proteins are known for a tertiary and quaternary structure, which is typical of the nucleoside monophosphate kinase family. The other PMK proteins are members of the galactokinase, homoserine kinase, MK, and phosphomevalonate kinase (GHMP) family. The tissue-isolated pig enzyme is reported to catalyze an ordered sequential reaction with mevalonate 5-phosphate assigned as the first substrate bound and ADP as the last product released. A recombinant form of *Streptococcus pneumoniae* has been characterized and reported to catalyze a random sequential bi-bi reaction. A recombinant form of *Enterococcus faecalis* PMK has also been isolated and characterized. The sequence of human PMK has been deduced.

Functional investigations of the recombinant human enzyme showed that the reaction catalyzed by PMK is a reversible reaction; kinetic constants of human PMK have been determined for both forward (formation of mevalonate 5-diphosphate) and reverse (formation of mevalonate 5-phosphate) reaction.

In the next step, mevalonate diphosphate decarboxylase; various abbreviations appear in the literature: MVD (in Fig. 1), also known as MDD, MPD, DPMDC, catalyzes the ATP-dependent decarboxylation of mevalonate 5-diphosphate to form isopentenyl 5-diphosphate (IPP), as indicated in the equation (Fig. 1).

This reaction is essential to the MP of polyisoprenoid and sterol synthesis. Activity has been measured in animals, plants, and yeast. Genetic complementation has implied activity in the *Staphylococcus aureus* and *Trypanosoma brucei* proteins. Highly purified active enzyme has been prepared from avian, porcine, and rat tissue and in recombinant form from bacteria expressing the yeast, human *Trypanosoma brucei*, and *Staphylococcus aureus* proteins. Characterization of the tissue-isolated protein implied the presence of an arginine that influences activity of the avian enzyme and documented the selectivity for divalent cation. The avian enzyme was also used to demonstrate that the transient phosphoryl transfer to the C3 oxygen proceeds with inversion of stereochemistry and, thus, no covalent E–P intermediate forms.

Farnesyl transferase (FTase) and geranylgeranyl transferases (GGTase) are two enzymes that carry out the process of prenylation in the cell. This process involves the covalent attachment of hydrophobic molecules (either the C-15 isoprene farnesyl or the C-20 isoprene geranylgeranyl groups) to the C-terminal end of some proteins including the γ-subunit of heterotrimeric G proteins, heme-A, nuclear lamins, and small GTP-binding proteins. Prenylation promotes the attachment of these proteins to internal cell membranes by means of a lipid anchors such as palmitate.

Such posttranslational modifications and activation GTP-binding proteins Rho, Rac, Rab, Rap, Ras play an important role in many important signaling cascades within the cell. Downstream from FPP the squalene synthase (FDFT1, also known as farnesyl-diphosphate farnesyl transferase 1) catalyzes the first committed step of the specific hepatic cholesterol biosynthesis at the final branch point of the cholesterol biosynthetic pathway, converting farnesyl-pyrophosphate into squalene. Squalene is then converted after a two-step cyclization into lanosterol, which is converted to cholesterol after a series of additional reactions.

Another downstream branch from FPP is catalyzed by the enzyme geranylgeranyl diphosphate synthase 1 (GGPS1) and leads to the synthesis of GGPP from farnesyl diphosphate and isopentenyl diphosphate. GGPP is an important molecule responsible for the C20-prenylation of proteins and for the regulation of a nuclear hormone receptor (**Fig. 1**).

Prenylation of Small GTPases and Brain Function

As mentioned earlier, FPP and GGPP are substrates for protein prenylation, and small GTP-binding proteins (sGTPases) represent the largest and most extensive characterized group of prenylated proteins. This superfamily is classified according to sequence and function similarity, into Ras, Rho, Rab, Sar1/Arf, and Ran families. The regulation of the activity of sGTPases is somewhat complex, and it has been extensively reviewed in greater detail, nonetheless, in general terms, sGTPases cycle between a guanosine diphosphate (GDP)- and GTP-bound conformation. The GDP-bound conformation is generally considered an inactive state, while the GTP-bound sGTPase activates downstream pathways by binding to specific effectors. sGTPases associate with the cytoplasmic leaflet of cellular membranes, where they perform their respective function. However, sGTPases are not transmembrane proteins, and in order to anchor to membranes they have to be covalently attached to lipid groups, with the exception of the so-called Ran (RAs-related nuclear protein) also known as GTP-binding nuclear proteins, for which no lipid modification has been reported. All the members of Ras, Rho, and Rab families undergo the lipid posttranslational modification of prenylation as the first committed step toward membrane association, while ARF family members undergo myristoylation. Both FPP and GGPP can be used as moieties for protein prenylation, catalyzed by the enzymes FTase and GGTase-I and -II, respectively.

Rho GTPases are essential players in neuronal development and function. Their activity is critical for neuronal cell migration, axon growth and guidance, dendritic arborization, dendritic spine formation and stabilization, growth cone motility and collapse, and synapse formation. Therefore, it is not surprising that studies in which statins or inhibitors of protein prenyltransferases were used, a decrease in neurite outgrowth and dendritic spines density was observed. In fact, inhibiting GGTase-I in vivo induces deficits in memory and learning in mice. However, there are also a number of studies showing that statins can actually increase neurite outgrowth, which might be explained by the different cell models, dosages, and statins that were used. Interestingly, neuronal survival has also been observed to diminish in the presence of statins, which was shown to be independent of a reduction on cholesterol levels, since the reversal of this phenotype was obtained by FPP/GGPP supplementation, but not by cholesterol addition.

Studies have shown that increased expression of CYP46A1 (= cholesterol 24-hydroxylase) in neurons enhanced prenylation and activation of sGTPases of the Rho and Rab family, and that this effect was dependent on the activation of the MP and GGTase-I activity, which is in accordance to previously mentioned studies. Moreover, it has been reported that this increase of sGTPases prenylation induced by CYP46A1 leads to an increase in neuronal dendritic outgrowth and dendritic protrusion density and elicits an increase of synaptic proteins in crude synaptosomal fractions, further highlighting the importance of how the activation of the MP and sustained production of isoprenoids are essential for neuronal development and function.

It is noteworthy that FPP and GGPP levels are much higher in the brain than in other tissues. Accordingly, in mouse brain cytosol, FPP and GGPP synthase activities are higher than those in the corresponding fractions from the liver, perhaps reflecting a higher demand for protein prenylation in the brain. Furthermore, it has been reported that in aged mouse brain, there is a reduction of GGTase-I activity, leading to a reduction of Rho GTPases membrane association, which is suggested to be one of the mechanisms underlying age-related cognitive dysfunction. There is a great lack of studies comparing the levels of nonsteroid products from the MP between neurons and astrocytes. It is noteworthy that CoQ10, which derives from the decaprenoide intermediary, has actually been measured and compared between both cell types (unpublished data reported in a PhD dissertation). Neurons were found to possess CoQ10 levels at around 68 pmol/mg protein, while in astrocytes the levels were significantly lower, about 38 pmol/mg protein. These results suggest that although neurons might have a lower cholesterol synthesis than astrocytes, an opposite profile might exist for isoprenoid synthesis, which supports our hypothesis that neurons undergo a shift from the postsqualene to the nonsterol branch of the MP. Based on those results and data presented in literature we hypothesize that the postsqualene branch and cholesterol synthesis might be downregulated in neurons to redirect the intrinsic MP toward the nonsterol branch, producing important isoprenoid intermediaries for neuronal function, while relying on external supply of cholesterol. Nevertheless, many questions remain unanswered: What are the underlying regulatory mechanisms involved the differential expression patterns of the MP enzymatic machinery in distinct neural cell types? Is the synthesis rate of isoprenoids different in different neural cell types? Is there a shuttle of isoprenoids from astrocytes to neurons? Is there a soluble factor released by astrocytes that signals for the shutdown of the postsqualene pathways in neurons? Are specific neuronal signaling pathways being modulated by isoprenoids levels, in a prenylation independent manner? Is there a specific deregulation of the nonsterol branch in neurons in pathological contexts? In order to answer these questions, the challenges ahead are also dependent on the development of new tools that facilitate imaging and quantification of the prenylome in neurons and astrocytes and to the identification their cognate protein prenyltransferases or unknown prenyl-binding proteins.

Evidence for a Redirection of the MP in Neurons

A compelling body of evidence suggests that, although neurons retain an active MP and can synthesize cholesterol, the efficiency of cholesterol synthesis is markedly lower when compared to astrocytes, possibly due to lower expression levels of enzymes belonging

to the postsqualene branch. There exists a hypothesis indicating that the postsqualene branch and cholesterol synthesis might be downregulated in neurons, and with this downregulation there is a redirection of the intrinsic MP toward the nonsterol branch, while neurons still rely on external supply of cholesterol. This hypothesis seems to be corroborated by data from other laboratories that published a RNA-Seq transcriptome of 7 days postnatal (P7) mice cortical neurons, astrocytes, oligodendrocytes, and vascular cells that corroborated a previous analysis of an array dataset using RNA isolated from forebrain cells of P7 and P16–17 (= 16–17 days old) mice. Based on data present in these two distinct datasets, the expression levels of genes involved in nonsterol, pre-, and postsqualene pathways in neurons and astrocytes and also between P7 and P16 neurons were compared. Both datasets suggest that the pre- and postsqualene branches in P7 neurons are substantially downregulated compared to astrocytes (blue and red, respectively). Nonetheless, the genes related to the nonsterol pathway have a similar or a higher-expression level in neurons when compared to astrocytes, with the exception of prenyl (solanesyl) diphosphate synthase, subunit 1 (Pdss1). Furthermore, the array data also enabled to compare the expression levels of genes involved in the MP in P16 neurons when compared to P17 astrocytes. Although there are two genes upregulated in the presqualene pathway (blue), the majority of genes have decreased mRNA levels in neurons when compared to astrocytes. Similar to P7 cells, while P16 neurons exhibit a clear downregulation of postsqualene pathway, they maintain or even increase the mRNA levels of genes belonging to the nonsterol branch, when compared to astrocytes. These expression patterns may indicate that while maintaining the efficiency of the nonsterol branch some what intact or even upregulated, there is a robust downregulation of the pre- and postsqualene pathway in neurons compared to astrocytes, which is a profile that seems to be conserved between these two cell types throughout CNS maturation. The fact that the nonsterol branch, in contrast to the pre- and postsqualene pathways, is not downregulated and might actually be somehow induced in neurons, is in line with the idea that the neuronal MP might be favoring the production of isoprenoids in detriment of cholesterol, which is ought to be supplied by astrocytes.

The decrease in mRNA levels from MP-associated genes in the presqualene pathway observed in neurons also favors the nonsterol pathway. Indeed, the affinity of GGPPS for FPP (*Km* value of 0.6 μM) is much higher than SQS (*Km* value of ~15 μM), thus under limited concentrations of FPP, isoprenoid synthesis will be favored. These kinetic findings together with the fact that during neuronal maturation the mRNA levels of Gggps increase while Fdft1 (the gene that codes for SQS) decrease argues that the nonsterol branch might be boosted in neurons in detriment of the postsqualene pathway. Accordingly, as previously mentioned, sGTPases activity is crucial during neuronal development and has been widely associated with neuronal dendritic development and synaptic regulation. Hence, as neurons mature, the demand for isoprenoids to prenylate sGTPases should increase, which is in agreement with the hypothesis of a redirection of the MP branch toward production of nonsterol intermediaries, while cholesterol needs are met by uptake from astrocytic-derived lipoproteins.

Role of the MP in Cancer

Cancer cells reprogram their metabolism to provide energy and the essential building blocks required to maintain their aberrant survival and growth. This reprogramming may occur through either mutations in metabolic enzymes (e.g., isocitrate dehydrogenases (IDHs)) or alterations in cell signaling owing to oncogenic events and/or the remodeled tumor microenvironment. These activated signaling cascades in turn deregulate the expression and/or the activity of enzymes in key metabolic pathways, including the MP.

The MP uses acetyl-CoA, NADPH, and ATP to produce sterols and isoprenoids that are essential for tumor growth (Fig. 1). The production of acetyl-CoA occurs following glucose, glutamine, or acetate consumption, which are often increased in cancer cells. NADPH is produced from a variety of sources, including the pentose phosphate pathway, malic enzyme, and IDHs. Therefore, the MP is highly integrated into the overall metabolic network of cancer cells. The transcription of genes encoding MP enzymes is primarily controlled by the SREBP family of basic helix–loop–helix leucine zipper transcription factors. When intracellular sterol levels are high, the SREBPs are maintained in an inactive state at the ER, where some MP enzymes are also localized. In response to sterol deprivation, a feedback response is initiated that leads to the SREBPs, along with their binding partner SREBP cleavage-activating protein (SCAP), dissociating from the insulin-induced genes (INSIGs) and translocating from the ER to the Golgi. At the Golgi, the SREBPs are sequentially cleaved by site-1 protease and site-2 protease, and they translocate to the nucleus where they bind to sterol regulatory elements (SREs) in the promoters of their target genes and activate the transcription of MP genes to restore sterol and isoprenoid levels. The importance of MP metabolites to the survival of cancer cells has been highlighted by recent studies that have identified a large number of MP enzymes as essential for the survival of several cancer cell lines. Additionally, numerous studies have shown that the statin family of drugs, which inhibit the initial flux-controlling enzyme of the MP, HMGCR, decrease growth and increase apoptosis in many cancer types in vitro and in vivo. These observations point to the MP as being a key dependency in tumors, and one that is readily targetable.

The MP has been suggested by some studies to be oncogenic. Early work in chronic lymphocytic leukemia showed that mevalonate can stimulate replication in primary leukemic cells. In another study, overexpression of the catalytic domain of HMGCR in primary mouse embryonic fibroblasts cooperated with HRASG12V to promote foci formation, suggesting that HMGCR is a metabolic oncogene. In addition, the direct infusion of MVA into mice harboring breast cancer cell xenografts caused an increase in tumor growth. Data from primary patient samples also suggest a role for the MP in promoting tumorigenesis, with a higher expression of MP genes correlating with poor prognosis in breast cancer. Collectively, this evidence indicates that the MP has a key role in cancer.

A series of evidences demonstrated that the MP is deregulated in cancer through aberrant cell signaling, which in turn establishes a tumor vulnerability that can be therapeutically targeted to improve outcomes for cancer patients.

MP-Derived Metabolites in Cancer

Initially, the regulation and function of the MP and its metabolites were studied in the context of normal and hypercholesterolemic tissues, which led to the Nobel prize-winning discoveries of Bloch and Lynen in 1964, and Brown and Goldstein in 1985. In recent years, the importance of MP-derived metabolites in cancer has become increasingly appreciated (discussed later).

Cholesterol

Cholesterol is an important component of most cellular membranes. Highly proliferative cancer cells need to produce membranes rapidly, and an increase in cholesterol synthesis contributes to this process. Cholesterol is also an integral component of lipid rafts, which are necessary to form signaling complexes. The cholesterol content of the ER has recently been linked to the antiviral type I interferon (IFN) response, with low ER cholesterol triggering an IFN response in macrophages that protects mice from viral challenge. Therefore, it is possible that high levels of cholesterol, produced by the MP, could have a role in protecting cancer cells from immune surveillance and various therapies. Cholesterol also serves as the precursor of downstream products, such as steroid hormones and oxysterols: steroid hormones drive the initiation and progression of various cancers, including breast and prostate carcinomas; increased oxysterol production can activate the liver X receptors, which have been proposed to be therapeutic targets in multiple cancer types. Therefore, cancer cells require cholesterol for growth and survival, and decreasing intracellular cholesterol biosynthesis is a promising anticancer strategy.

Isopentenyl-Diphosphate

In human cells, the MP is the sole intracellular source of isopentenyl-diphosphate (IPP) (**Fig. 1**). Aberrant activation of the MP in cancer results in increased intracellular levels of IPP, which has been shown to activate host γδ T cells that subsequently kill the IPP-overexpressing cells. These observations led to phase I clinical trials that evaluated the in vivo expansion of γδ T cells in response to zoledronate, a bisphosphonate (BP) that inhibits farnesyl diphosphate synthase (FDPS) and leads to the accumulation of IPP, in combination with interleukin-2 (IL-2) treatment in advanced-stage breast cancer and prostate cancer. In both studies, the therapy was well-tolerated and the number of sustained peripheral γδ T cells correlated with improved clinical outcome. Future phase II clinical trials will reveal whether combined zoledronate and IL-2 therapy is an effective anticancer strategy.

Farnesyl-Diphosphate and Geranylgeranyl-Diphosphate

Farnesyl-diphosphate (FPP) and geranylgeranyl-diphosphate (GGPP) are produced by sequential condensation reactions of dimethylallyl-diphosphate with two or three units of IPP, respectively. FPP and GGPP contain hydrophobic chains that are essential for the isoprenylation of proteins. This posttranslational modification tethers proteins to cell membranes, enabling proper protein localization and function. Most small GTPases—many of which are involved in tumorigenesis, such as RAS and RHO—are isoprenylated; inhibition of the MP can reduce the isoprenylation of these small GTPases and can induce the death of some cancer cells. This cell death can be reversed by the addition of GGPP, and sometimes FPP, suggesting that these MP metabolites are essential for tumor cell viability. Evidence suggests that it is unlikely that any one isoprenylated protein can be assigned functional responsibility for this cancer cell dependency on GGPP and FPP; instead, it seems that this is a "class effect," with the depletion of these isoprenoid pools potentially affecting the many proteins that are isoprenylated. Despite this dependency, directly inhibiting the isoprenylation of proteins using geranylgeranyl transferase inhibitors (GGTIs) or farnesyl transferase inhibitors (FTIs) has not been a successful anticancer strategy to date. The rationale behind these drug development programs was that key isoprenylated oncoproteins, such as RAS, could be targeted. However, the efficacy of FTIs was impeded by alternative isoprenylation using GGPP, and GGTIs have been disappointingly toxic. Further development of next-generation FTIs and GGTIs remains a fairly limited and focused area of research.

Dolichol

Dolichol is derived from 18 to 20 IPP molecules and is an essential component of the *N*-glycosylation of nascent polypeptides in the ER. Protein *N*-glycosylation is frequently altered in cancer and can contribute to tumor formation, proliferation, and metastasis. Not all *N*-glycans are associated with tumor progression; the complex branching of *N*-glycans leads to tumor-suppressive properties in some cancers. Glucose-derived *N*-acetylglucosamine has recently been shown to be necessary for the *N*-glycosylation of SCAP before ER-to-Golgi translocation. The SCAP–SREBP complex thus remains inactive in the ER when glucose is absent, even in the presence of low levels of sterols.

Coenzyme Q

Isoprenoids are also used to produce the quinone coenzyme Q (CoQ). The hydrophobic isoprenoid chain localizes CoQ to the inner membrane of the mitochondria, where the quinone group transfers electrons from complex I or II to complex III of the electron transport chain, thus enabling ATP production. Therefore, CoQ is crucial for ATP production in cancer cells that rely on oxidative phosphorylation to produce energy.

Oncogenic Regulation of the MP

Intracellular pools of MP metabolites are tightly regulated by modulating the expression and activity of the MP enzymes. MP gene expression is mainly controlled by the SREBP transcription factors (Fig. 2). There are three SREBP proteins, which are transcribed from two genes: SREBP2 is transcribed from the *SREBF2* gene and is the main transcription factor for MP-associated genes; SREBP1a and SREBP1c are transcribed from alternative start sites in the *SREBF1* gene, with SREBP1a regulating the expression of both MP and fatty acid metabolism genes, and SREBP1c predominantly regulating the expression of fatty acid metabolism genes.

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) studies have indicated some overlap in the target genes of each SREBP, including MP genes, indicating some redundancy. Most studies have also shown an overlap in the regulation of the SREBPs; however, the majority of studies limit full characterization to SREBP1, and most do not distinguish between SREBP1a and SREBP1c as available antibodies cannot differentiate between the two. Given the importance of the MP in cancer, a complete characterization of SREBP2 in transformed cells is needed.

In recent years, oncogenic and tumor-suppressive pathways have been shown to converge on the MP and its regulatory feedback loop. Cancer cells, with their aberrant growth and metabolism, are thus primed to upregulate the MP to provide essential building blocks for continued proliferation. The integration of cellular signaling from growth factors and essential metabolites, with the regulation of the MP and its SREBP-regulated feedback response, highlights the importance of this pathway in cancer.

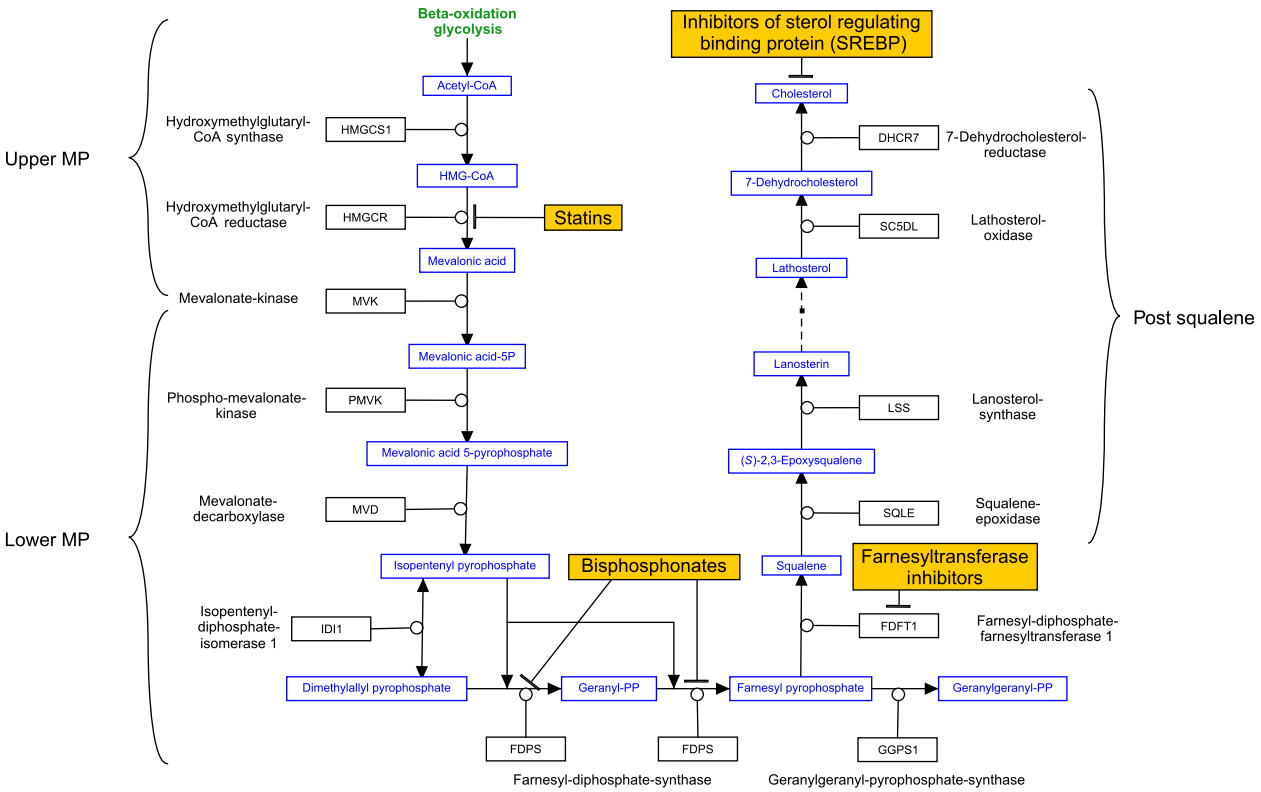

Fig. 2 Targets of inhibitors of the MP. *Statins* inhibit HMGCR, thereby reducing MP metabolites that are also essential for cancer cell growth and survival. This triggers sterol regulatory element-binding protein (SREBP) activation and the transcription of MP genes, thus restoring MP activity. Dipyridamole is one example of an agent that inhibits SREBP cleavage, preventing the restorative feedback response and increasing apoptosis in multiple cancer types. Combining SREBP cleavage inhibitors with statins may increase the therapeutic response compared with the use of statins alone. *Dashed boxes* represent metabolites or steps that are reduced by the indicated treatments. *Bisphosphonates* act downstream of statins and inhibit farnesyl pyrophosphate synthase, a key enzyme in the MP, with consecutive decrease of the formation of isoprenoid lipids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. This part of the MP is also targeted by *farnesyl transferase inhibitors*.

PI3K–AKT

The PI3K–AKT signaling pathway is a major regulator of cell survival and proliferation in response to growth factors. It is the single most frequently altered pathway in cancer, and the second most frequently mutated gene is *PIK3CA*, which encodes PI3K catalytic subunit alpha. Inactivating mutations in the PI3K–AKT pathway negative regulator PTEN and/or the hyperactivity of growth factor receptor tyrosine kinases are also common in cancer. Alterations in the PI3K–AKT pathway generally act to augment signaling and consequently increase the proliferation of cancer cells. PI3K–AKT can activate the MP through various mechanisms. For example, the stimulation of PI3K–AKT signaling by growth factors, such as insulin, platelet-derived growth factor, and vascular endothelial growth factor, can increase the mRNA and protein expression of SREBP1 and SREBP. It should be noted that although PI3K–AKT signaling strongly and consistently increases the mRNA and protein levels of SREBP1a and SREBP1c, its effects on SREBP2 expression are context dependent. AKT has also been suggested to increase the stability of nuclear SREBP1a, SREBP1c, and SREBP2 by preventing their proteasomal degradation mediated by the F-box and WD repeat domain containing 7 (FBXW7) E3 ubiquitin ligase. The importance of this degradation pathway is highlighted by an increase in cholesterol and fatty acid synthesis in FBXW7-deficient cells. The residues that are recognized by FBXW7 are phosphorylated by glycogen synthase kinase-3β; AKT, which inhibits this phosphorylation, may prevent FBXW7-mediated degradation of the SREBPs. Insulin also causes the dissociation of INSIG from SCAP–SREBP1c in a sterol-independent manner, leading to the increased transcription of MP genes. These studies were further validated through genetic approaches, in which SREBP1 and SREBP2 expression and activity were increased with the expression of constitutively active PI3K or AKT, and abrogated by dominant-negative AKT. The increase in lipid and cholesterol production that is mediated by the PI3K–AKT–SREBP axis promotes the proliferation of cancer cells and tumorigenesis in vitro and in vivo. Increased MP activity is inconsequential without the availability of both acetyl-CoA and NADPH, and PI3K–AKT signaling meets this requirement by increasing glucose uptake and the rate of glycolysis in cancer cells. Conversely, inhibition of the MP decreases PI3K activity, possibly through decreased RAS isoprenylation, thus demonstrating a two-way regulatory relationship between PI3K–AKT signaling and the MP.

mTOR Complex 1

Downstream of PI3K–AKT signaling, mTOR complex 1 (mTORC1), acts as a sensor of growth signals (such as insulin) and nutrients (such as amino acids) to regulate cellular growth. mTORC1 is often deregulated in cancer, and this supports aberrant growth. mTORC1 increases mRNA translation by phosphorylating and activating ribosomal S6 kinase 1 (S6K1; also known as RPS6KB1) and repressing the activity of the inhibitor of cap-dependent translation, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1; also known as EIF4EBP1). SREBPs are major downstream effectors of mTORC1 signaling, as evidenced by increased lipogenesis in response to mTORC1 activation. The observation that SREs are the most common regulatory elements in mTORC1-induced genes further strengthens the link between mTORC1 and the SREBPs. This link is also evident in samples from patients with primary breast cancer, as patients with high levels of phosphorylated S6K1 had correspondingly high expression of SREBP target genes, such as fatty acid synthase (*FASN*), low-density lipoprotein receptor (*LDLR*), and mevalonate kinase (*MVK*). This study also compared proteins from tumor samples and adjacent normal breast samples and described an increase in FASN protein levels in the tumors that had higher levels of phosphorylated S6K1.

mTORC1 can regulate the SREBP transcription factors at multiple levels although there are some cell- and tissue-type differences. For example, S6K1 has been shown to activate SREBP2 processing and increase the expression of MP genes in a hepatocellular carcinoma (HCC) cell line although the mechanism involved remains unclear. Greater understanding of the role of mTORC1 in SREBP activity came with the development of torins, which are mTOR catalytic site inhibitors. The original allosteric mTOR inhibitor, rapamycin, prevents the phosphorylation of S6K1 but does not inhibit 4EBP1 phosphorylation equally in all systems. By contrast, torins inhibit the phosphorylation of multiple mTOR targets, including S6K1 and 4EBP1. Recent work comparing torin and rapamycin action implicated a role for lipin 1 (LPIN1) in mediating the effects of mTORC1 on the SREBPs. LPIN1 is a nuclear phosphatidic acid phosphatase that is inhibited through direct phosphorylation by mTORC1, independently of S6K1. Active, unphosphorylated LPIN1 indirectly prevents the transcription of SREBP target genes by preventing the SREBPs from binding to chromatin although the mechanism involved remains unclear. A further link between LPIN1 and the MP was uncovered by studies using skeletal muscle, in which statins and LPIN1 were shown to increase autophagy. Given the role of SREBP2 in transcribing numerous autophagy genes, further work is needed to fully understand the interplay between mTORC1, LPIN1, and the SREBPs.

The position of the SREBPs as key effectors of mTORC1 signaling presents a potential vulnerability in tumors that have deregulated mTORC1 activity. Previous studies have linked the loss of SREBPs in breast cancer to the induction of ER stress, which induced apoptosis through mTOR. A separate study showed that genetic knockdown of *SREBF1* and/or *SREBF2* reduced proliferation and increased cell death in mTORC1-activated breast cancer cell lines. The observation that double knockdown of *SREBF1* and *SREBF2* showed the greatest proapoptotic effect suggests that small-molecule inhibitors that target both SREBP1 and SREBP2 may have the greatest therapeutic benefit.

AMP-Activated Protein Kinase

With an opposing role to that of mTORC1, AMP-activated protein kinase (AMPK) acts to dampen anabolic pathways when intracellular ATP levels are low. This role of AMPK as an energy sensor and central regulator of metabolism is crucial in metabolic

disorders such as type 2 diabetes and cancer. AMPK was discovered through its ability to phosphorylate and reduce the activity of microsomal HMGCR in rat liver extracts. Further studies showed that AMPK phosphorylates Ser872 within the catalytic domain of HMGCR, inhibiting its enzymatic activity in a manner that is independent of its feedback regulation by MP metabolites. The SREBPs are also direct targets of AMPK phosphorylation. Activated AMPK specifically interacts with both the precursor and the nuclear forms of SREBP1c and SREBP2, and phosphorylation by AMPK inhibits SREBP proteolytic processing and transactivation activity. Activation of AMPK in HepG2 liver cancer cells by either polyphenols or metformin stimulates this phosphorylation, which suppresses the accumulation of SREBPs in the nucleus under hyperglycemic and hyperinsulinemic conditions. Moreover, activation of AMPK in the livers of insulin-resistant mice inhibited the transcription of enzymes that are involved in lipid and cholesterol biosynthesis, including the MP enzymes 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) and HMGCR, which consequently resulted in a decrease in hepatic triglyceride and cholesterol levels. AMPK can thus inhibit MP activity both directly via the phosphorylation of HMGCR and indirectly through the phosphorylation and repression of SREBPs. However, the relevance of this regulation in the context of cancer is poorly understood.

The MP may also regulate AMPK activity, thereby forming a feedback loop. The tumor suppressor liver kinase B1 (LKB1; also known as STK11), which phosphorylates and activates AMPK, is farnesylated at a highly conserved carboxyterminal CAAX motif. Knock-in mice expressing a mutant form of LKB1, which could not be farnesylated, exhibited reduced membrane-bound LKB1 and impaired AMPK activity. This hints at a negative feedback loop, in which the activation of AMPK in response to decreased cellular energy results in the inhibition of the MP via the phosphorylation of HMGCR and the SREBPs. This in turn reduces the FPP pool within the cell, thereby hindering LKB1 farnesylation and inhibiting AMPK activation.

p53 and RB

TP53, which encodes the p53 tumor suppressor, is one of the most frequently altered genes in cancer, and mutations within the coding region of *TP53* can confer oncogenic properties to p53. Two gain-of-function mutations (*TP53*R273H and *TP53*R280K) enable p53 to functionally interact with nuclear SREBP2 and increase the transcription of MP genes. This MP gene activation was necessary and sufficient for mutant p53 to disrupt normal breast acinar morphology, and mutant TP53 expression in primary breast cancer tissues was correlated with the increased expression of sterol biosynthesis genes. Conversely, wild-type TP53 can reduce lipid synthesis under conditions of glucose starvation by inducing the expression of LPIN1, which, as described earlier, can prevent the association of SREBPs with chromatin. *TP53*R273H and *TP53*R280K mutations are also found in tumors from tissues other than the breast, for example, the ovaries, prostate, and lung.

The interplay between TP53 and the MP suggests that the MP may be a novel therapeutic target for tumors that harbor these specific p53 gain-of-function mutations.

The tumor suppressor protein RB has also been implicated as a regulator of the MP. In a mouse model of adenoma, loss of *Rb1* (which encodes RB) enhanced isoprenylation and activation of NRAS. Loss of RB relieved the suppression of the transcription factors E2F1 and E2F3, which were shown to bind and activate the promoters of numerous prenyltransferase genes, *FDPS* and *SREBF1*. Moreover, RB prevented the association of SREBP1 and SREBP2 with the *FDPS* promoter, suggesting that RB negatively regulates the MP at both the transcriptional and the posttranslational levels.

MYC

The MYC transcription factor is a potent oncogene that can drive transformation in multiple cancer types. It is deregulated in more than 50% of cancers and can reprogram cancer cell metabolism to enable the proliferation and survival of cancer cells. Like the SREBPs, MYC is a basic helix-loop-helix dimerizing protein and it has been shown to bind to SREBP1 to drive somatic cell reprogramming into induced pluripotent stem cells. Analysis of data from the Encyclopedia of DNA Elements (ENCODE) project also shows that MYC binds to promoters of MP genes in close proximity to SREBP1 and SREBP2 binding regions, suggesting that MYC can contribute to the expression of MP enzymes. As the MP is essential for cancer cells, and because MYC has a major role in metabolic regulation, deregulated MYC may ensure that MP metabolites are not limiting for tumorigenesis. The MP was also shown to be important in a MYC-driven transgenic model of HCC. In that study, atorvastatin reduced tumor initiation and growth, possibly through reduced isoprenylation of the RHO-family GTPase RAC1, leading to the activation of serine/threonine-protein phosphatase 2A, which is a negative regulator of MYC. More recently, *Myc*+/− mice (which are haploinsufficient) were shown to have an increased lifespan, which was associated with the decreased expression of MP genes, including *Hmgcr* and *Srebf2*. Given the importance of MYC in driving cancer, and the difficulty of targeting it therapeutically, further work is warranted to uncover the relationship between MYC and the MP.

Signaling From the MP

Altered metabolism in tumors not only fulfills the energetic and biosynthetic needs of a dividing cell but also produces metabolites that are important for downstream signaling. This is particularly true of the isoprenoid and sterol metabolites produced by the MP, which are also used by cancer cells to modulate multiple downstream signaling pathways that are important for tumor progression.

Yes-Associated Protein and TAZ

It was recently shown that the oncogenes Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ; also known as WWTR1) require the MP to be fully functional. YAP and TAZ are transcriptional coactivators that facilitate the transcriptional activation of progrowth genes and the repression of proapoptotic genes. The nuclear localization of YAP and TAZ is negatively regulated, partly by the activation of the tumor-suppressive Hippo signaling pathway. Activation of the Hippo cascade results in the phosphorylation and activation of large tumor suppressor kinase 1 (LATS1) and LATS2, which phosphorylate YAP and TAZ and retain them in the cytoplasm. YAP and TAZ nuclear localization requires the MP, as concurrent knockdown of *SREBF1* and *SREBF2* reduces nuclear localization of YAP and TAZ. These effects were mimicked by GGTIs (geranylgeranyl transferase inhibitors) and were prevented by a RHOA mutant that does not require geranylgeranylation. This suggests that SREBP-mediated induction of the MP maintains intracellular GGPP pools, which is necessary for RHOA upregulation and for downregulation of RHOB (which is essential for initiating protein degradation and recycling through an endolysosomal pathway), as well as YAP and TAZ nuclear localization. Although some studies showed that MP-mediated YAP and TAZ signaling is independent of LATS1 and LATS2 via RNA interference-knockdown experiments, one study demonstrated that both atorvastatin treatment and GGTI treatment increase the phosphorylation of LATS1 and LATS2, suggesting that geranylgeranylation regulates Hippo signaling. A separate study reported constitutive SREBP activation in the livers of mice with a liver-specific *Lats2* deletion, which corresponded to an increase in free cholesterol in the liver and protection from p53-mediated apoptosis. Activation of the MP and activation of YAP and TAZ are correlated with mutant TP53 expression in primary tumors, suggesting a dysfunctional mutant p53–SREBP–YAP–TAZ axis in cancer. Overexpression of *TP53*R280K in a *TP53*-null cell line activated YAP and TAZ only when the MP was active, suggesting that the MP is a crucial intermediate in the oncogenic activation of YAP and TAZ by mutant TP53.

Hedgehog

Cholesterol has a multifaceted role in the regulation of cell signaling. For example, the Hedgehog (HH) signaling pathway, which has important roles in vertebrate development and tumorigenesis, is regulated by sterols at multiple levels. Cholesterol itself can serve as a substrate for the posttranslational modification of HH ligands, which is required for their proper trafficking. Cholesterol and cholesterol-derived oxysterols can also activate HH signal transduction in medulloblastoma, whereas inhibition of the MP or downstream sterol biosynthesis decreased HH signaling and reduced cell proliferation.

Steroid Hormone Signaling

Cholesterol also serves as the precursor of steroid hormones, which drive the initiation and progression of cancers such as hormone-dependent breast cancer and prostate cancer. In breast cancer, patients with estrogen receptor-α (ERα)-positive disease are commonly treated with aromatase inhibitors to deprive the tumors of estrogen. Recent work demonstrated that long-term estrogen deprivation of ERα-positive breast cancers leads to the stable epigenetic activation of the MP and cholesterol biosynthesis. This is coupled with an enrichment of SREBP1 and SREBP2 DNA-binding motifs, as determined by DNase I footprinting analyses, suggesting that there is increased SREBP occupancy on open chromatin. The resulting increased levels of 27-hydroxycholesterol were sufficient to activate ER1 (estrogen receptor alpha) signaling in the absence of exogenous estrogen, driving the activation of genes that promote an invasive cell phenotype. Similarly, in prostate cancer, the de novo synthesis of androgens from cholesterol drives androgen receptor activity in castration-resistant disease. This finding, coupled with the observations that SREBP expression is increased in advanced-stage prostate cancer, suggests a role for the MP in prostate cancer progression. These findings warrant further investigation into the utility of inhibitors of the MP and/or SREBPs in the treatment of hormone-driven cancers.

Inhibitors

Statins

Statins competitively inhibit HMGCR (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase), the first committed enzyme of the MP, blocking the conversion of HMG-CoA to mevalonic acid (**Fig. 2**). The molecular mechanism of inhibition of HMGR by statins is a catalytic mechanism. The statins molecules occupy the catalytic portion of HMGCR, specifically the binding site of HMG-CoA, thus blocking access of this substrate to the active site. The structures of the catalytic domains of the enzyme in complex with statin molecules have been identified.

Efficacy of HMGCR inhibition by various compounds in the family of statin inhibitors is markedly dependent (e.g., nanomolar versus millimolar inhibitor affinity) on whether class I or class II enzyme is used. The inhibitors are characterized by hydroxymethylglutaryl (HMG)-like moieties linked to an extensive hydrophobic scaffold (e.g., a decalin ring in the case of mevastatin and simvastatin). Complexes of human enzyme catalytic domain with a variety of statins have been crystallized, and X-ray structures have been published. The structural results indicated binding of the HMG moiety in the active site pocket where the catalytic glutamate and lysine residues are located. In contrast, the NADPH substrate site is not occupied upon inhibitor binding. The structural results indicating that access to the substrate HMG-CoA is blocked by inhibitor binding are in accord with the observation of competitive inhibition with respect to HMG-CoA. A variety of additional polar interactions with the HMG moiety have also been documented.

The hydrophobic portion of the inhibitors is bound in a shallow hydrophobic groove of the human protein. A large number of van der Waals contacts between nonpolar amino acids in this groove and the diverse hydrophobic substituents that are a common feature of the various statins are proposed to represent the dominant contribution to high-affinity binding. A structure of lovastatin bound to the class II *P. mevalonii* enzyme has also been reported. As in the case of the class I enzyme, the structure indicates interactions with residues (e.g., lys, glu) identified in catalysis as well other polar residues in this pocket, with some hydrogen bonds mediated by water molecules. The hydrophobic decalin ring component of the inhibitor blocks a closure of the C-terminal flap domain of the protein, which includes the histidine residue that has been implicated in catalysis. Thus, inhibitor binding both blocks the active site and makes correct orientation of active site amino acids impossible. A large difference between class I and class II enzyme interactions with the statin inhibitor is suggested to involve a pocket formed, in part, by the alpha helix of the *P. mevalonii* enzyme. The structural results could expedite potential design of HMGCR inhibitors that can discriminate between class I and class II enzymes. Additionally, an approach proposed to increase inhibitor affinity involves derivatization of the parent inhibitory compound to incorporate chemical substituents effective in interaction with the NADPH site.

By interrupting cholesterol synthesis in the liver, statins activate the production of microsomal HMGCR and cell surface low-density lipoprotein (LDL) receptors. This results in a predictable increased clearance of LDL from the bloodstream and a decrease in blood LDL cholesterol levels that may range from 20% to 55%. Statins have shown strong evidence-based proved capacity of decreasing the cardiovascular morbidity and mortality in both primary and secondary prevention settings. Because of these properties, statins are among the most widely used pharmaceutical agents in the world, their role in the global battle against cardiovascular disease being compared with the crucial role of antibiotics in decreasing the mortality of infections. Subgroup analyses of several large clinical trials have suggested that changes in lipid levels alone may not explain all the beneficial effects of statins in human pathology. Evidence has emerged from both basic research and clinical trials that statins have many cholesterol-independent or so-called pleiotropic effects: improving endothelial function, atherosclerotic plaque-stabilizing effects, antiinflammatory and immunomodulatory effects, antithrombotic properties, effects on bone metabolism, on risk of dementia, induction of apoptosis, and antiproliferative effects.

Most of these pleiotropic effects result from inhibition of the synthesis of important isoprenoid intermediates of the MP, such of FPP and GGPP. Mevalonate-derived prenyl groups have been shown to have essential roles in many cellular functions including cell signaling, cell differentiation and proliferation, myelination, cytoskeleton dynamics, and endo−/exocytotic transport. In the last decade, substantial progress has been made in understanding the nonlipid-related pharmacological properties of statins and a growing interest in the potential therapeutical implications of these pleiotropic effects of statins has emerged. Recent experimental and clinical data indicate that statins may be of potential therapeutic use in a variety of nonvascular diseases, including autoimmune diseases, multiple sclerosis, rheumatoid arthritis, sepsis, dementia, and different types of cancer.

Many studies have shown that statins can directly and specifically inhibit the proliferation of tumor cells. For example, statins promote the apoptosis of cells derived from acute myeloid leukemia (AML), while normal myeloid progenitors do not undergo apoptosis and retain full proliferative potential. This tumor-normal therapeutic index may be due to the altered metabolic reprogramming of AML cells leading to an increased dependence on MP metabolites for growth and survival. The widespread use of statins for cholesterol management also demonstrates that these drugs cause minimal damage to normal cells. The side effects of these drugs are regularly treated by switching to a different statin or potentially by cotreating with CoQ although this cotreatment method is controversial owing to conflicting clinical evidence.

The data discussed earlier suggest that statins have a high therapeutic index to target tumors in vivo despite the ubiquitous expression of the MP. This rationale has led to multiple clinical trials investigating the efficacy of various statins as a therapeutic option in a variety of tumor types. Two recent breast cancer window-of-opportunity clinical trials, using atorvastatin and fluvastatin, showed reductions in the Ki67 index in a subset of patients who were administered with cholesterol-management doses of statins between cancer diagnosis and surgery. Statins have also been safely used in combination with other agents to increase efficacy. For example, pravastatin was combined with standard-of-care treatment in HCC and AML, resulting in significantly longer median survival in colorectal cancer and resulting in complete or partial response in 60% of patients with AML. In another study, combining lovastatin with thalidomide and dexamethasone in patients with relapsed or refractory multiple myeloma (MM) led to prolonged overall survival and progression-free survival.

Despite evidence of patient response to statins as anticancer agents, many patients remained nonresponsive to statin treatment in other cancer clinical trials. This is consistent with the current paradigm of interpatient tumor heterogeneity. This lack of response might also be expected considering the evidence that we discussed earlier showing that the MP is regulated by many key oncogenic signals. Similar to many anticancer agents, a personalized medicine approach is needed to implement statins, and/or other inhibitors of the MP, as a successful class of cancer therapeutics. To this end, a molecular signature of basal mRNA expression has been developed to predict statin response in breast cancer in vitro, and deregulated MYC expression has been a proposed indicator of statin response in specific tumor types; however, essential follow-up validation is required before these biomarkers can be used clinically. It is currently difficult to predict which cancers will be particularly sensitive to statin therapy. In addition to AML and MM, encouraging results from both clinical trials and epidemiological studies suggest that patients with hormone-dependent cancers, such as breast cancer and prostate cancer, may benefit from the addition of statins to their treatment regimen. This may be partly because the MP end-product cholesterol is the precursor of hormones such as estrogen and androgens, which have a major role in the development of these types of cancers. Colorectal cancer also seems to be particularly responsive to statins, perhaps because of the hepatotropic pharmacology of this family of drugs. Clinical trials are required in these and other cancers to further define the subset of cancers that are particularly statin-sensitive.

Targeting the SREBP in Combination With Statins

Crucial to the regulation of the MP is the tightly controlled, SREBP-mediated feedback mechanism, in which inhibition of the MP results in the activation of the SREBPs and an increase in the expression of MP genes, an effect that may be amplified in cancer cells. SREBP activation also increases the expression of the LDLR, which leads to the increased uptake of exogenous, lipoprotein-derived cholesterol: an effect that has been shown to be important in cancer cells. The SREBPs thus function to replenish MP metabolites, which can dampen the apoptotic response following statin treatment. This would be a classic resistance mechanism, similar to that seen with other anticancer therapeutics such as inhibitors of the BRAF protooncogene in BRAF-mutant melanoma. Cells treated with BRAF inhibitors, such as vemurafenib, can acquire an activating mutation in downstream kinases (e.g., the MEK1 mitogen-activated protein kinase kinase) or can have an increase in expression of receptor tyrosine kinases (e.g., epidermal growth factor receptor), bypassing the need for BRAF activity. These studies demonstrate that inhibiting both the cancer vulnerability and the resistance or feedback mechanism is crucial for maximum efficacy. Therefore, inhibiting the SREBP-regulated feedback response in conjunction with statin therapy could prevent resistance, thereby increasing the efficacy of statins as anticancer agents and the number of responsive patients.

Evidence that targeting the SREBPs in combination with statin therapy is a viable strategy has been provided by several studies. One study looking at breast and lung cancer cell lines used a short hairpin RNA screen to uncover genes that, when knocked down, potentiated the proapoptotic effects of statins. The MP genes *HMGCS1*, *GGPS1*, *SCAP*, and *SREBF2* all scored highly, adding credence to either inhibiting other enzymes in the MP or inhibiting the SREBP-mediated feedback response in combination with statin therapy. Another study showed that statin-induced SREBP processing can be blocked by another agent that has been approved for a noncancer indication, dipyridamole. Dipyridamole reduced the transcription of SREBP target genes such as *HMGCS1* and *HMGCR* and synergized with statins to increase apoptosis in AML and MM cell lines and patient samples. Other compounds, such as tocotrienols, have also been demonstrated to synergize with statins to induce cancer cell apoptosis, which is an effect that may be associated with their ability to degrade nuclear SREBP2 and inhibit its transcriptional activity. Although several other small molecules, including fatostatin, have been shown to inhibit SREBP processing, their lack of approval for use in patients limits their potential to immediately have an impact on cancer patient care.

Therefore, clinical investigation into the utility of combined statins and SREBP inhibitors for the treatment of cancer is currently warranted.

Bisphosphonates

BPs are potent inhibitors of osteoclast mediated bone resorption and are currently the most important and effective class of drugs used to treat metabolic bone disease. BPs bind avidly and achieve therapeutic concentration at sites of active bone metabolism in the mineralized bone matrix. By inhibiting bone resorption and inducing osteoclast apoptosis, BPs reduce bone turnover, increase bone mass, and improve bone mineralization. Recent experimental studies have shown that nitrogen-containing BPs may have interesting antitumor properties. As shown in **Fig. 2**, they inhibit FPP synthase, a key enzyme in the MP, with consecutive decrease of the formation of isoprenoid lipids such as FPP and GGPP. It has been demonstrated that the capacity of inhibiting FPP synthase is correlated to the synthesis from the accumulated isopentenyl diphosphonate of an ATP analogue, named Apppi. This, in turn, prevents the prenylation of a number of small GTPases, such as Rho, Ras, Rac, and Rab, which play a role in malignant transformation of cells by regulating intracellular signaling, cell growth, motility, and invasion. Interestingly, the ability of nitrogen-containing BPs to inhibit FPP synthase appears to be clearly related to the presence of a nitrogen atom at critical positions in the side chain. Consistent with their recently identified biochemical effects on protein prenylation, there is extensive evidence from preclinical research that BPs also exhibit antitumor activity in a variety of human cancers: myeloma, breast cancer, prostate cancer, pancreatic cancer, mesothelioma, mesenchymal tumors, and osteosarcoma. Summarizing, inhibition of the MP is the underlying molecular mechanism of many of these antitumor properties of nitrogen-containing BPs: inhibition of integrin-mediated tumor cells adhesion to bone. BPs act by inhibition of prenylation of small GTPases required for integrin activation–inhibition of cancer cells migration and invasion by inhibition of metalloproteinases and by inhibition of Rho activation by preventing geranylgeranylation. Inhibition of proliferation and induction of apoptosis of cancer cells were observed at high BP concentrations. Although inhibition of the MP was found to be the main molecular mechanism of promoting apoptosis, other mechanisms have also been described, that is, reduction of bcl-2 expression and activation of caspase. In addition, immunomodulatory effects mediated through accumulation of mevalonate metabolites in tumor cells, which stimulate T lymphocytes expressing the T cell receptor, were described.

Other Therapeutic Agents That Target MP

Extensive preclinical evidence suggested that manipulation of the MP through inhibition of FTase and GGTase, and, in consequence, inhibition of protein prenylation, can result in alteration of malignant cells' capacity of proliferation, growth, and migration. FTIs represent a new generation of signal transduction inhibitors, designed to target the critical posttranslational modification of Ras, a protooncogene that is believed to play a critical role in tumor growth or progression and may have prognostic significance.

Some studies suggest that FTIs inhibit not exclusively Ras proteins but also the farnesylation of additional cellular polypeptides that have not been identified, thereby exerting antitumor effects independent of the presence of activating Ras gene mutations. Lonafarnib SCH-66336 has been the first of these compounds to undergo clinical development. Several other FTIs have entered clinical trials for various cancer indications: examples are tipifarnib (R115777), BMS-214662, and L-778. Although phase I and II studies have shown that FTIs have a significant antitumor activity and an acceptable toxicity profile, the results of phase III trials have been disappointing, showing no overall significant amelioration of survival in solid cancers. The most promising activity to date has been demonstrated in patients with hematological malignancies, in particular AML and myelodysplastic syndrome. GGTIs are a novel class of drugs that are still in the experimental stage. It has been recently demonstrated that when FTase is blocked by FTIs, additional inhibition of geranylation by inhibitors of GGTase can augment antiproliferative properties of FTIs. Treatment of tumor cell lines in culture with varying doses of a GGTI in conjunction with a FTI can inhibit Ki-Ras prenylation and determine an apoptotic response that is greater than the apoptotic response elicited by either agent alone in vitro. However, the tolerability of this protocol in vivo was poor and suggested that the use of this approach in humans should be cautious because of a possible narrow therapeutic index. Recent preclinical research has identified a new inhibitor of protein prenylation, a selective, highly potent, and cell-active GGTase-I inhibitor, GGTI-DU40, and suggested that investigation of GGTIs, as potential new anticancer drugs should continue, in order to define their exact role and toxicity profile. In conclusion, in the last few years, there has been intense interest in understanding the clinical and therapeutical implications of MP, a metabolic pathway which plays a key role in regulation of cellular cholesterol synthesis and in controlling cell proliferation by generating prenyl intermediates. Since the discovery of MK deficiency as the direct biochemical and molecular cause of MVA and hyperimmunoglobulinemia D syndrome, considerable progress has been made in understanding the pathophysiology of these antiinflammatory disorders. However, it is still not known what is the causal relationship between the enzymatic defect and the inflammation and no effective treatment has been established so far. Also, MP is an important, attractive target for many areas of therapeutical research and application, through inhibition of the isoprenylation process of small intracellular proteins. Although extensively studied, many important issues remain unanswered, and this fascinating area of interference between biochemistry, clinical implications, and pharmacology remains a major focus for potential future investigation.

Outlook

Understanding tumor metabolism in the context of oncogenic signals has the potential to drive the development of targeted personalized therapies. The various signaling pathways that we describe in this book chapter are important drivers in many cancers, and they all have the ability to deregulate the MP, making these cancers potentially vulnerable to MP inhibition. Whether this occurs in every patient who presents with these lesions remains unclear. More work is needed to understand the extent to which driver mutations increase flux through the MP in patients. Rapidly developing technologies for the comprehensive flux-based analysis of MP metabolites will provide further advances in understanding how the MP receives and responds to oncogenic signals. In patients, it may be more feasible to determine pathway activity by mapping their oncogenic lesions to their sterol feedback response at the protein level (via SREBP localization) or mRNA expression level of MP genes, which may identify patients who will respond to MP inhibition. Designing clinical trials that will identify potential responders before treatment is required to prevent expensive failures of therapies that may still have benefits to a subset of patients. Improving reagents, particularly antibodies to HMGCR and SREBP2, will also aid trial design and interpretation.

The essentiality of the MP in many cancers, coupled with affordable and safe drugs that can target this pathway and its feedback response, provides a strong rationale for continuing to explore this key metabolic pathway in cancer and many other diseases.

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Further Reading

Buhaescu, I., Izzedine, H., 2007. Mevalonate pathway: a review of clinical and therapeutical implications. Clinical Biochemistry 40 (9–10), 575–584.

Miziorko, H.M., 2011. Enzymes of the mevalonate pathway of isoprenoid biosynthesis. Archives of Biochemistry and Biophysics 505 (2), 131–143.

Karlic, H., Thaler, R., Gerner, C., Grunt, T., Proestling, K., Haider, F., Varga, F., 2015. Inhibition of the mevalonate pathway affects epigenetic regulation in cancer cells. Cancer Genetics 208 (5), 241–252.

Moutinho, M., Nunes, M.J., Rodrigues, E., 2017. The mevalonate pathway in neurons: it's not just about cholesterol. Experimental Cell Research (Epub ahead of print).

Mullen, P.J., Yu, R., Longo, J., Archer, M.C., Penn, L.Z., 2016. The interplay between cell signalling and the mevalonate pathway in cancer. Nature Reviews. Cancer 16 (11), 718–731.

Räikkönen, J., Mönkkönen, H., Auriola, S., Mönkkönen, J., 2010. Mevalonate pathway intermediates downregulate zoledronic acid-induced isopentenylpyrophosphate and ATP analog formation in human breast cancer cells. Biochemical Pharmacology 79 (5), 777–783.

Microbiota and Colon Cancer: Orchestrating Neoplasia Through DNA Damage and Immune Dysregulation

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Glossary

Adenoma Describes a wide range of neoplastic nodules with glandular appearance that arise in the epithelial tissue and show no cytological features of malignancy.

Bacteriome Term used to define a bacterial community that inhabit a particular environment and is usually shaped by a symbiotic interaction with its host and other microbes.

Dysbiosis Perturbation of the natural balance in the gut microbiota, that is usually associated with pathogenesis.

Genotoxin A molecule that cause DNA damage. It could cause DNA mutations (mutagen), trigger cancer (carcinogen), or lead to birth defects (teratogen).

Polyp Is a typically benign abnormal growth of diffuse mucosal tissue. Some polyps have a stalk and are called pedunculated, while other polyps are flat and are called sessile.

Virome Term used to define a viral community that inhabit a particular environment and is usually shaped by a symbiotic interaction with its host and other microbes.

Nomenclature

AOM Azoxymethane
APC Adenomatous polyposis coli
***Apc*$^{min/+}$** APC multiple intestinal neoplasia
ATM Ataxia-telangiectasia mutated
BabA Blood group binding adhesin
BFT *Bacteroides fragilis* toxin
CAC Colitis-associated colon cancer
cagPAI *cag* pathogenicity island
CDT Cytolethal distending toxin
CEACAM Carcinoembryonic antigen-related cell adhesion molecule
CECs Colon epithelial cells
CHK Checkpoint kinase
CRC Colorectal cancer
DDR DNA damage response
DSBs Double strand breaks
EPEC Enterotoxigenic *Escherichia coli*
ETBF Enterotoxigenic *Bacteroides fragilis*
Gal-GalNac Galactose-*N*-acetyl-D-galactosamine
H_2O_2 Hydrogen peroxide
HNPCC Hereditary nonpolyposis colorectal cancer
HR Homologous recombination
IBD Inflammatory bowel disease
IFNγ Interferon gamma
iNOS Inducible nitric oxidase
MAP kinase Mitogen-activated protein kinase
MMR Mismatch repair
MPII Secretory metalloproteinase II
MSCRAMM Microbial surface component recognizing adhesive matrix molecules
MSI Microsatellite instability
NHEJ Nonhomologous end-joining
p53 Tumor protein P53
PGE2 Prostaglandin E2

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Rag2 Recombinase-activating gene-2
ROS Reactive oxygen species
SMO Spermine oxidase
T3SS Type III secretion system
T4SS Type IV secretion system
Tregs T regulatory cells
VacA Vacuolating cytotoxin
γH2AX Phosphorylated histone H2A.X

Healthy Human Enteric Microbiota

The gut microbiota is a dynamic structure that helps maintain host homeostasis by regulating metabolic and immune processes and establishing a network that keeps pathogenic microbes in line. Microbial composition is shaped by the host immune system, aging processes, dietary metabolites, oxygen levels, in situ microbial interactions, and other environmental factors. In the human colon, the microbiota is composed of archaea, eukarya, viruses, and bacteria. The *Methanobrevibacter* genus is the most prevalent archaea in healthy individuals and the phyla Ascomycota and Basidiomycota are the most prevalent fungi (Hoffmann et al., 2013). Intestinal bacteria are dominated by an inverse abundance ratio between Bacteroides and Firmicutes (Human Microbiome Project Consortium, 2012; Eckburg et al., 2005). However, the less abundant phylum Proteobacteria, which include pathogens such as *Escherichia coli* and *Helicobacter*, is the major source of gene variability between healthy hosts (Bradley and Pollard, 2017). Strict anaerobes such as *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Peptostreptococcus*, *Atopobium* and *Fusobacterium* constitute the major portion of the microbiota (Tlaskalova-Hogenova et al., 2004), whereas facultative anaerobes such as *Enterococci*, *Streptococci* and *Enterobacteriaceae* represent a minority. Both inflammation and antibiotic treatment can induce an increase in oxygen levels in the colon (Byndloss et al., 2017), which can favor dysbiosis by creating a niche for facultative anaerobes with potential pathogenic activity (Spees et al., 2013; Rivera-Chavez et al., 2016) (reviewed on Rivera-Chavez et al. (2017) and Litvak et al. (2017)).

Human Enteric Microbiota Associated With Carcinogenesis

Colorectal cancer (CRC) development is a multistep process that involves accumulation of mutations in colon epithelial cells (CECs), which lead to the formation of aberrant crypts (Irrazabal et al., 2014). Common genetic lesions that lead to colon carcinogenesis include loss of the *adenomatous polyposis coli* (*APC*) tumor suppressor gene, or mutations in the *β-catenin* gene, both of which lead to activation of β-catenin/TCF/LEF-1 signaling that in turn leads to uncontrolled CEC proliferation. In fact, 85% of sporadic CRC carry mutations in the *APC* gene (Medema and Vermeulen, 2011). These genetic changes are usually followed by mutations in *K-Ras*, *PIK3CA* and *TP53* genes (Raskov et al., 2014). In addition, mutations or epigenetic silencing that lead to mismatch repair (MMR) deficiency occur in approximately 20% of sporadic CRC (Poulogiannis et al., 2010) and are the most common mutation in patients with hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome.

Recently, several studies have shown that besides these genetics changes, differences in the composition and diversity on the human microbiota are also strongly associated with CRC development. Although findings have shown that CRCs are colonized with specific bacteria (Maddocks et al., 2009; Gagniere et al., 2017; Viljoen et al., 2015; Kostic et al., 2013; Purcell et al., 2017; Biarc et al., 2004), it is not clear whether such bacteria are CRC initiators or passengers (Tjalsma et al., 2012). This distinction is necessary to differentiate causality from association. CRC initiators induce DNA damage either directly through the production of genotoxins, or indirectly, by promoting inflammation that in turn leads to the production of genotoxic agents or by transforming diet-derived metabolites into secondary genotoxic molecules (Belcheva et al., 2015). On the other hand, passenger bacteria exploit the unique microenvironment provided by the tumor to grow and out-compete other microbes. In this case, bacteria could simply be along for the ride, or might promote the growth of the tumor.

Current evidence supporting bacterial causality in human CRC has been challenging, which is mainly due to three factors: bacterial genetic plasticity, host genetic variability, and the prolonged period between the initiation of carcinogenesis and the detection of the cancerous lesion in the clinic. First, the microbiota is a dynamic structure, the composition of which is affected by many factors. Due to the high mutation rates in viruses (Minot et al., 2013), the constant horizontal gene transfer between unrelated bacteria (Lloyd-Price et al., 2016), and the very long times that the microbiota develop and adapt to each other in an adult individual, it is likely that each person will ultimately possess a unique virome, composed primarily of phages, and a unique bacteriome. Hence, trying to precisely implicate a single bacterium at a determinate point to CRC initiation can be likewise compared to trying to measure with absolute precision the position and momentum of a quantum particle, as outlined by Heisenberg's uncertainty principle.

Second, most of the potentially carcinogenic bacteria can colonize humans asymptomatically, and are able to induce or promote carcinogenesis only after chronic infection in genetically susceptible hosts. Hence, such bacteria might be carcinogenic only under

certain conditions, and defining these conditions might prove to be challenging. Furthermore, considering that not all inflammatory processes are equally detrimental to the mucosa, a detailed characterization of the host inflammatory state is critical, since gross histological scores minimize the potential relevance of unique inflammatory responses.

Third, although a single acute exposure of a specific genotoxic bacterium might initiate genetic defects that facilitate cancer initiation, the bacterial pathogen that initiated the oncogenic process might have disappeared by the time the patient presents with an adenoma or CRC at the clinic. In this regard, more research should focus on how a single challenge with pathogenic bacteria could potentially lead to chronic dysbiosis, and/or intestinal disease.

Despite these caveats, there is nevertheless strong evidence for the involvement of specific microbes in colon cancer, and these are described below.

Helicobacter pylori

Helicobacter pylori (*H. pylori*) is a Gram-negative human pathogen found in approximately 50% of the population (Moayyedi and Hunt, 2004). This pathogen promotes chronic inflammation in the upper gastrointestinal tract and is a known inducer of gastric cancer. In fact, *H. pylori* is the only bacteria classified as a Group I carcinogen by the International Agency for Research on Cancer (Anon, 1994). This link has led to extensive research of a possible role of *H. pylori* in other gastrointestinal cancers, such as CRC.

Although *H. pylori* has been detected in the human colon (Keenan et al., 2010) and in human colonic tumors (Papastergiou et al., 2016), there is no clear mechanism by which this pathogen can initiate CRC. Furthermore, the association between *H. pylori* infection and CRC is conflicting (Breuer-Katschinski et al., 1999; Fujimori et al., 2005; Moss et al., 1995; Shmuely et al., 2001). Recently, an epidemiologic study in the United States showed that the ethnic distribution of sessile serrated polyps is inversely associated with *H. pylori* prevalence (Sonnenberg et al., 2017). In contrast, a study focused on the Korean population showed that the rate of *H. pylori* infection was higher in patients with adenoma compared to polyp-free controls (Nam et al., 2017). In addition, while some studies have shown that the presence of anti-*H. pylori* IgG antibodies is positively associated with advanced colon cancer (Lee et al., 2016), other studies have not found such associations (Blase et al., 2016). Although, none of these studies have demonstrated that this pathogen could directly induce colonic carcinogenesis, given the known mechanism by which *H. pylori* induces gastric cancer, it is reasonable to hypothesize that *H. pylori* might directly promote CRC. In this section, we will discuss the mechanisms by which *H. pylori* induce gastric cancer and potentially CRC.

H. pylori Virulence Factors

H. pylori virulence is related to the presence of a *cag* pathogenicity island (*cag*PAI) locus, which encodes for a type IV secretion system (T4SS) and the CagA virulence factor. T4SS binds to β integrins and injects CagA into host epithelial cells (**Fig. 1**a). T4SS also delivers peptidoglycan into gastric epithelial cells, which is recognized by Nod1 receptors, leading to IL-8 induction (**Fig. 1**b) (Viala et al., 2004). After CagA is injected into the epithelial cells, this oncoprotein hijacks key signaling pathways in the gastric cell, such as NF-κβ, MAP kinase and Jak/STAT pathways. These pathways control fundamental processes in gastric cells, such as proliferation, inflammation, cell cycle, and apoptosis (reviewed on Tegtmeyer et al. (2017)) leading to deregulation in cell homeostasis.

Another virulent factor produced by *H. pylori* is the vacuolating cytotoxin VacA, which is the only known *H. pylori* protein that targets the mitochondria. VacA is internalized into gastric epithelial cells in endosomes, a process that alters intracellular trafficking and inhibits antigen presentation (Molinari et al., 1998). Internalized VacA localizes to the mitochondrial inner membrane where it forms anion-conductive channels that reduce the mitochondrial membrane potential, resulting in a lower production of ATP, cytochrome *c* release and apoptosis (**Fig. 1**c) (McClain et al., 2017). VacA channel-forming activity also leads to autophagy, which seems to be a host defense mechanism to reduce cellular damage (Terebiznik et al., 2009). On the other hand, VacA can trigger reactive oxygen species (ROS)-induced autophagy by decreasing intracellular levels of glutathione, one of the main antioxidants in the cell (Tsugawa et al., 2012). Additionally, VacA can inhibit the function and proliferation of several adaptive and innate immune cells (McClain et al., 2017). VacA activity differs between *H. pylori* strains due to gene variability (Blaser, 2002). Nevertheless, *H. pylori* strains that possess a functional T4SS, a *CagA* gene, and a cytotoxic *VacA* gene, are positively associated with induction of gastric tumor lesions (Blaser, 2002).

Once *H. pylori* reaches the stomach, it adheres to gastric epithelial cells by binding to some members of the CEACAM (carcinoembryonic antigen-related cell adhesion molecule) family expressed by epithelial cells (Koniger et al., 2016). Curiously, while CEACAM1 is a recognized tumor marker in the colon, this protein is downregulated during colon carcinogenesis (Neumaier et al., 1993), and its deficiency contributes to colon carcinogenesis in mice (Leung et al., 2006). These data suggest that the environment generated after tumor formation in the colon do not favor *H. pylori* colonization, but doesn't rule out such a role for *H. pylori* in tumor initiation.

Another protein that facilitate *H. pylori* adherence to gastric epithelial cells is the blood group binding adhesin (BabA), which binds to ABO/Le^b blood group antigens and fucosylated carbohydrates, such as the ones present in mucus. Interestingly, BabA binding to epithelial cells is reduced by low pH, and increased in neutral environments (Bugaytsova et al., 2017). Since the acidic gastric content is neutralized in the intestine, *H. pylori* could potentially bind mucus in the small and large intestine through BabA-mediated interactions. Furthermore, it is unknown whether an increase in mucus production, which is a phenotype of colitis and colitis-associated colon cancer (CAC), could affect *H. pylori* potential for intestinal colonization.

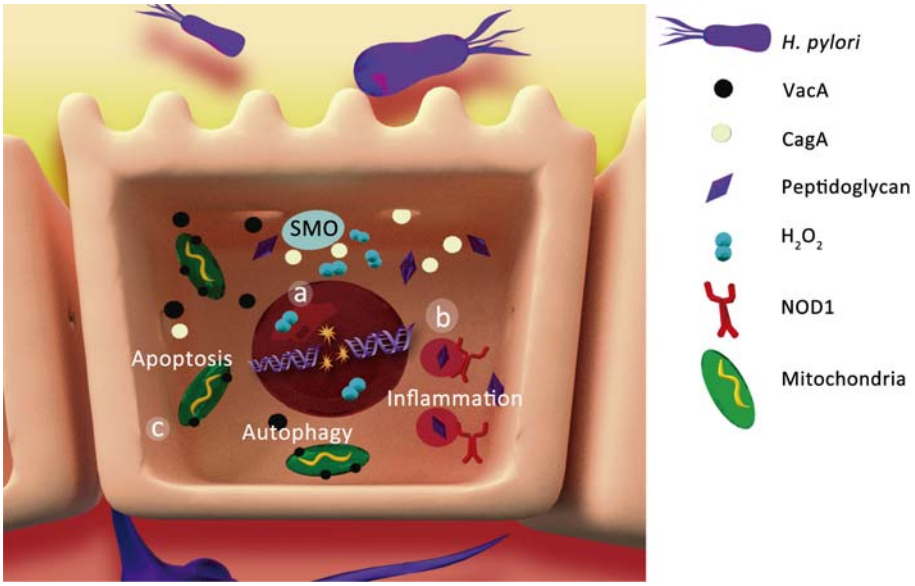

Fig. 1 (a) *H. pylori* injects the toxin CagA into the gastric epithelial cell using a type IV secretion system. CagA induces the expression of spermine oxidase (SMO), which produces H_2O_2 damaging the DNA. Notice that the enterotoxigenic *B. fragilis* toxin also induces SMO and DNA damage (b) *H. pylori* also delivers peptidoglycan into gastric epithelial cells, which is recognized by Nod1 receptors, leading to inflammation. (c) *H. pylori* produces VacA, which targets the mitochondria. VacA is internalized in endosomes, and localizes to the mitochondrial inner membrane where it forms pores that reduce the mitochondrial membrane potential, resulting in a lower production of ATP, and apoptosis. VacA also leads to autophagy as a response to antioxidant depletion.

Mechanisms by Which *H. pylori* Could Induce Colon Carcinogenesis

After *H. pylori* colonizes the gastric epithelial cells, it induces several physiological changes that have been associated with the induction of either gastritis or gastric cancer. These changes include: hypergastrinemia, inflammation, destruction of gastric cells, hypochlorhydria, and DNA damage, all of which could potentially promote colon carcinogenesis.

Hypergastrinemia

Chronic infection with *H. pylori* can increase the release of gastrin, which is a known inducer of hyperproliferation in human CECs (Renga et al., 1997); however, the mitogenic effects of gastrin are not related to the prevalence of adenomas in the colon of patients presenting hypergastrinemia (Sobhani et al., 1993). Since cell hyperproliferation is not sufficient for tumor formation, it is possible that *H. pylori* promotes neoplasia initiation under certain circumstances, such as in DNA repair-deficient hosts. Furthermore, given that mutations on DNA damage repair proteins appear early during colon carcinogenesis, it is possible that under these circumstances *H. pylori* accelerates cancer development.

Inflammation

Chronic inflammation induced by *H. pylori* leads to destruction of gastric glandular cells, a process that can facilitate microbial dysbiosis across the gastrointestinal tract (Kanno et al., 2009). Dysbiosis, in turn, could generate an environment suitable for overgrowth of other cancer-promoting pathogens and/or exacerbate inflammation. Chronic inflammation can also favor cell transformation by silencing some genes through epigenetic modifications, or by leading to the production of ROS which in turn induces DNA damage.

Hypochlorhydria

H. pylori also cause hypochlorhydria, which can cause a decrease in stomach protein hydrolysis and an increase in colonic protein fermentation (Cater 2nd, 1992). An increase in colonic protein availability could potentially provide an advantage to pathogenic bacteria that can ferment proteins and/or use nitrogen as a source of energy (Byndloss et al., 2017; Spees et al., 2013; Winter et al., 2013); under these circumstances *H. pylori* could act as a passenger that facilitate colonic tumor development.

DNA damage

H. pylori might promote DNA damage by at least three mechanisms: increasing ROS production (Chaturvedi et al., 2011), inducing mutations in mitochondrial and nuclear DNA, and reducing DNA repair (Machado et al., 2009). One of the main factors related to *H. pylori*-induced DNA damage is the toxin CagA. This virulence factor could potentially induce CRC by deregulating Wnt (Yong et al., 2016; Gnad et al., 2010) and p53 pathways (Buti et al., 2011; Wei et al., 2010), by inducing the expression of inflammatory mediators that are known CRC promoters, such as IL-8 (Ferreira et al., 2016; Chang et al., 2015) or by inducing DNA damage

(Chaturvedi et al., 2011; Xu et al., 2004). CagA *positive H. pylori* infection of gastric epithelial cells induces DNA double-strand breaks (DSBs) and telomere shortening. Specifically, *H. pylori* strains that express the oncogenic protein CagA induce upregulation of spermine oxidase (SMO) (Chaturvedi et al., 2011). SMO produces hydrogen peroxide (H_2O_2) as a by-product, leading to oxidative stress, DNA damage, and apoptosis of gastric epithelial cells (**Fig. 1**a) (Chaturvedi et al., 2011). Surprisingly, after *H. pylori* infection, a subset of gastric epithelial cells with high levels of DNA damage do not undergo apoptosis, a phenomenon that has been associated with the activation of the EGF receptor by *H. pylori* (Chaturvedi et al., 2014). Another mechanism that could increase survival of cells with high levels of DNA damage could take place in cells that harbor mutations in the DNA damage response (DDR). When the DDR is not active, cell cycle checkpoints are not activated, and cells with high levels of damaged DNA will proliferate. For example, ATM (ataxia-telangiectasia mutated)-deficient cells proliferate after irradiation due to its inability to activate the cell cycle checkpoints in response to double-stranded DNA breaks (Painter and Young, 1980; Falck et al., 2001).

Although CagA could induce CRC by several mechanisms, and some studies have shown that CagA seropositivity in patients infected with *H. pylori* is associated with a higher incidence of CRC (Shmuely et al., 2001), other reports have found no such correlations (Papastergiou et al., 2016; Chen et al., 2016). In the same way, it has also been shown that seropositivity against the toxin VacA is strongly associated with CRC risk (Epplein et al., 2013), but although both CagA (Chaturvedi et al., 2011) and VacA (Tsugawa et al., 2012) induce ROS and DNA damage in gastric cells, no reports have shown a direct effect of these toxins on CECs.

In conclusion, although it is possible that *H. pylori* could induce CRC, more research is required with specific focus on specific susceptible subjects. For example, *H. pylori* can directly induce DNA damage, and the potential role of *H. pylori* on DNA repair-deficient subjects have not been fully studied.

Helicobacter hepaticus

Other *Helicobacter* species, such as *Helicobacter hepaticus* (*H. hepaticus*), have also been associated with carcinogenesis. In 1992, *H. hepaticus* was shown to cause hepatocellular and hepatocholangiolar adenomas and carcinomas in genetically susceptible mice (Falsafi and Mahboubi, 2013). Subsequent work showed that *H. hepaticus* infection induced colitis and CRC in immunodeficient mice (Erdman et al., 2003, 2009; Ge et al., 2017; Nagamine et al., 2008a, 2008b; Wang et al., 2017).

Mechanisms by Which *H. hepaticus* Could Induce Colon Carcinogenesis

Although *H. hepaticus* has been found in patients with chronic liver disease (Nilsson et al., 2000; Yang et al., 2013; Hamada et al., 2009), there is no data that demonstrate that *H. hepaticus* colonize the human intestinal tract and therefore a direct causality on inflammatory bowel disease (IBD) or CRC in humans is nonexistent. Nevertheless, current research suggests that *H. hepaticus* can induce CRC in mice by at least two mechanisms: inflammation and DNA damage.

Inflammation

Lymphocyte homeostasis directly affects the progress of intestinal disease during *H. hepaticus* infection; SMAD3-deficient mice develop colitis after infection with *H. hepaticus*, a process that is dependent on the activation and dysregulation of effector lymphocytes in the gut (McCaskey et al., 2012). Although, one might expect that lymphocyte-deficient mice will not develop colitis after infection with *H. hepaticus*, recombinase-activating gene-2-deficient ($Rag2^{-/-}$) mice inoculated with *H. hepaticus* develop colitis and colonic polyps, which is attenuated by providing mice with T regulatory cells (Tregs) (Erdman et al., 2003, 2009). In the same line, mice deficient in the immunoregulatory molecule IL-10, also develop colitis after infection with *H. hepaticus*, and it has been suggested that monocyte recruitment into the lamina propria lead to CAC (Bain et al., 2018). While, the pathogenesis of *H. hepaticus* was thought to be due to its capacity to activate inducible nitric oxidase (iNOS) in macrophages, recent data suggest that *H. hepaticus* induce iNOS directly on the apical side of cecal epithelial cells (**Fig. 2**a). In $Rag2^{-/-}$ mice, *H. hepaticus*-mediated nitric oxide production leads to cecal epithelial cell hyperproliferation and DNA damage; phenotypes that are reverted after IL-22 depletion. *H. hepaticus*-mediated IL-22 induction was not only associated with nitric oxide production, but also with dysbiosis (Wang et al., 2017).

DNA damage

H. hepaticus also produces the cytolethal distending toxin (CDT), which is formed by three subunits encoded by the genes *cdtA*, *cdtB* and *cdtC. The* subunit CdtA allows the toxin to attach to the cell membrane, while CdtC promotes the translocation of the DNase CdtB into the nucleus (**Fig. 2**b) (Avenaud et al., 2004). Once this toxin is translocated into the nucleus, it catalyzes the formation of DSBs during S-phase of the cell cycle (Fedor et al., 2013), inducing DNA damage (Ge et al., 2017) and G2/M cell cycle arrest (Young et al., 2000). The mechanism by which CDT induces colitis and CRC has been extensively investigated in the context of other pathogens that can produce this toxin, such as enterotoxigenic *Escherichia coli* (see below), however the mechanisms by which *H. hepaticus* CDT induces disease in vivo has not been fully investigated. In vitro studies have shown that the DNA repair pathways activated by *H. hepaticus* CDT are very similar to those activated after irradiation (IR)-induced DNA damage. CDT activates both ATM-CHK2 and ATR-CHK1 pathways, and leads to the formation of persistent yH2AX foci in human fibroblasts (Fahrer et al., 2014) and colon epithelial cells from *Rag2*-deficient mice (Ge et al., 2017). When CDT-induced DNA damage is detected, cells undergo G2/M cell cycle arrest (Young et al., 2000), which is consistent with the protection against CDT-induced DSBs conferred

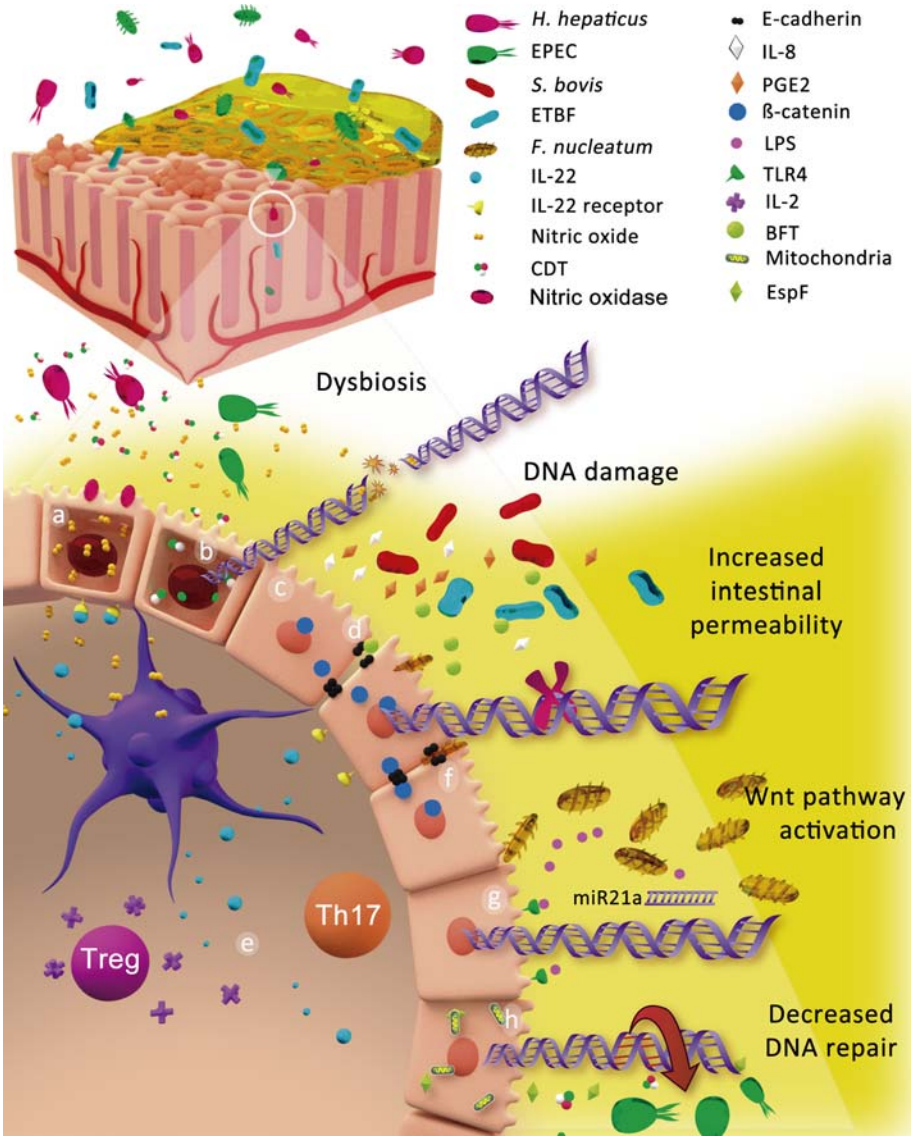

Fig. 2 (a) *H. hepaticus* induce IL-22 production by immune cells, which triggers iNOS expression on the apical side of the epithelial cells and nitric oxide production. Nitric oxide leads to cell hyperproliferation, DNA damage and dysbiosis. (b) *H. hepaticus* and EPEC produce the cytolethal distending toxin (CDT), which induces the formation of DSBs. (c) *S. bovis* can directly induce hyperproliferation of CECs and increase release of CXC chemokines, IL-8 and prostaglandins E2 (PGE2). PGE2 can activate the transcription of genes that regulate proliferation in the colon, such as the Wnt-pathway. (d) ETBF toxin (BFT) cleaves E-cadherin increasing intestinal permeability, and leading to the translocation of β-catenin into the nucleus, which induces proliferation. (e) After ETBF infection, Tregs are recruited into the lamina propria, creating a microenvironment that facilitates Th17 polarization, which promotes IL-17 mediated carcinogenesis. (f) *F. nucleatum's* FadA adhesin binds to E-cadherin, which increases free β-catenin, thereby activating the Wnt-pathway. (g) *F. nucleatum* also activates TLR4, which induces the expression of miR21a leading to the activation of the Wnt-pathway. (h) EPEC secretes EspF, which targets the mitochondria leading to the downregulation of the MMR activity.

by both homologous recombination (HR) and nonhomologous end-joining (NHEJ) in human fibroblasts (Fahrer et al., 2014; Bezine et al., 2016). Interestingly, since it is known that the MMR proteins MLH1 and MSH2 are necessary for DNA repair and the activation of the ATM-CHK2 pathway (Brown et al., 2003), it is possible that patients with MMR-deficiencies could be especially susceptible to CDT-induced DNA damage, although this would need to be investigated.

In conclusion, while *H. hepaticus* is clearly linked to CRC development in mice, the mechanism by which it does so in vivo is lacking. Other pathogens that produce CDT and have been associated with induction of CRC in humans, do not colonize mice efficiently, and therefore, although *H. hepaticus* might not colonize the human colon, it easily colonizes the mouse intestine without the need of antibiotic pretreatment, providing an excellent model to study the effects of CDT on colitis and CRC. Furthermore, different pathogens could lead to distinct immune responses resulting in divergent phenotypes. Therefore, one cannot assume that the effect of one toxin produced by a specific pathogen would be the same when the toxin is produced by another distant pathogen.

Streptococcus bovis Biotype I

Streptococcus bovis (*S. bovis*) is a gram-positive commensal bacterium that belongs to the Firmicutes phylum, and is found in 2.5%–15% of healthy humans (Hooper and Gordon, 2001). Surprisingly, when this opportunistic pathogen causes endocarditis, its infection is linked to CRC (McCoy and Mason 3rd, 1951; Hoppes and Lerner, 1974; Klein et al., 1977). Specifically, there is a striking association between endocarditis induced by *S. bovis* biotype I (*S. gallolyticus* subsp. *gallolyticus*, referred after as *S. bovis*) and CRC (Ruoff et al., 1989; Corredoira et al., 2005). In fact, both ulcerative carcinomas and precancerous polyps are associated with this microbe (Burns et al., 1985; Tjalsma et al., 2006). However, it is still under debate whether *S. bovis* infection can directly induce CRC.

S. bovis Adherence and Colonization of Epithelial Cells

Although *S. bovis* usually do not infect intestinal tissue, some reports suggest that ulceration in colorectal carcinomas facilitate the entry of *S. bovis* into the blood stream leading to bacteremia and endocarditis. *S. bovis* express several surface proteins that belong to the Microbial Surface Component Recognizing Adhesive Matrix Molecules (MSCRAMM) family (Sillanpaa et al., 2009). These proteins can bind to collagen-rich surfaces, which are found in both dysplastic colonic epithelium and damaged cardiac tissue. In vitro studies suggest that after *S. bovis has* attached to the epithelium, these bacteria can cross the epithelial layer via a paracellular mechanism, without inducing IL-1β or IL-8 responses that would otherwise clear the infection (Boleij et al., 2011). In contrast, in vivo studies have found that *S. bovis* infection lead to inflammation of the colonic mucosa (Ellmerich et al., 2000), which is associated with cancer development (Biarc et al., 2004).

Mechanisms by Which *S. bovis* Could Induce Colon Carcinogenesis

Animal models have shown that *S. bovis* can directly induce cellular transformation in vivo. Rats treated with the carcinogen azoxymethane (AOM) and either *S. bovis* or bacterial antigens extracted from its cell wall developed preneoplastic lesions characterized by hyperproliferation of CECs and increased release of CXC chemokines, IL-8 and prostaglandins E2 (PGE2) in the colonic epithelium (Biarc et al., 2004; Ellmerich et al., 2000). PGE2 can activate the transcription of genes that are usually under the control of the Wnt-pathway (**Fig. 2**c) (Shao et al., 2005), which when dysregulated can enhance tumor formation. Therefore, *S. bovis*-induced PGE2 could be a link between inflammation and carcinogenesis.

Potential Role of *S. bovis* in Human CRC

A causative association between *S. bovis* infection and CRC seems to be restricted to certain scenarios such as when the host is chronically infected and the colonic epithelium is inflamed, the latter of which could lead to an increased rate of genetic mutations and/or hyperproliferation. At least two studies have found that IBD patients have a trend toward an increase in the frequency of fecal *S. bovis*, results that were likely not significant due to the size of the sample (Klein et al., 1977; Dubrow et al., 1991; Moshkowitz et al., 1992). Nevertheless, further work is required to establish *S. bovis* in CRC in both humans and colitis-prone animal models.

In conclusion, it is not clear whether *S. bovis* is a CRC initiator or passenger, however given that this bacterium can induce inflammation and cell transformation in vivo, it is reasonable to hypothesize that *S. bovis* could generate an environment that can exacerbate colon carcinogenesis.

Enterotoxigenic *Bacteroides fragilis*

Bacteroides are one of the major phylum found in the colon, and compromise several symbionts, such as the human anaerobe *Bacteroides fragilis*, which constitute 1%–2% of cultured fecal bacteria. The presence of a 6 kb pathogenicity island that encodes the secretory metalloproteinase II (MPII) and one of the three homologous toxins named *B. fragilis* toxins (BFT), distinguishes two classes of *B. fragilis*. Strains that secrete BFT are called enterotoxigenic *Bacteroides fragilis* (ETBF) while strains that do not produce this toxin are called nontoxigenic *B. fragilis* (Sears et al., 2014). Although, ETBF can asymptomatically colonize the human colon (Sears, 2009), several reports linked ETBF infection to acute diarrhea in young children (Sack et al., 1992) and CRC (Viljoen et al., 2015; Purcell et al., 2017; Zhou et al., 2016; Wu et al., 2009; Goodwin et al., 2011; Housseau et al., 2016; Geis et al., 2015), which will be discussed in this section.

Mechanisms by Which *B. fragilis* Toxin Could Induce Colon Carcinogenesis

The mechanisms of action of BFT are not fully understood, however current research suggests that BFT can cleave E-cadherin, increase intestinal permeability, trigger inflammation and DNA damage, and induce morphological changes and cell proliferation in intestinal epithelial cells.

E-cadherin cleavage

The adhesion molecule E-cadherin, which usually enables cell-to-cell contacts in the epithelium, is a target for both MPII and BFT. While MPII binds to E-cadherin (Remacle et al., 2014), BFT cleaves it thereby weakening tight junctions, and increasing cell permeability, which lead to a series of morphological changes in human intestinal cells (Wu et al., 1998). E-cadherin is not only an adhesion molecule, its cytoplasmic domain associates with β-catenin. Binding of E-cadherin can lead to the translocation of β-catenin into the nucleus and transcriptional activation of several genes that control cell proliferation (**Fig. 2**d). Accordingly, when human CECs (HT29/C1) are treated with BFT, cleavage of membrane associated-E-cadherin leads to nuclear localization of β-catenin, and transcriptional upregulation of c-myc, triggering cell proliferation (Wu et al., 2003).

Some studies have suggested that BFT induces IL-8 secretion by cleaving E-cadherin in human intestinal cells. In fact, E-cadherin deficient cells do not express IL-8 after exposure to BFT. However, disruption of cell-to-cell interactions after EDTA-treatment is enough to induce IL-8 production by HT29/C1 cells. Furthermore β-catenin activation alone can induce IL-8 secretion, suggesting that E-cadherin cleavage is not required to induce IL-8 secretion (Hwang et al., 2013).

Inflammation

ETBF has also been shown to trigger either colitis or CAC depending on the host's T-cell polarization after infection. For example, $Apc^{min/+}$ (multiple intestinal neoplasia) mice infected with ETBF develop colonic neoplasia via activation of inflammatory Th17 cells (Wu et al., 2009). After ETBF infection, anti-inflammatory Tregs are recruited into the lamina propria, limiting IL-2 availability, and creating a microenvironment that facilitates pathogenic Th17 polarization, which promotes IL-17 mediated carcinogenesis (**Fig. 2**e) (Housseau et al., 2016; Geis et al., 2015). In agreement with this finding, ETBF-infected Treg deficient mice do not develop CAC, however in this scenario IL-2 is available for Th1 cell polarization, which produce interferon gamma (IFNγ) inducing colitis but not CRC. In this case, EBTF infection could result in two different inflammatory pathways, which depend on the immune status of the host. These findings highlight the importance of characterizing the nature of the inflammatory response after microbial infection (Irrazabal and Martin, 2015), information that is lacking with other CRC-associated bacteria.

DNA damage

BFT has also been shown to increase ROS production by inducing SMO, similar to that observed with the *H. pylori* toxin *CagA*, which in turn can lead to DNA damage (**Fig. 2**d). BFT upregulates the enzyme SMO in CEC cell lines (i.e., HT29/c1) and colonic tissue of C57BL/6 mice (Goodwin et al., 2011). SMO is a polyamine catabolic enzyme that leads to increased ROS production after inflammatory stimuli. BFT-mediated SMO-induction increases the levels of y-H2AX in colon cancer cell lines. On the other hand, chronic inflammation and CEC hyperproliferation in ETBF-infected mice is reduced when mice are treated with a SMO-inhibitor (Goodwin et al., 2011). In fact, colonic polyps can be reduced with administration of a SMO-inhibitor in BFT-infected $Apc^{min/+}$ mice.

Evidence Supporting a Role for ETBF in Human Colon Carcinogenesis

Given the evidence that ETBF could induce CRC in mice, research has now focused on studying the prevalence and association of ETBF with colon carcinogenic lesions in humans. Indeed, the presence and abundance of the BFT gene in mucosal tissue from patients undergoing colonoscopy is positively associated with early-stage tumoral lesions (Purcell et al., 2017). In addition, studies focused on different populations have found that ETBF is significantly increased in tumoral tissue compared to both adjacent normal tissue and healthy controls (Viljoen et al., 2015; Zhou et al., 2016).

In conclusion, extensive research supports a role of ETBF in CAC induction in genetically susceptible mouse models. ETBF is also increased in tumoral tissue in human subjects. However, there is not enough evidence to conclude that ETBF is linked to tumor initiation in healthy subjects. ETBF is found in normal tissue and control subjects, suggesting that ETBF might have pathological effects only under certain conditions.

Fusobacterium nucleatum

Fusobacterium nucleatum (*F. nucleatum*) is an invasive, adherent and proinflammatory gram-negative anaerobe that is normally found in the oral cavity, and rarely detected in the gut of healthy humans. However, *F. nucleatum* can be isolated from the intestines of individuals suffering with IBD (Strauss et al., 2011) which has stimulated further research into *F. nucleatum*, inflammatory intestinal disease, and CAC.

Mechanisms by Which *F. nucleatum* Could Induce Colon Carcinogenesis

F. nucleatum adheres to CECs, which has been reported to be mediated by binding of the fusobacterial protein Fap2 to Gal-GalNAc lectin that is expressed on CECs, and overexpressed in CRC cells (Abed et al., 2016). In fact, binding of Fap2-deficient *F. nucleatum* to CRC cells that express Gal-GalNAc is reduced (Abed et al., 2016), suggesting that colonic tumor microenvironment favors *F. nucleatum* attachment to epithelial cells. It has been proposed that *F. nucleatum* can induce CRC mainly by inducing CEC proliferation and inhibiting inflammatory cells.

Proliferation inducer

Cancer cell lines (Caco-2 and SW480) infected with an invasive *F. nucleatum* strain extracted from human CRC tissue have increased protein levels of activated β-catenin and its targets c-myc and cyclin D1 (Chen et al., 2017) suggesting that invasive *F. nucleatum* can directly induce hyperproliferation of CRC cells. There are a number of reports that suggest that the effects of *F. nucleatum* on proliferation could be mediated by the action of the adhesin FadA and through TLR4 activation. First, *F. nucleatum's* FadA adhesin binds to E-cadherin, which increases free β-catenin, thereby activating the Wnt-pathway and inducing cell proliferation (**Fig. 2f**) (Rubinstein et al., 2013). This adhesin could potentially be used as a diagnostic marker for CRC since the levels of the *fadA* gene in the colon of patients with adenomas are >10–100 times higher compared to controls. Second, inhibition of the Toll-like receptor 4 in CRC cells decreases *F. nucleatum*-induced activation of the β-catenin pathway (Chen et al., 2017). Interestingly, microRNA (miRNA) 21, which is expressed under the control of TLR4, can also affect CEC proliferation since inhibitors of miR21 reduce *F. nucleatum*-induced proliferation and invasion in cultured cells (**Fig. 2g**) (Yang et al., 2017). Indeed, *F. nucleatum*-infected miR21a-deficient mice have less tumors and increased survival compared to control mice. In addition, it has also been reported that elevated levels of *F. nucleatum* DNA in human tumors correlate with high MiR21 expression and worse clinical outcome (Yang et al., 2017).

Immunosuppresor

Besides being necessary for adherence, *F. nucleatum* Fap2 protein can inhibit specific immune responses. Fap2 interacts with the inhibitory receptor TIGIT, which is expressed in all human natural killer (NK) cells and on some T-cell populations, and therefore *F. nucleatum* can inhibit the activity of NK cells and tumor infiltrating lymphocytes (Gur et al., 2015). In fact, there is an inverse correlation between CRCs associated with *F. nucleatum* and $CD3^+$ T-cell density (Mima et al., 2015), suggesting that this bacterium could promote CRC by downregulating antitumoral T-cell cytotoxicity. In addition, *F. nucleatum*-induced CRCs in $Apc^{Min/+}$ mice have an elevated number of infiltrating myeloid-derived suppressor cells, which have immunosuppressive activity and allow tumor growth (Kostic et al., 2013). $APC^{min/+}$ mice gavaged with *F. nucleatum* develop more tumors in the colon (Kostic et al., 2013; Yang et al., 2017), and have higher mortality than vehicle treated controls (Yang et al., 2017). Tumors from *F. nucleatum*-treated $APC^{min/+}$ mice show a proinflammatory profile similar to human *Fusobacterium*-positive tumors, however *F. nucleatum* does not worsen colitis and CAC in mice (Kostic et al., 2013), suggesting that this bacterium can induce an antiinflammatory environment that facilitates tumor progression but not tumor initiation. Moreover, *F. nucleatum* produce butyrate, and butyrate can induce Treg differentiation (Furusawa et al., 2013), which could facilitate tumor growth by hampering antitumoral immune responses.

Evidence Supporting a Role of *F. nucleatum* in Human Colon Carcinogenesis

F. nucleatum is significantly increased in tumors compared to adjacent normal tissue and tissue from healthy controls (Viljoen et al., 2015; Zhou et al., 2016; Castellarin et al., 2012; Kostic et al., 2012). The presence of *F. nucleatum* is also significantly associated with microsatellite instability (MSI) high tumors (Nosho et al., 2016). In addition, *F. nucleatum* strains extracted from inflamed mucosa of IBD patients are more invasive than strains extracted from control patients (Strauss et al., 2011), and invasive *F. nucleatum* infection is positively associated with β-catenin activation in CRC tissues (Chen et al., 2017).

Overall there is substantial evidence supporting a positive association between *F. nucleatum* infection and CRC in humans. However, in contrast to other bacteria associated to CRC, *F. nucleatum* seems to have antiinflammatory capacities that facilitate tumor escape from immune surveillance, and therefore might promote tumor progression rather than initiate tumors.

Escherichia coli

Escherichia coli (*E. coli*) are commensal gram-negative bacteria found in normal gut microbiota and rarely cause disease. However, a subgroup of these bacteria, named enteropathogenic *E. coli* (EPEC), can attach to the intestinal mucosa and produce toxins that induce bowel disease in humans.

Mechanisms by Which EPEC Could Induce Colon Carcinogenesis

Some reports have found that EPEC incidence is significantly higher in CRC patients than in healthy controls (Magdy et al., 2015), prompting research in search for a possible link between EPEC and CRC. Current research indicates that inflammation might favor EPEC carcinogenesis, which could potentially be induced by two mechanisms: DNA damage, and MMR downregulation.

DNA damage

EPEC uses a type 3 secretion system (T3SS) to inject various effector proteins into the host cells, and it also express the genotoxins colibactin and CDT (**Fig. 2b**, see *H. hepaticus*). Colibactin is encoded within the *polyketide synthase* (*pks*) gene island, and crosslinks DNA (Vizcaino and Crawford, 2015), promoting genomic instability in vitro (Cuevas-Ramos et al., 2010), and DSBs in mouse enterocytes (Cuevas-Ramos et al., 2010; Arthur et al., 2012). In a colitis AOM/$IL\text{-}10^{-/-}$ model of CRC, infection with pks+ *E. coli* increased tumor counts (Arthur et al., 2012). This finding was recapitulated in an AOM/DSS treated mice models (Cuevas-Ramos et al., 2010; Cougnoux et al., 2014; Dalmasso et al., 2014). Additionally, inflammation seems to accelerate *pks*+ *E. coli*-induced carcinogenesis: AOM-treated $IL\text{-}10^{-/-}$ mice infected with *pks*+ *E. coli* exhibit significantly more tumors than

lymphocyte-deficient AOM-treated Rag2$^{-/-}$ IL-10$^{-/-}$ mice infected with *pks*+ *E. coli* (Arthur et al., 2014). The *E. coli* pks island is one of the top five operons upregulated in AOM-treated IL-10$^{-/-}$ mice compared to untreated mice (Arthur et al., 2014), suggesting that during carcinogenesis, the inflamed intestinal environment induce *pks* transcription hastening carcinogenesis in infected mice.

The EPEC genotoxin CDT also induces DSBs in cultured cells, which leads to ATM-mediated induction of G2/M cell cycle arrest (Fedor et al., 2013). Moreover, CDT accelerates growth in p53-deficient but not in KRAS-deficient human CECs (Graillot et al., 2016), suggesting that CDT genotoxicity could be exacerbated in DNA repair-defective cells.

MMR downregulation

EPEC has been found to downregulate the expression of the MMR proteins MSH2 and MLH1 in cultured CRC cell lines (**Fig. 2**h) (Maddocks et al., 2009). MMR activity downregulation is mediated by EPEC secretion of the protein EspF, which targets the mitochondria (Maddocks et al., 2013). Interestingly, *H. pylori* VacA also targets the mitochondria and reduce the MMR function, suggesting that these toxins may disrupt similar mitochondrial pathways.

Evidence Supporting a Role of EPEC on Human Colon Carcinogenesis

Sixty seven percentage of CRC patients harbor *pks*+ *E. coli* compared to 21% in normal controls (Arthur et al., 2012). Furthermore, although there is a significant increase in total *E. coli* colonization in human MSI tumors, colibactin-producing *E. coli* have been found to be enriched in microsatellite stable (MSS) tumors (Gagniere et al., 2017). In contrast, another report found that there is no significant difference in the concentration of *pks*-positive bacterial DNA between CRC, adenoma, and control patient groups (Shimpoh et al., 2017).

In summary, EPEC can induce CRC in inflammatory mouse models. In addition, EPEC encode two genotoxins that control the cell cycle and induce DNA damage. Moreover, current data indicate that there is a positive association between EPEC infection and CRC in humans (Magdy et al., 2015; Arthur et al., 2012), supporting a role for EPEC in CRC initiation in humans.

Conclusion

It is evident that most bacteria that have been linked to CRC seem to exploit similar mechanisms to induce carcinogenesis: DNA damage, immune dysregulation, and hyperproliferation (**Fig. 2**). All the bacteria previously described, with exception of *S. bovis* and *F. nucleatum*, have the capacity to induce DNA damage (Chaturvedi et al., 2011; Xu et al., 2004; Wang et al., 2017; Goodwin et al., 2011; Cuevas-Ramos et al., 2010). Remarkably, as with irradiation-induced DNA damage, most bacteria seem to trigger ROS production, which in turn leads to DSB formation. Oxidative stress induces DNA oxidation which is usually repaired by the MMR and BER (base excision repair) pathways (Russo et al., 2007). Therefore, DNA repair-deficient subjects might be more susceptible to the effects of ROS. Furthermore, it has been implied that *E. coli* can create the perfect storm for colon carcinogenesis by both inducing DNA damage and downregulating DNA damage repair proteins (Maddocks et al., 2013).

Immune dysregulation is another factor exploited by many microbes. *S. bovis* induce an inflammatory response that facilitate uncontrolled cell proliferation, while carcinogenesis induced by *H. pylori*, *H. hepaticus*, ETBF, and *pks*+ *E. coli* is facilitated by inflammation. Furthermore, ETBF infection in mice could lead to either colitis or CAC depending on the inflammatory response mounted by the host (Geis et al., 2015). In contrast, *F. nucleatum* seems to induce an immunosuppressive environment that facilitate tumor progression (Mima et al., 2015).

Many microbes implicated in CRC also induce CEC hyperproliferation. The most common CRC-associated mutations are in genes that control CEC proliferation (i.e., APC and β-catenin), and some microbes seem to exploit this pathway to induce proliferation of CECs. On the other hand, hyperproliferation induced by microbes could be a consequence of deficiencies in DDR in the host, since genetic mutations induced by microbes would not lead to the activation of cell cycle checkpoints, allowing damaged DNA to be propagated to daughter cells, facilitating carcinogenesis.

Finally, current research suggests that *H. pylori* is the only bacterium that can induce carcinogenesis in any host, irrespective of genetic background, while most of the aforementioned bacteria cannot induce CEC transformation in healthy subjects and need a "first hit," such as a specific genetic deficiency, that sensitizes the host to the oncogenic effects of the microbe. Indeed, this pathogen + susceptibility gene formula has been previously proposed for IBD (Cadwell et al., 2010), and seems applicable to many cases of microbial-induced CRC or CAC.

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See also: Carcinogenesis: Role of Reactive Oxygen and Nitrogen Species. Colorectal Cancer: Pathology and Genetics.

References

Abed, J., Emgard, J.E., Zamir, G., Faroja, M., Almogy, G., Grenov, A., et al., 2016. Fap2 mediates fusobacterium nucleatum colorectal adenocarcinoma enrichment by binding to tumor-expressed Gal-GalNAc. Cell Host & Microbe 20 (2), 215–225.

Anon, 1994. Infection with *Helicobacter pylori*. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 61, 177–240.

Arthur, J.C., Perez-Chanona, E., Muhlbauer, M., Tomkovich, S., Uronis, J.M., Fan, T.J., et al., 2012. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338 (6103), 120–123.

Arthur, J.C., Gharaibeh, R.Z., Muhlbauer, M., Perez-Chanona, E., Uronis, J.M., McCafferty, J., et al., 2014. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. Nature Communications 5, 4724.

Avenaud, P., Castroviejo, M., Claret, S., Rosenbaum, J., Megraud, F., Menard, A., 2004. Expression and activity of the cytolethal distending toxin of *Helicobacter hepaticus*. Biochemical and Biophysical Research Communications 318 (3), 739–745.

Bain CC, Oliphant CJ, Thomson CA, Kullberg MC, Mowat AM. Pro-inflammatory role of monocyte-derived CX3CR1int macrophages in *Helicobacter hepaticus*-induced colitis. Infection and Immunity 2018, 86(2). pii: e00579-17.

Belcheva, A., Irrazabal, T., Martin, A., 2015. Gut microbial metabolism and colon cancer: Can manipulations of the microbiota be useful in the management of gastrointestinal health? BioEssays 37 (4), 403–412.

Bezine, E., Malaise, Y., Loeuillet, A., Chevalier, M., Boutet-Robinet, E., Salles, B., et al., 2016. Cell resistance to the Cytolethal distending toxin involves an association of DNA repair mechanisms. Scientific Reports 6, 36022.

Biarc, J., Nguyen, I.S., Pini, A., Gosse, F., Richert, S., Thierse, D., et al., 2004. Carcinogenic properties of proteins with pro-inflammatory activity from *Streptococcus infantarius* (formerly *S. bovis*). Carcinogenesis 25 (8), 1477–1484.

Blase, J.L., Campbell, P.T., Gapstur, S.M., Pawlita, M., Michel, A., Waterboer, T., et al., 2016. Prediagnostic *Helicobacter pylori* antibodies and colorectal cancer risk in an elderly, Caucasian population. Helicobacter 21 (6), 488–492.

Boleij, A., Muytjens, C.M., Bukhari, S.I., Cayet, N., Glaser, P., Hermans, P.W., et al., 2011. Novel clues on the specific association of *Streptococcus gallolyticus* subsp *gallolyticus* with colorectal cancer. The Journal of Infectious Diseases 203 (8), 1101–1109.

Bradley, P.H., Pollard, K.S., 2017. Proteobacteria explain significant functional variability in the human gut microbiome. Microbiome 5 (1), 36.

Breuer-Katschinski, B., Nemes, K., Marr, A., Rump, B., Leiendecker, B., Breuer, N., et al., 1999. *Helicobacter pylori* and the risk of colonic adenomas. Colorectal Adenoma Study Group. Digestion 60 (3), 210–215.

Brown, K.D., Rathi, A., Kamath, R., Beardsley, D.I., Zhan, Q., Mannino, J.L., et al., 2003. The mismatch repair system is required for S-phase checkpoint activation. Nature Genetics 33 (1), 80–84.

Bugaytsova, J.A., Bjornham, O., Chernov, Y.A., Gideonsson, P., Henriksson, S., Mendez, M., et al., 2017. *Helicobacter pylori* adapts to chronic infection and gastric disease via pH-responsive BabA-mediated adherence. Cell Host & Microbe 21 (3), 376–389.

Burns, C.A., McCaughey, R., Lauter, C.B., 1985. The association of *Streptococcus bovis* fecal carriage and colon neoplasia: Possible relationship with polyps and their premalignant potential. The American Journal of Gastroenterology 80 (1), 42–46.

Buti, L., Spooner, E., Van der Veen, A.G., Rappuoli, R., Covacci, A., Ploegh, H.L., 2011. *Helicobacter pylori* cytotoxin-associated gene a (CagA) subverts the apoptosis-stimulating protein of p53 (ASPP2) tumor suppressor pathway of the host. Proceedings of the National Academy of Sciences of the United States of America 108 (22), 9238–9243.

Byndloss, M.X., Olsan, E.E., Rivera-Chavez, F., Tiffany, C.R., Cevallos, S.A., Lokken, K.L., et al., 2017. Microbiota-activated PPAR-gamma signaling inhibits dysbiotic Enterobacteriaceae expansion. Science 357 (6351), 570–575.

Cadwell, K., Patel, K.K., Maloney, N.S., Liu, T.C., Ng, A.C., Storer, C.E., et al., 2010. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell 141 (7), 1135–1145.

Castellarin, M., Warren, R.L., Freeman, J.D., Dreolini, L., Krzywinski, M., Strauss, J., et al., 2012. *Fusobacterium nucleatum* infection is prevalent in human colorectal carcinoma. Genome Research 22 (2), 299–306.

Cater 2nd, R.E., 1992. The clinical importance of hypochlorhydria (a consequence of chronic Helicobacter infection): Its possible etiological role in mineral and amino acid malabsorption, depression, and other syndromes. Medical Hypotheses 39 (4), 375–383.

Chang, W.L., Yang, H.B., Cheng, H.C., Yeh, Y.C., Kao, C.Y., Wu, J.J., et al., 2015. Intracellular osteopontin induced by CagA-positive *Helicobacter pylori* promotes beta-catenin accumulation and interleukin-8 secretion in gastric epithelial cells. Helicobacter 20 (6), 476–484.

Chaturvedi, R., Asim, M., Romero-Gallo, J., Barry, D.P., Hoge, S., de Sablet, T., et al., 2011. Spermine oxidase mediates the gastric cancer risk associated with *Helicobacter pylori* CagA. Gastroenterology 141 (5), 1696-708 e1-2.

Chaturvedi, R., Asim, M., Piazuelo, M.B., Yan, F., Barry, D.P., Sierra, J.C., et al., 2014. Activation of EGFR and ERBB2 by *Helicobacter pylori* results in survival of gastric epithelial cells with DNA damage. Gastroenterology 146 (7), 1739-51 e14.

Chen, X.Z., Schottker, B., Castro, F.A., Chen, H., Zhang, Y., Holleczek, B., et al., 2016. Association of *Helicobacter pylori* infection and chronic atrophic gastritis with risk of colonic, pancreatic and gastric cancer: A ten-year follow-up of the ESTHER cohort study. Oncotarget 7 (13), 17182–17193.

Chen, Y., Peng, Y., Yu, J., Chen, T., Wu, Y., Shi, L., et al., 2017. Invasive *Fusobacterium nucleatum* activates beta-catenin signaling in colorectal cancer via a TLR4/P-PAK1 cascade. Oncotarget 8 (19), 31802–31814.

Corredoira, J.C., Alonso, M.P., Garcia, J.F., Casariego, E., Coira, A., Rodriguez, A., et al., 2005. Clinical characteristics and significance of *Streptococcus salivarius* bacteremia and *Streptococcus bovis* bacteremia: A prospective 16-year study. European Journal of Clinical Microbiology & Infectious Diseases 24 (4), 250–255.

Cougnoux, A., Dalmasso, G., Martinez, R., Buc, E., Delmas, J., Gibold, L., et al., 2014. Bacterial genotoxin colibactin promotes colon tumour growth by inducing a senescence-associated secretory phenotype. Gut 63 (12), 1932–1942.

Cuevas-Ramos, G., Petit, C.R., Marcq, I., Boury, M., Oswald, E., Nougayrede, J.P., 2010. *Escherichia coli* induces DNA damage in vivo and triggers genomic instability in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 107 (25), 11537–11542.

Dalmasso, G., Cougnoux, A., Delmas, J., Darfeuille-Michaud, A., Bonnet, R., 2014. The bacterial genotoxin colibactin promotes colon tumor growth by modifying the tumor microenvironment. Gut Microbes 5 (5), 675–680.

Dubrow, R., Edberg, S., Wikfors, E., Callan, D., Troncale, F., Vender, R., et al., 1991. Fecal carriage of *Streptococcus bovis* and colorectal adenomas. Gastroenterology 101 (3), 721–725.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al., 2005. Diversity of the human intestinal microbial flora. Science 308 (5728), 1635–1638.

Ellmerich, S., Scholler, M., Duranton, B., Gosse, F., Galluser, M., Klein, J.P., et al., 2000. Promotion of intestinal carcinogenesis by *Streptococcus bovis*. Carcinogenesis 21 (4), 753–756.

Epplein, M., Pawlita, M., Michel, A., Peek Jr., R.M., Cai, Q., Blot, W.J., 2013. *Helicobacter pylori* protein-specific antibodies and risk of colorectal cancer. Cancer Epidemiology, Biomarkers & Prevention 22 (11), 1964–1974.

Erdman, S.E., Rao, V.P., Poutahidis, T., Ihrig, M.M., Ge, Z., Feng, Y., et al., 2003. CD4(+)CD25(+) regulatory lymphocytes require interleukin 10 to interrupt colon carcinogenesis in mice. Cancer Research 63 (18), 6042–6050.

Erdman, S.E., Rao, V.P., Poutahidis, T., Rogers, A.B., Taylor, C.L., Jackson, E.A., et al., 2009. Nitric oxide and TNF-alpha trigger colonic inflammation and carcinogenesis in *Helicobacter hepaticus*-infected, Rag2-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 106 (4), 1027–1032.

Fahrer, J., Huelsenbeck, J., Jaurich, H., Dorsam, B., Frisan, T., Eich, M., et al., 2014. Cytolethal distending toxin (CDT) is a radiomimetic agent and induces persistent levels of DNA double-strand breaks in human fibroblasts. DNA Repair (Amst) 18, 31–43.
Falck, J., Mailand, N., Syljuasen, R.G., Bartek, J., Lukas, J., 2001. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 410 (6830), 842–847.
Falsafi, T., Mahboubi, M., 2013. *Helicobacter hepaticus*, a new pathogenic species of the Helicobacter genus: Similarities and differences with *H. pylori*. Iranian Journal of Microbiology 5 (3), 185–194.
Fedor, Y., Vignard, J., Nicolau-Travers, M.L., Boutet-Robinet, E., Watrin, C., Salles, B., et al., 2013. From single-strand breaks to double-strand breaks during S-phase: A new mode of action of the *Escherichia coli* cytolethal distending toxin. Cellular Microbiology 15 (1), 1–15.
Ferreira, R.M., Pinto-Ribeiro, I., Wen, X., Marcos-Pinto, R., Dinis-Ribeiro, M., Carneiro, F., et al., 2016. *Helicobacter pylori* cagA promoter region sequences influence CagA expression and interleukin 8 secretion. The Journal of Infectious Diseases 213 (4), 669–673.
Fujimori, S., Kishida, T., Kobayashi, T., Sekita, Y., Seo, T., Nagata, K., et al., 2005. *Helicobacter pylori* infection increases the risk of colorectal adenoma and adenocarcinoma, especially in women. Journal of Gastroenterology 40 (9), 887–893.
Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., et al., 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504, 446–450.
Gagniere, J., Bonnin, V., Jarrousse, A.S., Cardamone, E., Agus, A., Uhrhammer, N., et al., 2017. Interactions between microsatellite instability and human gut colonization by *Escherichia coli* in colorectal cancer. Clinical Science (London, England) 131 (6), 471–485.
Ge, Z., Feng, Y., Ge, L., Parry, N., Muthupalani, S., Fox, J.G., 2017. *Helicobacter hepaticus* cytolethal distending toxin promotes intestinal carcinogenesis in 129Rag2-deficient mice. Cellular Microbiology 19 (7).
Geis, A.L., Fan, H., Wu, X., Wu, S., Huso, D.L., Wolfe, J.L., et al., 2015. Regulatory T-cell response to enterotoxigenic *Bacteroides fragilis* colonization triggers IL17-dependent colon carcinogenesis. Cancer Discovery 5 (10), 1098–1109.
Gnad, T., Feoktistova, M., Leverkus, M., Lendeckel, U., Naumann, M., 2010. *Helicobacter pylori*-induced activation of beta-catenin involves low density lipoprotein receptor-related protein 6 and Dishevelled. Molecular Cancer 9, 31.
Goodwin, A.C., Destefano Shields, C.E., Wu, S., Huso, D.L., Wu, X., Murray-Stewart, T.R., et al., 2011. Polyamine catabolism contributes to enterotoxigenic *Bacteroides fragilis*-induced colon tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America 108 (37), 15354–15359.
Graillot, V., Dormoy, I., Dupuy, J., Shay, J.W., Huc, L., Mirey, G., et al., 2016. Genotoxicity of cytolethal distending toxin (CDT) on isogenic human colorectal cell lines: Potential promoting effects for colorectal carcinogenesis. Frontiers in Cellular and Infection Microbiology 6, 34.
Gur, C., Ibrahim, Y., Isaacson, B., Yamin, R., Abed, J., Gamliel, M., et al., 2015. Binding of the Fap2 protein of *Fusobacterium nucleatum* to human inhibitory receptor TIGIT protects tumors from immune cell attack. Immunity 42 (2), 344–355.
Hamada, T., Yokota, K., Ayada, K., Hirai, K., Kamada, T., Haruma, K., et al., 2009. Detection of *Helicobacter hepaticus* in human bile samples of patients with biliary disease. Helicobacter 14 (6), 545–551.
Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D., et al., 2013. Archaea and fungi of the human gut microbiome: Correlations with diet and bacterial residents. PLoS One 8 (6), e66019.
Hooper, L.V., Gordon, J.I., 2001. Commensal host–bacterial relationships in the gut. Science 292 (5519), 1115–1118.
Hoppes, W.L., Lerner, P.I., 1974. Nonenterococcal group-D streptococcal endocarditis caused by *Streptococcus bovis*. Annals of Internal Medicine 81 (5), 588–593.
Housseau, F., Wu, S., Wick, E.C., Fan, H., Wu, X., Llosa, N.J., et al., 2016. Redundant innate and adaptive sources of IL17 production drive colon tumorigenesis. Cancer Research 76 (8), 2115–2124.
Human Microbiome Project Consortium, 2012. Structure, function and diversity of the healthy human microbiome. Nature 486 (7402), 207–214.
Hwang, S., Gwon, S.Y., Kim, M.S., Lee, S., Rhee, K.J., 2013. Bacteroides fragilis toxin induces IL-8 secretion in HT29/C1 cells through disruption of E-cadherin junctions. Immune Network 13 (5), 213–217.
Irrazabal, T., Martin, A., 2015. T regulatory cells gone bad: An oncogenic immune response against enterotoxigenic *B. fragilis* infection leads to colon cancer. Cancer Discovery 5 (10), 1021–1023.
Irrazabal, T., Belcheva, A., Girardin, S.E., Martin, A., Philpott, D.J., 2014. The multifaceted role of the intestinal microbiota in colon cancer. Molecular Cell 54 (2), 309–320.
Kanno, T., Matsuki, T., Oka, M., Utsunomiya, H., Inada, K., Magari, H., et al., 2009. Gastric acid reduction leads to an alteration in lower intestinal microflora. Biochemical and Biophysical Research Communications 381 (4), 666–670.
Keenan, J.I., Beaugie, C.R., Jasmann, B., Potter, H.C., Collett, J.A., Frizelle, F.A., 2010. Helicobacter species in the human colon. Colorectal Disease 12 (1), 48–53.
Klein, R.S., Recco, R.A., Catalano, M.T., Edberg, S.C., Casey, J.I., Steigbigel, N.H., 1977. Association of *Streptococcus bovis* with carcinoma of the colon. The New England Journal of Medicine 297 (15), 800–802.
Koniger, V., Holsten, L., Harrison, U., Busch, B., Loell, E., Zhao, Q., et al., 2016. *Helicobacter pylori* exploits human CEACAMs via HopQ for adherence and translocation of CagA. Nature Microbiology 2, 16188.
Kostic, A.D., Gevers, D., Pedamallu, C.S., Michaud, M., Duke, F., Earl, A.M., et al., 2012. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Research 22 (2), 292–298.
Kostic, A.D., Chun, E., Robertson, L., Glickman, J.N., Gallini, C.A., Michaud, M., et al., 2013. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host & Microbe 14 (2), 207–215.
Lee, J.Y., Park, H.W., Choi, J.Y., Lee, J.S., Koo, J.E., Chung, E.J., et al., 2016. *Helicobacter pylori* infection with atrophic gastritis is an independent risk factor for advanced colonic neoplasm. Gut Liver 10 (6), 902–909.
Leung, N., Turbide, C., Olson, M., Marcus, V., Jothy, S., Beauchemin, N., 2006. Deletion of the carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1) gene contributes to colon tumor progression in a murine model of carcinogenesis. Oncogene 25 (40), 5527–5536.
Litvak, Y., Byndloss, M.X., Tsolis, R.M., Baumler, A.J., 2017. Dysbiotic Proteobacteria expansion: A microbial signature of epithelial dysfunction. Current Opinion in Microbiology 39, 1–6.
Lloyd-Price, J., Abu-Ali, G., Huttenhower, C., 2016. The healthy human microbiome. Genome Medicine 8 (1), 51.
Machado, A.M., Figueiredo, C., Touati, E., Maximo, V., Sousa, S., Michel, V., et al., 2009. *Helicobacter pylori* infection induces genetic instability of nuclear and mitochondrial DNA in gastric cells. Clinical Cancer Research 15 (9), 2995–3002.
Maddocks, O.D., Short, A.J., Donnenberg, M.S., Bader, S., Harrison, D.J., 2009. Attaching and effacing *Escherichia coli* downregulate DNA mismatch repair protein in vitro and are associated with colorectal adenocarcinomas in humans. PLoS One 4 (5), e5517.
Maddocks, O.D., Scanlon, K.M., Donnenberg, M.S., 2013. An *Escherichia coli* effector protein promotes host mutation via depletion of DNA mismatch repair proteins. MBio 4 (3), e00152-13.
Magdy, A., Elhadidy, M., Abd Ellatif, M.E., El Nakeeb, A., Abdallah, E., Thabet, W., et al., 2015. Enteropathogenic *Escherichia coli* (EPEC): Does it have a role in colorectal tumourigenesis? A Prospective Cohort Study. International Journal of Surgery 18, 169–173.
McCaskey, S.J., Rondini, E.A., Clinthorne, J.F., Langohr, I.M., Gardner, E.M., Fenton, J.I., 2012. Increased presence of effector lymphocytes during *Helicobacter hepaticus*-induced colitis. World Journal of Gastroenterology 18 (13), 1459–1469.
McClain, M.S., Beckett, A.C., Cover, T.L., 2017. *Helicobacter pylori* vacuolating toxin and gastric cancer. Toxins (Basel) 9 (10).
McCoy, C.W., Mason 3rd., J.M., 1951. Enterococcal endocarditis associated with carcinoma of the sigmoid; report of a case. Journal of the Medical Association of the State of Alabama 21 (6), 162–166.

Medema, J.P., Vermeulen, L., 2011. Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. Nature 474 (7351), 318–326.
Mima, K., Sukawa, Y., Nishihara, R., Qian, Z.R., Yamauchi, M., Inamura, K., et al., 2015. *Fusobacterium nucleatum* and T cells in colorectal carcinoma. JAMA Oncology 1 (5), 653–661.
Minot, S., Bryson, A., Chehoud, C., Wu, G.D., Lewis, J.D., Bushman, F.D., 2013. Rapid evolution of the human gut virome. Proceedings of the National Academy of Sciences of the United States of America 110 (30), 12450–12455.
Moayyedi, P., Hunt, R.H., 2004. Helicobacter pylori public health implications. Helicobacter 9 (Suppl. 1), 67–72.
Molinari, M., Salio, M., Galli, C., Norais, N., Rappuoli, R., Lanzavecchia, A., et al., 1998. Selective inhibition of ii-dependent antigen presentation by *Helicobacter pylori* toxin VacA. The Journal of Experimental Medicine 187 (1), 135–140.
Moshkowitz, M., Arber, N., Halpern, Z., Gilat, T., 1992. Fecal carriage of *Streptococcus bovis* and inflammatory bowel disease. Gastroenterology 102 (5), 1829.
Moss, S.F., Neugut, A.I., Garbowski, G.C., Wang, S., Treat, M.R., Forde, K.A., 1995. *Helicobacter pylori* seroprevalence and colorectal neoplasia: Evidence against an association. Journal of the National Cancer Institute 87 (10), 762–763.
Nagamine, C.M., Rogers, A.B., Fox, J.G., Schauer, D.B., 2008a. *Helicobacter hepaticus* promotes azoxymethane-initiated colon tumorigenesis in BALB/c-IL10-deficient mice. International Journal of Cancer 122 (4), 832–838.
Nagamine, C.M., Sohn, J.J., Rickman, B.H., Rogers, A.B., Fox, J.G., Schauer, D.B., 2008b. *Helicobacter hepaticus* infection promotes colon tumorigenesis in the BALB/c-Rag2(−/−) Apc(Min/+) mouse. Infection and Immunity 76 (6), 2758–2766.
Nam, J.H., Hong, C.W., Kim, B.C., Shin, A., Ryu, K.H., Park, B.J., et al., 2017. *Helicobacter pylori* infection is an independent risk factor for colonic adenomatous neoplasms. Cancer Causes & Control 28 (2), 107–115.
Neumaier, M., Paululat, S., Chan, A., Matthaes, P., Wagener, C., 1993. Biliary glycoprotein, a potential human cell adhesion molecule, is down-regulated in colorectal carcinomas. Proceedings of the National Academy of Sciences of the United States of America 90 (22), 10744–10748.
Nilsson, I., Lindgren, S., Eriksson, S., Wadstrom, T., 2000. Serum antibodies to *Helicobacter hepaticus* and *Helicobacter pylori* in patients with chronic liver disease. Gut 46 (3), 410–414.
Nosho, K., Sukawa, Y., Adachi, Y., Ito, M., Mitsuhashi, K., Kurihara, H., et al., 2016. Association of *Fusobacterium nucleatum* with immunity and molecular alterations in colorectal cancer. World Journal of Gastroenterology 22 (2), 557–566.
Painter, R.B., Young, B.R., 1980. Radiosensitivity in ataxia-telangiectasia: A new explanation. Proceedings of the National Academy of Sciences of the United States of America 77 (12), 7315–7317.
Papastergiou, V., Karatapanis, S., Georgopoulos, S.D., 2016. Helicobacter pylori and colorectal neoplasia: Is there a causal link? World Journal of Gastroenterology 22 (2), 649–658.
Peek Jr., R.M., Blaser, M.J., 2002. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nature Reviews Cancer 2 (1), 28–37.
Poulogiannis, G., Frayling, I.M., Arends, M.J., 2010. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. Histopathology 56 (2), 167–179.
Purcell, R.V., Pearson, J., Aitchison, A., Dixon, L., Frizelle, F.A., Keenan, J.I., 2017. Colonization with enterotoxigenic *Bacteroides fragilis* is associated with early-stage colorectal neoplasia. PLoS One 12 (2), e0171602.
Raskov, H., Pommergaard, H.C., Burcharth, J., Rosenberg, J., 2014. Colorectal carcinogenesis—Update and perspectives. World Journal of Gastroenterology 20 (48), 18151–18164.
Remacle, A.G., Shiryaev, S.A., Strongin, A.Y., 2014. Distinct interactions with cellular E-cadherin of the two virulent metalloproteinases encoded by a *Bacteroides fragilis* pathogenicity island. PLoS One 9 (11), e113896.
Renga, M., Brandi, G., Paganelli, G.M., Calabrese, C., Papa, S., Tosti, A., et al., 1997. Rectal cell proliferation and colon cancer risk in patients with hypergastrinaemia. Gut 41 (3), 330–332.
Rivera-Chavez, F., Zhang, L.F., Faber, F., Lopez, C.A., Byndloss, M.X., Olsan, E.E., et al., 2016. Depletion of butyrate-producing clostridia from the gut microbiota drives an aerobic luminal expansion of Salmonella. Cell Host & Microbe 19 (4), 443–454.
Rivera-Chavez, F., Lopez, C.A., Baumler, A.J., 2017. Oxygen as a driver of gut dysbiosis. Free Radical Biology & Medicine 105, 93–101.
Rubinstein, M.R., Wang, X., Liu, W., Hao, Y., Cai, G., Han, Y.W., 2013. *Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. Cell Host & Microbe 14 (2), 195–206.
Ruoff, K.L., Miller, S.I., Garner, C.V., Ferraro, M.J., Calderwood, S.B., 1989. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius*: Clinical correlates of more accurate identification of isolates. Journal of Clinical Microbiology 27 (2), 305–308.
Russo, M.T., De Luca, G., Degan, P., Bignami, M., 2007. Different DNA repair strategies to combat the threat from 8-oxoguanine. Mutation Research 614 (1–2), 69–76.
Sack, R.B., Myers, L.L., Almeido-Hill, J., Shoop, D.S., Bradbury, W.C., Reid, R., et al., 1992. Enterotoxigenic *Bacteroides fragilis*: Epidemiologic studies of its role as a human diarrhoeal pathogen. Journal of Diarrhoeal Diseases Research 10 (1), 4–9.
Sears, C.L., 2009. Enterotoxigenic *Bacteroides fragilis*: A rogue among symbiotes. Clinical Microbiology Reviews 22 (2), 349–369. Table of Contents.
Sears, C.L., Geis, A.L., Housseau, F., 2014. Bacteroides fragilis subverts mucosal biology: From symbiont to colon carcinogenesis. The Journal of Clinical Investigation 124 (10), 4166–4172.
Shao, J., Jung, C., Liu, C., Sheng, H., 2005. Prostaglandin E2 stimulates the beta-catenin/T cell factor-dependent transcription in colon cancer. The Journal of Biological Chemistry 280 (28), 26565–26572.
Shimpoh, T., Hirata, Y., Ihara, S., Suzuki, N., Kinoshita, H., Hayakawa, Y., et al., 2017. Prevalence of pks-positive *Escherichia coli* in Japanese patients with or without colorectal cancer. Gut Pathogens 9, 35.
Shmuely, H., Passaro, D., Figer, A., Niv, Y., Pitlik, S., Samra, Z., et al., 2001. Relationship between *Helicobacter pylori* CagA status and colorectal cancer. The American Journal of Gastroenterology 96 (12), 3406–3410.
Sillanpaa, J., Nallapareddy, S.R., Qin, X., Singh, K.V., Muzny, D.M., Kovar, C.L., et al., 2009. A collagen-binding adhesin, Acb, and ten other putative MSCRAMM and pilus family proteins of *Streptococcus gallolyticus* subsp. *gallolyticus* (*Streptococcus bovis* group, biotype I). Journal of Bacteriology 191 (21), 6643–6653.
Sobhani, I., Lehy, T., Laurent-Puig, P., Cadiot, G., Ruszniewski, P., Mignon, M., 1993. Chronic endogenous hypergastrinemia in humans: Evidence for a mitogenic effect on the colonic mucosa. Gastroenterology 105 (1), 22–30.
Sonnenberg, A., Turner, K.O., Genta, R.M., 2017. The ethnic distribution of sessile serrated polyps in the United States is inversely associated with *Helicobacter pylori* prevalence. Colorectal Disease 19 (11), 996–1002.
Spees, A.M., Wangdi, T., Lopez, C.A., Kingsbury, D.D., Xavier, M.N., Winter, S.E., et al., 2013. Streptomycin-induced inflammation enhances *Escherichia coli* gut colonization through nitrate respiration. MBio 4 (4).
Strauss, J., Kaplan, G.G., Beck, P.L., Rioux, K., Panaccione, R., Devinney, R., et al., 2011. Invasive potential of gut mucosa-derived *Fusobacterium nucleatum* positively correlates with IBD status of the host. Inflammatory Bowel Diseases 17 (9), 1971–1978.
Tegtmeyer, N., Neddermann, M., Asche, C.I., Backert, S., 2017. Subversion of host kinases: A key network in cellular signaling hijacked by *Helicobacter pylori* CagA. Molecular Microbiology 105 (3), 358–372.
Terebiznik, M.R., Raju, D., Vazquez, C.L., Torbricki, K., Kulkarni, R., Blanke, S.R., et al., 2009. Effect of *Helicobacter pylori*'s vacuolating cytotoxin on the autophagy pathway in gastric epithelial cells. Autophagy 5 (3), 370–379.
Tjalsma, H., Scholler-Guinard, M., Lasonder, E., Ruers, T.J., Willems, H.L., Swinkels, D.W., 2006. Profiling the humoral immune response in colon cancer patients: Diagnostic antigens from *Streptococcus bovis*. International Journal of Cancer 119 (9), 2127–2135.
Tjalsma, H., Boleij, A., Marchesi, J.R., Dutilh, B.E., 2012. A bacterial driver-passenger model for colorectal cancer: Beyond the usual suspects. Nature Reviews. Microbiology 10 (8), 575–582.

Tlaskalova-Hogenova, H., Stepankova, R., Hudcovic, T., Tuckova, L., Cukrowska, B., Lodinova-Zadnikova, R., et al., 2004. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunology Letters 93 (2–3), 97–108.

Tsugawa, H., Suzuki, H., Saya, H., Hatakeyama, M., Hirayama, T., Hirata, K., et al., 2012. Reactive oxygen species-induced autophagic degradation of *Helicobacter pylori* CagA is specifically suppressed in cancer stem-like cells. Cell Host & Microbe 12 (6), 764–777.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., et al., 2004. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. Nature Immunology 5 (11), 1166–1174.

Viljoen, K.S., Dakshinamurthy, A., Goldberg, P., Blackburn, J.M., 2015. Quantitative profiling of colorectal cancer-associated bacteria reveals associations between *Fusobacterium* spp., enterotoxigenic *Bacteroides fragilis* (ETBF) and clinicopathological features of colorectal cancer. PLoS One 10 (3), e0119462.

Vizcaino, M.I., Crawford, J.M., 2015. The colibactin warhead crosslinks DNA. Nature Chemistry 7 (5), 411–417.

Wang, C., Gong, G., Sheh, A., Muthupalani, S., Bryant, E.M., Puglisi, D.A., et al., 2017. Interleukin-22 drives nitric oxide-dependent DNA damage and dysplasia in a murine model of colitis-associated cancer. Mucosal Immunology 10 (6), 1504–1517.

Wei, J., Nagy, T.A., Vilgelm, A., Zaika, E., Ogden, S.R., Romero-Gallo, J., et al., 2010. Regulation of p53 tumor suppressor by *Helicobacter pylori* in gastric epithelial cells. Gastroenterology 139 (4), 1333–1343.

Winter, S.E., Winter, M.G., Xavier, M.N., Thiennimitr, P., Poon, V., Keestra, A.M., et al., 2013. Host-derived nitrate boosts growth of *E. coli* in the inflamed gut. Science 339 (6120), 708–711.

Wu, S., Lim, K.C., Huang, J., Saidi, R.F., Sears, C.L., 1998. *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. Proceedings of the National Academy of Sciences of the United States of America 95 (25), 14979–14984.

Wu, S., Morin, P.J., Maouyo, D., Sears, C.L., 2003. *Bacteroides fragilis* enterotoxin induces c-Myc expression and cellular proliferation. Gastroenterology 124 (2), 392–400.

Wu, S., Rhee, K.J., Albesiano, E., Rabizadeh, S., Wu, X., Yen, H.R., et al., 2009. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nature Medicine 15 (9), 1016–1022.

Xu, H., Chaturvedi, R., Cheng, Y., Bussiere, F.I., Asim, M., Yao, M.D., et al., 2004. Spermine oxidation induced by *Helicobacter pylori* results in apoptosis and DNA damage: Implications for gastric carcinogenesis. Cancer Research 64 (23), 8521–8525.

Yang, J., Ji, S., Zhang, Y., Wang, J., 2013. *Helicobacter hepaticus* infection in primary hepatocellular carcinoma tissue. Singapore Medical Journal 54 (8), 451–457.

Yang, Y., Weng, W., Peng, J., Hong, L., Yang, L., Toiyama, Y., et al., 2017. *Fusobacterium nucleatum* increases proliferation of colorectal cancer cells and tumor development in mice by activating toll-like receptor 4 signaling to nuclear factor-kappab, and up-regulating expression of MicroRNA-21. Gastroenterology 152 (4), 851–866.e24.

Yong, X., Tang, B., Xiao, Y.F., Xie, R., Qin, Y., Luo, G., et al., 2016. *Helicobacter pylori* upregulates Nanog and Oct4 via Wnt/beta-catenin signaling pathway to promote cancer stem cell-like properties in human gastric cancer. Cancer Letters 374 (2), 292–303.

Young, V.B., Knox, K.A., Schauer, D.B., 2000. Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus*. Infection and Immunity 68 (1), 184–191.

Zhou, Y., He, H., Xu, H., Li, Y., Li, Z., Du, Y., et al., 2016. Association of oncogenic bacteria with colorectal cancer in South China. Oncotarget 7 (49), 80794–80802.

Mitogen-Activated Protein Kinases (MAPK) in Cancer

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Glossary

Apoptosis Programmed cell death.
Cell signaling Ability of the cell to perceive changes in the environment and to produce a correct response.
Differentiation Process by which a less specialized cell evolves to a more specialized cell type.
Kinase Enzyme that catalyzes the transfer of a phosphate group (phosphorylation) from a phosphate donating molecule to specific substrates.
Oncogene Gene that encodes a protein that promote tumor development.
Phosphatase Enzyme that catalyzes the removal of a phosphate group from its substrate.
Proliferation Process by which the cell number increases. It is the balance between cell division and cell loss.
Scaffold protein Protein that bring together two or more proteins in a relatively stable configuration.
Tumorigenesis The process of tumor development.
Tumor suppressor gene Gene that encodes a protein that suppresses the development of tumor.

Introduction

Mitogen-activated protein kinases (MAPKs) are a family of kinases, which are ubiquitous and highly conserved in all eukaryotes. They belong to the CMGC group of protein kinases that also includes glycogen synthase kinases (GSK), cyclin-dependent kinases (CDKs) and CDK-like kinases. The different members of the MAPK family share a number of common structural and regulatory features. MAPKs exhibit more than 40% amino acid identity across the kinase domain, but also have unique characteristics. All MAPKs are integrated in signaling modules called MAPK pathways. Several molecular intermediaries compose MAPK pathways: a MAPK, a MAPK activator (MAPK kinase, MKK, MAP2K or MEK), and a MKK activator (MKK kinase (MKKK, MAP3K or MEKK)) that become activated sequentially in response to vast amount of intra- and extracellular stimuli (Fig. 1). The different MAPK pathways are involved in the transduction of extracellular changes into coordinated and integrated adaptive intracellular responses. They, therefore, control many cellular functions including gene transcription, protein biosynthesis, proliferation, cellular differentiation, metabolism rate, apoptosis, migration or cellular interaction among others.

MAPKs are activated by dual phosphorylation of Thr and Tyr residues, in a conserved Thr-X-Tyr motif in the activation loop. This phosphorylation is catalyzed by the dual-specificity kinases MEKs. MAPK phosphatases reverse this phosphorylation and return the MAPK to their inactive state. Further MAPK regulation is achieved by scaffold proteins, which are usually specific for each of the MAPK pathways, and collect specific MEKK–MEK–MAPK core into organized signaling modules. Scaffold proteins are spatial regulators of MAPK signals, and depending on the subcellular localization from which the activating signals arise, defined scaffolds determine which substrates are phosphorylated (Fig. 2). After activation MAPKs phosphorylate specific Ser and Thr (followed by Pro) residues of target substrates, which include many transcription factors, and other kinases and proteins. MEKs are highly selective in phosphorylating specific MAPKs, and they are in turn activated upon phosphorylation of Ser/Thr residues in a conserved motif catalyzed by MEKKs. MEKKs are the first component activated in the MAPK signaling module (Fig. 1) and have distinct motifs in their sequences that confer selectivity to their activation in response to different stimuli; however, their regulation by a range of multiple upstream components is still poorly understood.

MAPKs have been extensively studied and, in mammals, subdivided mainly into four subfamilies, also known as "conventional MAPK" subfamilies: extracellular signal-regulated protein kinase 1/2 (ERK1/2), c-Jun N-terminal kinases 1–3 (JNK1, JNK2 and JNK3), p38 MAPKs (p38α, p38β, p38γ and p38δ) and ERK5 (also known as BMK) (Fig. 1). There are other MAPKs so called "atypical MAPK" that include ERK3/ERK4, Nemo-like kinase (NLK) and ERK7, which are not activated by MEKs and their biological function remains largely unknown.

ERK1/2 Pathway

ERK1 and ERK2 (ERK1/2) were the first MAPKs to be identified and are encoded by two genes *MAPK3* and *MAPK1*, respectively. Both are ubiquitously expressed, although their relative levels varies in different tissues. ERK1/2 are activated by a wide range of growth factors and mitogens, but also by cytokines, certain stresses, transforming agents and G protein couple-receptors among others. ERK1/2 activation is implicated in many cellular processes such as cell motility, proliferation, survival or differentiation. As all conventional MAPKs, ERK1/2 require dual phosphorylation on Thr185 and Tyr187 (for ERK2), in a Thr-Glu-Tyr motif in

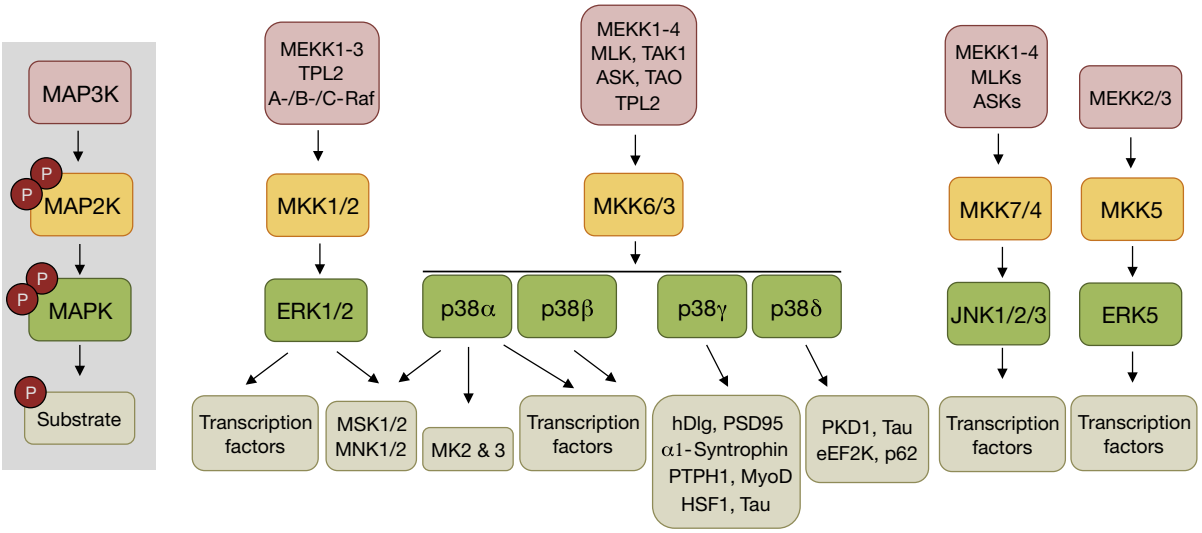
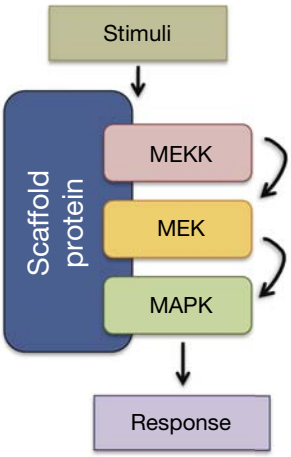


Fig. 1 Schematic representation of conventional MAPK pathways in mammals. *ASK*, apoptosis signal-regulating kinase; *MLK*, mixed lineage kinase; *TAK1*, transforming growth factor beta-activated kinase-1; *TAO*, thousand and one amino acid kinase. See text for more details.



Signalling	Scaffold	Binding partners		
		MEKK	MEK	MAPK
ERK1/2	KRS1/2	Raf	MEK1/2	ERK1/2
	IQGAP1/2/3	Raf	MEK1/2	ERK1/2
	β-Arrestin	Raf	MEK1/2	ERK1/2
	Paxillin	Raf	MEK1/2	ERK1/2
JNK	JIP1	MLK3/DLK	MKK7	JNK1
	JIP2	MLK2/3	MKK7	JNKs
	JIP3	MLK2/3/DLK	MKK7	JNKs
	JIP4	MEKK3	MKK4	JNKs
	POSH1/2/3	MLK	MKK4/7	JNKs
p38 MAPK	JIP4	MEKK3	MKK3/6	p38MAPKs
	OSM	MEKK3	MKK3	p38MAPKs
ERK5	MEK5	MEKK2/3	-	ERK5

Fig. 2 MAPKs scaffold proteins. The figure shows some of the most studied scaffold proteins in the different MAPK pathways. Scaffold proteins bring together two or more proteins (from specific MAPK signaling pathways) in a relatively stable configuration and facilitate their activation in response to different stimuli to trigger the cellular response. *KSR*, kinase suppressor of Ras; *IQGAP*, IQ-motif-containing GTPase-activating protein; *JIP*, JNK interacting partner; *POSH*, plenty of SH3 domain; *OSM*, osmosensing scaffold for MEKK3.

the activation loop of subdomain VIII to be activated. More than 150 substrates for ERK1/2 have been described so far, and among them, ERK1/2 phosphorylate the transcription factors c-Myc, c-Fos, Elk1 and Ets, and also the protein kinases p90 ribosomal S6 kinase (RSK), mitogen- and stress-activated kinase 1 and 2 (MSK1/2) and the MAPK interacting kinase 1 and 2 (MNK1/2). ERK1/2 phosphorylation is catalyzed by two MEKs (MEK1 and MEK2), which are in turn activated mainly by the MAP3Ks Raf (Fig. 1), although in certain cell types and in response to specific stimuli MEK1/2 can also be phosphorylated by other MAP3K such as TPL2 (tumor progression locus 2; also known as cancer Osaka thyroid, COT). There are three Raf family members: ARaf, BRaf and CRaf, also called Raf-1, which is the most studied. Raf-1 is activated by a combination of cellular localization, phosphorylation and binding to Ras. Scaffold proteins also play a crucial role as ERK1/2 regulators, maintaining the pathway integrity and efficiency. At least 15 proteins have been identified as scaffolds for ERK1/2 pathway, which fine-tune signal amplitude and duration, and provide signal fidelity by isolating these complexes from external interferences. Scaffold proteins of the ERK1/2 pathway include kinase suppressor of Ras (KRS), MEK partner 1 (MP1), β-arrestins and IQGAP (Fig. 2).

ERK1/2 pathway regulates processes that are central for cellular transformation; for example, they are implicated in cell-cycle progression by controlling the expression of cyclin D1 and cyclin-dependent kinase (CDK) inhibitors p21 and p27. The intensity and the duration of ERK1/2 signaling determine whether cell undergoes differentiation, proliferation or cell-cycle arrest. In addition, ERK1/2 signaling controls cell survival and apoptosis by regulating both the expression and the activity of proapoptotic proteins.

ERK1/2 signaling is upregulated in cancer cells and in human tumors, which has led to the development of inhibitors of this pathway for cancer therapy. There is a broad spectrum of human tumors known to display aberrant ERK1/2 pathway activation caused by activating mutations in Ras (KRas, NRas and HRas) and Raf (BRaf). Among these tumors are: breast, colon, pancreatic, ovarian, prostate, non-small-cell lung and papillary thyroid cancer, as well as melanoma, glioma, acute myeloid leukemia or acute lymphocytic leukemia. Therefore, there is a need for exploring the potential of ERK1/2-pathway inhibitors as anticancer agents. Ras, Raf and MEK are better therapeutic targets than ERK1/2, since they are proteins that posses unique structural features. Very potent small-molecule inhibitors have been generated for Ras, Raf and MEK, and have been tested in several clinical trials, being so far Raf and MEK inhibitors more effective than Ras inhibitors. The treatment of cutaneous melanoma is one example in which MAPK pathway inhibitors have been used. The development of MEK inhibitors PD908059 and U0126 demonstrate the role of MEK-ERK1/2 pathway in human melanoma cell proliferation, survival and invasion. Approximately 50% and 28% of melanoma patients display mutations in BRaf (BRafV600E is the most predominant) and NRas, respectively. The Braf inhibitors vemurafenib (PLX4032) and dabrafenib (GSK2118436) have been used in the treatment of BRafV600E mutant melanoma patients, showing very good clinical responses in phase I to III trials (overall response of 80%). However, the response to the inhibitors is transient in most patients due to the development of drug-resistance caused by various mechanisms, most of them involving re-wiring of the MAPK pathway. In addition, approximately 30% of the patients treated with the BRaf inhibitors developed Ras-driven secondary cancers such as squamous cell carcinomas, colon cancer or leukemia, probably due to CRaf activation. This has prompted to the development of combination therapies using both BRaf and MEK inhibitors. The combination of Raf and MEK inhibitors in melanoma therapies reduces the development of secondary cancers and prolongs the response, but unfortunately patients still develop resistance. Nonetheless, therapies with BRaf and MEK inhibitors, such as trametinib/dabrafenib and cobimetinib/vemurafenib are currently accepted for the treatment of BRaf-mutant advanced melanomas. Additionally, alternative strategies are being tested nowadays, and drugs targeting ERK, RTK or BRaf and CRaf have been described.

JNK Pathway

There are three JNKs encoded by different genes JNK1 (*MAPK8*), JNK2 (*MAPK9*) and JNK3 (*MAPK10*). Additionally, the JNKs are alternatively spliced giving rise to at least 10 different JNK isoforms. JNK1 and JNK2 are expressed ubiquitously, whereas JNK3 is mainly expressed in the nervous system. JNKs are activated by dual phosphorylation of Thr183 and Tyr185 residues in the Thr-Pro-Tyr motif of the activation loop. This phosphorylation is catalyzed by the MAP2Ks, MKK4 and MKK7. The main targets of JNK are members of the activator protein 1 (AP1) group of transcription factors, such the transcription factors c-Jun, JunB, JunD and related proteins as Fos. JNK phosphorylates the N-terminal part of these molecules causing their activation and therefore promoting gene transcription. Other JNK targets are proteins involved in the control of the cell cycle, survival, cell death, migration, invasion and gene transcription. As ERK1/2, JNKs are also activated by mitogens, although they are preferentially and strongly activated by environmental stresses, toxins, translational inhibitor (cycloheximide and anisomycin), pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), and by proinflammatory cytokines. As all MAP2Ks, MKK4 and MKK7 are regulated by Ser/Thr phosphorylation catalyzed by a variety of MEKK depending on the stimulus and on the cell type (Fig. 1). The specificity of JNK activation is achieved in part by scaffold protein such as the JNK interacting protein (JIP) family or the plenty of SH3 domains (POSH) family (Fig. 2).

It is well established that JNKs are involved in cell proliferation, survival, cell death and differentiation. However, their specific effect and degree of implication in those processes depends on the JNK family member, the cellular context, the stimuli, or on the strength and duration of the JNK activation. Therefore, to define the implication of the JNKs in cancer development is complex. It is well documented that JNK pathways are implicated in both pro- and antioncogenic processes. The oncogenic function of the JNKs is mainly based on their ability to activate AP1 by phosphorylating and stabilizing c-Jun, and their role as negative regulators of the tumor suppressor p53. On the other hand, tumor-suppressive functions are probably related to their proapoptotic activity. The role of JNK in cancer development is being investigated using mouse models. Studies using JNK1-null mice and JNK2-null mice have

further demonstrated that JNK can cause negative and positive effects in tumor development. For example, the deletion of JNK1 and JNK2 has opposite effects in the development of chemical-induced skin cancer. JNK2 deletion increases skin cancer, whereas the lack of JNK1 decreases the formation of skin tumors. Moreover, chemical-induced hepatocellular carcinoma (HCC) and Bcr-Abl-induced lymphoma are suppressed by JNK1 deletion, but not by the lack of JNK2. In contrast, studies of glioblastoma, prostate cancer and lung carcinoma cell lines have identified important roles for JNK2, but not for JNK1 in oncogenic processes. These data confirm that JNK pathways play a role in cancer development, and that the relative roles of each JNK isoform depend on type of cancer.

One clear example of the implication of the JNK pathway in cancer development is the HCC, which is the third most common cause of death from cancer in the world. Studies using knockout mice have demonstrated that JNK1 deletion, but not JNK2 deletion, decreases the development of chemical-induced HCC with the compound N-nitrosodiethylamine (DEN). In the liver, DEN causes hepatocyte death followed by compensatory regeneration, and maintained administration of DEN leads to the development of HCC. Compared to wild-type mice, JNK1-null mice exhibit decreased liver cell proliferation and tumor formation after DEN treatment, which is caused by a reduction in the expression of c-Myc and an increase in the levels of the cyclin-dependent kinase (CDK) inhibitor p21. These effects have also been found when JNK1 is depleted in human HCC cell lines. Thus JNK1 is central for HCC tumorigenesis. Pharmacological inhibition of JNK reduced HCC development in both DEN-induced liver cancer in mice and in xenografted human HCC cells, indicating that JNK1 is a promising therapeutic target for the HCC treatment.

The contribution of different JNKs to skin cancer development has also been described using the DMBA (7,12-dimethylbenz(*a*) anthracene)/TPA (12-*O*-tetradecanoylphorbol-13-acetate) model. In this model, JNK1 acts as a tumor suppressor as JNK1-null mice are more susceptible to skin tumor development than wild-type mice, whereas in JNK2-null mice the development of skin tumors is suppress, indicating an oncogenic role for JNK2 in this particular cancer model. The molecular mechanisms by which JNKs regulate skin cancer development are still under study. Nonetheless, it has been suggested that the altered AP1 transcription factor activity, together with the differential regulation of essential signaling pathways such as ERK1/2 or Akt, could account for the specific functions of JNK1 and JNK2 in skin cancer development. Akt pathway is a signal transduction pathway regulated by phosphoinositide 3-kinase and the mTOR (mammalian target of rapamycin) that promotes survival and growth in response to extracellular stimuli. JNKs have also been suggested to be targets for antimelanoma therapy as they regulate the survival of melanoma cells. Cutaneous melanoma is an extremely aggressive tumor with a high tendency to relapse and high metastasis rate, its represent only 5% of skin cancers, but it causes approximately the 75% of skin cancer deaths. In melanoma JNKs are frequently active; however their role is not clear as they can act as either oncogenes or tumor suppressors, depending on the upstream or the downstream JNK pathway components presents in a given cell. JNK inhibition causes cell-cycle arrest or apoptosis depending on the cell line. For example, in the human melanoma WM983B cells JNK inhibition causes apoptosis mediated by p53, Bad (Bcl-2-associated death promoter) and Bax (Bcl-2-associated X protein), whereas in 1205Lu cells causes the arrest of the cell cycle mediated by p21.

Additionally, somatic mutations in the JNK pathway (JNK1, JNK2, JNK3 and their upstream activators MKK4 and MKK7) have been identified in large-scale sequencing analyses of protein kinases in human tumors suggesting their involvement in cancer development. These mutations could cause either the loss of activity or the activation of the kinase; thus, the loss of JNK3 function promotes tumor formation, and mutations in the *MAPK10* gene were found in $\sim$53% of human brain tumor examined. Also, sustained activation of JNK1 is associated with altered histone H3 methylation in human HCC, and several members of the JNK pathway are upregulated in prostate cancer.

The role of JNKs in cell proliferation and apoptosis is being used for the development of therapeutic inhibitors. Several peptide inhibitors have been generated, such as JNKI1, which has been successfully used in mouse models for HCC; or BI-78D3, which inhibits JNK activity by interfering with the binding to JIP1 scaffold. The application of JNK inhibitors has been proven to work in human cells and in vivo in animal models; however, JNK pathway has not been translated into clinical use.

p38 MAPK Pathways

There are four p38MAPKs encoded by different genes: p38α (*MAPK14*), p38β (*MAPK11*), p38γ (*MAPK12*) and p38δ (*MAPK13*). Alternative spliced variants of p38α has been identified: CSBP1 (cytokine suppressive antiinflammatory drug-binding protein), Mxi2 (Max-interacting protein) or Exip (for exon skip), although their physiological functions have been poorly studied. Most of the literature on p38MAPK refers to p38α. This was the first p38MAPK identified, and therefore is the best characterized. Based on sequence homology, substrate specificities, and sensitivity to chemical inhibitors, the p38MAPKs can be further divided into two subgroup: p38α/p38β and p38γ/p38δ. p38γ and p38δ are also known as alternative p38MAPKs. Although p38MAPKs are widely expressed, their expression pattern varies in tissues. For instance, p38α is ubiquitously expressed in all cell types and tissues; p38β is highly expressed in the brain, thymus and spleen; p38γ is very abundant in skeletal muscle; and p38δ levels are high in pancreas, intestine, adrenal gland, kidney and heart.

The p38MAPKs are strongly activated by a wide variety of environmental and cellular stresses and by inflammatory cytokines, but they are poorly activated by growth factors. p38MAPKs and JNKs are activated by the same stimuli, and together are also called stress-activated protein kinase pathways. All p38MAPKs are activated by dual phosphorylation on Thr180 and Tyr182 of the The-Gly-Tyr activation motif mediated by the MAP2Ks MKK3 and MKK6, and in the case of p38α also by MKK4. p38α may also be activated by autophosphorylation in cardiomyocytes in ischemic heart, and in T lymphocytes stimulated through the T-cell antigen receptor (TCR). The activation of MKK3 and MKK6 is by phosphorylation of Ser/Thr residues mediated by different MEKKs, which are stimulus and cell type specific (**Fig. 1**).

The use of kinase inhibitors that specifically inhibit p38α and p38β, but do not block p38γ and p38δ activity, and the genetic deletion of specific p38MAPK isoforms has showed that they have some overlapping substrates and functional redundancy. However, there are particular proteins that are better substrates for p38α/p38β than for p38γ/p38δ. More than 100 proteins can be phosphorylated by p38α/p38β and a significant proportion of them is involved in the regulation of gene expression such as the transcription factors ATF2 (activating transcription factor 2), MEF2 (myocyte enhancer factor 2) or ELK1 (Ets like gene 1). Other p38α/p38β substrates are protein kinases such as MAPK-activated protein kinase 2 (MK2) or MK3; MSK1/2 or MNK1/2 (Fig. 1), which control numerous cellular processes. p38δ phosphorylates proteins such as the eukaryotic elongation factor 2 kinase (eEF2K), the protein kinase D1 (PKD1) or the signal adaptor p62; and p38γ the transcription factor MyoD. p38γ associates with PDZ-domain containing proteins, such as α1-syntrophin, SAP (synapse-associated protein) 90/PSD (postsynapse density) 95, hDlg (human disk large also known as SAP97) and the protein tyrosine phosphatase PTPH1 and under stress conditions it is able to phosphorylate them and modulate their activity. p38γ is unique among the MAPKs and contains a short sequence at its C-terminal that associates with PDZ-domains, whose function is facilitate protein–protein interaction (Fig. 3). Also, p38γ and p38δ are the principal p38 MAPKs phosphorylating the neuronal microtubule-associated protein Tau and the heat shock factor 1 (HSF-1).

p38 MAPK pathway are involved in processes that promote cell transformation and tumor development. They control the cell cycle, cell growth, apoptosis, angiogenesis, tissue invasion and metastasis. p38 MAPK pathway regulates the checkpoint controls and cell cycle at G0, G1/S and G2/M transitions. Depending on the cell type, p38α can either induce progression or inhibition at G1/S transition by differential regulation of specific cyclin levels (cyclin A or D1) as well as by phosphorylation of the retinoma protein (which is a hallmark of G1/S progression), and by phosphorylation of the p53 tumor suppressor and the upregulation of p16 tumor suppressor. On the other hand, activation of p38 MAPK in mammalian cells in response to various environmental stresses initiates G2/M checkpoint, which induces cell-cycle arrest and facilitate DNA repair. DNA damage checkpoints function as surveillance

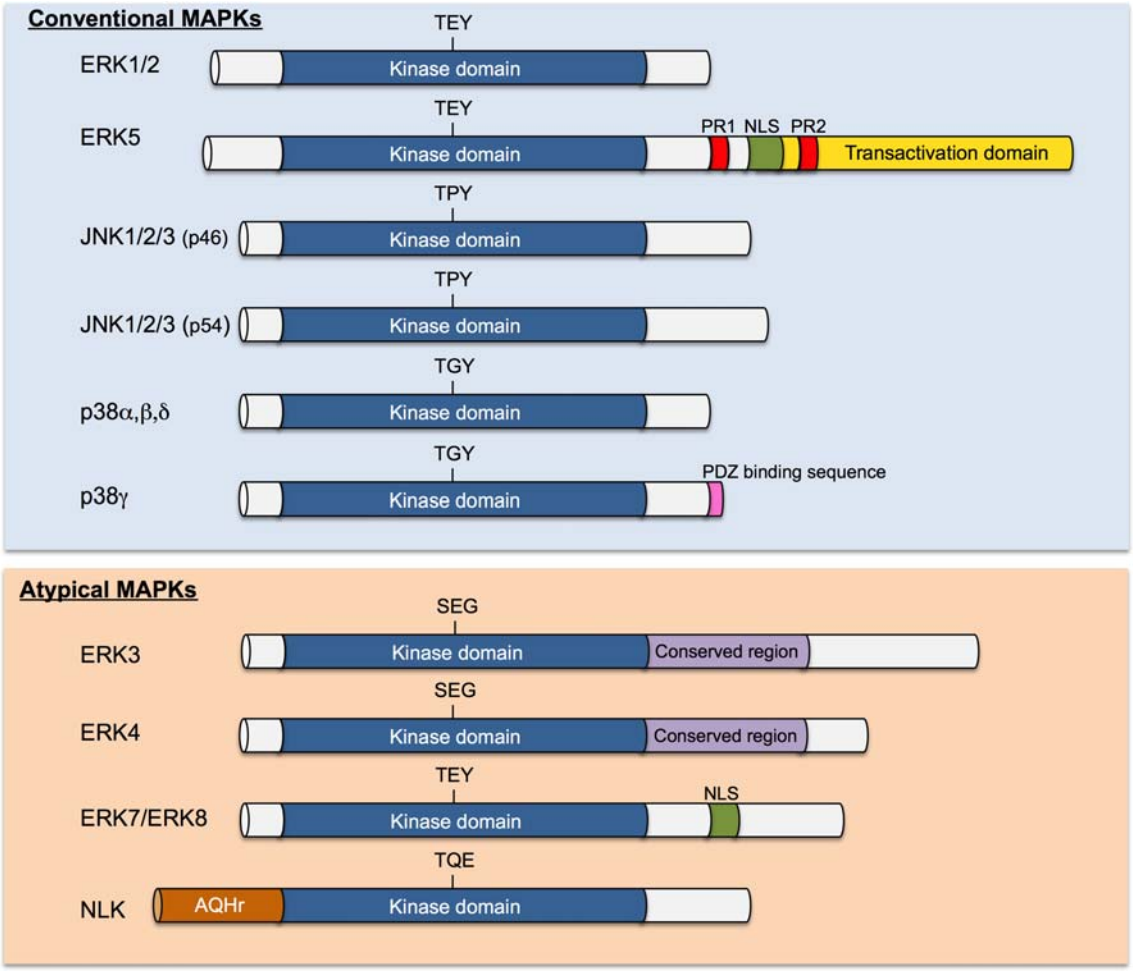


Fig. 3 Schematic representation of the structures of MAPKs. All MAPKs contain a kinase domain, and N- and C-terminal regions of different lengths. Additionally, some MAPK have specific domains/regions/sequences: NLS is a nuclear localization sequence; PR1 and PR2 are Pro rich regions; in p38γ there is a PDZ binding sequence; in ERK3 and ERK4 are C-terminal conserved region; AQHr is an Ala, Glu and His rich domain.

mechanisms during cell division to ensure that each step is completed properly, thus maintaining genetic integrity. Most of the work published on cell-cycle regulation by p38 MAPK pathway has been focused on studying the role of the isoforms p38α and p38β. In the case of p38γ, one report indicates its implication in G2 arrest after ionizing radiation. Although p38α is normally associated with negative regulation of proliferation, there are reports indicating that this kinase can also positively control cellular proliferation in several cancer cell lines. These opposite effects could be due to the strength of the p38 MAPK activation and on the crosstalk with different signaling pathways.

p38α is also implicated in the induction of apoptosis by modulating survival pathways and pro- and antiapoptotic proteins; whereas p38β seems to have antiapoptotic functions. Of particular interest is the key role of p38α in preventing tumorigenesis by promoting growth arrest and apoptosis in response to reactive oxygen species (ROS) in immortalized cells. In contrast, p38α can also facilitate the survival and proliferation of tumor cells supporting tumor growth and metastasis; it regulates angiogenesis and cell invasion by controlling the production, secretion and signaling of angiogenic factors such as VEGF (vascular endothelial growth factor) or MMPs (matrix metalloproteinases). Increased levels of phosphorylated p38α have been correlated with malignancy and poor prognosis in several types of cancers, such as breast carcinoma, glioma, head and neck squamous cell carcinoma, as well as in follicular, lung and thyroid carcinoma. In addition, p38α activation helps tumor cells to survive chemotherapeutic treatments, for example, the tamoxifen resistance in breast cancer patients.

The use of: p38α-deficient mice, p38α-inhibitors (such as SB203580, LY2228820 or PH797804), and the deletion of p38α-regulators (such as MKK6/MKK3 or the phosphatase Wip1), have provided evidence of the tumor suppressor role of p38α in mammary epithelial cells, lung, liver and colon. Studies using mice deficient in Wip1, which is a phosphatase that targets p38α, show reduced breast tumorigenesis upon expression of the oncogenes Erbb2 or HRas; this correlates with higher p38 MAPK phosphorylation. Contrary, pharmacological inhibition of p38α using the compounds LY2228820 and PH797804 impairs tumor growth in xenografts of human breast cancer cell lines and in mice with breast tumors induced by polyoma middle T (PyMT) expression. p38α-deficient mice are more sensitized to KrasG12V-induced lung tumorigenesis, and the specific deletion of p38α in hepatocytes facilitates the DEN-induced HCC by activating the JNK pathway and enhancing the accumulation of ROS.

Not only p38α but also other isoforms could mediate tumorigenesis. Studies in cancer patient samples, human cancer cell lines, cells from knockout mice, and in mouse cancer models indicate that p38γ and p38δ have both pro- and antioncogenic roles in tumor development. Thus, the tumor suppressor role of p38γ and p38δ has been shown in mouse embryonic fibroblasts, in which the deficiency in these kinases increases cell migration and MMP-2 secretion, and impairs cell contact inhibition. Additionally, the loss of p38γ in KRas-transformed fibroblasts leads to increased cell proliferation and tumorigenesis, both in vitro and in vivo. In contrast, it has been described that p38δ is overexpressed in cholangiocarcinoma, invasive human cutaneous squamous cell carcinoma (SCC), head and neck SCC, and in prostate cancer, whereas p38γ is overexpressed in triple-negative breast cancer cells, supporting the potential pro-oncogenic role of these kinases. This was confirmed in the two-step 7,12-dimethylbenz(*a*)anthracene (DMBA)/TPA chemical skin carcinogenesis model, in which p38γ and p38δ deletion blocks skin tumor development, probably by enabling formation of a proinflammatory microenvironment that fosters epidermal hyperproliferation and tumorigenesis. TPA-induced epidermal hyperproliferation and inflammation is reduced in p38γ/p38δ-deficient mice.

Inflammation has long been associated with cancer development. During tumorigenesis, innate and adaptive immune cells infiltrate the tumor microenvironment and regulate tumor cell fate either directly or via the production of inflammatory molecules. p38α, p38γ and p38δ control multiple functions of the immune cells and also the production of cytokines and chemokines, which may either promote or suppress tumor growth. Colitis-associated cancer (CAC) is a clear example of tumor development associated to inflammation. CAC is a colon cancer subtype associated with inflammatory bowel disease (IBD) such as ulcerative colitis or Crohn's disease. IBD confer an increased risk for colorectal cancer, one of the most common fatal malignancies worldwide. Patients with long-standing ulcerative colitis are particularly prone to CAC, the major cause of death in these patients. It has been shown that p38α controls colon homeostasis and that the loss of p38α in intestinal epithelial cells (IEC) increases proliferation, reduces the number of mucus-producing goblet cells and affects epithelial barrier function by altering tight junction assembly. The intestinal epithelia serve as a barrier that protects the intestinal tract against luminal invading pathogens and ingested toxin, which can promote inflammatory responses. p38α in IEC also controls the expression of chemokines that are essential for the recruitment of immune cells, which protect against mucosal infection and dextran sodium sulfate (DSS)-induced colitis. As a result, mice that do not express p38α in IEC are more susceptible to colitis-associated colon tumorigenesis in an azoxymethane (AOM)/DSS colitis-associated colon cancer model. In contrast, the downregulation of p38α in colon tumor cells reduces tumor number in mice. Pharmacological inhibition of p38α using the compounds PH797804 or SB202190 also reduces colon tumor growth either in AOM/DSS model, in xenografts of human colon cancer cell lines or in mice that express APC^{min}. These studies suggest a dual role for p38α in IEC, supporting colon tumor progression and suppressing the initiation of inflammation-associated colon tumor.

p38γ and p38δ also regulate inflammatory signaling that promote colon tumorigenesis in an AOM/DSS mouse model of CAC. Mice that do not express p38γ and p38δ are less susceptible to colitis-associated colon tumorigenesis compared to wild-type mice. These mice show a decreased number of colon tumors, which correlates with reduced inflammation, cytokine and chemokine production and inflammatory cell infiltration in the colon. Experiments using bone marrow transplants demonstrated that p38γ and p38δ in hematopoietic cells are essential for CAC development. Other study nonetheless shows that p38γ in IEC is also important for CAC development, as colon tumor formation is reduced in mice with IEC-specific p38γ deletion. These studies highlight the importance of p38γ and p38δ in cancer, although their specific functions in different cells are yet to be determined.

ERK5 Pathway

Extracellular signal-regulated kinase (ERK) 5, also known as Big MAPK (BMK), is twice the size of other MAPKs and is encoded by the *MAPK7* gene. Unlike the classical MAPKs, ERK5 has two distinct functional domains, a catalytic N-terminal domain, which is very similar to that of ERK1/2 and is responsible for conventional protein kinase activity; and a unique C-terminal half with no homology to any other protein, and responsible for ERK5 transcriptional transactivation activity. C-terminal domain contains a transcriptional transactivation domain, two proline-rich regions and a nuclear localization sequence (NLS) (**Fig. 3**). Interaction between the C- and N-terminal domains has an important role in regulating ERK5 localization and activity. ERK5 is activated selectively by dual phosphorylation on Thr218 and Tyr220 in the TEY motif by its only upstream kinase, MEK5 (**Fig. 1**). ERK5 is activated in cells by oxidative and osmotic stress, but also by several growth factors and cytokines. Some downstream targets of ERK5 have been identified, including the myocyte enhancer factor-2 (MEF2) transcription factor family members MEF2A, MEF2C and MEF2D, and other transcription factors such as SAP1 (switch-activating protein 1), c-Myc and cAMP response element-binding (CREB).

ERK5 plays a central role in cellular survival, differentiation and proliferation, and also in cell-cycle progression. Additionally, in the last decade the ERK5 signaling has been proposed to play a central role in the regulation of tumor angiogenesis, the development and aggressiveness of several types of cancer and in the function of a wide variety of oncogenes. Thus, the ERK5 pathway has been associated with advanced-stages of prostate adenocarcinomas in patients. Prostate cancer (PCa) is the second-leading diagnosed cancer in men in the world. ERK5 and MEK5 are over expressed in prostate cancer cells, which correlate with aggressiveness and more metastasis. For example, in the prostate cancer cell line PC-3 MEK5/ERK5 expression increases the proliferation of these cells by inducing DNA replication, whereas the silencing of ERK5 inhibits PC-3 proliferation and invasion. In addition, ERK5 has been suggested as a therapeutic target in patients with FOXF1-positive PCa. FOXF1 (Forkhead box F1) is a transcription factor that normal prostate tissue does not express, and it has been proposed that FOXF1 regulates ERK5 phosphorylation by increasing the expression of ERK5 upstream activators. Using murine orthotopic models of PCa, it has been shown that overexpression of FOXF1 in the Myc-CaP and TRAMP PCa cell lines induces tumor and metastasis progression, which is associated with increased ERK5 phosphorylation. Also, the silencing of ERK5 in Myc-CaP and TRAMP cells decreased proliferation and metastasis. On the other hand, the implication of ERK5 pathway in metastasis is also supported by its involvement in the regulation of key proteins and pathways, such as the MMP or the regulation of integrins/FAK signaling, that are implicated in invasion and metastasis. Deletion of ERK5 in PTEN-null mice increases T-cell infiltration in PCa tumors compared to single PTEN-deficient mice. The combined loss of PTEN and ERK5 reduce PCa tumor size and cell proliferation, and increased the expression of T-cell chemoattractants CCL5 and CXCL10, and the infiltration of CD4+ T cells in the tumors, which indicate that ERK5 can be a good target for PCa immunotherapy. PTEN (from phosphatase and tensin homolog) is a phosphatase that regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells.

Additional in vivo (using mouse models) and clinical data indicate that abnormal ERK5 signaling is also implicated in many others type of cancers such as colorectal cancer, esophageal cancer, osteosarcoma, pancreatic, neuroblastoma, bladder, lung, HCC, leukemia or in triple-negative breast cancer where ERK5 is considered a potential therapeutic target. ERK5 silencing and ERK5 kinase inhibitors, such as the compounds TG02 or XMD8-92, block cell proliferation of different cancer cell lines and tumor growth in xenografts models. For example, it has been shown that the treatment with XMD8-92 blocks the growth of skin tumor, lung carcinoma, pancreatic ductal adenocarcinoma, HCC or neuroblastoma in xenograft models; whereas in triple-negative breast cancer, the administration of XMD8-92 increase the sensitization of cancer cells to chemotherapy agents, such as doxorubicin, cisplatin, etoposide or bortezomib among others, potentiating their antitumor activity in vitro and in vivo.

Atypical MAPK

In addition to the "conventional MAPKs" (ERK1/2, JNK, p38MAPK and ERK5), there are "atypical MAPKs," which are not activated by MEKs. Atypical MAPKs include ERK3/ERK4, Nemo-like kinase (NLK) and ERK7 (also known as ERK8).

ERK3/ERK4

ERK3 and ERK4 are MAPKs encoded by the genes *MAPK6* and *MAPK4*, respectively. ERK3 is ubiquitously expressed, although its expression is higher in brain, skeletal muscle and the gastrointestinal tract; whereas ERK4 expression is more restricted to colon, heart, kidney, eye, lung, placenta, prostate, skin, ovary, pancreas and brain (where its expression is higher).

ERK3/ERK4 have very similar structure and their activation loops contain a single phospho-acceptor residue in the Ser-Glu-Gly motif that lacks the phospho-acceptor residue Tyr. Therefore, ERK3/ERK4 are very poor MEKs substrates. The identity of ERK3/ERK4 upstream activator kinase is unclear, although there are some reports identifying the group 1 of p21 activated kinases (PAKs) as ERK3/ERK4 activation-loop kinases. Ser189 residue in ERK3 and Ser186 in ERK4 are constitutively phosphorylated in vivo in growing cells, and so far no stimuli have been found to promote ERK3/ERK4 phosphorylation or activation in vivo. The only known ERK3/ERK4 substrate is the MAPK-activated protein kinase MK5.

ERK3 is a very unstable protein, with a half-life of 30–45 min depending on the cellular context, contrary to ERK4, which is highly stable. The biological role of ERK4 is largely unknown; however, several studies show that ERK3 participates in a number of biological processes. It has been suggested that ERK3 is a negative regulator cell proliferation and a positive regulator of cell differentiation. Is possible that ERK3 is implicated in oncogenic processes since its expression is induces in Raji lymphoma cells or in squamous cell carcinoma after stimuli that inhibit proliferation. Additionally, ERK3 phosphorylation and protein stability is regulated by CDK1 and the phosphatase Cdc14, indicating that ERK3 has a role in cell-cycle progression through mitosis. Also, ERK3 overexpression in various cell lines was found to inhibit the progression through S phase. Unfortunately, mice lacking ERK3 are not viable, and conditional knockout mouse models will be required to perform studies of the implication of this kinase (and of ERK4) in tumor development.

Nemo-Like Kinase (NLK)

The NLK kinase was originally identified as a vertebrate ortholog of Drosophila nemo, its catalytic domain shows a 45% amino acid identity with ERK2. NLK is ubiquitously expressed, but its expression is higher in brain and lymphoid organs. NLK lacks the phospho-acceptor residue Tyr in the Thr-Gln-Glu motif in the activation loop, and possesses unique N- and C-terminal extensions, that may contribute to the interaction with its substrates (**Fig. 3**). The exact MEKs that directly phosphorylate NLK are still unknown, but a potential kinase that could lead to NLK activation is the kinase homeodomain-interacting protein kinase 2 (HIPK2). Also, NLK could autophosphorylate itself in vitro. In cells, NLK can be activated by stimuli of the Wnt signaling pathway, and also by interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF) and transforming growth factor β (TGF-β). Transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family and STAT3 are NLK substrates.

Increasing evidences show that NLK can either act as an oncogene or as a tumor suppressor depending on the cell context. NLK has been described to be important for tumor cell proliferation, migration and apoptosis. Downregulation of NLK decreases the proliferation of gallbladder cancer cells and of HCC cells, by inducing G1 arrest. Contrary, NLK induction inhibits cell proliferation and increases apoptosis in human colon carcinoma cells, prostate and breast cancer cell, and in glioblastoma cells. In addition, altered expression of NLK has been found in different types of cancers. For example, the expression of NLK is increased in cervical squamous cell carcinoma, HCC or gallbladder; and decreased in prostate, ovarian, lung, breast cancer or glioma. Also, it has been suggested that NLK expression is associated with the risk of invasive epithelial ovarian cancer. Further studies are needed for the elucidation of molecular mechanisms mediated by NLK in tumor development.

ERK7

ERK7 (encoded by the gene *MAPK15*) was cloned from a rat brain cDNA library, and some years later the human kinase was identified and called ERK8. Surprisingly, ERK7 and ERK8 only show approximately 69% amino acid identity, which is lower than the typical identity found between other human and rodents MAPK orthologs. From now, I will refer to the human kinase as human ERK7, which is predominantly expressed in lung and kidney.

ERK7 activation loop contains the Thr-Glu-Tyr motif, being Thr and Tyr residues constitutively phosphorylated, which indicates that a MEK is involved in its activation. However, when inactive ERK7 mutant is expressed in cells, this is not phosphorylated in the Thr-Glu-Tyr motif suggesting that ERK7 phosphorylation is regulated by autophosphorylation. Human ERK7 can also be regulated by stimuli such as serum, amino acid starvation or hydrogen peroxide. So far no physiological substrates have been identified for ERK7.

Although the implication of ERK7 in cancer development has not been described, it is known that this kinase plays a role in cell proliferation, autophagy and in the response to glucocorticoids and estrogens.

Conclusions

Members of the MAPK family function in a cell context- and cell type-specific manner to integrate signal that affect numerous processes that are essential in tumorigenesis. The involvement of MAPK family members in cancer development has supported the potentially beneficial role of MAPK inhibitors with current cancer therapy. In addition, given the redundancy in MAPK signaling pathways and the adaptive capacity of cancer cells, drug combinations are increasingly being investigated for therapy. For instance, the incorporation of several Raf/MEK inhibitors in combination therapies are currently being tested in clinical trials. The toxic effect that MAPK inhibitors could have in normal proliferating cells is a concern, so the development of small-molecule inhibitors with sufficient potency and selectivity to inhibit MAPK signaling pathway specifically in cancer cells is a real challenge. Studies in mouse models have been essential to better understand how MAPKs control cancer development. The development of new MAPK mouse models, structural analysis and mutational analysis by next generation genomic sequencing are expected to provide new strategies for the design of improved therapeutic approaches.

See also: Mutational Signatures and the Etiology of Human Cancers.

Further Reading

Cargnello, M., Roux, P.P., 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiology and Molecular Biology Reviews 75, 50–83.
Casar, B., Crespo, P., 2016. ERK signals: Scaffolding scaffolds? Frontiers in Cell and Development Biology 4, 49.
Chen, F., Castranova, V., 2009. Beyond apoptosis of JNK1 in liver cancer. Cell Cycle 8, 1145–1147.
Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., Cobb, M.H., 2001. MAP kinases. Chemical Reviews 101, 2449–2476.
Coulombe, P., Meloche, S., 2007. Atypical mitogen-activated protein kinases: Structure, regulation and functions. Biochimica et Biophysica Acta 1773, 1376–1387.
Cuenda, A., Rousseau, S., 2007. p38 MAP-kinases pathway regulation, function and role in human diseases. Biochimica et Biophysica Acta 1773, 1358–1375.
Cuenda, A., Sanz-Ezquerro, J.J., 2017. p38gamma and p38delta: From spectators to key physiological players. Trends in Biochemical Sciences 42, 431–442.
Escos, A., Risco, A., Alsina-Beauchamp, D., Cuenda, A., 2016. p38gamma and p38delta mitogen activated protein kinases (MAPKs), new stars in the MAPK galaxy. Frontiers in Cell and Development Biology 4, 31.
Garcia-Cano, J., Roche, O., Cimas, F.J., Pascual-Serra, R., Ortega-Muelas, M., Fernandez-Aroca, D.M., Sanchez-Prieto, R., 2016. p38MAPK and chemotherapy: We always need to hear both sides of the story. Frontiers in Cell and Development Biology 4, 69.
Gomez, N., Erazo, T., Lizcano, J.M., 2016. ERK5 and cell proliferation: Nuclear localization is what matters. Frontiers in Cell and Development Biology 4, 105.
Huang, Y., Yang, Y., He, Y., Li, J., 2015. The emerging role of Nemo-like kinase (NLK) in the regulation of cancers. Tumour Biology 36, 9147–9152.
Igea, A., Nebreda, A.R., 2015. The stress kinase p38alpha as a target for cancer therapy. Cancer Research 75, 3997–4002.
Imperial, R., Toor, O.M., Hussain, A., Subramanian, J., Masood, A., 2017. Comprehensive pancancer genomic analysis reveals (RTK)-RAS-RAF-MEK as a key dysregulated pathway in cancer: Its clinical implications. Seminars in Cancer Biology. https://doi.org/10.1016/j.semcancer.2017.11.016.
Inesta-Vaquera, F.A., Campbell, D.G., Tournier, C., Gomez, N., Lizcano, J.M., Cuenda, A., 2010. Alternative ERK5 regulation by phosphorylation during the cell cycle. Cellular Signalling 22, 1829–1837.
Kidger, A.M., Keyse, S.M., 2016. The regulation of oncogenic Ras/ERK signalling by dual-specificity mitogen activated protein kinase phosphatases (MKPs). Seminars in Cell & Developmental Biology 50, 125–132.
Kyriakis, J.M., Avruch, J., 2012. Mammalian MAPK signal transduction pathways activated by stress and inflammation: A 10-year update. Physiological Reviews 92, 689–737.
Li, S., Jang, H., Zhang, J., Nussinov, R., 2018. Raf-1 cysteine-rich domain increases the affinity of K-Ras/Raf at the membrane, promoting MAPK signaling. Structure 26 (513–525), e512.
Loveridge, C.J., Mui, E.J., Patel, R., Tan, E.H., Ahmad, I., Welsh, M., Galbraith, J., Hedley, A., Nixon, C., Blyth, K., et al., 2017. Increased T-cell infiltration elicited by Erk5 deletion in a Pten-deficient mouse model of prostate carcinogenesis. Cancer Research 77, 3158–3168.
Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S., 2002. The protein kinase complement of the human genome. Science 298, 1912–1934.
McKay, M.M., Morrison, D.K., 2007. Integrating signals from RTKs to ERK/MAPK. Oncogene 26, 3113–3121.
Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., Cobb, M.H., 2001. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocrine Reviews 22, 153–183.
Perander, M., Keyse, S.M., Seternes, O.M., 2016. New insights into the activation, interaction partners and possible functions of MK5/PRAK. Frontiers in Bioscience (Landmark Edition) 21, 374–384.
Raman, M., Chen, W., Cobb, M.H., 2007. Differential regulation and properties of MAPKs. Oncogene 26, 3100–3112.
Risco, A., Cuenda, A., 2012. New insights into the p38gamma and p38delta MAPK pathways. Journal of Signal Transduction 2012, 520289.
Sebolt-Leopold, J.S., English, J.M., 2006. Mechanisms of drug inhibition of signalling molecules. Nature 441, 457–462.
Sebolt-Leopold, J.S., Herrera, R., 2004. Targeting the mitogen-activated protein kinase cascade to treat cancer. Nature Reviews. Cancer 4, 937–947.
Simoes, A.E., Rodrigues, C.M., Borralho, P.M., 2016. The MEK5/ERK5 signalling pathway in cancer: A promising novel therapeutic target. Drug Discovery Today 21, 1654–1663.
Uzdensky, A.B., Demyanenko, S.V., Bibov, M.Y., 2013. Signal transduction in human cutaneous melanoma and target drugs. Current Cancer Drug Targets 13, 843–866.
Wagner, E.F., Nebreda, A.R., 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. Nature Reviews. Cancer 9, 537–549.
Wellbrock, C., Arozarena, I., 2016. The complexity of the ERK/MAP-kinase pathway and the treatment of melanoma skin cancer. Frontiers in Cell and Development Biology 4, 33.

Mod Squad: Altered Histone Modifications in Cancer

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Introduction

It is now understood that "An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" or in other words epigenetics is the heritable molecular determinants of a trait that are independent of DNA sequence. The principle mechanisms by which this is achieved are through DNA methylation, covalent histone tail modifications, and noncoding RNAs. These epigenetic modifications work in concert with the genetic sequence to determine cellular physiology and identity in human cells. It is now well understood that perturbations to these marks can lead to pathologies such as cancer. Each class of epigenetic mark, for example, DNA methylation, represents large fields of research. Here we will focus solely on the role of covalent modifications of histone tails to the appearance and progression of cancers. Excellent reviews for DNA methylation and noncoding RNAs can be found elsewhere.

The DNA molecules that comprise the genome are packaged within a cell by winding about octomers of histone proteins to form nucleosomes, the building block of chromatin. The histone proteins H2A, H2B, H3, and H4 are small positively charged proteins with exposed N-terminal tails that dimerize to form the core of the nucleosome particle. Approximately 147 bp of superhelicial DNA wraps around each octomer to form the core particle. The exposed N-terminal tails are the substrate for posttranslational modifications and are the basis for the "histone code," the molecular logic behind chromatin-based inheritance. The principle histone modifications are acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation and lead to chromatin compaction and transcriptional expression or repression. Notable examples are listed in **Table 1**. Canonical nomenclature for the modification is to list the histone protein number, then the modified amino acid and its position in the protein, and finally the type of modification. For example, H3K27me3 represents trimethylation of a lysine at the 27th residue in histone H3.

Histone modifications are synthesized by an array of enzymes such as histone deacetylases and methyl transferases. These enzymes incorporate or remove the modifications or "marks" and are the target of a number of oncogenic mutations. While there are a large number of characterized chromatin marks, and advances in proteomics promises the discovery of more to come, a few classical marks such as H3K9me3 are well studied. While the functions of the best-studied marks are remarkably context dependent certain patterns have emerged, such as H3K9me3 being associated to chromatin compaction and transcriptional repression, and canonical marks are summarized in **Fig. 1**. Most tumors harbor both genetic and epigenetic defects with complex interactions between the two. Indeed, efforts to sequence the human cancer genome found some of the most frequent mutations were in genes encoding the subunits of protein complexes that read and write histone modifications like ATP-dependent remodeling complexes SWI/SNF and BAF and suggest that more than 20% of human cancers bear one of these remodeling mutations. In response there have been a number of international epigenome mapping projects such as the NIH's Roadmap Epigenomics Mapping Consortium, International Human Epigenome Consortium, The Cancer Genome Atlas Network, BLUEPRINT, and the International Cancer Genome Consortium. The result of these consortiums has been an ability to subclassify tumors based on their molecular etiology rather than clinical features. This ability allows for the advancement of precision medicine and patient specific treatments and will be discussed further at the end of the article.

Cancer is principally unrestrained cell division and is typically thought to be caused by dysregulation of gene expression. While transcription factors such as p53 or MYC are essential to tumor formation, they represent difficult drug targets as protein:protein and protein:DNA interactions are difficult to target with any specificity. Epigenetic proteins represent much better therapeutic targets given than many are enzymes. It should be noted that epigenome targeting drugs such as HDACis (histone deacetylase inhibitors) have been FDA approved and in use for years. Here we will describe how disruptions in histone modifications caused by HDACs, BETs, and methyltransferases lead to cancer.

Table 1 List of important histone modifications and their impact on transcription

Modification	*Position*	*Transcriptional effect*	*Associated enzymes*
Methylation	H3K4	Activation	MLL1-5, SETD1
	H3K9	Repression	SUV39H1, G9A, SETDB1
	H3K37	Repression	EZH1,2
	H3K36	Activation	SETD2, ASH1L
	H3K79	Activation	DOT1L
	H3R2, H3R17, H3R26	Activation	PRMT4
	H3R8	Repression	PRMT5
	H2AR3	Activation	PRMT1 PRMT5
	H4K20	Repression	SETD8, SUV4
Acetylation	H3K9, H3K14, H3K18, H3K23, H3K27	Activation	KAT2A, KAT2B, p300, CBP
	H4K5, H4K8, H4K12, H4K16	Activation	KAT5, KAT7, p300, CBP

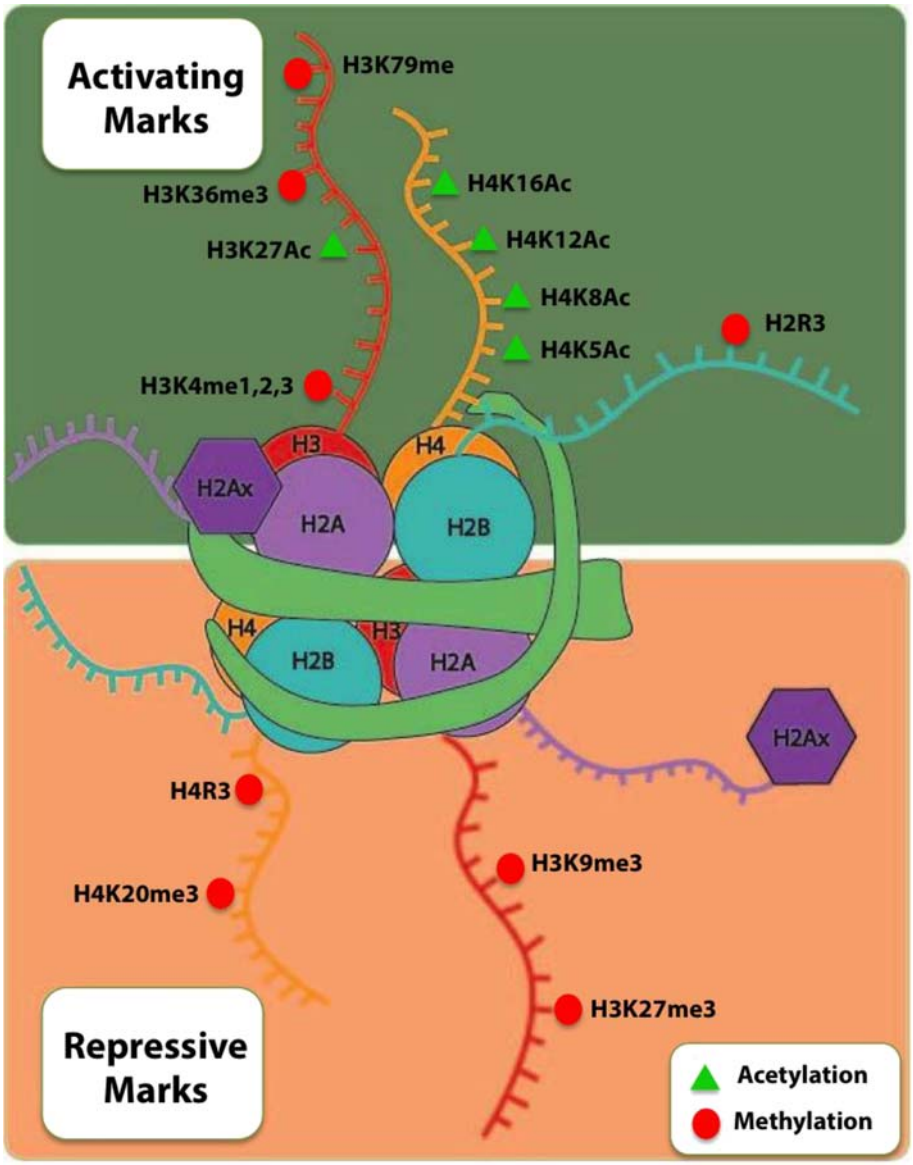

Fig. 1 Illustration of the nucleosome with selected important histone modifications. Schematic of the nucleosome with four pairs of histone proteins and important N-tail histone modifications. Methylation events are represented with a red circle, acetylation by a green triangle.

Altered Histones in Cancer

A hallmark study of altered histone modifications in cancer examined posttranslational modifications to histone H4 in a comprehensive panel of normal tissues, cancer cell lines and primary tumors. The study found a loss of monoacetylated and trimethylated forms of histone H4 characterized cancer cells. These changes appeared early and accumulated during the tumorigenic process, and were also observed in a mouse model of skin carcinogenesis. The losses occurred predominantly at H4K16Ac and H4K20me3 and were associated with the hypomethylation of DNA repetitive sequences, a well-known characteristic of cancer cells. This study was the first to suggest that the global loss of monoacetylation and trimethylation of histone H4 is a common hallmark of human tumor cells and alerted the field to the importance of altered histone modifications in cancer.

Chromatin Remodelers as Tumor Suppressors

Chromatin-remodeling complexes are large multisubunit complexes that mediate the compaction and expansion of chromatin in the nucleus while allowing for precise gene expression, DNA repair, and replication. The mammalian BAF (Brg/Brahma associated

factors) or SWI/SNF complex can be imagined as a single unit comprised of 15 subunits encoded by 29 genes. A mutation to one of these genes is found in at least 20% of cancers. The genes were first identified and characterized in yeast studies where they were discovered in screens attempting to identify signal transduction pathway members. Their role in chromatin compaction was confirmed by phenotypic rescue with secondary mutations in histone proteins, demonstrating that they remodeled chromatin rather than participated in signal transduction. In human cells they appear to primarily act to oppose the effects of the PRC2 complex, which is best known for its role in depositing the H3K27me3 mark.

There are many lines of evidence linking mutations to the BAF complex to cancer. Initial observations of malignant cell lines found that these lines often had deletions or mutations to critical subunits of the complex, suggesting a role as a tumor suppressor. Further study revealed subunits were able to bind RB protein and repress E2F function. Their role as tumor suppressor was confirmed through a study of malignant rhabdoid tumor (MRT), a rare cancer that occurs in children. Versteege et al. found that MRT tumors feature uniform loss of BAF47 (i.e., *INI1*, *hSNF5*, *SMARCB1*), typically from a germline loss of one allele followed by the loss of a second allele in the tumor tissue. This indicated that BAF47 mutations act as classic tumor suppressors, resembling the genetics of retinoblastoma. Further studies found cancer cell lines that featured inactivation of both Brg and Brm subunits, a finding that was replicated in several breast cancer cell lines and led to the common view that tumor suppression likely required at least one functional allele of either subunit.

The mechanisms by which mutations to chromatin remodelers caused cancer remained mysterious until the advent of genome-wide mapping of chromatin marks. The advent of genome sequencing allowed the sensitive detection of mutations, however this revealed that loss of both alleles of particular subunits were not necessary. For example, initial reports of ovarian cancers revealed that while 57% of ovarian clear cell carcinoma and 46% of endometriosis-associated ovarian carcinoma contained inactivating mutations to BAF250A (*ARID1A*), nearly all of the cases the mutations were in only a single allele. This suggested that at least in the case of ovarian cancers, BAF250A operated as a dominant tumor suppressor subunit, a finding that was confirmed by repeated studies. Further confounding mechanistic insight was the observation that BAF250 is not necessary for chromatin remodeling, at least not with respect to remodeling events that can be observed from in vitro nucleosomal template experiments. In addition, many subunits do not appear to be involved in tumor suppression as they are not observed to be mutated at particularly high rates in any known sequenced tumor.

So how can the BAF complex be both extremely sensitive to the loss of a given allele while at the same time having neither its function nor other subunits altered? Standford biologist Gerald Crabtree observed that this places three constraints on the mechanism for BAF tumor suppression. Firstly, it is dosage-sensitive, as evidenced by the observed genetic dominance. Secondly, the fundamental mechanism cannot be detected in an in vitro chromatin-remodeling assay. Lastly, the tumor-suppression function manifests itself in a highly tissue specific manner. A clear example of this is seen within small cell cancers: 90% of small cell ovarian cancers feature mutations to the ATPase BRG (*SMARCA4*) while less than 5% of small cell lung cancers carry mutations to the gene.

So how do mutations in BAF complex subunits mediate oncogenesis? BAF complexes target repressive Polycomb targets and work to oppose transcriptional repression. Mutations apparently cause mis-targeting and improper gene regulation. For example, mutation of BAF subunit ATPase Brg1 (*Smarca4*) in embryonic stem cells causes accumulation of H3K27me3 and the subsequent repression of genes underlying the mark. A similar mechanism, the inability for the BAF complex to properly regulate the placement and function of the PRC (Polycomb repressive complex) is at work in cancer. For example, tissue specific deletion of BAF47 in mice leads to rapid rise of T cell lymphoma. In these mutant cells, BAF deletion causes improper removal of Polycomb from the Ink4a (*Cdkn2a*) locus, which results in an aberrant accumulation of H3K27me3 and repression of Ink4a. Thus, altered modification of H3K27 causes the repression of a potent tumor suppressor and proliferation regulator. A similar mechanism is thought to be at play in MRTs albeit with different tissue specificity. That MRTs have the lowest mutational burden observed in any tumor suggests the tumors are induced into oncogenesis solely through changes to histone marks and epigenetic regulation.

Another beautiful illustration of altered histone modifications driving cancer progression is seen in human synovial sarcoma, a soft-tissue tumor featuring a precise translocation of the BAF SS18 (C90) subunit. This translocation fuses 78 amino acids of the C terminus of SSX to the C terminus of the SS18 subunit to create a well-studied oncogenic fusion protein termed SS18-SSX. This fusion protein outcompetes wild type SS18 in BAF complexes and results in the mistargeting of BAF47 and SS18-SSX complexes to loci containing oncogenic genes like SOX2 and PAX6. This retargeting results in the loss of PRC complexes at these loci and the subsequent loss of H3K27me3, thus allowing the expression of the oncogenes.

Not all BAF driven tumors operate through these mechanisms and even in the case of MRT and synovial tumors where BAF47 is mistargeted, the molecular mechanism is very distinct. These examples however clearly show the importance of correctly modified histones and the ability of epigenetic changes to induce cancer.

Interpreting Histone Acetylation

ε-N-acetylation of lysine residues in the N-terminal tails of histone proteins is one of the predominant and best-studied histone modifications. Acetylation is regulated by histone acetyltransferases (HATs) which act as "writers" adding acetyl groups to the histone tail, while histone deacetylases (HDACs) act as "erasers" and remove the mark. There are many examples of these enzymes suffering inactivating mutations that drive cancer. For example, mutations in CBP (KAT3A) and p300 (KAT3B) are found in B cell lymphomas, monoallelic loss of KAT5 in observed in human lymphomas, breast and head and neck cancers and homozygous

deletion of KAT6B in small cell lung cancer. Additionally, HDAC deregulation results in the silencing of tumor-suppressor genes or overexpression of oncogenes. For example, HDAC1, HDAC3 and HDAC6 are overexpressed in tumors. These studies have provided the basis for the development of HAT11 and HDAC inhibitors, some of which had already proved successful in clinical oncology and will be discussed further below.

Bromodomains (BRDs) are "readers" of acetyl marks in histone tails, targeting chromatin-modifying enzymes and other protein machinery to specific sites in the chromatin, thus regulating gene transcription. Bromodomains are a family of evolutionarily conserved motifs identified for in the *brahma* gene of *Drosophila melanogaster*. BRDs bind the acetylated lysines in histone tails allowing recruitment of other chromatin factors and transcriptional machinery. A total of 61 bromodomains were found in 46 different proteins of the human proteome. The bromodomain and extra-terminal (BET) family has been thoroughly investigated and holds great potential as a chemotherapeutic target in cancer therapy. The BET family is comprised of ubiquitously expressed BRD2, BRD3, BRD4, and BRDT, and BRDT, which is only expressed in testis. BET proteins are characterized by their two bromodomains in tandem (BD1 and BD2) in the N-terminal and a C-terminal extra-terminal (ET) domain.

It was elucidated some years ago that BRD4 and BRD2 play an important role in transcription elongation by recruiting the positive transcription elongation factor complex (P-TEFb) to acetylated chromatin. P-TEFb is composed of cyclin-dependent kinase-9 (CDK9) and its activator, cyclin T and mediates phosphorylation of the C-terminal repeat domain (CTD) of RNA polymerase II (RNAP II) to ensure efficient transcription. BRD4 recruits P-TEFb to acetylated chromatin through its BRD, allowing activation of the CDK9 kinase subunit, phosphorylation of RNAPII and efficient transcript elongation. BRD4 also controls the release of active P-TEFb from its inactive complex with HEXIM1 protein and 7S snRNA. Thus, correct histone acetylation is essential for the proper recruitment of P-TEFb by BRD4, which is then critical for proper transcriptional initiation and elongation of genes controlling cell proliferation.

BET proteins and their interactions with acetylated histone tails are now strongly implicated in cancer, not the least because BETs have been show to directly regulate the expression of oncogenes such as c-MYC. Indeed, inhibition of BET protein binding at the MYC locus with BET inhibitors leads to a reduction in cell proliferation.

The BET family can function as cell cycle regulators as well by controlling the expression of genes required for M to early G1 phase transition. BRD2 recruits the key transcriptional cell cycle-regulatory genes E2F1 and E2F2 and acts as a scaffold on which they assemble.

BRD4 regulates gene transcription globally, so its inhibition would be expected to cause downregulation of activity throughout the genome. Surprisingly, the impact of BRD4 inhibition is modest and somewhat specific as only a few hundred genes are downregulated. However, most of these genes are very important in tumorigenesis. The molecular basis for this surprising discrepancy is explained by the observation that BRD4 has a strong preference for binding enhancers and super-enhancers in key genes of hematological and solid tumors, such as MYC.

Brd4 acts as a general regulator that couples the acetylation state of chromatin with Pol II elongation. Midline carcinoma is generated when *BRD4* is mutated by chromosomal translocation to form in-frame fusions with the *NUT* gene. The resulting Brd4-NUT oncoprotein is an aberrant transcriptional regulator.

Targeting BET Bromodomains With Small-Molecules for Therapy

Mediating the interaction between histone modifications and transcription with enzymatic activity make BET a desirable target for small molecule therapies. Indeed, the field is proceeding rapidly as in 2010 two independent groups reported the antitumor efficacy of small-molecule inhibitors of BET bromodomains, called JQ1 and I-BET, which have a similar chemical structure and mode of target inhibition. JQ1 and I-BET are notable for their high affinity for bromodomains of the BET family (BD1 and BD2 of Brd2/Brd3/Brd4/Brdt) over other bromodomain subfamilies. BET inhibitors also have suitable pharmacokinetics for in vivo application, which has enabled a rapid evaluation of their potential therapeutic activity in various disease models.

BETs can become oncogenes upon translocational mutation. For example, *BRD4* can be mutated by chromosomal translocation to form in-frame fusions with the *NUT* gene to initiate an aggressive cancer called midline carcinoma. The resulting Brd4-NUT oncoprotein is an aberrant transcriptional regulator that relies on the bromodomains of Brd4 for its oncogenic function. As such, this malignancy provides a clear initial test case for the evaluation of therapeutic benefit of BET inhibitors. In midline carcinoma cell lines, JQ1 treatment led to the release of Brd4-NUT from chromatin and triggered terminal squamous cell differentiation and apoptosis. In patient-derived xenograft models of midline carcinoma, it was shown that daily exposure to JQ1 extended the survival of cancer-bearing mice at doses that had minimal toxicity to normal tissues. This pivotal study provided the first demonstration of efficacy for a BET inhibitor in a preclinical cancer model.

HDACi Demonstrate Utility of Altered Histone Modifications to Fight Cancer

HDAC inhibitors (HDACi) are a class of small molecules that interfere deacetylation and were first approved for the treatment of cutaneous T-cell lymphoma in 2006. HDACi's now have shown efficacy, either alone or in combination, in multiple myeloma, acute myelogenous leukemia and myelodysplastic syndrome. Treating solid tumors with HDACi has been challenging, although some efficacy was recently reported in non-small cell lung cancer when combined with a second epigenetic therapy.

Mapping the Histone Modifications of the Epigenome

The advent of whole genome and high-throughput sequencing technologies has enabled fast, accurate genome-wide maps of histone modifications at rapidly diminishing costs. This has led to international epigenome mapping projects such as the NIH's Roadmap Epigenomics Mapping Consortium, International Human Epigenome Consortium, The Cancer Genome Atlas Network, BLUEPRINT, and the International Cancer Genome Consortium. These projects aim to map the epigenomes of healthy and cancer cells and have led to a surplus of data and new insights.

Most strikingly, these epigenomic maps can be used to subclassify tumor samples allowing for precision and personalized medicine. There are myriad examples to choose from. H3K4 acetylation, H3K9 acetylation and H3K27 methylation can be used to identify breast tumor molecular subtypes. Indeed, most every major tumor type has now been profiled and study epigenetically, see for example malignant melanoma.

Targeting Altered Histone Modifications for Therapy

Drugs targeting the epigenome are now being extensively developed given their promise for halting tumor progression and preventing chemoresistance. They are considered broadly to act either in a broad pan-genome manner, termed genome medicine, or are developed to treat specific tumor subsets for precision or personalized medicine. HDACi were initially discovered based on drug screens for differentiation inducers in leukemias. There are now several prominent HDACi used in the clinic: for example, vorinostat (Zolinza; Merck & Co.), belinostat (Beleodaq; Spectrum Pharmaceuticals) and romidepsin (Istodax; Celgene) have all been approved for the treatment of cutaneous or peripheral T cell lymphomas, and panobinostat (Farydak; Novartis) was recently approved for the treatment of drug-resistant multiple myeloma when used in combination with the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals). Notable examples of drugs targeting histone modification pathways are listed in Table 2. HDACi efficacy has been examined in other malignancies have shown limited single agent activity. iBETs, which reversibly bind to the bromodomains of BET proteins, are another prominent class of genome medicines that have shown promise. Most iBETs target BRD4, which is translocated in some cancers and is key in correct interpretation of histone acetylation (see above).

There are several notable examples of targeted therapies showing efficacy. Specific genetic defects in epigenetic pathways can be targeted using small molecules. One such example is apparent given mutation in the H3K27 histone *N*-methyltransferase EZH2 which is activated by mutations in lymphomas. Researchers found the application of a selective EZH2 inhibitor induced the specific death of cells harboring the EZH2 mutation in cell lines.

Synthetic lethality approaches can also be used to achieve subtype specificity for targeted therapy and precision medicine. For instance, inhibitors of H3K79 *N*-methyltransferase DOT1L have shown effective in vitro study of leukemias with activation of MLL (mixed-lineage leukemia; also known as histone-lysine *N*-methyltransferase 2A (KMT2A)), whereas a drug targeting lysine-specific histone demethylase 1 (LSD1; also known as KDM1A) is active in vitro only in malignancies with specific DNA methylation patterns. Preclinical studies have shown that these targeted therapies have particular efficacies in specific patient subsets, which is to be expected from targeted therapies and is different from broad genomic medicine approaches. Indeed, in the field of DNA methylation, epigenetics can aid in assigning the correct treatment regimen to Cancers of Unknown Origen suggesting that accurate epigenome profiling of histone modifications may also aid in developing precision medicine.

Table 2 Selection of drugs targeting regulators of histone modifications currently FDA approved or in advanced clinical trials

Inhibitor class	*Mechanism*	*Drug*	*Target*	*Cancer*
HDACi	Inhibition of histone deacetylation	Belinostat	Class I & II HDACs	Peripheral T-cell lymphoma
	Inhibition of histone deacetylation	Panobinostat	Class I, II, & IV	Multiple myeloma
	Inhibition of histone deacetylation	Romidepsin	Class I	Cutaneous T-cell lymphoma
	Inhibition of histone deacetylation	VOrinostat	Class I, II, & IV	Cutaneous T-cell lymphoma
	Inhibition of histone deacetylation	Entinostat	Class I	Breast cancer
	Inhibition of histone deacetylation	Givinostat	Class I & II	Hematological malignancies
	Inhibition of histone deacetylation	Mocetinostat	Class I	Hematological malignancies
	Inhibition of histone deacetylation	Resminostat	HDAC1,3,6	Hepatocellular carcinoma
	Inhibition of histone deacetylation	Ricolinostat	HDAC6	Solid tumors and hematological malignancies
iBET	Inhibition of BET binding to acetylated histones	OTX015	Pan-BET	Hematological malignancies
	Inhibition of BET binding to acetylated histones	INCB054329	Pan-BET	Leukemias and solid tumors
	Inhibition of BET binding to acetylated histones	BMS-986158	Pan-BET	Solid tumors
	Inhibition of BET binding to acetylated histones	GSK525762	Pan-BET	Solid tumors and hematological malignancies
EZH2 inhibitor	Inhibition of H3K27 methylation	CPI-1205	EZH2	Lymphoma
	Inhibition of H3K27 methylation	Tazemetostat	EZH2	Lymphomas & sarcomas

Concluding Remarks

As our understanding of the interactions between histone modifications and cell identity become clearer so too does our appreciation for the ways that altered histone modifications can lead to cancer. It stands to reason that deeper study of these covalent modifications will shed light on to the basic biology of how epigenetics can govern cellular functions in addition to the discovery of therapeutically relevant targets for chemotherapy development.

Acknowledgments

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See also: Environmental Exposures and Epigenetic Perturbations. Mutations in Chromatin Remodeling Factors. Mutations in Histone Lysine Methyltransferases and Demethylases.

Further Reading

Bae, J.-B., 2013. Perspectives of international human epigenome consortium. Genomics & Informatics 11, 7–14.
Berger, S.L., Kouzarides, T., Shiekhattar, R., Shilatifard, A., 2009. An operational definition of epigenetics. Genes & Development 23, 781–783.
Bernstein, B.E., Stamatoyannopoulos, J.A., Costello, J.F., Ren, B., Milosavljevic, A., Meissner, A., Kellis, M., Marra, M.A., Beaudet, A.L., Ecker, J.R., et al., 2010. The NIH roadmap epigenomics mapping consortium. Nature Biotechnology 28, 1045–1048.
Bolden, J.E., Peart, M.J., Johnstone, R.W., 2006. Anticancer activities of histone deacetylase inhibitors. Nature Reviews. Drug Discovery 5, 769–784.
Cancer Genome Atlas Research Network, Weinstein, J.N., Collisson, E.A., Mills, G.B., Shaw, K.R.M., Ozenberger, B.A., Ellrott, K., Shmulevich, I., Sander, C., Stuart, J.M., 2013b. The Cancer Genome Atlas Pan-Cancer Analysis project. Nature Genetics 45, 1113–1120.
Chadwick, L.H., 2012. The NIH roadmap epigenomics program data resource. Epigenomics 4, 317–324.
Costa, A.L., Lobo, J., Jerónimo, C., Henrique, R., 2017. The epigenetics of testicular germ cell tumors: Looking for novel disease biomarkers. Epigenomics 9, 155–169.
Delmore, J.E., Issa, G.C., Lemieux, M.E., Rahl, P.B., Shi, J., Jacobs, H.M., Kastritis, E., Gilpatrick, T., Paranal, R.M., Qi, J., et al., 2011. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell 146, 904–917.
Esteller, M., 2011a. Epigenetic changes in cancer. Biology of Reproduction 3, 9.
Esteller, M., 2011b. Non-coding RNAs in human disease. Nature Reviews. Genetics 12, 861–874.
Esteller, M., 2017. Epigenetic drugs: More than meets the eye. Epigenetics 12, 307.
Fernández, J.M., de la Torre, V., Richardson, D., Royo, R., Puiggròs, M., Moncunill, V., Fragkogianni, S., Clarke, L., Consortium, B.L.U.E.P.R.I.N.T., Flicek, P., et al., 2016. The BLUEPRINT data analysis portal. Cell Systems 3, 491-495.e495.
Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., et al., 2010. Selective inhibition of BET bromodomains. Nature 468, 1067–1073.
Fraga, M.F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K., et al., 2005. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nature Genetics 37, 391–400.
Jones, P.A., Issa, J.-P.J., Baylin, S., 2016. Targeting the cancer epigenome for therapy. Nature Reviews. Genetics 17, 630–641.
Kadoch, C., Crabtree, G.R., 2015. Mammalian SWI/SNF chromatin remodeling complexes and cancer: Mechanistic insights gained from human genomics. Science Advances 1, e1500447.
Kulis, M., Esteller, M., 2010. DNA methylation and cancer. Advances in Genetics 70, 27–56.
Mohammad, H.P., Smitheman, K.N., Kamat, C.D., Soong, D., Federowicz, K.E., Van Aller, G.S., Schneck, J.L., Carson, J.D., Liu, Y., Butticello, M., et al., 2015. A DNA hypomethylation signature predicts antitumor activity of LSD1 inhibitors in SCLC. Cancer Cell 28, 57–69.
Moran, S., Martínez-Cardús, A., Sayols, S., Musulen, E., Balañá, C., Estival-Gonzalez, A., Moutinho, C., Heyn, H., Diaz-Lagares, A., de Moura, M.C., et al., 2016. Epigenetic profiling to classify cancer of unknown primary: A multicentre, retrospective analysis. The Lancet Oncology 17, 1386–1395.
Moran, S., Martínez-Cardús, A., Boussios, S., Esteller, M., 2017b. Precision medicine based on epigenomics: The paradigm of carcinoma of unknown primary. Nature Reviews. Clinical Oncology 14, 682–694.
Pérez-Salvia, M., Simó-Riudalbas, L., Llinàs-Arias, P., Roa, L., Setien, F., Soler, M., de Moura, M.C., Bradner, J.E., González-Suárez, E., Moutinho, C., et al., 2017. Bromodomain inhibition shows antitumoral activity in mice and human luminal breast cancer. Oncotarget 8, 51621–51629.
San-Miguel, J.F., Hungria, V.T.M., Yoon, S.-S., Beksac, M., Dimopoulos, M.A., Elghandour, A., Jedrzejczak, W.W., Günther, A., Nakorn, T.N., Siritanaratkul, N., et al., 2014. Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: A multicentre, randomised, double-blind phase 3 trial. The Lancet Oncology 15, 1195–1206.
Shi, J., Vakoc, C.R., 2014. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. Molecular Cell 54, 728–736.
Simó-Riudalbas, L., Esteller, M., 2014. Cancer genomics identifies disrupted epigenetic genes. Human Genetics 133, 713–725.
Stunnenberg, H.G., International Human Epigenome Consortium, Hirst, M., 2016. The international human epigenome consortium: A blueprint for scientific collaboration and discovery. Cell 167, 1897.
Yang, Z., Yik, J.H.N., Chen, R., He, N., Jang, M.K., Ozato, K., Zhou, Q., 2005. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Molecular Cell 19, 535–545.
Yardley, D.A., Ismail-Khan, R.R., Melichar, B., Lichinitser, M., Munster, P.N., Klein, P.M., Cruickshank, S., Miller, K.D., Lee, M.J., Trepel, J.B., 2013. Randomized phase II, double-blind, placebo-controlled study of exemestane with or without entinostat in postmenopausal women with locally recurrent or metastatic estrogen receptor-positive breast cancer progressing on treatment with a nonsteroidal aromatase inhibitor. Journal of Clinical Oncology 31, 2128–2135.
Zhang, Y., Lv, J., Liu, H., Zhu, J., Su, J., Wu, Q., Qi, Y., Wang, F., Li, X., 2010. HHMD: The human histone modification database. Nucleic Acids Research 38, D149–D154.
Zöllner, S.K., Rössig, C., Toretsky, J.A., 2015. Synovial sarcoma is a gateway to the role of chromatin remodeling in cancer. Cancer Metastasis Reviews 34, 417–428.

Molecular Epidemiology and Cancer Risk☆

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Glossary

Biomarker Any biological indicator that serves as an objective measure of physiological or pathogenic processes used to detect exposure to carcinogens and assess carcinogenic hazard.

Causal inference The process of deciding whether existing evidence is sufficient to conclude there is a causal relationship between a perturbation and an effect.

Exposome All environmental (nongenetic) exposures in a lifetime intended to explore the role of the environment in human health.

Molecular epidemiology A scientific field that seeks to study the contribution of risk factors, identified through biological indicators (i.e., biomarkers), to the etiology of pathological processes.

Omics The field that seeks to characterize and quantify large-scale data considered collectively from different biological fields such as genomics or proteomics.

Primary risk prevention Intervention applied before evidence of disease with the aim of preventing the onset of disease.

Reverse causation Observed when the outcome of interest precedes the exposure instead of the common presumption that exposure occurs before the outcome of interest.

Subphenotypes A subset of the phenotype with characteristics specific to a part of the population.

Introduction

Cancer continues to be a dire predicament worldwide. Established as the second leading cause of death after cardiovascular diseases in 2015; cancer represents a substantial personal and social burden with serious impact in both economy and quality of life (Fitzmaurice et al., 2017). Classic epidemiology has greatly contributed to the study of cancer risk. Illustrative of this is that most of what we initially learned from smoking and lung cancer resulted from traditional epidemiologic studies. During the early 1950's evidence based on questionnaire information started to surface linking smoking with lung cancer. The consistent evidence mounting over the years by different studies and the replicability of results across investigations made causal inference conceivable (Samet, 2016). More recently, molecular epidemiology approaches have enabled to further explore and understand carcinogenic mechanisms of tobacco compounds through the identification of biomarkers of smoking exposure and susceptibility.

The term molecular cancer epidemiology was first reported in 1982 by Perera and Weinstein who proposed combining the concepts of classic epidemiology with the use of advanced laboratory technology to identify specific environmental hazards and host factors that modify susceptibility, and to elucidate mechanisms of carcinogenesis. For this purpose, four categories of molecular markers (i.e., biomarkers) were described: internal dose, biologically effective dose, response, and susceptibility. The ultimate goal of implementing biomarkers in epidemiology is to improve the characterization of exposure providing an objective, quantifiable measure for a more precise evaluation of human cancer risk. This is particularly useful when evaluating complex exposures and weak effects which are difficult to assess through traditional epidemiology approaches (Perera and Weinstein, 1982; Perera, 1987, 2000).

Molecular epidemiology aims to gather evidence that specific compounds pose carcinogenic hazard to human health through the definition of exposure-cancer associations and the identification of populations at greater risk. Conversely, factors that protect against carcinogenesis could be defined. Therefore, based on the notion that up to some extent an important proportion of cancer could be prevented, modifiable risk factors represent a great opportunity for primary cancer prevention through avoidance or minimization of key exposures or through the promotion of protective factors. Moreover, molecular epidemiology represents a unique opportunity of increasing our understanding of the mechanisms underlying carcinogenesis hence shaping our knowledge towards the biological plausibility behind carcinogenic exposure.

The fast development of technology, particularly high-throughput technologies, has resulted in the rapid growth and evolution of molecular epidemiology in the past decades. More sophisticated molecular platforms have allowed to refine exposure assessment and to improve the definition of disease phenotype. Therefore, progress in the field has provided us with the valuable opportunity

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This article is an update of Frederica P. Perera, Molecular Epidemiology and Cancer Risk, in Encyclopedia of Cancer (Second Edition), edited by Joseph R. Bertino, Academic Press, 2002, Pages 213–220.

of increasing our knowledge and understanding of cancer risk. However, we have also encountered several difficulties that hamper their practical application in risk assessment. In the present article we discuss key contributions of molecular epidemiology in cancer risk assessment, prediction and prevention as well as some of its most relevant challenges in the current scientific landscape. In addition, while the role of genetic factors in disease is formally studied under the umbrella of genetic epidemiology, we will briefly comment on genetic susceptibility to cancer due to important interactions with other cancer risk factors, as well as their relevance in the *omics* and data integration fields.

Epidemiologic Study Designs for the Evaluation of Biomarkers

Molecular epidemiology builds upon classic epidemiology designs. Among these, the case-control studies are the most commonly used, entailing the retrospective recruitment of disease and disease-free subjects. While ascertainment of the diagnosis of cases before recruitment is one of the main advantages of case-control studies, these suffer from different drawbacks, one of them specifically pertaining to the difficulty of establishing reverse causation. For this purpose, prospective studies are ideal because information about exposure and biological samples are collected at baseline from a group of "healthy" subjects who are followed-up during a period of time in order to identify those who develop the disease of interest. Since exposure is assessed before disease development, prospective studies are less likely to introduce selection and information bias such as recall bias. Moreover, due to the prospective nature of this design reverse causation is precluded. Prospective studies also offer the possibility of collecting information and samples at different time points during the follow-up time. However, these studies are costly and time consuming particularly for uncommon diseases for which larger study populations and longer time periods are necessary to identify sufficient number of subjects with the disease of interest.

For different reasons, many large cohort studies are unable to measure the biomarker(s) of interest in the whole study population. Under this circumstance, nested case-control studies are an efficient sampling scheme. In this design, all cases identified up to a particular point in time are included in the study while controls are composed from a random sample of subjects free of the disease of interest by the time of the case diagnosis or by the end of the follow-up time. Similarly useful is the case-cohort design, in which disease-free subjects are sampled as controls at baseline from the pool of all subjects enrolled having the same disease risk as anyone at time zero of the study. This type of design requires specific statistical considerations since some controls could also be included as cases in the study (García-Closas et al., 2006, 2011).

Contributions of Molecular Epidemiology

Assessment of Exposure

The use of biomarkers in molecular epidemiology

The use of molecular markers to assess the precise contribution of individual factors, their interactions and those with the host result in a more objective measure of exposure, one that is exempt from the individual's perception or recall. Moreover, biological markers measured with laboratory methods more closely reflect the level of exposure that affects the target cells or organs (i.e., biological effective dose).

Risk assessment has particularly benefited from the advancement of refined laboratory techniques such as those used to measure metabolites of carcinogens and their interaction with DNA or proteins (adducts). DNA adducts are quantitative indicators of exposure used to evaluate carcinogenicity of specific compounds like those contained in tobacco. Tobacco was initially identified as a potent carcinogen in the mid-twentieth century and it is known to contain at least 70 human carcinogens including polycyclic aromatic hidrocarbons (PAHs). PAHs in tobacco smoke are metabolized and activated in the human body forming DNA adducts that have the potential to induce mutations and ultimately initiate a carcinogenic process (IARC, 2004). Consequently, DNA adducts are suitable biomarkers of carcinogenesis since they indicate a genetic damage that is relevant to such process not only reflecting exposure but also the host's ability to metabolize these compounds (Perera and Weinstein, 1982).

Levels of PAH DNA adducts have been reported to be higher in smokers than nonsmokers in different human tissues including those not directly exposed to tobacco smoke such as bladder, liver or pancreas (Phillips and Venitt, 2012). Accordingly, higher DNA adduct levels have been suggestive of increased risk of lung, bladder and breast cancer (Agudo et al., 2017; Jin et al., 2017; Munnia et al., 2017). Moreover, the role of PAHs in cancer is highlighted by reports that show that single nucleotide polymorphisms (SNPs) in key metabolism and conjugation genes are associated with PAH-DNA adducts levels (Etemadi et al., 2013).

As a result of the extensive research performed in the field during the past decades, different countries worldwide have applied smoking bans that along with smoking cessation strategies have had an impact in lowering the incidence of smoking related cancers (McAfee et al., 2015).

The emergence of omics data in molecular epidemiology

Cancer is a complex condition that entails many biological changes within the affected cells and the surrounding microenvironment. These include mutations and modifications of gene and protein expression through different mechanisms. Advances in high-throughput technology during the past two decades coupled with sound biostatistics and bioinformatics methodology

have allowed exploring the collective of different small biological molecules such as genes (genomics), gene expression (transcriptomics), epigenetic modifications (epigenomics), and metabolites (metabolomics), among others. These technologies represent an invaluable tool for a more comprehensive insight of human exposure, which is particularly useful in the inquiry of molecular mechanism of carcinogenesis.

During decades, researchers have been focusing in the study of single factors. Based on an a priori knowledge of the molecule's biological function, candidate studies were pioneer in the identification of association with disease (Zhu and Zhao, 2007). However, given that the available functional information is limited elaborating a priori hypothesis may be impaired. As a result, after its emergence, high-throughput technology has been widely used as an agnostic approach in an attempt to identify risk factors for diseases such as cancer. In genomics, through the analysis of thousands of genetic variants in a single run, genome-wide association studies (GWAS) have identified common low-penetrance alleles providing evidence of polygenic susceptibility. Among the different discoveries reached through GWAS, the region 8q24.21 is perhaps one of the most intriguing, harboring multiple independent risk loci for several tumor types including breast, bladder, colorectal, ovarian, pancreatic and prostate cancer (Al Olama et al., 2014; Michailidou et al., 2015; Sud et al., 2017).

DNA methylation, a form of epigenetic mechanism that does not alter DNA sequence but may affect gene expression, has also been identified as an important mechanism of carcinogenesis. As such, aberrant DNA methylation has been reported in different cancers. Methylation arrays allow the rapid assessment of global methylation patterns through the analysis of thousands of CpGs throughout the genome. Studies have reported that such epigenome-wide DNA methylation analysis could have a potential use in the identification of risk markers in cancers such as breast cancer (Severi et al., 2014).

Other *omics* platforms have been similarly applied in an attempt to better characterize cancer leading to advances in risk assessment through a greater understanding of this disease.

The exposome and its potential use in risk assessment

The exhaustive consideration of exposure in risk assessment was initially addressed by Wild in the early 2000s, who proposed the concept of exposome as a means to account for all exposures an individual encounters during their lifetime. The concept encompasses internal exposure, to which molecular and *omics* data contribute to; specific external exposure that includes pollutants, infectious agents, lifestyle factors or diet; and generalized external exposure such as social, economic and psychological exposures (Wild, 2005, 2012). Therefore, while molecular information can greatly contribute to a better definition of exposure it is unable to cover the totality of a subject's exposure. The integration of epigenomics, metabolomics and microbiome (microbial load and diversity data), with exposure information collected via epidemiological questionnaires and biological markers such as vitamin D or cholesterol, is likely to provide a more precise characterization of the exposome (López de Maturana et al., 2016).

The accurate assessment of the exposome would require prospective studies in which multiple repetitive measures are collected throughout the participants' lifetime. Therefore, to date, it is still debatable to what extent the assessment of the exposome and its common use in scientific research and risk assessment is pragmatic.

Definition of Phenotype

The multi-factorial etiology of cancer is highlighted by its great heterogeneity of both clinico-pathological and molecular features. Accordingly, there have been numerous reports on the wide diversity of the natural course of tumors and the way these respond to treatments. Consequently, the use of a single phenotype to describe a mixture of closely related subtypes of diseases (i.e. subphenotypes) is gradually becoming recognized as an unsuitable approach for the proper assessment of disease risk.

Improving the phenotyping accuracy has been an important focus of molecular epidemiology in recent years. Innovative technologies have given place to the definition of novel molecular tumor subtypes in different cancers. In this section, we focus in inter-patient tumor heterogeneity. For information about intra-tumor heterogeneity, the reader can refer to further reading.

Cancer subphenotyping was first explored in breast cancer, one of the most commonly diagnosed cancers in women worldwide and for which different treatment responses and clinical outcomes have been widely reported (Zardavas et al., 2015). Four main breast tumor subtypes were established through genome-wide expression: luminal A, luminal B, HER2 enriched and basal like, which reflect relevant biological entities regarding variation of growth rate, activation of specific signaling pathways and cellular compositions (Perou et al., 2000). Following its initial characterization, the evolution of technology allowed exploring these subtypes through a broad array of molecular platforms as mRNA expression microarrays, DNA methylation chips, SNP arrays or miRNA sequencing providing a deeper insight into each subtype like specific mutational processes or signaling pathways (TCGA, 2012). Accurate molecular subphenotyping of breast cancer has an important clinical impact, particularly in therapeutic decision making (Zardavas et al., 2015). Moreover, tumor subtypes might potentially reflect etiological differences with important implications in risk prediction. Up to date however, there has been a lack of consensus on the characterization of molecular breast cancer subtypes in epidemiological studies that, along with the limited statistical power in the less common subtypes, have hampered their use in etiological research (Ellingjord-Dale et al., 2017). Regarding risk, at present, the most consistent findings show a stronger protective effect of breastfeeding on the basal-like subtype; while, other factors such as parity have been consistently associated with decreased risk of luminal subtypes (Holm et al., 2017).

The effort of utilizing molecular information and its translation to specific tumor subtypes has expanded beyond breast cancer. With support of The Cancer Genome Atlas and The International Cancer Genome Consortiums different cancers have

been sub-phenotyped in the past years including colorectal, cervical and urothelial (Guinney et al., 2015; TCGA, 2017; Robertson et al., 2017). Along with the clinical impact of the subclassification of tumor types, the vision of a more precise definition of cancer etiologies and risk gradually becomes more tangible. However, there is still a long way ahead before establishing the practical use of this wealth of information in the public health arena.

High-Risk Populations and Primary Prevention Strategies

Measure of inter-individual variability

The concept of human variability, that is, diverse responses to similar exposures to carcinogens, is not new. Classic epidemiology has laid the groundwork for discerning between vulnerable groups. Illustrative is that initial work in the field was paramount to establish that intrauterine and preconceptional exposure to ionizing radiation was associated with the development of childhood cancer (Wakeford, 1995). Molecular epidemiology has also helped in the identification of interactions that explain a certain fraction of variability in risk. In addition to familial aggregation and high penetrance genetic variants, low penetrance variants can affect the way a person processes and reacts to an exposure thus affecting their vulnerability to a carcinogen. For example, arsenic, an environmentally ubiquitous element and whose metabolites are detected through mass spectrometry in urine, is classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC). Since the discovery of the enzyme involved in arsenic metabolism (arsenic 3 methyl transferase, AS3MT), polymorphisms in the coding gene have been associated with arsenic methylation efficiency and consequently with cancer risk, helping in the identification of subsets of the population with different degrees of vulnerability to arsenic-associated carcinogenesis (Engström et al., 2015; Gomez-Rubio et al., 2010). Additionally, other nongenetic factors have been associated with susceptibility to arsenic toxicity such as sex and body mass index (Vahter et al., 2007; Gomez-Rubio et al., 2011).

The definition of key factors modifying cancer risk is essential for a better understanding of interindividual variability. Failure to account for this variation can result in overlooking important sensitive subgroups leading to inadequate health policies and measures.

Definition of high risk populations

One of the main goals of molecular epidemiology is disease prevention through the better understanding of exposure-susceptibility interactions. The appropriate definition of high-risk populations has been pivotal for risk assessment guiding the implementation of numerous health prevention measures. For instance, smoking cessation strategies have been implemented by different healthcare systems worldwide with the final aim of reducing smoking-related health effects such as lung and oropharyngeal cancers (McAfee et al., 2015). Moreover, molecular epidemiology studies associating occupational exposures with carcinogenicity have helped in the implementation of more healthy work policies and practices (Espina et al., 2013). For instance, in Sweden, incidence of mesothelioma linked to occupational exposure to asbestos, leveled off after being one of the first countries restricting asbestos use in the 70s (Hemminki and Shehnaz, 2008). Other characteristics defining cancer high-risk populations include genetic predisposition, age, sex or ethnicity. Appropriate screening and informed decisions of lifestyle modifications or even participation in chemoprevention programs could be expected as a result of proper risk stratification.

Though the definition of high-risk populations is not a substitute for general prevention efforts applicable to the whole populations (e.g., promotion of a healthy weight, or reduction of alcohol and tobacco consumption), certain interventions to high-risk individuals could be more effective, particularly when those interventions result in hazardous side effects (e.g., chemoprevention agents) and/or are too expensive to be applied in the whole population.

Improving the definition of risk

The underlying interplay of multiple etiological factors in human carcinogens is a reality scientists recognize and attempt to address. Up to date, the most common type of analysis performed has independently considered each biological process and their relationship with cancer status. While we have gained important insight with this approach, a great deal of disease complexity might be hidden behind networks of interactions between several disease processes. For example, higher risk of cancer has been reported when sets of morbidities are jointly analyzed in comparison with their individual analysis (Gomez-Rubio et al., 2017). Moreover, innovative statistical approaches have been implemented to integrate more than two types of *omics* data (e.g., genetic variants, DNA methylation and gene expression) with the aim of pinpointing potential carcinogenic mechanisms in different malignancies such as bladder and breast cancer (Sun et al., 2011; Pineda et al., 2015). Hence, the combination of multiple sources of data including molecular markers as well as lifestyle factors could provide with a more thorough and comprehensive definition of risk by accounting for the interplay among different risk factors. In breast cancer, the integration of genetic risk factors with the Gail's risk model based on demographic and clinical data conferred a modest improvement in classification of breast cancer risk compared to either the Gail's risk or the genetic score alone in a population of white non-Hispanic postmenopausal women (Mealiffe et al., 2010).

The clinical utility of risk models for disease prediction depends in their ability to accurately define population strata with different risk levels. Despite significant efforts devoted to developing integrative approaches for risk assessment, its transition into clinics is not currently regular practice. Therefore, risk prediction tools properly validated in prospective cohorts are urgently needed in order to adequately incorporate risk stratification in primary prevention strategies.

Challenges of Molecular Epidemiology in the New Era

In complex diseases, *omics* data can help to achieve a better and more accurate characterization of the exposure, genetic factors and phenotype providing a holistic view of the human health states by including both the combined exposure of all sources that reach the internal chemical environment and the response of our body to environmental influences, including metabolic processes (Rappaport and Smith, 2010; Miller and Jones, 2014). Technological progress in high-throughput biological data generation have provided with massive molecular *omics* data from different layers of the biological processes of the host (e.g., genomics, epigenomics, transcriptomics, proteomics or metabolomics, among others) and the nonhost, such as microbiome. Unfortunately, this substantial data production has not been accompanied with the development of next generation statistical methods to integrate in a computationally timely manner all the *omics* data needed to better characterize the phenotype, the exposome and the genome (Lopez de Maturana et al., 2014). The integration of this type of data is crucial and can be as costly as *omics* data generation (Mardis, 2010).

Although some progress has been achieved in the field, there is still a need of developing sophisticated analytical strategies that can handle the most common characteristic to *omics*-based studies: the vast number of variables versus a limited number of individuals, the so-called large p small n problem. Apart from volume, computational approaches should consider the complexity of each *omics* data type jointly with potential interactions among *omics* variants of the same or different type, correlated variants and possible (non)linear relationships (Green and Guyer, 2011; Tini et al., 2017). Moreover, these analytical strategies should also consider the integration of *omics* data with epidemiological data from questionnaires. Integration of both types of data represents a new challenge requiring of both sufficient understanding of the underlying biological concepts and of analytical algorithms to build hierarchical models and theories of disease causation.

Two main analytical strategies have been applied for data integration: the multistage and the meta-dimensional analyses (Ritchie et al., 2015). While meta-dimensional analysis combines multiple types of data for simultaneous analysis producing complex models, multistage (or multistep) approach first looks for the relationship between the data types and then, between the data types and the trait of interest. At present, multistage approach is adopted in most of the integrative studies; however, its main drawback is that due to its step-wise nature, this approach is not able to capture interactions. Therefore, despite the current efforts devoted to keeping up with the high biostatistics and bioinformatics demands of the existing data, there are still many issues that need to be addressed in order to take full advantage of this information.

Summary and Prospective Vision

Molecular epidemiology has experienced an important evolution during the past 15 years. Improved molecular technologies have given place to the opportunity to refine our accuracy of cancer risk estimation contributing in the implementation of proper prevention strategies. However, despite great efforts devoted to cancer risk assessment, and some important advances, the full potential of cancer prevention is yet to be attained. Practical application of cancer risk assessment is further hampered by the complexity of newly available high-dimensional data. As technology evolves and massive amounts of biological information are being generated, new methodology for data integration and analysis is demanded. Along with better methods, cancer consortia will prove to be fundamental for testing and validation of more complex risk assessment models.

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See also: Aspirin and Cancer. Enhancers in Cancer: Genetic and Epigenetic Deregulation. Environmental Exposures and Epigenetic Perturbations. Epigenetic Therapy.

References

Agudo, A., Peluso, M., Munnia, A., et al., 2017. Aromatic DNA adducts and breast cancer risk: A case-cohort study within the EPIC-Spain. Carcinogeneisis 38 (7), 691–698.

Al Olama, A.A., Kote-Jarai, Z., Berndt, S.I., et al., 2014. A meta-analysis of 87,040 individuals identifies 23 new susceptibility loci for prostate cancer. Nature Genetics 46 (19), 1103–1109.

Ellingjord-Dale, M., Vos, L., Tretli, S., et al., 2017. Parity, hormones and breast cancer subtypes- results from a large nested case-control study in a national screening program. Breast Cancer Research 19 (1), 10.

Engström, K.S., Vahter, M., Fletcher, T., et al., 2015. Genetic variation in arsenic (+3 oxidation state) methyltransferase (AS3MT), arsenic metabolism and risk of basal cell carcinoma in a European population. Environmental and Molecular Mutagenesis 56 (1), 60–69.

Espina, C., Porta, M., Schüz, J., et al., 2013. Environmental and occupational interventions for primary prevention of cancer: A cross-sectorial policy framework. Environmental Health Perspectives 121 (4), 420–426.

Etemadi, A., Islami, F., Phillips, D.H., et al., 2013. Variation in PAH-related DNA adduct levels among non-smokers: The role of multiple genetic polymorphisms and nucleotide excision repair phenotype. International Journal of Cancer 132 (12), 2738–2747.

Fitzmaurice, C., Allen, C., Barber, R.M., et al., 2017. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: A systematic analysis for the global burden of disease study. JAMA Oncology 3 (4), 524–548.
García-Closas, M., Varmeulen, R., Sherman, M.E., et al., 2006. Application of biomarkers in cancer epidemiology. In: Schottenfeld, D., Fraumeni Jr., J.F. (Eds.), Cancer epidemiology and prevention, 3rd edn. Oxford University Press, New York (NY), pp. 70–80.
García-Closas, M., Vermeulen, R., Cox, D., et al., 2011. Population-based study designs in molecular epidemiology. IARC Scientific Publications 163, 241–259.
Gomez-Rubio, P., Meza-Montenegro, M.M., Cantu-Soto, E., Klimecki, W.T., 2010. Genetic association between intronic variants in AS3MT and arsenic methylation efficiency is focused on a large linkage disequilibrium cluster in chromosome 10. Journal of Applied Toxicology 30 (3), 260–270.
Gomez-Rubio, P., Roberge, J., Arendell, L., et al., 2011. Association between body mass index and arsenic methylation efficiency in adult women from soythwest U.S. and Northwest Mexico. Toxicology and Applied Pharmacology 252 (2), 176–182.
Gomez-Rubio, P., Rosato, V., Márquez, M., et al., 2017. A systems approach identifies time-dependent associations of multimorbidities with pancreatic cancer risk. Annals of Oncology 28 (7), 1618–1624.
Green, E.D., Guyer, M.S., 2011. Charting a course for genomic medicine from base pairs to bedside. Nature 470, 204–213.
Guinney, J., Dienstmann, R., Wang, X., et al., 2015. The consensus molecular subtypes of colorectal cancer. Nature Medicine 21 (11), 1350–1356.
Hemminki, K., Shehnaz, H., 2008. Mesothelioma incidence has leveled off in Sweden. International Journal of Cancer 122, 1200–1201.
Holm, J., Eriksson, L., Ploner, A., et al., 2017. Assessment of breast cancer risk factors reveals subtype heterogeneity. Cancer Research 77 (13), 3708–3717.
IARC working group on the evaluation of carcinogenic risk to humans, 2004. Tobacco smoke and involuntary smoking. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 83, 1–1438.
Jin, F., Thaiparambil, J., Donepudi, S.R., et al., 2017. Tobacco-specific carcinogens induce hypermethylation, DNA adducts, and DNA damage in bladder cancer. Cancer Prevention Research 10 (10), 588–597.
Lopez de Maturana, E., Ibanez-Escriche, N., Gonzalez-Recio, O., et al., 2014. Next generation modeling in GWAS: Comparing different genetic architectures. Human Genetics 133 (10), 1235–1253.
López de Maturana, E., Pineda, S., Brand, A., Van Steen, K., Malats, N., 2016. Toward the integration of Omics data in epidemiological studies: Still a "long and winding road". Genetic Epidemiology 40 (7), 558–569.
Mardis, E.E., 2010. The $1,000 genome, the $100,000 analysis? Genome Medicine 2 (11), 84.
McAfee, T., Babb, S., MnNabb, S., Fiore, M.C., 2015. Helping smokers quit: Opportunities created by the affordable care act. The New England Journal of Medicine 372 (1), 5–7.
Mealiffe, M.E., Stokowski, R.P., Rhees, B.K., et al., 2010. Assessment of clinical validity of a breast cancer risk model combining genetic and clinical information. Journal of the National Cancer Institute 102 (21), 1618–1627.
Michailidou, K., Beesley, J., Lindstrom, S., et al., 2015. Genome-wide association analysis of more than 120,000 individuals identifies 15 new susceptibility loci for breast cancer. Nature Genetics 47 (4), 373–380.
Miller, G.W., Jones, D.P., 2014. The nature of nurture: Refining the definition of the exposome. Toxicological Sciences 137 (1), 1–2.
Munnia, A., Giese, R.W., Polvani, S., et al., 2017. Bulky DNA adducts, tobacco smoking, genetic susceptibility, and lung cancer risk. Advances in Clinical Chemestry 81, 231–277.
Perera, F.P., Weinstein, I.B., 1982. Molecular epidemiology and carcinogen-DNA adduct detection: New approaches to studies of human cancer causation. Journal of Chronic Diseases 35 (7), 581–600.
Perera, F.P., 1987. Molecular cancer epidemiology: A new tool in cancer prevention. Journal of the National Cancer Institute 78, 887–898.
Perera, F.P., 2000. Molecular epidemiology: On the path to prevention? Journal of the National Cancer Institute 92, 602–612.
Perou, C.M., Sørlie, T., Eisen, M.B., et al., 2000. Molecular portraits of human breast tumours. Nature 406 (6797), 747–752.
Phillips, D.H., Venitt, S., 2012. DNA and protein adducts in human tissues resulting from exposure to tobacco smoke. International Journal of Cancer 131 (12), 2733–2753.
Pineda, S., Real, F.X., Kogevinas, M., et al., 2015. Integration analysis of three omics data using penalized regression methods: An application to bladder cancer. PLoS Genetics 11 (12), e1005689.
Rappaport, S.M., Smith, M.T., 2010. Epidemiology. Environment and disease risk. Science 330 (6003), 460–461.
Ritchie, M.D., Holzinger, E.R., Ruowang, L., Pendergrass, S.A., Kim, D., 2015. Methods of integrating data to uncover genotype-phenotype interactions. Nature Reviews Genetics 16, 85–97.
Robertson, A.G., Kim, J., Al-Ahmadie, H., et al., 2017. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell **171** (3), 540–556.e25.
Severi, G., Southey, M.C., English, D.R., et al., 2014. Epigenome-wide methylation in DNA from peripheral blood as a marker of risk for breast cancer. Breast Cancer Research and Treatment 148 (3), 665–673.
Samet, J.M., 2016. Epidemiology and the tobacco epidemic: How research on tobacco and health shaped epidemiology. American Journal of Epidemiology 183 (5), 394–402.
Sud, A., Kinnersley, B., Houlston, R.S., 2017. Genome-wide association studies of cancer: Current insights and future perspectives. Nature Reviews Cancer 17 (11), 692–704.
Sun, Z., Asman, Y.W., Kalari, K.R., et al., 2011. Integrated analysis of gene expression, CpG island methylation, and gene copy number in breast cancer cells by deep sequencing. PLoS One 6 (2), e17490.
Tini, G., Marchetti, L., Priami, C., Scott-Boyer, M.P., 2017. Multi-omics integration-a comparison of unsupervised clustering methodologies. Briefing in Bioinformatics. https://doi.org/10.1093/bib/bbx167.
The Cancer Genome Atlas Network, 2012. Comprehensive molecular portraits of human breast tumours. Nature 490 (7418), 61–70.
The Cancer Genome Atlas Network, 2017. Integrated genomic and molecular characterization of cervical cancer. Nature 543 (7645), 378–384.
Vahter, M., Akesson, A., Lidén, C., Ceccatelli, S., Berglund, M., 2007. Gender differences in the disposition and toxicity of metals. Environmental Research 104 (1), 85–95.
Wakeford, R., 1995. The risk of childhood cancer from intrauterine and preconceptional exposure to ionizing radiation. Environmental Health Perspectives 103 (11), 1018–1025.
Wild, C.P., 2005. Complementing the genome with an "exposome": The outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiology, Biomarkers & Prevention 14 (8), 1847–1850.
Wild, C.P., 2012. The exposome: From concept to utility. International Journal of Epidemiology 41 (1), 24–32.
Zardavas, D., Irrthum, A., Swanton, C., Piccart, M., 2015. Clinical management of breast cancer heterogeneity. Nature Reviews Clinical Oncology 12 (7), 381–394.
Zhu, M., Zhao, S., 2007. Candidate gene identification approach: Progress and challenges. International Journal of Biological Sciences 3 (7), 420–427.

Further Reading

Chatterjee, N., Shi, J., García-Closas, M., 2016. Developing and evaluating polygenic risk prediction models for stratified disease prevention. Nature Reviews Genetics 17 (7), 392–406.
McGranahan, N., Swanton, C., 2017. Clonal heterogeneity and tumor evolution: Past, present, future. Cell 168 (4), 613–628.
Rothman, K.J., Sander, G., Lash, T.L. (Eds.), 2008. Modern epidemiology, 3rd edn. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia (PA).

Relevant Websites

http://cancergenome.nih.gov/—The cancer genome atlas.
http://www.exposomicsproject.eu/—The EXPOsOMICS project.
http://icgc.org—The International Cancer Genome Consortium project.

Multiple Myeloma: Pathology and Genetics

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Introduction

Multiple myeloma (MM) is a hematologic malignancy that originates from plasma cells in the bone marrow (BM). According to the National Cancer Institute, the prevalence of MM was estimated at 89,650 people in the United States in 2012, with an annual incidence of 6.3 new cases per 100,000 individuals. All MM are preceded by a monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM). The diagnosis of MM is often suspected because of the presence of clinical symptoms such as bone pain with lytic lesions, an increased total serum protein concentration, the presence of a monoclonal protein in the urine or serum, anemia or hypercalcemia. MM is incurable, due to the lack of treatment able to target specific oncogenes involved in the pathogenesis of the disease. Genomic events such as chromosomal translocations, copy number variation and somatic mutations as well as epigenetic modifications are responsible of deregulation of specific oncogenes or tumor suppressor genes and their corresponding pathways, leading to MM proliferation.

Plasma Cell Differentiation and Myeloma Initiation

As MM is characterized by the production of a monoclonal immunoglobulin (Ig), its initiation is linked to B cell development. B-cells undergo rearrangement of their Ig genes into the bone marrow, generating a functional B cell receptor precursor, and migrate to lymph nodes where they encounter antigens. B cells are then activated and reach the germinal center where they undergo affinity maturation in response to antigen presented on antigen-presenting cells. This process is called somatic hypermutation (SHM) and induces mutations of the hypervariable regions of the immunoglobulin heavy chain locus (IGH), thus producing highly specific and avid antibodies. The functionality of these antibodies is increased by class switch recombination (CSR), which produces antibodies of different immunoglobulin isotypes. SHM and CSR require the expression of activation-induced deaminase (AID), which induces double strand DNA breaks (DSBs) in the Ig loci. AID-induced DSBs are mostly repaired locally, however they can be joined to DSBs occurring elsewhere in the genome, resulting in aberrant chromosomal translocations. These translocations occur preferentially into actively transcribed genes; however, the range of CSR-driven translocations in MM is limited. This may explain why only a subset of these translocations causes a proliferation advantage.

Once SHM and CSR completed, selected B-cells leave the germinal center and migrate to the bone marrow where they become mature plasma cells, secreting antibodies. This process requires cell cycle arrest, compaction of chromatin, silencing of cellular functions that are unnecessary for antibody production and activation of key programs that are required to generate and secrete antibodies. Deregulation of these pathways could potentially lead to malignant transformation. Normal plasma cell differentiation is regulated by different transcription factors. IRF4 downregulates BCL-6, resulting in the upregulation of BLIMP1, which in turn leads to downregulation of PAX5 and upregulation of XBP1. Expression of IRF4, BLIMP1 and XBP1 is necessary for survival of plasma cells. After exiting the germinal center, plasma cells migrate to the bone marrow where either they rapidly undergo apoptosis, or reside in specialized niches where they can survive for many years as memory plasma cells.

Inherited Variants

Although lifestyle or environmental exposures have not been consistently linked to the incidence of MM, there seems to be a two- to fourfold elevated risk of MM in relatives of individuals with the disease. This has been postulated to be a consequence of the co-inheritance of multiple low-risk variants. Investigating these families further and performing genome-wide association studies (GWAS) on large patient populations, three genetic loci were associated with a modest but increased risk of developing MM. These include 3p22.1 (rs1052501, in ULK4), 7p15.3 (rs4487645, surrounding by DNAH11 and CDCA7L) and 2p23.3 (rs6746082, surrounding by DNMT3A and DTNB). A follow-up study by the same group, including 4692 individuals with MM and 10,990 controls, revealed four new loci: 3q26.2 (rs10936599, surrounding by MYNN and TERC), 6p21.33 (rs2285803 in PSORS1C2), 17p11.2 (rs4273077 in TNFRSF13B) and 22q13.1 (rs877529 in CBX7). These seven identified loci provide further evidence for an inherited genetic susceptibility to MM and reportedly account for $\sim$13% of the familial risk of MM. The complete functional role of each of these candidate genes remains to be elucidated. The authors found no association between genotypes and the expression level of their genes. Interestingly, in another GWAS study, the same team identified a strong association between the variant rs603965, responsible for c807G>A polymorphism in *CCND1* and the translocation t(11;14)(q13;q32), in which CCND1 is placed under the control of the immunoglobulin heavy (IGH) chain enhancer. In this model, a constitutive genetic factor is associated with risk of a specific chromosomal translocation. Based on these initial studies, it is likely that more susceptibility loci will be identified in the future and possibly correlated with specific MM subtypes. For example, African-Americans have a higher risk of

developing MM than Caucasians; however, no potential genetic variants have been identified to date. Moreover, uncovering the functional role of these seven SNPs significantly associated with MM might help to advance our understanding of MM oncogenesis.

Chromosomal Translocations

In MM, most chromosomal translocations involve chromosome 14, and specifically the IGH locus on 14q32.33, placing a partner gene under the control of the IGH enhancer. These translocations are generated by abnormal class switch recombination (CSR) events and are usually present in all clonal cells. They are also detectable in monoclonal gammopathy of unknown significance (MGUS), consistent with their early development in MM oncogenesis. Five major chromosomal partners—t(4;14), t(6;14), t(11;14), t(14;16), and t(14;20)—seem to impart a selective advantage to the clone by up regulating expression of specific oncogenes—*MMSET* and *FGFR3*, *CCND3*, *CCND1*, *MAF* and *MAFB*, respectively. It is likely that all these translocations lead to deregulation in the cell cycle G1/S transition, which has been described as a key early molecular abnormality in MM. This can be direct through t(11;14) and t(6;14) deregulating CCND1 and CCND3 respectively. In t(14;16), this is modulated through MAF which up regulates CCND2 by directly binding to its promoter while in t(4;14), the exact mechanism is still uncertain but the translocation of FGFR3 and MMSET to the IGH enhancer is known to also up regulate CCND2. Recently mutations involving the *MYC* locus have been identified in MM.

Translocation (4;14) is observed in about 15% of MM cases and has been associated with adverse prognosis in a variety of clinical settings. The juxtaposition results in deregulation of the expression of FGFR3 and MMSET/WHSC1. The breakpoints all reside between FGFR3 and MMSET, resulting in overexpression of FGFR3 in 70% of cases and MMSET in all cases. MMSET is a methyl-transferase protein, whose up regulation leads to methylation of histone H3K36, which regulates expression of several genes. MMSET has been shown also to regulate histone H4K20 methylation and recruit 53BP1 at DNA damage sites. FGFR3 is a tyrosine kinase receptor oncogene activated by mutations in several solid tumor types. Notably, FGFR3 is up regulated in only 70% of patients with the translocation because of an unbalanced translocation with loss of the telomeric part of chromosome 4, bearing FGFR3. This suggests that MMSET is the main molecular target of the translocation. Interestingly, despite the poor prognosis associated with t(4;14), a survival advantage in these patients has been demonstrated through early treatment with the proteasome inhibitor Bortezomib.

Translocation (6;14) is a rare translocation present in only about 2% of MM patients and results in the direct up regulation of *CCND3* via juxtaposition to the *IGH* enhancers. The breakpoints are all located 5′ of the gene. The overall prognostic impact of this translocation is neutral.

Translocation (11;14) is the most frequent translocation cited as being present in about 15%–20% of patients with MM. Normally B cells express cyclin D2 and D3 but not D1. However, due to the translocation juxtaposing *CCND1* to the *IGH* enhancer, its expression is deregulated. The breakpoints seem to be located 5′ of *CCND1*. In terms of prognosis, this translocation is considered as neutral, however it has been shown recently that in 10% of t(11;14) a *CCND1* mutation co-occurs and the combination is associated with a poor prognosis when compared with non-mutated t(11;14) patients.

Translocation (14;16) is estimated to be present in about 5%–10% of patients with MM and results in overexpression of the *MAF* oncogene splice variant c-MAF, a transcription factor which up regulates a number of genes, including *CCND2*, by binding directly to its promoter. Breakpoints are located 3′ of *MAF* within the last exon of *WWOX*, a known tumor suppressor. Though t(14;16) was associated with poor prognosis in several studies. A more recent retrospective multivariable analysis on 1003 newly diagnosed MM patients showed that t(14;16) is not associated with poor prognosis.

Translocation (14;20) is present in about 1% of patients and is the rarest translocation of the major five. It results in upregulation of the *MAF* gene paralog *MAFB*. According to microarray studies, *MAFB* overexpression results in a similar gene expression profile (GEP) as that seen with *c-MAF*, implying common downstream targets including CCND2. The translocation is associated with poor prognosis when present in MM, but interestingly correlates to long-term stable disease when found in precursor conditions like MGUS and smoldering MM (SMM). This suggests that the translocation itself is not responsible for the poor prognosis, but additional genetic events are likely required to accumulate imparting this negative prognosis.

MYC translocations have been recently identified in a cohort of 463 whole exome sequencing including extra baits on the *MYC* locus. MYC translocations were found in 85 patients (18.4%). Partner genes include *IGH*, *IGL* and *IGK* loci, as well as *FAM46C*, *FOXO3*, *BMP6* and rarely *XBP1*, *TXNDC5*, *CCND1* and *CCND3*. These translocations lead to significant overexpression of *MYC*, probably resulting from juxtaposition of super-enhancers surrounding the partner gene to *MYC* locus. *MYC* translocations are associated with a poor outcome.

Hyperdiploidy

Hyperdiploidy (HRD) is defined as a chromosome number between 48 and 74. HRD MM are characterized by multiple chromosomal gains, preferentially trisomy of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. The mechanism underlying this is not known but one hypothesis suggests that the gain of multiple whole chromosomes occurs during a single catastrophic mitosis rather than through the serial gain of chromosomes over time. Nearly half of MGUS and MM tumors are hyperdiploid. Only a few HRD tumors have a co-existing primary IgH translocation—about 10% of the cases—whereas non-HRD tumors usually have an IgH

translocation. Interestingly, when HRD and IGH translocations coexist, HRD may precede IGH translocations in a proportion of patients, as revealed by single cell sequencing analysis. In terms of signaling pathways, HRD tumors display biological heterogeneity. Some harbor high expression of proliferation-associated genes while others are characterized by genes involved in tumor necrosis factor/nuclear factor-κB (TNF/NFκB) signaling pathway. HRD is associated with a more favorable outcome in general, however coexistent adverse cytogenetic lesions (del 17p, t(4;14) and gain of 1q) shorten survival in MM patients with HDR tumors.

Copy Number Variations

Copy number variations (CNVs) represent a common feature of MM and are thought to be secondary events, involved in tumor progression. CNVs result from gain and loss of DNA, which can be focal or involve an entire chromosome arm. Like single nucleotide mutations, CNVs are probably both driver and passenger events. Highly frequent and recurrent CNVs are likely to be driver, suggesting that the minimal amplified or deleted regions contain important genes involved in the development and progression of MM.

1q gain: Duplication of the long arm of chromosome 1 is present in 35%–40% of patients. This is known to have an adverse effect on overall survival. Gain of 1q21, detected with a specific probe for *CKS1B*, is an independent prognostic factor and remains when other adverse cytogenetic lesions that frequently coexist are removed. Though the relevant genes on 1q have not yet been fully explored, a minimally amplified region was identified between 1q21.1 and 1q23.3 containing 679 genes. Among these candidate oncogenes are *CKS1B*, *ANP32E*, *BCL9*, and *PDZK1*. Of these genes, *ANP32E*, a protein phosphatase 2A inhibitor involved in chromatin remodeling and transcriptional regulation, is particularly interesting and has been shown to be independently associated with shortened survival. These findings reinforce the role of gain of 1q in MM pathogenesis and suggest that patients with this type of CNV may benefit from specific inhibitors of these candidate genes and pathways that have been identified.

1p deletion: Deletions of 1p are observed in approximately 30% of MM patients and are associated with poor prognosis. Two regions of the 1p arm are of interest in MM pathogenesis when deleted: 1p12 and 1p32.3. 1p12 contains the candidate tumor suppressor gene *FAM46C*, of which expression has been correlated to that of ribosomal proteins and eukaryotic initiation/elongation factors involved in protein translation. This gene is frequently mutated in MM and this has been independently correlated with poor prognosis. Region 1p32.3 may be hemi- and homozygously deleted and contains the two target genes *CDKN2C* and *FAF1*. *CDKN2C* is a cyclin-dependent kinase 4 inhibitor involved in negative regulation of the cell cycle, whereas *FAF1* encodes a protein involved in initiation and enhancement of apoptosis through the Fas pathway. Deletion 1p is associated with adverse overall survival.

13q deletion: Monosomy of the long arm of chromosome 13 is present in about 45%–50% of patients and is commonly associated with non-hyperdiploid tumors. In approximately 85% of cases, deletion of chromosome 13 constitutes monosomy or loss of the q arm, whereas in the remaining 15% various interstitial deletions occur. Chromosome 13 has been extensively investigated as prognostic factor and as a location of tumor suppressor genes. The minimally deleted region lies between 13q14.11-13q14.3 and contains 68 genes including *RB1*, *EBPL*, *RNASEH2B*, *RCBTB2*, and the microRNA miR-16-1 and miR-15a. Molecular studies have shown that the tumor suppressor gene *RB1* is significantly under-expressed in these deletions, which may result in inferior negative cell cycle regulation. Establishing a prognostic significance of deletion 13 is challenging because it is frequently associated with other high risk cytogenetic lesions such as t(4;14). As such, the historic link between deletion 13 and poor prognosis is a surrogate of its association with high-risk lesions.

17p deletion: Most chromosome 17 deletions are hemizygous and involve the whole p arm, a genetic event observed in around 10% of newly diagnosed MM cases, with an increasing frequency in later stages of the disease. The minimally deleted region includes the tumor suppressor gene *TP53*. While cases without del(17p) have a rate of *TP53* mutation that is $<1\%$, cases with the deletion show a higher rate of mutation at 25%–37%—suggesting that mono-allelic 17p deletion contributes to the disruption of the remaining allele. The *TP53* gene, which has been mapped to 17p13, is known to function as a transcriptional regulator influencing cell cycle arrest, DNA repair, and apoptosis in response to DNA damage. Loss of 17p is associated with an adverse overall survival. The deletion is also linked to an aggressive disease phenotype, more extra-medullary disease, and shortened survival.

Somatic Mutations

The generalization of next generation sequencing a few years ago has enabled high throughput whole-exome sequencing in several cancers, including MM. The frequency of somatic mutations in MM is at the median across cancer types, with an average of 1.6 mutations per Mb, as compared to <0.5 per Mb in pediatric cancer, such as rhabdoid tumor or Ewing sarcoma, and about 10/Mb in melanoma and lung cancer. Studies by whole genome sequencing (WGS) and whole exome sequencing (WES) of patients with MM, comparing sequences from each tumor to its corresponding normal germline sample, allowed identification of tumor-specific mutations. Significantly mutated genes included three that were previously reported as being implicated in MM: *KRAS*, *NRAS*, and *TP53* as well as two newly described genes *FAM46C* and *DIS3*.

Several new oncogenic mechanisms were suggested by the pattern of somatic mutations across this data set. Nearly half the patients showed mutations of genes involved in protein translation. One of these is the *DIS3* gene, also known as *RRP44*, which encodes a highly conserved RNA exonuclease and serves as the catalytic component of the exosome complex involved in regulating

the processing and abundance of all RNA species. *DIS3* mutations, postulated to be loss of function, cluster in the catalytic pocket of the enzyme and deregulate protein translation as an oncogenic mechanism. Another significantly mutated gene, *FAM46C*, is less well characterized but thought to be functionally related to translation regulation.

The same team (Lohr et al., 2014) next reported massively parallel sequencing of a large cohort of patients with MM—including those previously studied. Beyond the five significantly mutated genes previously described, they identified another six significantly mutated genes (*BRAF*, *TRAF3*, *PRDM1*, *CYLD*, *RB1*, and *ACTG1*). Overall in this study, 65% of the patients had mutations in one or more of the 11 recurrently mutated genes.

Like *KRAS* and *NRAS*, *BRAF* is a known oncogene playing a role in regulating the MAP kinase pathway. Strikingly, mutations in *KRAS*, *NRAS*, and *BRAF* can be both clonal and sub-clonal. However, if mutations in these genes sometimes co-exist in the same tumor, they are almost never simultaneously clonal, indicating that they probably rarely occur in the same clone but rather in different sub-clones. In contrast, *KRAS* and *DIS3* mutations are reported to be often simultaneously clonal and therefore probably co-occurring in the same clone.

TRAF3 and *CYLD* are part of the NFkB pathway, which is also the case for nine other mutated genes of significance in this cohort (*BTRC*, *CARD11*, *IKBKB*, *MAP3K1*, *MAP3K14*, *RIPK4*, *TLR4*, and *TNFRSF1A*), which reaffirms the central role of the NFkB pathway in MM.

Another significantly mutated gene is *PRDM1* (also called *BLIMP1*), a transcription factor involved in plasma cell differentiation. Loss of function mutations of *BLIMP1* occur in diffuse large B cell lymphoma. The oncogene *IRF4*, a transcriptional regulator of *PRDM1* was also frequently mutated, in addition to mutations of *PRDM1*.

In parallel, a WES and copy number analysis of a series of MM samples led to the identification of two new recurrently mutated genes, *SP140* and *LTB*. *SP140* is a lymphoid restricted homologue of *SP100* that encodes a nuclear body protein implicated in antigen response of mature B cells, and is truncated in several cases. *LTB*, a type II membrane protein of the TNF family involved in lymphoid development, also harbor truncated mutations.

Finally, WES of a large number of patients enrolled in a phase III clinical trial (Walker et al., 2015) brought the list to 15 significantly mutated genes, comprising *KRAS*, *NRAS*, *TP53*, *FAM46C*, *DIS3*, *BRAF*, *HIST1H1E*, *RB1*, *EGR1*, *TRAF3*, *LTB*, *CYLD*, *IFR4*, *MAX*, and *FGFR3*. Interestingly, mutations in RAS (43% of the cases) and NFkB (17% of the cases) were prognostically neutral. In contrast, mutations in *CCND1* and the DNA repair pathway (*TP53*, *ATM*, *ATR*, and *ZFHX4*) were associated with a negative impact on survival in contrast to those in *IRF4* and *EGR1* that are associated with a favorable overall survival.

Identification of driver mutations in MM holds great promise for personalized medicine, whereby patients with particular mutations would be treated with the appropriate targeted therapy. However, if the mutation is present in only a fraction of the cells, one might doubt whether such targeted therapy would be clinically efficacious.

Clonal Heterogeneity and Clonal Evolution in Multiple Myeloma

In addition to the genetic complexity of MM, intra-clonal heterogeneity has emerged as a further level of complexity. Analysis of clonal heterogeneity by WES (Lohr et al., 2014) showed that most patients harbor at least three detectable sub-clones, some having as many as seven, which reaffirms that MM tumors are highly heterogeneous. The finding that tumors contain on average at least five sub-clones is even an underestimation of the clonal diversity in MM as the applied method only allows for the detection of sub-clones representing at least 10% of the entire tumor sample.

It has become clear that following disease initiation, the steps necessary for MM development do not occur through a linear fashion but rather via branching, non-linear pathways, as proposed by Darwin in explaining the evolution of species. This idea is based on the notion that mutations occur randomly and are selected and propagated based on the clonal survival advantage that they confer. A phenomenon of parallel evolution, whereby independent but not distantly related clones might acquire similar mutations conferring important growth or survival advantages, is revealed in single-cell level studies showing more than one alteration of same genetic pathway (RAS/MAPK) within the same tumor, but in separately evolving divergent clones. In a series of t(11;14) MM, evidence for the persistence of the earliest MM progenitor cell clone was found in two cases, characterized by the presence of a sub-clone carrying t(11;14) as the sole abnormality, validating that this translocation is an early event in myeloma pathogenesis. The clonal diversity is present at all stages of the disease. Although less genetically complex than MM, the pre-malignant stages MGUS and SMM harbor clonal heterogeneity. By studying sequential samples of SMM and overt MM, it was shown that the predominant clone of MM is already present at the SMM stage.

Treatment Implications

One of the main areas of development in genomics of MM is the critical need to comprehensively implement precision medicine. Perhaps more importantly, precision medicine aims to tailor the appropriate therapy to a patient in a personalized fashion, based on the disease genomic information. To this end, identification of driver mutations in MM holds great promise for precision medicine, whereby patients with particular mutations would be treated with the appropriate targeted therapy. A recent report describes a durable response to the mutation-specific BRAF inhibitor Vermurafenib in a patient harboring an activating mutation of *BRAF*. Several of the recurrently mutated genes in MM are potentially "actionable," meaning they can be targeted by therapies specifically

inhibiting the mutated or activated oncogene. However, this targeted therapy approach must be considered in light of the clonal heterogeneity and clonal competitions co-occurring in the cancer cell population. In fact, *BRAF* inhibitors are known to paradoxically activate the MAPK pathway in the event of co-existent *KRAS*-, *NRAS*-mutated or *BRAF*-WT sub-clones. This could be abrogated by the combination of *BRAF* and *MEK* inhibitors. Clonal heterogeneity poses a significant challenge; in fact, in most cases relapsed disease shows marked differences in genetic makeup compared with pre-treatment disease. This brings up the challenge of determining the most effective combinations of therapy. Thus, the comprehensive understanding of clonal heterogeneity is crucial to further develop targeted therapies in MM.

Conclusion

MM is a genetically complex and heterogeneous disease, combining primary events, secondary events and clonal diversity, leading to tumor development and progression from MGUS to late stages of MM. It is likely that many driver events need to co-occur for MM development and progression. This genomic complexity presents a challenge towards the cure of MM. In recent years, a tremendous amount of information has been obtained by next generation sequencing of MM tumors. Now that we have a good comprehension of the genomic landscape in MM at presentation, the near future should provide some insights regarding pre-malignant stages and resistance to treatment.

Further Reading

Lohr, J.G., et al., 2014. Widespread genetic heterogeneity in multiple myeloma: Implications for targeted therapy. Cancer Cell 25, 91–101.
Manier, S., et al., 2017. Genomic complexity of multiple myeloma and its clinical implications. Nature Reviews. Clinical Oncology 14, 100–113.
Morgan, G.J., et al., 2012. The genetic architecture of multiple myeloma. Nature Reviews. Cancer 12, 335–348.
Palumbo, A., Anderson, K., 2011. Multiple myeloma. New England Journal of Medicine 364, 1046–1060.
Walker, B.A., et al., 2015. Mutational spectrum, copy number changes, and outcome: Results of a sequencing study of patients with newly diagnosed myeloma. Journal of Clinical Oncology 33, 3911–3920.

Mutational Signatures and the Etiology of Human Cancers

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Glossary

Mutational process A mixture of DNA damaging and repair mechanisms that act collectively and have the ability to cause mutations in cells.
Mutational catalogue The conglomeration of all detected somatic mutations in the genome of a cell.
Mutational signature A characteristic pattern of somatic mutations imprinted by a mutational process on the genome of a cell.
Nonnegative matrix factorization A mathematical approach that allows unscrambling mixed signals and identifying meaningful components, such as, mutational signatures.
Somatic mutation Any change in the nucleotide sequence of DNA that is present in the genome of a somatic cell and has occurred after conception.

Nomenclature

AA Aristolochic acid
AFB1 Aflatoxin B1
APOBEC Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
BaP Benzo[*a*]pyrene
BSS Blind source separation
DNA Deoxyribonucleotide acid
EBV Epstein Barr virus
HBV Hepatitis B virus
HCC Hepatocellular carcinoma
HMEC Human mammary epithelial cell
HPV Human papilloma virus
Hupki Human *TP53* knock-in
ICGC International Cancer Genome Consortium
Indels Small insertions and deletions
MEF Mouse embryonic fibroblast
MMR DNA mismatch repair
mRNA Messenger RNA
NGS Next-generation sequencing
NMF Nonnegative matrix factorization
PCR Polymerase chain reaction
ROS Reactive oxygen species
SBS Single-base substitutions
TSG Tumor suppressor gene
UTUC Upper tract urothelial carcinoma
UV Ultraviolet light

Introduction

The term "cancer" encompasses a broad group of over two hundred different diseases characterized by an abnormal cellular growth. The transformation from a normally functioning cell to a neoplastic cell is due to the accumulation of changes in the genetic material of the cell. These changes are known as somatic DNA mutations. Analogous to Darwinian evolution, some mutations can have no effect on the cell, some can be deleterious thus killing the cell in which they arouse, and others can provide a selective growth advantage. Mutations providing growth advantage ultimately allow cancer cells to survive, to proliferate, to invade, and to metastasize to other tissues in the body.

Somatic mutations can be caused by many different mutational processes. These mutational processes can be triggered by the internal molecular mechanisms of cells (e.g., mutations occurring due to normal cellular division), tissues-specific processes (e.g., inflammation), external environmental sources (e.g., exposure to radiation), and lifestyle choices (e.g., tobacco smoking). Each mutational process imprints a characteristic pattern of somatic mutations on the genome of cancer cells, termed, mutational signature. Mutational signatures can be deciphered from genomes of cancer cells by using next-generation sequencing data and advanced computational and mathematical approaches. This article reviews our current understanding of mutagenesis, mutational signatures in human cancer, and mathematical approaches for deciphering mutational signatures from next-generation sequencing data.

Somatic Mutations

By definition, a somatic mutation is a change in the DNA sequence of a somatic cell. This change can be a single base substitution, multi-base tandem substitutions, small insertion, small deletion, complex insertion and/or deletion, chromosomal rearrangement, copy number variation, or introduction of a foreign DNA (Fig. 1).

A single base substitution (SBS) modifies one DNA base pair to another. For example, a C:G base pair is substituted with an A:T base pair. Multi-base tandem substitutions simultaneously change multiple adjacent DNA base pairs. For example, a CC:GG sequence is substituted with a TT:AA sequence. Small insertions and/or deletions, commonly termed indels, result from the loss or the gain of a small number of adjacent nucleotide base pairs (Fig. 1). Most indels in a cancer genome have a length of less than 10 base pairs but some can be as long as a few hundred base pairs. Depending on the base changes and positions, single base substitutions and indels can have distinct effects on cellular functionality. For example, silent or synonymous mutations do not alter the translated protein sequence of the transcripted RNA. However, they may affect the translation fidelity of the transcripted RNA in the ribosomal subunits. Conversely, non-synonymous mutations change the protein sequence by introducing amino-acid changes, frameshifts, or stop codons. Further, many indels and SBSs occur in the non-coding region of the genome where they can disrupt gene expression by, for example, affecting the binding affinity of transcription factors.

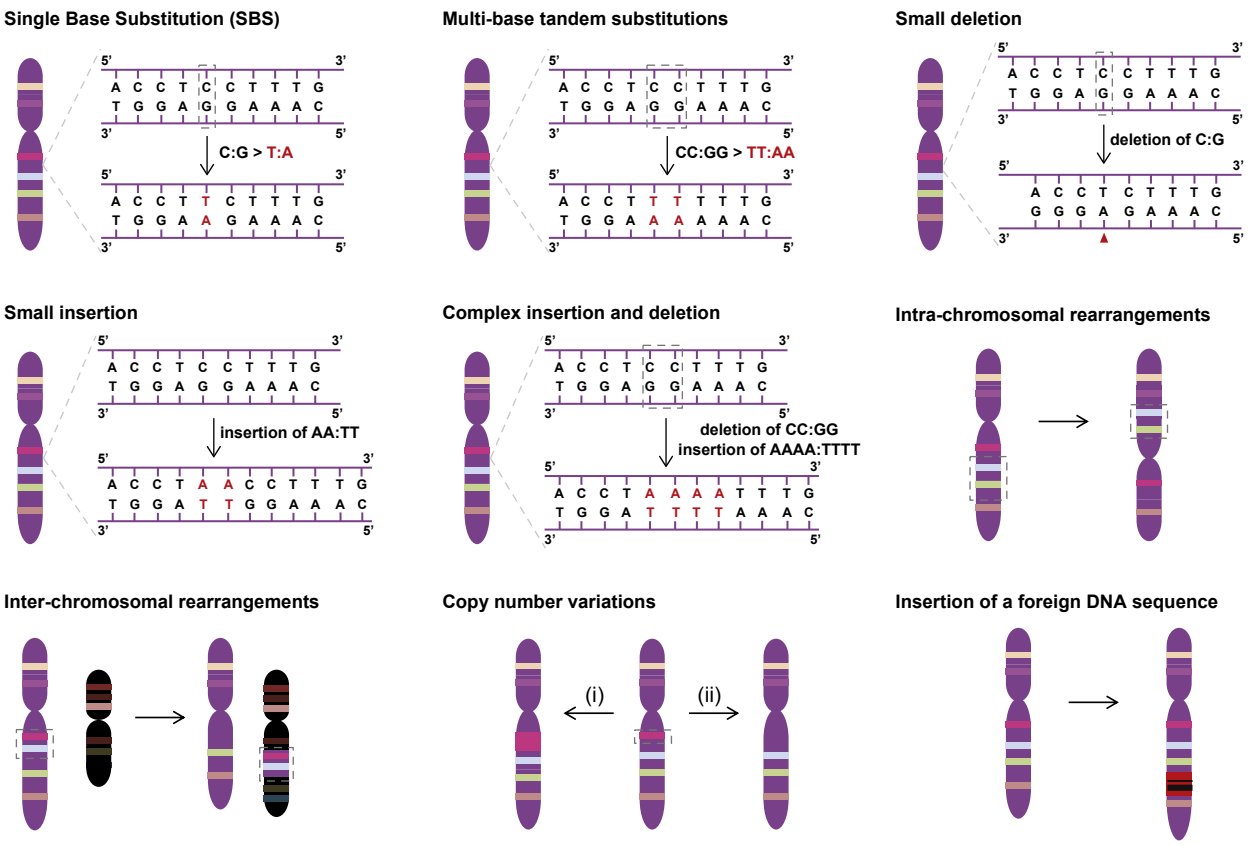

Fig. 1 Illustrative examples of the different types of genomic alterations present in cancer genomes. Small changes of the genome consist of single base substitutions, multi-base tandem substitutions, small insertions, small deletions, and complex insertions and deletions. Chromosomal rearrangements comprise intra-chromosomal translocations and inter-chromosomal translocations. Copy number variation of a specific DNA sequence leading to an increased (i) or a depleted (ii) copy of genome region. Insertion of new DNA sequence (e.g., viral DNA). Each alteration is represented relative to its position on chromosomes.

In addition to indels and SBSs, chromosomal rearrangements and copy number variations are commonly found in many types of human cancer. Chromosomal rearrangements occur when DNA segments break off and re-attach at different genomic locations. These locations can be on the same chromosome or on a different chromosome, resulting in intra- or inter-chromosomal rearrangements, respectively (Fig. 1). Chromosomal rearrangements have many functional implications and can lead, for example, to gene disruption, to fusion of two distinct genes, or to abnormal gene expression.

Copy number changes of a DNA sequence can increase its two copies in a normal diploid genome to several hundred copies in a cancer genome. This increase is referred to as genomic amplification, which is a common mechanism for the activation of many proto-oncogenes. Copy number changes can also reduce the copies of a DNA sequence due to large chromosomal deletions. These deletions may induce the complete absence of a DNA segment resulting in the loss of an associated gene; a commonly observed mutational mechanism for tumor suppressor genes.

Insertion of a foreign DNA sequence, originating from an exogenous source, is another important mechanism unambiguously implicated in the development of different types of cancer. Notable examples include incorporation of the sequence of the human papilloma virus (HPV), Epstein Barr virus (EBV), or hepatitis B virus (HBV).

Driver and Passenger Somatic Mutations

Mutations accumulate progressively during the lifespan of an individual in every single cell of the body. In general, it is accepted that mutations occur somewhat randomly across the genome and that they can be broadly separated into two categories—(i) mutations that provide selective advantage for clonal expansion and (ii) mutations that do not result in any growth advantage. The latter have been termed passenger mutations, while the former are referred to as driver mutations. It is widely believed that the number of driver mutations in a cancer genome is limited to a handful, usually between two and ten mutations. In contrast, the genome of a cancer can harbor more than a million somatic mutations most of which are considered passengers. Passenger mutations are not per se involved in cancer development but are rather the residual molecular fingerprints of the operative mutational processes.

Mutational Catalogues of Cancer Genomes

Even before the official start of the Human Genome Project, it was hypothesized that systematically analyzing the genetic information of cancer cells at a single base resolution will provide significant insights into the mechanisms of cancer development. While previous approaches allowed identification of large genomic events (e.g., copy number changes, chromosomal translocations, etc.) examining cancer genes by interrogating their sequence at a single-base resolution held the promise for observing previously unseen mutational events. At first, such sequencing examinations were performed using polymerase chain reaction (PCR)-based capillary sequencing for a targeted set of genes; however, the development of next-generation sequencing methods allowed rapidly sequencing of the complete set of exons in a cancer genome (i.e., an approach known as whole-exome sequencing) and, currently, even whole-genome sequences of cancer samples can be generate at a low cost.

Regardless of the experimental approach, the idea behind sequencing cancer genomes (or parts of these genomes) is simple. Genomic DNA is extracted from both the cancer and the normal tissue (which is usually but not always blood) and then these genomic DNAs are sequenced separately. The identified normal and cancer nucleotide sequences are aligned to the reference human genome, they are compared to it, and then they are compared to each other. The nucleotides that differ from the reference genome and that are found in both the normal and the cancer tissues are attributed to germline polymorphisms. Conversely, DNA sequence changes identified only in the cancer tissue, but not in the normal tissue, are attributed to somatic mutations specific for the cancer. All DNA changes identified only in the cancer tissue constitute the mutational catalogue of the cancer genome. An illustrative example identifying a somatic base substitution and a single nucleotide polymorphism from next generation sequencing (NGS) reads is provided in Fig. 2.

The majority of somatic mutations identified in the mutational catalogues of cancer genomes are passenger mutations. The ability to examine hundreds and even thousands of mutational catalogues of cancer genomes has resulted in the development of advanced statistical methods that allow pinpointing a handful of driver mutations from an ocean of passenger mutations. In simple terms, these algorithms evaluate which genes are mutated more often than purely expected by chance while correcting for a multitude of different factors.

Using a targeted capillary sequencing approach, an early cancer genomics study demonstrated that mutations in the BRAF gene are found in ~70% of melanomas. This was followed by subsequent studies identifying PIK3CA and EGFR as genes commonly mutated in human cancer. These early successes and their clinical significance made the identification of cancer genes through the systematic sequencing of cancer genomes, one of the main topics in cancer research. The emergence of NGS technologies allowed rapid and cheap examination of the genetic material of cancer cells. This led to the formation of the International Cancer Genome Consortium (ICGC). The goal of the ICGC is the identification of novel cancer genes through the molecular characterization of 50 types of human cancer (and their matched-normal tissues) from more than 25,000 patients. Nowadays, large-scale initiatives, such as the ICGC, continue to identify genes causally implicated in tumorigenesis and the census of genes driving human cancer gets updated on nearly a monthly basis.

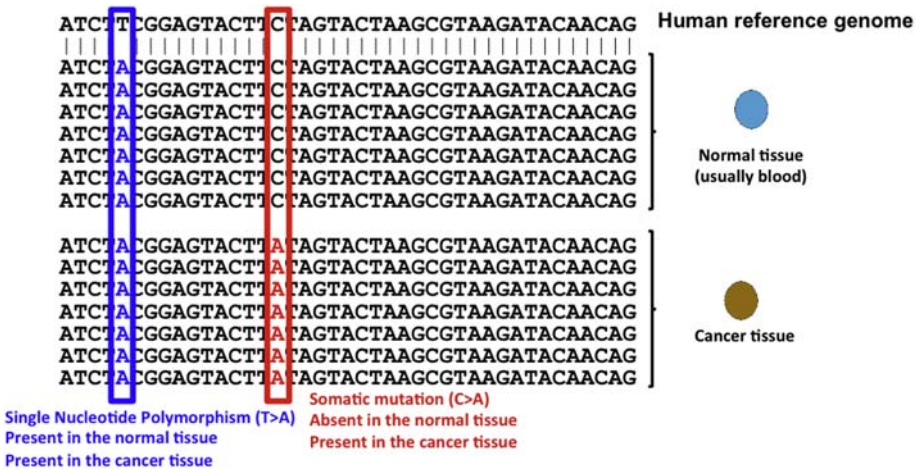

Fig. 2 Somatic mutations in cancer and nucleotide polymorphisms in the germline. Illustrated example demonstrating the identification of germline polymorphisms and somatic mutations from sequencing data.

Endogenous and Exogenous Mutational Processes

The genome of a cancer cell can be examined as a historical record that bears the imprints of the different mutagens to which the lineage of this cell has been exposed. Mutations can be caused by a plethora of endogenous factors, such as defects in the DNA repair machinery, or a variety of exogenous factors, such as dietary compounds, smoking, viruses, as well as some occupational and environmental carcinogens.

Differences in incidence rates across cancer types have stimulated a discussion on whether stochastic DNA changes (i.e., somatic mutations due to the variations in stem cell divisions in different organs) could explain these variations. Correlative analyses of the lifetime risk for developing a certain cancer with the number of stem cells divisions within the same organ implied that replication-related mutations are associated with neoplastic development. Mathematical analyses leveraging DNA sequencing and epidemiological data from 69 countries, representing 4.8 billion people, estimated that at least 29% of mutated driver genes are linked to environmental factors, whereas 66% can be attributed to replicative errors. However, it is impossible to completely separate replication-related mutations from exogenous factors. For example, consumption of very hot beverages, which has been linked to oesophageal carcinogenesis, induces severe damage to the cellular lining of the esophagus, which in turn will trigger stem cells located in the deep layers to divide in order to replace the damaged cells. Therefore, divisions of stem cells, caused by an exogenous factor can introduce replication-related mutations. Moreover, the endogenous production of reactive oxygen species (ROS), which can trigger replicative mutations, has been associated with several environmental compounds that act indirectly on DNA. Examples of such compounds are pesticides, mycotoxins, and heavy metals.

Numerous epidemiological studies emphasize the contribution of the environment to the cancer burden observed in particular populations. For instance, hepatocellular carcinoma (HCC) shows a high incidence rate in regions with a high risk of exposure to aflatoxin B1 (AFB1) compared to other regions where AFB1 exposure is minimal. In addition, comparing cancer incidence of Japanese subjects residing in Hawaii versus those living in Japan, especially ones living in Okinawa, revealed a dramatic decrease in cancers of the mouth, pharynx, and esophagus in all Japanese migrants. This result suggests that Japanese migrants have escaped exposure to mysterious environmental cancer risk factor(s) particular to the Okinawa region. Multiple epidemiological studies employing different approaches have estimated that extrinsic factors contribute 70%–90% of the risk for cancer development, with the rest being due to intrinsic factors. Thus, current epidemiological estimates indicate that the majority of global cancer risk is preventable. Taking the ongoing discussion regarding the contribution of replication errors into account, a smaller proportion of cancers would be amenable to a reduction of environmental exposures, while the majority would require cancer prevention measures based on early detection and early intervention. Regardless of the cancer prevention approach, uncovering the causes that lead to cancer will allow developing measures that seek to reduce or completely eradicate the exposure factors.

Cancer Etiology and Approaches to Identify the Sources of Somatic Mutations

All cancer genomes harbor mutational patterns that reflect tumor heterogeneity and that stem from exposures to multiple mutagenic carcinogens. Some mutagenic carcinogens leave a specific SBS imprint on DNA, exemplified by tobacco smoke carcinogens in lung (viz., C:G > A:T), ultraviolet light in most cancers of the skin (viz., C:G > T:A), and AFB1 in liver cancers from certain regions around the world (viz., C:G > A:T). A further refined classification of these SBS mutations can be applied by taking into consideration the immediate 5′ and 3′ nucleotide bases flanking the somatic mutation. This elaborated classification makes it possible to discriminate between the mutational patterns of C:G > A:T transversions observed in lung and liver cancers. Currently, the analysis of human cancer mutational spectra offers the possibility to study cancer etiology with much greater resolution. In the next few

sections, we discuss leveraging somatic mutations in single genes as well as somatic mutations across larger parts of the genome for elucidating cancer etiology.

Single gene approaches

Previously, mutagenicity and genotoxicity evaluation of compounds relied on simple assays employing prokaryotic systems, such as the Ames test, as well as assays that are laborious, such as the comet and micronucleus assays. However, these assays do not provide insights regarding the specific base changes and their sequence context. Leveraging single-gene sequencing in experimental models and primary human tumors provides an alternative to study the mutagenic processes associated with specific carcinogenic exposures. The experimental systems used for this purpose depend either on a phenotypic selection method (e.g., bacterial reporter genes) or on genes that are frequently mutated in human cancers.

Commonly utilized in vitro reporter genes rely on endogenous genes (e.g., *HPRT*, *DHFR*, or *TK*) to convert certain media supplements to toxic metabolites implying the occurrence of genetic changes in the encoded genes. In contrast, animal in vivo model systems include the genomic integration of a transgene consisting of a reporter gene (e.g., *lacI*, *lacZ*, *gpt*, *gpa*, *hprt*, *aprt*, *supF*, or *cII*) and a viral shuttle vector. After exposure, the transgene is packaged into phage particles ensuring the efficient delivery of the target gene into a bacterial host. Mutation detection is examined using chromogenic or viability selection. Reporter gene assay allowed the assessment of the mutagenicity of a number of carcinogens, for instance, the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, a common dietary carcinogen in cooked meat, which was associated with an increased rate of C:G > A:T transversions. Other examples include the dietary carcinogen acrylamide (viz., T:A > A:T and C:G > G:C), AFB1 (viz., C:G > A:T), and the chemotherapeutic agent 8-methoxypsoralen (viz., T:A > A:T).

Sequencing of cancer genes facilitates the identification of driver mutations in a cancer type. This approach also provided the first evidence of the molecular mechanisms by which environmental carcinogens leave characteristic imprints on DNA. Examples of the most frequently mutated genes in human tumors include TP53, KRAS, and BRAF genes. Single cancer gene sequencing in skin and lung tumors identified mutational patterns characteristic of exposures to ultraviolet light (UV-light) and benzo[*a*]pyrene (BaP), respectively. UV-light induces C:G > T:A transitions at dipyrimidines in skin cancers, and tobacco smoke prompts C:G > A:T transversions in lung tumors (**Fig. 3**). These tumor-associated mutational patterns are in agreement with results from in vitro controlled experimental exposure studies of UV-light and BaP. Indeed, sequencing of the TP53 gene in cancer patients with different exposures history (i.e., exposed vs. non-exposed) strengthens the link between environmental factors and cancer development. Lung tumors from smokers display a predominant C:G > A:T mutational pattern which is not evident in lung tumors from non-smokers, and the amount of the C:G > A:T imprint correlates with the level of tobacco consumption.

Human exposure to the plant carcinogen aristolochic acid (AA) has been linked to the endemic Balkan nephropathy as well as upper tract urothelial carcinoma (UTUC) and cancers of the liver, bladder, and kidneys. Indeed, TP53 mutation screening of these

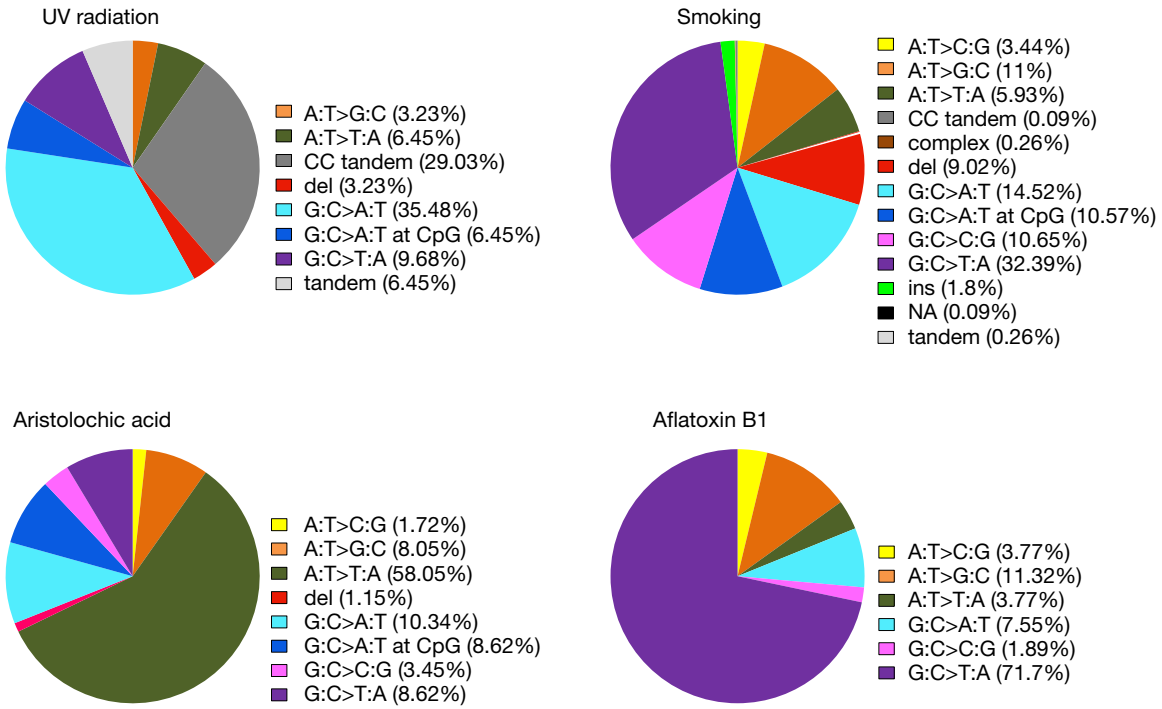

Fig. 3 Data extracted from the International Agency for Research on Cancer's TP53 database. Pie chart representations of the proportion of TP53 single base substitutions observed in human cancers of the skin, lung, kidney, and liver. Each of the pie charts corresponds to a specific exposure linked with one or more of these cancer types. The database can be found at: http://p53.iarc.fr/TP53SomaticMutations.aspx.

cancers identified a pronounced T:A>A:T mutational fingerprint associated with the unique mutation pattern of AA observed in laboratory experiments. Finally, the TP53 gene exhibits a unique mutational profile characterized by predominant C:G>A:T transversions in HCC cases from regions where AFB1 exposure is prevalent. Liver cancers from other populations, where AFB1 exposure is minimal and other risk factors prevail, exhibit other distinct TP53 mutational fingerprints.

Different in vivo and in vitro experimental systems contributed to the identification of TP53 mutational patterns, representing "rudimentary signatures" of mutagens, and their putative associations to specific cancer types. These models include a genetically engineered mouse system, harboring the human TP53 gene, from which embryonic fibroblasts were derived. These Hupki (Human TP53 knock-in) mice were exposed to UV-light inducing characteristic TP53 gene mutations similar to those predominantly observed in human skin cancer (viz., C:G>T:A). Moreover, Hupki mouse embryonic fibroblasts (Hupki MEFs) were exposed to a number of carcinogens revealing an analogy between the Sanger sequenced TP53 genes from the in vitro assays and human tumors associated with the same exposures.

Yeast systems were also exploited for TP53 mutagenesis using a strain transfected with an expression vector harboring human TP53 cDNA that had been in vitro irradiated with UV-light. The results revealed CC:GG>TT:AA mutations which is consistent with the observations from data derived from sequencing UV-light associated skin cancers. Normal human fibroblasts were also treated with other known carcinogens, such as BaP, AFB1, and acetaldehyde. The observed mutational patterns of TP53 were evaluated and they were consistent to the mutational patterns observed in some human cancers. Overall, the experimental identification of carcinogen-specific mutational patterns has demonstrated a convergence between mutational data and epidemiological studies. These mutational data can be effectively utilized for establishing of causal associations between environmental exposures and human cancers.

Despite their significant contribution to understanding the sources of somatic mutations in cancer, single gene sequencing studies harbor major limitations. First, many samples from a specific cancer type are needed to accumulate enough alterations to extract a specific mutational profile. This profile is specific for a cancer type and, due to the small numbers of mutations in a gene, one cannot examine individual samples. Second, the patterns of mutations in single genes are usually affected by selection as, in many cases, these genes provide a selective growth advantage. The selection imprint can severely bias the observed mutational spectrum. Third, single genes have specific nucleotide sequences that are not representative of the human genome. The nucleotide structure of genes affects the opportunity for observing a somatic mutation and it further biases the observed mutational spectrum. Advances in NGS and bioinformatics analyses have addressed these challenges and allowed efficient testing of hypotheses regarding putative cancer-risk factors.

Genome-wide mutational patterns and deciphering mutational signatures from human cancers

Massively parallel sequencing has revolutionized many aspects of biology, including mutation research, due to high speed sequencing capacities and the reduction in the overall sequencing cost. NGS enables examining the complete genomes of cancer cells at a single-base level, providing an unprecedented resolution of the mutational processes that have molded these genomes through the lineage of the cancer cells. The generation of large mutational datasets required the development of novel mathematical models and computational frameworks that allow meaningful interpretation of these data. Below, we provide a description of a mathematical model that was previously used to identify the signatures of the mutational processes operative in human cancer by examining thousands of cancer genomes.

A mutational catalogue of a cancer genome can encompass a diverse set of mutation classes including single base substitutions, insertions/deletions, structural rearrangements, copy number changes, and others (Fig. 1). Each class of mutation can then be further sub-classified. For example, SBSs can be sub-classified according to the six unique types of single base substitutions. For simplicity, the pyrimidine of the Watson–Crick base pair is usually used as a reference for a SBS mutation, thus, a C:G>A:T mutation will be written as a C>A mutations. Using this abbreviate nomenclature the six unique SBS types are: C>A, C>T, C>G, T>A, T>C, and T>G. This classification can be further elaborated to include a variety of mutational features such as the sequence context of the mutated base and the transcriptional strand on which the substitution has arisen.

For the purpose of mathematical modeling, a limited number of features of a mutational catalogue need to be selected. The choice of features may be influenced by prior biological knowledge. The choice is also often constrained by statistical considerations and the available data. Mathematically, a set of mutational features can be expressed as a finite alphabet Ξ with K letters, where each letter corresponds to a mutational feature. The simplest alphabet for SBSs, Ξ_6 contains $K = 6$ letters. Ξ_6 is based on the six types of single base substitutions and it has six letters: C>A, C>T, C>G, T>A, T>C, and T>G. It should be noted that this alphabet of mutation types could be easily extended by, for example, including other mutation types such as doublet base substitutions. Here, we focus on mathematical alphabets representing SBSs as these alphabets have been the ones most commonly used to examine mutational signatures.

The Ξ_6 alphabet is perhaps the simplest possible alphabet as it considers only the six types of somatic substitutions. This alphabet is generally not used in any analysis but, rather, its simplicity is leveraged to provide examples and visual representations that clarify mathematical concepts.

The Ξ_{96} alphabet provides a greater resolution for examining the six types of single nucleotide variants (i.e., the Ξ_6 alphabet) by including the immediate sequence context of each mutated base. In this alphabet, a mutation type contains a somatic substitution and both the 5′ and 3′ base immediately next to the somatic mutation. For example, a C>T mutation can be characterized as …Tp<u>C</u>pG… > …Tp<u>T</u>pG… (mutated base underlined and presented as the pyrimidine partner of the mutated base pair in the

trinucleotide sequence) generating 96 possible mutation types—(six types of substitutions) * (four possible types of 5′ base pairs) * (four possible types of 3′ base pairs).

The Ξ_{1536} further extends Ξ_{96} by including two base pairs 5′ and 3′ to the mutated base resulting in 1536 possible mutated pentanucleotides—(six types of substitutions) * (16 possible types of the two immediate 5′ base pairs) * (16 possible types of the two immediate 3′ base pairs). For example, using the Ξ_{1536} alphabet, one of the 256 subclasses of a C>T mutation is …ApTpCpGpC… > …ApTpTpGpC… (mutated base underlined and presented as the pyrimidine partner of the mutated base pair in the pentanucleotide sequence).

Lastly, Ξ_{192} elaborates Ξ_{96} by considering the transcriptional strand on which a substitution resides. In contrast to all previously discussed alphabets, Ξ_{192} is defined only in the regions of the genome where transcription occurs, which in most analyses has been limited to the genomic footprints of protein coding genes. Thus, the previously defined 96 substitution types are extended to 192 mutation types. For example, C>T mutations at TpCpA are split into two distinct categories: C>T mutations at TpCpA occurring on the untranscribed strand of genes and C>T mutations at TpCpA occurring on the transcribed strand of genes. In general, one would expect that these two numbers are approximately the same unless the mutational processes are influenced by the activity of the transcriptional machinery. This could happen, for example, due to recruitment of the transcription-coupled component of nucleotide excision repair. For example, if a mutational process has a higher number of C>T substitutions on the transcribed strand compared to C>T substitutions on the untranscribed strand (note that a C>T mutation on the untranscribed strand is the same as a G>A mutation on the transcribed strand), this could indicate that the mutations caused by this process are being repaired by transcription-coupled nucleotide excision repair, although other explanations are also possible. A known example of such strand-bias due to an interplay between a DNA damaging process and a repair mechanism is the formation of photodimers due to ultraviolet light exposure that are repaired by transcription-coupled nucleotide excision repair, resulting in a higher number of C>T mutations on the untranscribed strand of UV-light associated cancers.

Quantitatively, a mutational catalogue of a cancer genome is a vector, m, containing the number of somatic mutations of a genome, g, defined over a finite alphabet of mutational types Ξ. Mathematically, a mutational catalogue is a morphism between the pre-defined finite alphabet, Ξ, and a set of K nonnegative integers, ${\mathbb{N}_0}^K$, that is, $m : \Xi \rightarrow {\mathbb{N}_0}^K$. Thus, for a given genome, its mutational catalogue can be expressed as a K-tuple of natural numbers, $m = [m^1, m^2, \ldots, m^K]^T$. Comparing the mutational catalogues of two cancer genomes, $m_1 = [{m_1}^1, {m_1}^2, \ldots, {m_1}^K]^T$ and $m_2 = [{m_2}^1, {m_2}^2, \ldots, {m_2}^K]^T$, requires that both mutational catalogues are defined over the same mutational alphabet Ξ. A comparison can be performed either by using a cosine distance to compare the difference between the mutational patterns in two catalogues or by using a Euclidean distance to compare the absolute mutational difference between two catalogues.

Different cancer genomes can be exposed to a particular mutational process at different intensities. For example, a mutational process may cause 350 mutations in one cancer genome while causing 350,000 in another. We refer to this number of mutations as a *mutational exposure* (or simply *exposure*) of a signature of a mutational process in a cancer genome. Hence, one may say that a mutational process with a signature P has an exposure e,corresponding to the number of mutations caused by this process in a mutational catalogue m of a cancer genome.

Multiple mutational processes can be operative in a single cancer genome and each of these processes can have a distinct mutational exposure. Thus, the mutational catalogue of a cancer genome $m = [m^1, m^2, \ldots, m^K]^T$,defined over the mutation alphabet Ξ with K letters, is a superposition of the signatures of the N operative mutational processes $P_i = [{p_i}^1, {p_i}^2, \ldots, {p_i}^K]^T$, $i = 1 \ldots N$,each defined over the mutation alphabet Ξ, with their respective exposures e^i, $i = 1 \ldots N$. This superposition is never perfect as DNA sequencing, bioinformatics analyses, and other pre- and post-processing of the data causes non-systematic noise, n. Taking into account this noise, the number of the j-the mutational type in an m mutational catalogue can be examined as:

$$m^j = \sum_{i=1}^{N} P_i^j e^i + n^j \tag{1}$$

Note that in this definition, m, e, and n are vectors, while P is expressed as a matrix. Indeed, the signature P_1 of a mutational process, defined over an alphabet Ξ with K letters, can also be expressed as a nonnegative K-tuple, $P_1 = [{p_1}^1, {p_1}^2, \ldots, {p_1}^K]^T$, where $\sum_{i=1}^{K} p_1^i = 1$ and ${p_1}^i$ is the probability of the mutational processes P_1 to cause the mutation type corresponding to the i-th letter of the alphabet Ξ. As previously described, a set of N mutational signatures can be expressed as a nonnegative mutational signature matrix $P = \begin{bmatrix} p_1^1 & p_2^1 & \cdots & p_{N-1}^1 & p_N^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ p_1^K & p_2^K & \cdots & p_{N-1}^K & p_N^K \end{bmatrix}$ with size $K \times N$,where K is the number of mutation types and N is the number of signatures. The subscript index indicates the signature, while the superscript index corresponds to the mutation type.

The mutational catalogue of a cancer genome, defined over the alphabet of mutation types Ξ, is represented by a morphism m, where $m : \Xi \rightarrow {\mathbb{N}_0}^K$. For a given genome,$i$, its mutational catalogue can be expressed as a nonnegative K-tuple, $m_i = [{m_i}^1, {m_i}^2, \ldots, {m_i}^K]^T$. Hence, the mutational catalogues of G cancer genomes can be expressed as a nonnegative matrix of mutational catalogues $M = \begin{bmatrix} m_1^1 & m_2^1 & \cdots & m_{G-1}^1 & m_G^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ m_1^K & m_2^K & \cdots & m_{G-1}^K & m_G^K \end{bmatrix}$ of size $K \times G$. In this case, the mutational catalogues are the columns of the matrix, where K is the

number of mutation types and G is the number of genomes. The subscript index indicates the mutational catalogue while the superscript index corresponds to the mutation type.

The exposure to a mutational process with a signature $P_1 = [p_1{}^1, p_1{}^2, \ldots, p_1{}^K]^T$ is a scalar describing the number of mutations, $e^1 \in \mathbb{N}_0$, attributed to that signature in a given mutational catalogue. In this notation, the product $p_1{}^2 \times e_g{}^1$ is the number of mutations of type corresponding to the second letter of alphabet Ξ caused by the mutational process P_1 in a cancer genome with number g. Hence, one can define a set of exposures of G genomes to a set of N processes as a nonnegative matrix $E = \begin{bmatrix} e_1^1 & e_2^1 & \cdots & e_{G-1}^1 & e_G^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ e_1^N & e_2^N & \cdots & e_{G-1}^N & e_G^N \end{bmatrix}$ with size $N \times G$. Here, the subscript index indicates the genome while the superscript index corresponds to the signature. In addition to the signatures of the operative mutational processes, the mutational catalogue of a cancer genome also reflects the effect of random error processes, which may occur due to the used experimental approach (e.g., DNA sequencing) and/or bioinformatics methods (e.g., algorithms for identifying somatic mutations from next-generation sequencing data). This noise term n reflects an additive white Gaussian noise that occurs due to non-systematic errors. The noise term is specific to each mutational catalogue and it is defined over the alphabet Ξ of the mutational catalogue, where $n : \Xi \rightarrow \mathbb{R}^K$. Hence, for a set of mutational catalogues of G cancer genomes, the noise term can be expressed as a matrix $N = \begin{bmatrix} n_1^1 & n_2^1 & \cdots & n_{G-1}^1 & n_G^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ n_1^K & n_2^K & \cdots & n_{G-1}^K & n_G^K \end{bmatrix}$ of real numbers with size $K \times G$. The subscript index indicates the noise term for the mutational catalogue while the superscript index corresponds to the mutation type.

The signatures of N different mutational processes and their respective exposures need to be extracted from the mutational catalogues of M cancer genomes. Using the introduced notations, this could be expressed as:

$$\begin{bmatrix} m_1^1 & m_2^1 & \cdots & m_{G-1}^1 & m_G^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ m_1^K & m_2^K & \cdots & m_{G-1}^K & m_G^K \end{bmatrix} = \begin{bmatrix} p_1^1 & p_2^1 & \cdots & p_{N-1}^1 & p_N^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ p_1^K & p_2^K & \cdots & p_{N-1}^K & p_N^K \end{bmatrix} \times \begin{bmatrix} e_1^1 & e_2^1 & \cdots & e_{G-1}^1 & e_G^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ e_1^N & e_2^N & \cdots & e_{G-1}^N & e_G^N \end{bmatrix} + \begin{bmatrix} n_1^1 & n_2^1 & \cdots & n_{G-1}^1 & n_G^1 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ n_1^K & n_2^K & \cdots & n_{G-1}^K & n_G^K \end{bmatrix} \quad (2)$$

or one can simplify Eq. (2) using matrix notations as:

$$M = P \times E + N \quad (3)$$

In practice, one knows only the mutational catalogues in the matrix M as M corresponds to the mutations identified by NGS. To identify the signatures of the operative mutational processes one needs to decompose M and identify P and E such that these matrices best describe the original matrix without over-fitting the data.

Finding an optimal solution for Eq. (3) can be achieved by leveraging a blind source separation (BSS) technique. BSS problems involve unscrambling latent (i.e., not observed) signals from a set of mixtures of these signals, without knowing or assuming anything about the mixing process. In the cases of mutational signatures, each cancer genome can be examined as a mixture of distinct mutational processes that have imprinted their mutational signatures on this genome. In principle, BSS algorithms are capable of revealing hidden features and dependencies in large sets of observed data. Then, these features can be used to build a representation of the data that can contribute to understanding of the underlying mechanisms behind these data. The unmixing and reconstruction of the original signals is usually based on some constrained and/or regularized optimization procedure minimizing an objective (i.e., cost) function together with a few imposed constraints, such as: maximum variability, statistical independence, nonnegativity, smoothness, sparsity, simplicity, etc. The choice of the optimization constraints is usually based on a priori knowledge about the examined data, and, hence, constraints are usually specifically tailored for a particular problem and/or a specific mathematical model.

Unscrambling mutational signatures from the genomes of cancer cells can be effectively done by leveraging nonnegative matrix factorization (NMF). NMF is a BSS approach that assumes simple nonnegativity of the different signals. In turn, the nonnegativity makes each of the identified latent variable (i.e., mutational signature) a component of the original data, thus, resulting in easily identifiable and interpretable latent variables. Moreover, NMF contains a natural sparsity constraint that allows separating distinct latent variables only when there are sufficient data for such separation.

In 2013, a computational framework, based on NMF, was first developed and later used by Alexandrov and colleagues to identify signatures of mutational processes from 7042 cancer patients with 30 different types of human cancer. More than 20 distinct mutational signatures were identified, many of which were attributed to either exogenous or endogenous mutational processes. In the past few years, the map of mutational signatures in human cancer has been further refined and more than 30 distinct mutational signatures have been discovered. The newest set of mutational signatures in human cancer can be found on the COSMIC website: http://cancer.sanger.ac.uk/cosmic/signatures.

Signatures of Mutational Processes With Proposed Etiology

The analysis of thousands of cancer genomes has revealed more than 30 signatures of mutational processes in human cancer. From some of these signatures, previous analyses have been able to identify or, at least, to propose a putative etiology. Tobacco-associated

lung cancers are overwhelmed by signature 4 (Fig. 4), which displays a predominant C>A pattern with transcription strand bias indicative of damage to guanine. This signature matches well the in vitro pattern of mutations induced by tobacco smoke carcinogens and, most specifically, the pattern induced by in vitro exposure to BaP. Bladder, kidney, and liver cancers from certain regions around the world exhibit a unique mutational signature known as signature 22 (Fig. 4). Signature 22 is characterized by T>A mutations and it has been ascribed to exposures to AA. Skin-cancers associated with ultraviolet-light harbor large-numbers of mutations that are attributed to signature 7 (Fig. 4). Signature 7 is predominantly characterized by C>T mutations at dipyrimidine nucleotides with a transcriptional strand bias that is consistent with the formation of photodimers. Importantly, the hitherto described mutational signatures (and others) confirmed prior observations derived from single-gene sequencing of TP53 and provided a strong mutational link between a specific cancer type and its main aetiological factor.

The mutational catalogue of a cancer is a composite of superimposed mutational signatures left by various mutagenic insults. In HCC, many individual cancer samples exhibit more than five distinct mutational signatures, reflecting the variety of carcinogens that can cause mutations in hepatocytes. Some of the known risk factors attributed to liver cancer are HBV infection, HCV infection, alcohol consumption, and exposure to AFB1. Amongst the identified signatures, signature 16, characterized by T>C transitions at ApTpN sites, is observed exclusively in the majority of liver tumors. Nevertheless, its etiology is still unknown. Signature 24 was uncovered in AFB1-exposed liver cancers and it is characterized by a transcription asymmetry of C>A transversions (Fig. 4). Integrated experimental analysis across human cell lines, animal models, and primary HCC tumors further confirmed the association between signature 24 and exposure to AFB1.

Interestingly, endogenous mutational processes constitute almost half of the identified mutational signatures. Signature 1, attributed to the spontaneous deamination of 5′-methylcytosine, is seen in almost all tumors and it contributes up to 80% of mutations in some cancer types, for example, in myeloid leukemia. Together, signatures 1 and 5 has been termed clock-like mutational signatures as the number of mutations attributed to each of these signatures in cancer (and normal somatic) cells are proportionate to the chronological age of the person from which the cancer (or normal cells) were taken. A number of mutational signatures have been attributed to the disruption of processes regulating DNA homeostasis. Signature 9 reflects infidelity of the DNA polymerase eta, while signature 10 is due to a malfunction of the DNA polymerase epsilon. Defects in DNA mismatch repair (MMR) were found to generate at least five distinct mutational signatures depending on the nature of MMR failure: signatures 6, 15, 20, 21, and 26. Failure of DNA double strand repair by homologous recombination also causes a specific mutational signature, termed, signature 3. Signature 30 was attributed to failure of base-excision repair due to defects in the cancer predisposition gene NTHL1. Notably, in more than half of the cancer types analyzed to date, mutational signatures attributed to the APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family of deaminase, known as signatures 2 and 13, have been identified. The patterns of the APOBEC signatures have been further supported by extensive experimental work utilizing various model systems. The APOBEC deaminases have been implicated in virus restriction and suppression of retrotransposition. Signatures 2 and 13 have been overwhelmingly found in cervical cancer as well as in cancers of the head and neck, both of which are related to HPV infection. Moreover, signatures 2 and 13 are highly elevated in samples with prior evidence for HPV infection, implying the recurrent activation of APOBEC upon viral infection. In addition to mutational signatures with known etiology, the mechanisms underlying many of the identified mutational signatures still remain mysterious. A comprehensive information of the current set of mutational signatures in human cancer can be found on the COSMIC website.

Orphan Mutational Signatures and Approaches for Understanding Them

Genome-wide mutational analysis of thousands of human cancers highlighted a number of mutational signatures attributed to endogenous and exogenous mutagens. In order to reveal the causal factors underlying a mutational signature, the convergence of multiple lines of evidence from different areas of research is required including experimental studies, epidemiology data, and individual patient history including exposomics data.

Amongst the 30 distinct mutational signatures thus far identified, 40% remain with unknown etiology reflecting the need for controlled experimental studies. In vivo exposure bioassays as well as in vitro exposure assays are two roads that can lead to a controlled assessment of the genotoxicity, mutagenicity, and carcinogenicity of a compound. Ideally, such exposure studies would use model systems that enable the testing of a large number of compounds within a reasonable timeframe.

Normal primary cells, both human and rodent, have a limited lifespan when transferred from their in vivo environment to culture. These cells undergo stress-associated senescence, with a permanent exit from the cell cycle and eventual death of the cell population. Occasionally, one cell may bypass this fate due to genetic changes and resume the cell cycle producing a clone of immortalized cells that replicates indefinitely in vitro. Clonal expansion is a prerequisite property of the system allowing the investigation of the acquired somatic mutations in more or less homogeneous cell populations by deep sequencing analysis.

Cellular models suitable for mutational signatures analyses should include a bottleneck step followed by a clonal expansion, thus, mimicking key steps of carcinogenesis: (i) initiation via exposures, (ii) promotion, (iii) and progression. There are two approaches that should be considered for an in vitro system: (i) bypassing a biological barrier, such as crisis or senescence, and emergence of an immortalized clonal population; or (ii) single cell subcloning after exposure for cells for which a selective biological bypass step is not applicable; also known as clonal expansion. Moreover, these experimental models should recapitulate key aspects of human biology (e.g., metabolic activity as well as activity of DNA repair pathways).

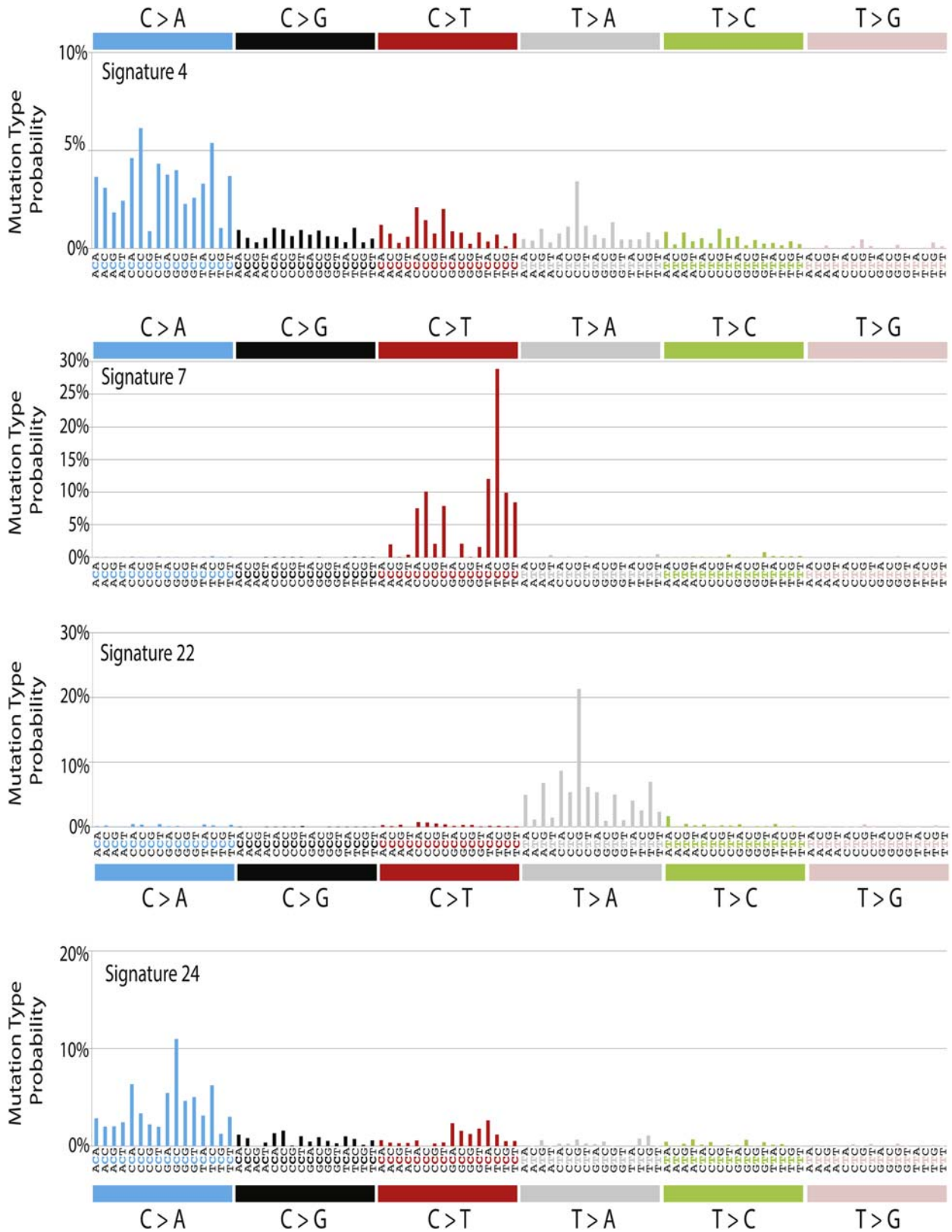

Fig. 4 Examples of mutational signatures identified from examining next-generation sequencing data from human cancers. Signature 4 has been overwhelmingly found in lung cancer from smokers and it has been attributed to smoking tobacco cigarettes. Signature 7 generates the majority of mutations in cancers of the skin and it has been attributed to exposure to ultraviolet light. Signature 22 and 24 has been found in cancers of the liver from specific regions around the world and they have been attributed to exposure to aristolochic acid and aflatoxin B1, respectively.

Through extensive laboratory work, multiple mouse-derived and human-derived cells have been employed to experimentally investigate the genome-wide mutagenic effects of candidate cancer-risk factors. First, Hupki MEFs have proven suitable for cell immortalization due to their sufficiently long telomeres. Interestingly, the scope of overlap between the genome-wide mutational patterns observed in human datasets and immortalized MEF cell lines, exposed to a number of strong carcinogens, includes: (i) the SBS mutational pattern based on the six possible types of SBS mutations, (ii) the transcription strand bias of specific SBSs, and (iii) the immediate sequence context of the dominant mutation type. Second, a number of human cell line models have been explored for the analysis of mutational patterns, namely a proximal tubule human kidney cell line, viz., HK-2, as well as HepaRG and HepG2 cells derived from HCC, and the human mammary epithelial cells (HMEC). As for most human model systems, their use requires long-term exposure and the number of compounds that can be tested is a limiting factor. Nevertheless, the use of human cancer target cell models is an elegant strategy to identify the mutational pattern of a carcinogen triggering cancer development in a specific site. Other emerging models such as induced pluripotent stem cells and organoids are versatile systems capable of clonal expansion that can be generated from different cell and tissue types. However, these models lack the biological barrier step of HMEC and Hupki MEFs and it is not clear whether this will influence the imprinted mutational signatures. Further, taking into consideration in vivo carcinogen-animal models may provide new avenues for a better understanding of the molecular alterations observed in human diseases, and thus improve our knowledge on cancer initiation, progression, diagnosis, and treatment.

In addition to experimental examination, future epidemiological studies can be used to identify novel mutational signatures as well as to reveal the identity of known mutational signatures with mysterious etiology. Coupling of epidemiology and cancer genomics approaches can provide synergistic results by allowing association of epidemiological features with mutational signatures or other molecular cancer data. Further, cancer risk assessment through the establishment of new epidemiological studies with thorough follow-up of human subjects can enable the exploration of carcinogenic risk associated to specific carcinogens and eventually the categorization of mutational signatures to cancer risk factors.

Prospective Vision

Characterization of novel mutational signatures specific to cancer-risk agents may ultimately contribute to the overall interdisciplinary mission of cancer research and provide novel avenues for cancer prevention.

Multiple national and international initiatives have started with the goal for better understanding mutational signatures in human cancer. Notably, the *Mutographs of Cancer* project is investigating whether different mutational signatures can explain geographical differences in cancer incidence across the world. This international project will collect cancer samples and information about cancer-causing exposures from 5000 patients with colorectal, oesophageal, pancreatic, and kidney cancer living in regions of high or low cancer incidence across five continents. The complete DNA of these cancers (and their matched normal tissues) will be sequenced and the mutational signatures will be comparted and linked to cancer risk factors. Further, *Mutographs* will also identify specific causes of mutational signatures by sequencing the DNA of rodent cancers and cultured human cells experimentally exposed to more than 150 cancer-causing agents, thus assembling a compendium of mutational signatures associated with known causes of cancer. The overall outcome of the *Mutographs* project will provide a comprehensive understanding of mutational signatures in cancer and may lead to new approaches to prevent cancer as well as provide opportunities for more effective application of existing cancer therapies.

Interestingly, in recent years, evidence has emerged linking epigenetics modifications to environmental factors and human malignancies. Cancer genomes frequently undergo epigenetic changes that follow the Darwinian natural selection process and favor the growth of cells with characteristically altered chromatin structure and deregulated gene expression. These changes are brought through epigenetic modifier genes that are frequently mutated in human cancer. Some examples include DNA methyltransferases, DNA demethylases, histone modifiers, ATP-dependent chromatin remodelers, and other. The results of epigenetic deregulation can range from dysregulated expression of individual oncogenes or tumor suppressor genes to large-scale chromatin structure alterations and genomic instability.

Well-established cancer-risk agents and lifestyle factors have been studied in terms of epigenome deregulation, improving the understanding of their long-lasting effects on cancer outcome. Tobacco smoking, diet, infections, inflammation and age are known to affect epigenetic states and can play a role in the early onset of cancers through different mechanisms. Smoking, which is the strongest exposure factor causing lung cancer, harbors an epigenetic signature characterized by consistent methylation changes in the Aryl-hydrocarbon receptor repressor gene. Age is the strongest demographic risk factor for cancer and, interestingly, DNA methylation profiles of chronological age established an "epigenetic clock" that can be affected by different external and endogenous factors.

Progress in epigenetic research can open the door to a new era where epigenetic signatures can serve as surrogate for diagnostics and risk stratification of cancer in tissues and can provide evidence on the interactive role of epigenetic deregulation in the roadmap between environmental exposures and cancer.

See also: Aflatoxins. Carcinogen-DNA Adducts. Dietary Factors and Cancer. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics.

Further Reading

Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Campbell, P.J., Stratton, M.R., 2013. Deciphering signatures of mutational processes operative in human Cancer. Cell Reports 3, 246–259.

Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A.J.R., Behjati, S., Biankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Børresen-Dale, A.-L., et al., 2013. Signatures of mutational processes in human cancer. Nature 500, 415–421.

COSMIC database of mutational signatures, http://cancer.sanger.ac.uk/cosmic/signatures.

Hollstein, M., Alexandrov, L.B., Wild, C.P., Ardin, M., Zavadil, J., 2017. Base changes in tumor DNA have the power to reveal the causes and evolution of cancer. Oncogene 36, 158–167.

Zhivagui, M., Korenjak, M., and Zavadil, J. (2017). Modeling mutation spectra of human carcinogens using experimental systems. Basic and Clinical Pharmacology and Toxicology, 121 Suppl 3: 16-22: https://doi.org/10.1111/bcpt.12690, Epub 2017 Feb 3.

Mutations in Chromatin Remodeling Factors

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Glossary

ATPase A motor enzyme that performs a reaction by ATP hydrolysis.

Chromatin The multilayered protein-DNA structure that packages DNA inside a nucleus.

Chromatin remodeling An ATP-dependent process that mobilizes nucleosomes either by sliding them through DNA or the removal of histone proteins from chromatin. Facilitated by chromatin remodeler complexes.

DNA accessibility Refers to how open or relaxed the chromatin structure is. More "open" chromatin has longer linker DNA between nucleosomes or has a higher nucleosome turnover and is permissible for DNA regulatory factors to bind.

DNA repair The process by which an error in DNA is corrected. There are several pathways for DNA repair that is dependent on the type of damage.

Histone modification The addition of chemical moieties to histone proteins, such as methylation or acetylation.

Nucleosome The basic repeating unit of chromatin that consists of an octamer of histone proteins that accommodates 147 bp of DNA in approximately two helical turns.

Transcription RNA polymerase transcribes or "copies" the coding strand of the DNA into an RNA molecule.

Introduction

DNA does not exist as a linear molecule within a cell; instead it is wrapped around proteins into a dynamic multilayer structure called chromatin. The basic repeating unit of chromatin is the nucleosome, the core of which is comprised of an octamer of histone proteins (two of each histone H2A, H2B, H3 and H4) that accommodates 147 base pairs (bp) of DNA in approximately two helical turns. The H1 linker histone sits outside of the core and influences the orientation of DNA at the entry and exit points of the nucleosome, locking them into position. The length of the linker (inter-nucleosomal) DNA between two nucleosomes varies from ~20–90 bp depending on the degree of chromatin compaction and physiological conditions. A single piece of linker DNA coupled with the nucleosome forms the chromatasome and an array of these structures forms the characteristic "beads on a string" chromatin fiber in the presence of low salt. Chromatin protects DNA from damage and naturally represses gene transcription, unless this state is actively circumvented. Therefore, nucleosomes must be mobilized so that the DNA strand becomes physically accessible to transcription factors, the transcriptional machinery and other chromatin binding factors in order for gene expression to be activated. To do so, two major subclasses of protein complexes are actively recruited to specific genomic regions to control transcription. These are: "chromatin posttranslational modifiers" and "ATP-dependent chromatin remodelers."

The first broad group of chromatin modifiers refers to those that alter histone protein posttranslational modifications (PTMs). These complexes are the epigenomic "writers" and "erasers" that add or remove chemical groups for other complexes to "read" and interpret. PTMs include, but are not limited to: acetylation, methylation, phosphorylation, ubiquitination, sumoylation and poly-ADP-ribosylation. Most of the currently known modifications occur on the histone tails that project out from the nucleosome core, although the globular domains can also be modified, and contribute to the epigenetic landscape of a genomic locus. These epigenetic histone modifications play a critical role in many DNA-based processes and in chromatin stability. There are four broad classes of proteins that are able to "read" histone modifications including; chromatin architectural proteins, ATP-dependent chromatin remodeling enzymes, chromatin modifiers (as many of the enzymes that "write" and "erase" these modifications also contain domains to "read" them) and adapter proteins that recruit molecular machinery for processes such as transcription. For example, the ATP-dependent chromatin remodeler, CHD1, utilizes its tandem chromodomains to recognize and remodel nucleosomes carrying histone 3 lysine 4 is tri- methylation (H3K4me3), a modification found at active gene promoters. When recruited, CHD1 localizes with transcription elongation factors to positively promote transcription at highly transcribed genes. While histone posttranslational modifications are important in chromatin biology, this chapter will instead focus on the role of ATP-dependent chromatin remodelers.

ATP-dependent chromatin remodelers mobilize nucleosomes and alter their physical positions. First, by loosening the DNA around the nucleosome, then by either translocating the nucleosome through the DNA or completely removing the nucleosome from the DNA strand (Fig. 1). Conversely, these complexes can re-position nucleosomes during the process of gene repression and chromatin compaction (Fig. 1). Nucleosome movement is not only essential for transcriptional control, but also for normal cellular processes including cell cycle progression, DNA replication and DNA repair. For example, progression through the cell cycle requires nucleosome compaction for DNA condensation and sister chromatid segregation. During DNA replication, histone cores are loaded onto newly synthesized DNA while the DNA repair process is impaired by the presence of

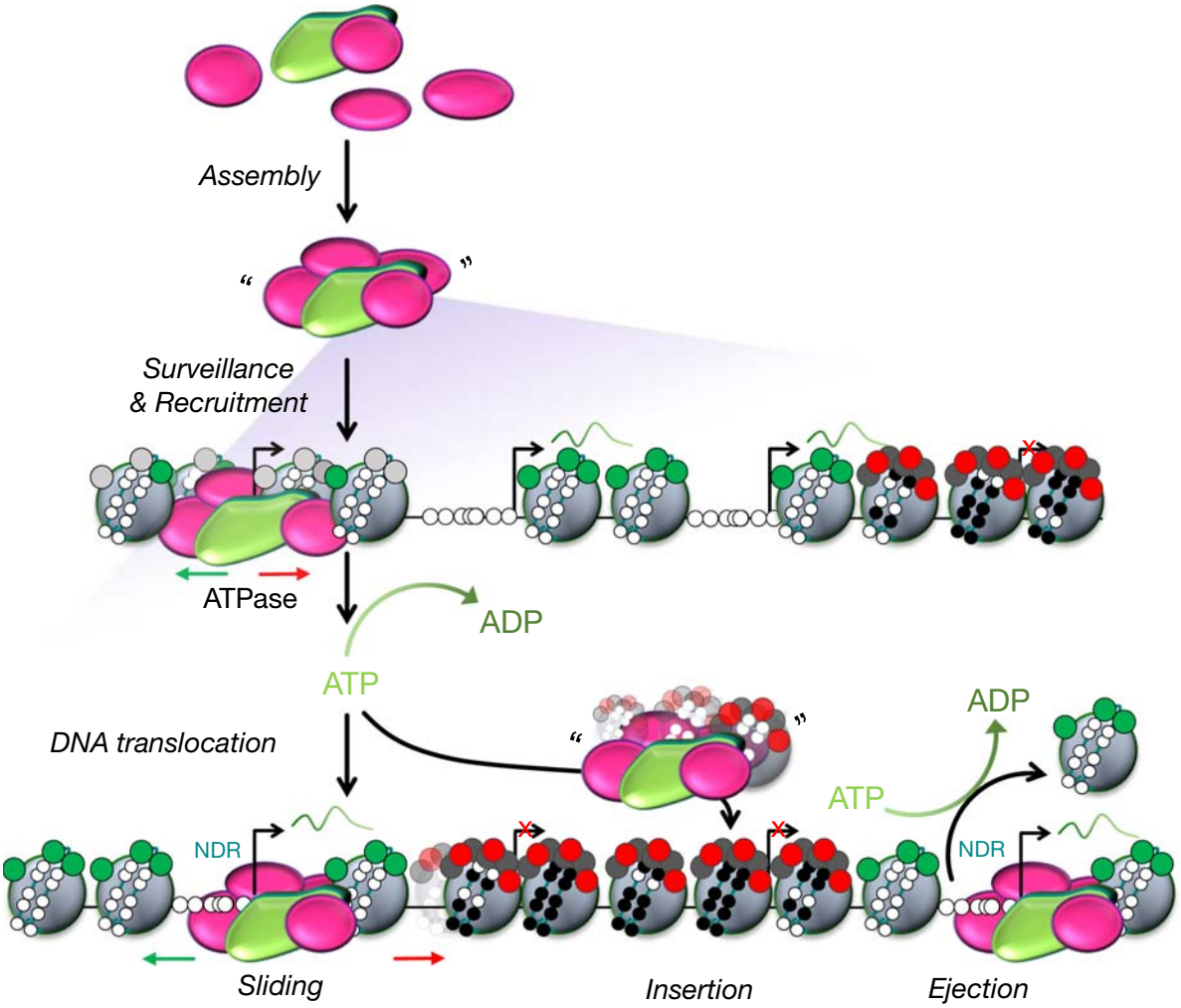

Fig. 1 *Mechanisms of ATP-dependent chromatin remodeling for the alteration of DNA accessibility.* Utilizing ATP hydrolysis, chromatin remodeler protein complexes act on chromatin to modulate the physical positioning of nucleosomes to allow or inhibit the binding of regulatory factors to DNA. Chromatin remodelers do not recognize a particular consensus sequence and continually "survey" chromatin for suitable substrates. On recruitment, chromatin remodelers may slide, insert of eject nucleosomes from chromatin. Mobilization by *sliding* nucleosomes alters the position of the nucleosome and simultaneously changes the length of the internucleosomal DNA fragment. This may involve lengthening (depicted by the *green arrow*), when a nucleosome depleted region (NDR) is formed, or shortening (depicted by the *red arrow*), causing loss of the detectable NDR. Alternatively, nucleosome positions may be altered by *deposition* or *eviction*. In some cases, such as with the INO80-like family, remodelers are able to exchange canonical histones for histone variants, such as dimer exchange of H2A-H2B for H2A.*Z*-H2B. A generic chromatin remodeling complex (pink) with an ATPase subunit (*green*) is depicted. The complex may be recruited to active chromatin (unmethylated at CpG dinucleotides (*small white circles*) and enriched with active histone posttranslational modifications (*green circles*)), poised chromatin (unmethylated and enriched with permissive histone posttranslational modifications (*gray circles*)) or silenced chromatin (methylated and enriched with repressive histone posttranslational modifications (*red circles*) to coordinate sliding, insertion or ejection of nucleosomes (*large gray circles*). Transcriptional start sites are indicated by arrows, and may be active (*green line*) or inactive (*red cross*).

nucleosomes, which must be removed to make DNA accessible to the repair machinery. Chromatin remodelers are also responsible for exchange of canonical histones for variant histones. These histone variants, such as H3.3 and H2A.Z, provide additional resolution at which chromatin structure is controlled by altering the affinity between DNA and the nucleosome core on a finer scale. Hence, chromatin remodelers may be described as the "gate-keepers" of chromatin structure that facilitate or impede access to DNA.

There are four structural families of chromatin remodelers: Switch/Sucrose Non-Fermenting (SWI/SNF), Imitation Switch (ISWI), Inositol 80 (INO80) and chromodomain-helicase-DNA binding (CHD) (Fig. 2). Common features of these complexes include: that they have a high affinity for the nucleosome, have an essential catalytic subunit that utilizes ATP hydrolysis to mobilize nucleosomes, a high degree of similarity between their helicase ATPase domain structures (DExx and HELICc), and that they interact with other regulatory factors (both protein and RNA) to facilitate chromatin control. Despite their common properties, each remodeling family has evolved their own unique mechanistic properties and functions (Fig. 3). SWI/SNF ATPases have a bromo-domain

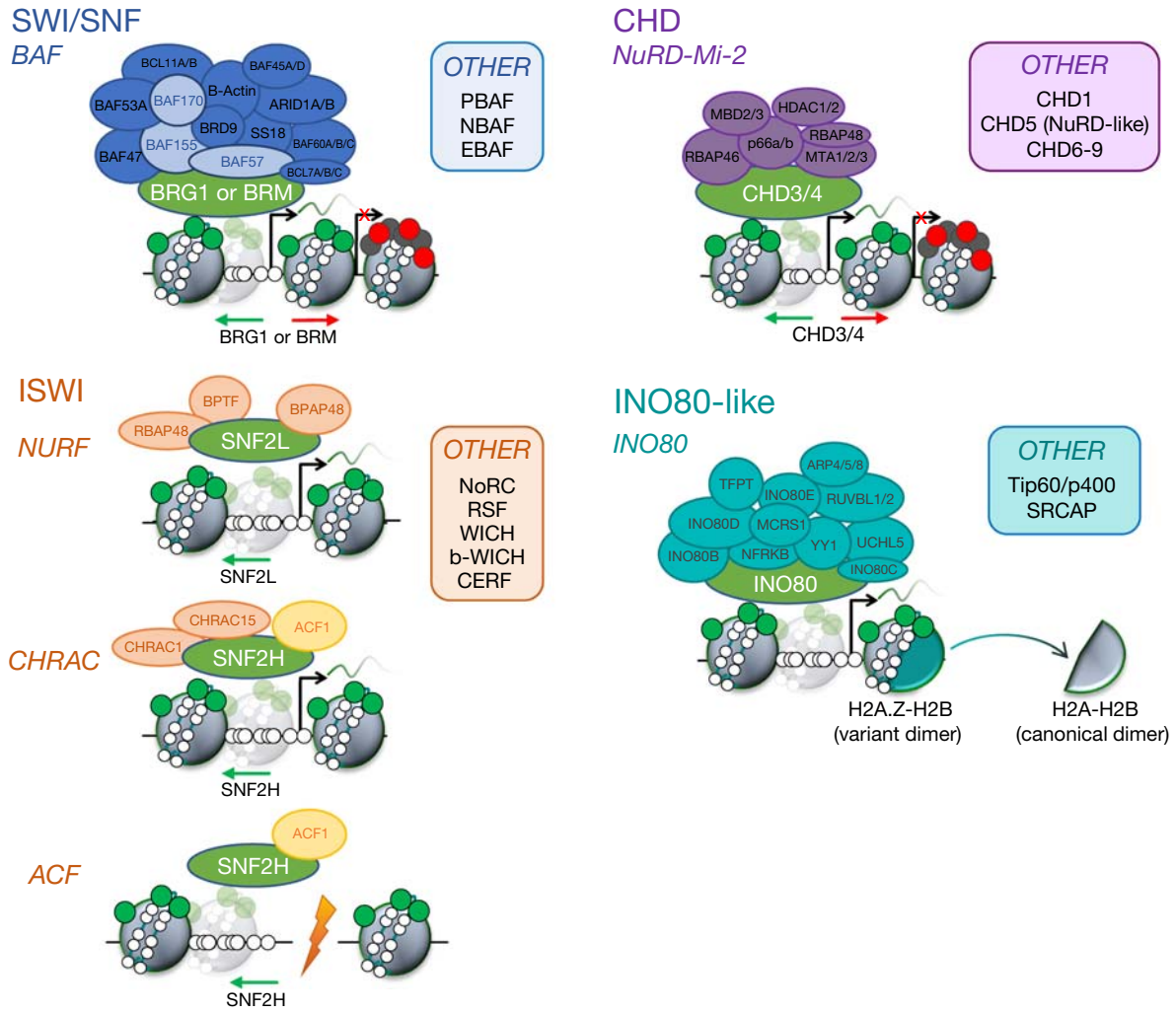

Fig. 2 *The classical organization of ATP-dependent chromatin remodeling families based on the SNF2 ATPase catalytic subunit structure.* Typical subunit composition is shown for common SWI/SNF, ISWI, CHD and INO80-like complexes. *BAF* is a SWI/SNF complex containing either BRG1 or BRM as the ATPase, and is present in most cell types. Other SWI/SNF complexes are present only in certain cell types, such as embryonic BAF (EBAF) in embryonic stem cells. Most SWI/SNF complexes can remodel chromatin ready for transcription activation or repression. *NURF* and *CHRAC* are both ISWI complexes with known roles in transcriptional activation. *NURF* contains the SNF2L ATPase and aberrantly activates oncogenes in cancer cells while *CHRAC* contains the SNF2H ATPase and is known to activate transcription in both normal and cancer cells. *ACF* contains the SNF2H ATPase and remodels nucleosomes after DNA damage (orange lightning bolt). The *NuRD/Mi-2* complex containing the CHD3/4 ATPase is representative of the CHD family that has roles in remodeling chromatin for transcriptional activation or repression. *INO80* from the INO80-like complexes contains INO80 as the ATPase and is activates transcription by nucleosome mobilization and facilitating exchange of the canonical H2A-H2B dimer for the variant H2A.Z-H2B. Nucleosomes, *large gray circles*; CpG sites, *small white circles*; active histone posttranslational modifications, *green circles*; repressive posttranslational histone modifications, *red circles*; transcriptional start site, *arrow*.

recognizing acetylated histones; ISWI ATPases have a HAND/SLANT/SLIDE domain that recognizes nucleosomes and inter-nucleosomal DNA; INO80 complexes have a longer peptide chain between their DExx and HELICc domains that has been proposed to accommodate replication forks and Holliday junction DNA structures, and CHD ATPases have tandem chromo-domains recognizing methylated histones (Fig. 3). The inclusion of divergent additional domains within the ATPase subunit, as well as the variety of accessory subunits recruited, suggests that each remodeler complex can uniquely decode chromatin, accounting for their differing roles within the cell.

Chromatin remodelers are known to utilize two primary mechanisms to identify a target nucleosome. Firstly, they can continually scan chromatin for an appropriate nucleosome substrate, relying on their recognition of histone PTMs and inter-nucleosomal DNA. Alternatively, a remodeler can be recruited to and interact with a sequence-specific protein or noncoding RNA (ncRNA) species at a particular genomic locus when required. Since ATP-dependent chromatin remodelers act generically in the absence of consensus sequences, these cofactors provide an additional layer of chromatin regulation that ensures remodeler complexes are recruited to the right place at the right time.

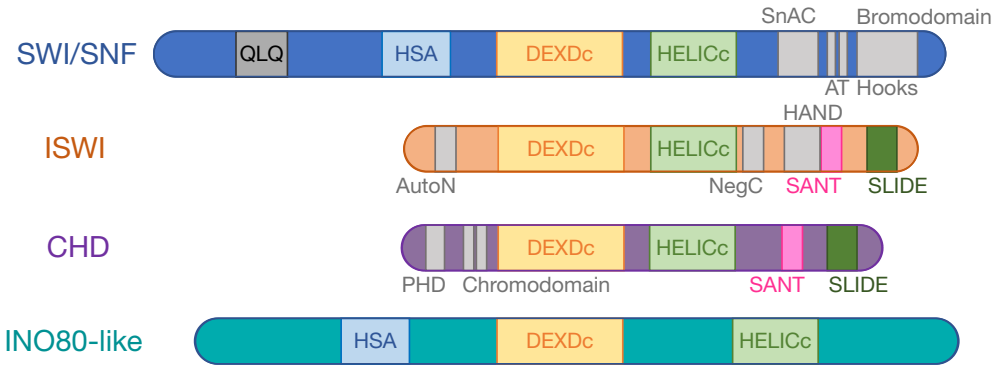

Fig. 3 *Domain structures of the ATPase catalytic subunit of the remodeller families containing their signature motifs.* Each chromatin remodeler contains a core SNF2 ATPase domain consisting of an N-terminal DEXDc and C-terminal HELICc domains (*yellow/green*). The SWI/SNF remodelers also contain a N-terminal helicase-SANT (HSA) domain (*blue*) for actin binding and a C-terminal bromodomain (*gray*) for the recognition of acetylated histones. The ISWI family also contain a C-terminal HAND/SANT/SLIDE domain (*gray/pink/green*) for the recognition of nucleosomes and regulatory domains N-terminal AutoN (*gray*) and NegC (*gray*) C-terminal to the ATPase domain. The CHD family each contain tandem chromodomains (*gray*) and a PHD domain (*gray*, Class II only) for recognizing methylated histones at the N-terminal, and various other domains depending on whether they fall into subclass I, II and III (not all shown). The ISWI remodelers have a split ATPase with a spacer between the DEXDc and HELICc domains, and a N-terminal HSA domain (*blue*), similar to the SWI/SNF family.

The outcomes of recent genomic sequencing studies have revealed that chromatin remodelers are frequently mutated in multiple cancer types. Indeed, the frequency of mutation of the SWI/SNF complex alone rivals that of *TP53*, which is otherwise known as the "guardian of the genome." There are conflicting reports of chromatin remodelers functioning as both tumor suppressors and oncogenes, highlighting the diverse roles that they are likely to play in tumor biology. Modulating chromatin is critical for cell proliferation and it is unsurprising that the factors involved play an important role in cancer initiation and disease progression. Sequencing of cancer genomes has demonstrated that chromatin organization is directly associated with genome-wide mutation rates, where open chromatin as a result of remodeling is more likely to contain indels and substitutions than closed chromatin. As informative links between chromatin remodelers and their role in tumor development have been established, a desire for further insight into both the normal function of these complexes and the mechanisms by which cancer develops as a consequence of their mutation is rising.

SWI/SNF

Of all the remodeling complexes, SWI/SNF has been the most comprehensively studied. It is a large multisubunit complex initially identified in *Saccharomyces cerevisiae* and highly conserved from yeast to humans. Core subunits of this complex include a catalytic ATPase, either the mutually exclusive Brahma (BRM, encoded by *SMARCA2*) or Brahma-related gene 1 (BRG1, encoded by *SMARCA4*), SNF5 (also known as BAF47 and INI1, encoded by *SMARCB1*), BAF170 (encoded by *SMARCC2*) and BAF155 (encoded by *SMARCC1*). Accessory subunits such as the mutually exclusive ARID1A and ARID1B that bind to AT-rich, nonsequence specific DNA are responsible for the targeting, assembly and regulation of SWI/SNF. SWI/SNF mutant cancers demonstrate a role for the complex in DNA repair, proliferation, motility, invasion and metastasis, and genes involved in these processes are generally up-regulated in SWI/SNF deficient tumors. Therefore, it is not unexpected that the expression of SWI/SNF subunits and activity of the complex is altered in several cancer types.

The SWI/SNF complex has a well-characterized role in the normal DNA repair process; defective DNA repair directly results in genome instability and cancer development. There are several reports demonstrating that loss of functional SWI/SNF leads to an increased susceptibility to DNA damage, most likely mediated through interactions between SWI/SNF and the histone variant, γH2A.X. BRG1 normally promotes double stranded break (DSB) repair by binding to acetylated H3 histones at the site of damage in the presence of γH2A.X; thus, loss of BRG1 slows the repair process leading to inefficient repair. Contrary, loss of other subunits within the SWI/SNF complex does not necessarily affect DSB repair pathways. SNF5 and ARID1A deficient tumors exhibit little genome instability, remain diploid and are characteristically normal on single nucleotide polymorphism (SNP) arrays, again highlighting the multifaceted functions that each subunit can have within this complex.

SWI/SNF interacts with and regulates several known cancer associated genes, including genes involved in cell cycle progression (*RB, E2Fs, CCND1, MYC, GLI1*), nuclear receptor signaling (glucocorticoid (*GR*), estrogen (*ER*), androgen (*AR*), vitamin D (*VDR*) and retinoic acid (*RARA*)), and cell-to-cell interaction/adhesion proteins (integrin's and *CD44*). Interestingly, SWI/SNF can both up- and down- regulate the same gene; the outcome being dependent on cellular context. For example, in response to estrogen, SWI/SNF is bound by ER and recruits p300 to activate ER-responsive genes, while estrogen antagonists, such as the tumor suppressor protein Prohibitin, recruit SWI/SNF and HDAC1 to repress these same genes. Similarly, SWI/SNF has a complex role in regulating *MYC* oncogene expression. *MYC* over-expression in a variety of cancers disrupts the expression of genes involved in cell cycle progression, apoptosis and differentiation. SWI/SNF can normally directly repress *MYC*, but in certain cancers such as leukemia, over expression

of the SWI/SNF complex causes chromatin de-condensation at cell-specific enhancer regulatory elements to increase *MYC* expression. The following section outlines the roles played by significant subunits of the SWI/SNF complex in cancer.

SNF5

Some of the strongest evidence linking chromatin remodeling and cancer comes from studies of rhabdoid tumors (RT). RTs are an aggressive, soft-tissue childhood cancer with a poor response to therapy and bi-allelic loss of SNF5 is a defining feature—occurring in 95% of these cancers (**Table 1**). RTs can derive from both somatic and familial mutations in *SMARCB1* (encoding SNF5), which causes gene inactivation by deletion, nonsense, missense and frameshift mutations. Schwannomatosis and occasionally other sarcomas also report a bi-allelic loss of SNF5, and other cancers demonstrating high mutation frequency in SNF5 include colorectal, gastric and bladder (**Table 1**). The use of knockout mouse models further confirm the functional role of SNF5 in RT initiation; SNF5 knockout mice develop RT with a rapid onset, indicating that SNF5 is likely a driver mutation in these cancers.

Loss of SNF5 in RT occurs concomitant with an increase in expression of the Polycomb group (PcG) protein, EZH2. EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which contributes to the repression of genes through the tri-methylation of histone 3 lysine 27 (H3K27me3). Depletion of EZH2 in SNF5 deficient tumors is associated with a decrease in cellular proliferation, suggesting that EZH2 is required for the growth of these tumors. This is in part due to the role that SNF5 and the PcG proteins play in the regulation of key tumor suppressor genes. For example, SNF5 is required for activation of the *INK4b-ARF-INK4a* locus and in the absence of SNF5, epigenetic repression of this region is initiated by EZH2 and the PcG proteins. This study also demonstrated that with re-expression of SNF5 into a SNF5-deficient RT human cell line, PcG proteins were evicted from the *INK4b-ARF-INK4a* locus and that the activity of the SWI/SNF complex was restored. Moreover, the authors noted that the mixed lineage leukemia 1 (MLL1) histone posttranslational modifier is recruited following PcG eviction, in order to restore an active epigenetic signature on histone 3 lysine 4 (H3K4).

Precisely how atypical SNF5 expression disrupts the function of SWI/SNF is unclear. It is possible that aberrant SNF5 expression alters the composition of the SWI/SNF complex, driving atypical interactions with other proteins in a context dependent manner to promote tumorigenesis. In SNF5-deficient cell lines, continued growth is reliant on increased expression of BRG1, which could explain why loss of SNF5 in cancers activates gene expression pathways to promote growth whilst in primary cells it triggers cell cycle arrest. Indeed, several cell cycle dependent proteins are consequently disrupted following loss of SNF5. One example is of p16INK4A, a cyclin-dependent kinase (CDK) inhibiting cyclinD1-CDK4 phosphorylation, which the downregulated concomitant with SNF5 inactivation. Conversely, expression of cyclinD1 itself, MYC and E2F target genes are elevated.

SNF5 loss has been linked to the disruption of cancer promoting signaling pathways. Hedgehog signaling is essential for the growth of RTs and is up-regulated in SNF5-deficient tumors. *RHOA* stimulates cell migration, actin stress fiber formation and contractility. When SNF5 is lost, *RHOA* expression increases which is correlated with poor prognosis in cancer. These data suggest that SNF5 has broad roles in establishing the cancer phenotype, potentially by driving aberrations in histone posttranslational modification marking at gene regulatory regions.

BRG1 and BRM

A SWI/SNF complex always contains either BRG1 or BRM as two possible alternative ATPases. While they share approximately 75% sequence homology, BRG1 has a unique N-terminal domain not existent in BRM, through which it can bind to zinc-finger proteins. BRG1 and BRM have partially overlapping functions and therefore may offer some compensation when one of them is lost or disrupted in cancer. However, the ATPases also have clearly defined roles since BRG1, but not BRM, is embryonic lethal. Both BRG1 and BRM suppress cancer development through control of the cell cycle and cell-to-cell adhesion. In cancer cell lines deficient of both functional BRG1 and BRM, re-expression of either ATPase can induce Rb-mediated cell cycle arrest. Similarly, loss of BRG1 and/or BRM in lung cancer drives cancer development and alters cell-to-cell contacts through an increased *VIM* expression and down-regulation of *CDH1*, *CD44* and *CSF-1*. Furthermore, BRG1 has a synthetic lethal relationship with BRM and research has shown that cancers depend on BRM for growth and survival. Loss of BRM in these cancers results in cell cycle arrest, senescence and increased tri-methylation of H3K9 (H3K9me3). The cancer-specific differences in SWI/SNF activity may be partially explained by the variance in expression of BRG1 and BRM in tumors, which likely alters feedback loops that balance tumor suppressive and oncogenic activity of SWI/SNF.

SMARCA4 (encoding BRG1) is down-regulated in bladder, colon, nontriple negative breast cancers, head and neck, esophageal, melanoma, pancreatic and ovarian cancers; where mutation rates have been reported between 4% and 13% in these cancers (**Table 1**). Conversely, *SMARCA4* is over-expressed in cancers of the prostate, triple negative breast cancers and some leukemias (**Table 1**). Interestingly, no *SMARCA4* mutations have been reported in cancers over-expressing BRG1, suggesting that atypical *SMARCA4* expression may have an epigenetic basis, although this has not been experimentally confirmed. The context-dependent function of BRG1 makes it difficult to fully define its role in cancer; however, there is sufficient evidence that the balance between tumor suppressive and oncogenic activity of BRG1 is important.

Functional studies have assessed the outcomes of both depletion and restoration of the BRG1 protein. Aberrant BRG1 expression disrupts proliferation, cell motility and epithelial to mesechymal transition (EMT). For example, in combination with lung-specific carcinogens, haploinsufficiency of *SMARCA4* increases the likelihood of lung cancer development while conversely, re-expression of BRG1 protein in lung cancer cells reduces proliferation, metastasis and EMT via up-regulation of miR-148b. Mechanistic studies in cell lines demonstrate changes in cell motility occur concomitant with BRG1 depletion and similarly, the loss of BRG1 in a pancreatic cancer cell line stimulates actin stress fibers and motility. Conversely, in a cervical cancer cell line deficient in BRG1, restoration

Table 1 Summary of SWI/SNF subunits with an identified role in cancer as either a tumor suppressor and/or an oncogene

Gene	*Protein*	*Function*	*Tumor suppressor*	*Oncogene*
SMARCB1	SNF5/INI1/BAF47	Binds to lateral surface of the nucleosome, scaffold	Bladder, colorectal, gastric, rhabdoid tumors, sarcoma, Schwannomatosis	
SMARCA4	BRG1	ATPase, mutually exclusive with BRM, interacts with acetylated histones	Bladder, breast (except triple negative), cholangiocarcinoma, colorectal, esophagus, gastric, head and neck, lung, medulloblastoma, melanoma, ovarian, pancreatic, uterine	Colorectal, leukemia, prostate, triple negative breast cancer
SMARCA2	BRM	ATPase, mutually exclusive with BRG1, interacts with acetylated histones	Adenoid, bladder, colorectal, gastric, lung, prostate, uterine	
ARID1A	ARID1A/BAF250a	Nonsequence specific DNA binding, mutually exclusive with ARID1B	Bladder, breast, Burkett's lymphoma, cervical, cholangiocarcinomas, colorectal, endometrial, esophagus, gastric, heptoblastoma, liver, lung, medulloblastoma, melanoma, neuroblastoma, ovarian clear cell, pancreatic, prostate, renal, sarcoma	
ARID1B	ARID1B/BAF250b	Nonsequence specific DNA binding, mutually exclusive with ARID1A	Bladder, breast, cervical, colorectal, esophagus, gastric, melanoma, lung, sarcoma	
SMARCE1	BAF57	Interacts with nuclear receptors and DNA, HMG domain		Breast, endometrial, ovarian, prostate
BRD7	BRD7	Interacts with acetylated histones	Breast, colorectal, gastric, lung, ovarian, prostate	
ARID2	ARID2	Role in NER damage response pathway	Bladder, colorectal, esophagus, gastric, hepatocellular, lung, melanoma, NSCLC, uterine	
PBRM1	BAF180	Interacts with acetylated histones	Bladder, breast, cholangiocarcinoma, clear cell renal cell carcinoma, gastric, head and neck	
SMARCC2	BAF170	Role in neural differentiation	Bladder, gastric	
SMARCC1	BAF155	Interacts with SNF5 through SWIRM domain	Colorectal, gastric, lung	
SMARCD1/2/3	BAF60A/B/C	Role in cellular senescence, lipid, nutrient and energy, metabolism	Colorectal	
ATRX	ATRX	ATRX/DAXX complex, ATPase, DNA damage response, heterochromatin formation, deposits H3.3 into chromatin	Brain, osteosarcomas	

of BRG1 resulted in reduced actin stress fibers and increased expression of *ROCK1*, a major driver of cell motility. By contrast, over-expression of BRG1 promotes proliferation and cancer development. Several studies have reported that *SMARCA4* over-expression in prostate cancer is a feature correlated with disease progression and invasiveness. In leukemia, BRG1 plays a role in regulating the actin cytoskeleton and proliferation, where loss of BRG1 down-regulates hedgehog signaling, decreases proliferation via down-regulation of *MYC*.

Unlike the divergent BRG1 expression profiles observed between cancers and cancer subtypes, the BRM ATPase is typically depleted. This is consistent with the observations that BRG1 has both tumor suppressive and oncogenic roles, while all studies of BRM in tumors suggest that it acts solely as a tumor suppressor. This is supported by the observation that BRM loss correlates with advanced disease in prostate and lung cancers (**Table 1**). Indeed, there is an anticorrelation between the proliferative markers, ki-67 and E2F1, and BRM in these cancers. In prostate cancer, BRM loss occurs concomitant with an increased rate of EMT and perturbed cell cycle progression. Moreover, decreased BRM expression leads to increased androgen-dependent growth, and homozygous deletion of BRM in prostate epithelial cells causes acquisition of lobe-specific, castration-resistant proliferation. BRM is silenced in 15% of nonsmall cell lung cancer (NSCLC), and lung tumors with loss of BRM have a lepidic growth pattern pathology and *EGFR* mutations. Two studies conducted in cancer cell lines found no evidence of deleterious BRM mutations, and in both of these studies, BRM expression was restored following treatment with histone deacetylase (HDAC) inhibitors. These data suggest that BRM is epigenetically silenced and is supported further by work showing that certain mutations, BRM-741 (rs34480940) and BRM-1321 (rs3832613), in the BRM promoter facilitate the binding of HDACs. Interestingly, tumors with these genetic variants are associated with worse overall survival.

BRG1 and BRM expression changes can at least be partially attributed to epigenetic mechanisms, indicating that epigenetic therapies could be effective for cancers deficient in either BRG1 or BRM. Epigenetic therapies to alter BRG1 expression are of particular interest in cancers traditionally treated with the chemotherapeutic agent, cisplatin; cancers with reduced BRG1 are more sensitive to this line of treatment. Work in head and neck cancer cell lines with knockdown of either BRG1 or BRM found the enhanced sensitivity of cancer cells to cisplatin was through the reduced repair of intrastrand adducts and interstrand crosslink formation and cisplatin-induced DSB repair was impaired from ineffective recruitment of ERCC1 for later stage repair. Furthermore, this study demonstrated that altered cell cycle checkpoint activation and enhanced apoptosis in these cancers. In BRM depleted cancers, there is potential use for HDAC inhibitors. Treatment of lung cancer cells with HDAC inhibitors restored BRM expression, but inhibited BRM function. Removal of HDAC inhibitors kept BRM expression elevated for a few days and the complex was functional before BRM levels again were depleted. Future targeting of this pathway must refine the HDAC inhibitors to reduce the impact on BRM function.

ARID1A and ARID1B

The AT-rich interactive domain (ARID) subunits, ARID1A and ARID1B, are mutually exclusive within the SWI/SNF complex. They facilitate interaction with DNA that is not sequence specific. While these subunits share approximately 60% sequence homology, they are nonredundant. SWI/SNF complexes containing ARID1A repress *MYC*, but complexes with ARID1B increase *MYC* expression. Additionally, as cells progress through the cell cycle the expression of ARID1A decreases and ARID1B increases.

ARID1A is one of the most frequently mutated genes in a variety of primary tumor types. Its down-regulation positively correlates with tumor progression, indicating that its main role is that of a tumor suppressor. Loss of function mutations in *ARID1A* have been identified in bladder, colorectal, breast, Burkitts Lymphoma, cervical, endometrial, gastric, melanoma, medulloblastoma, uterine corpus endometrioid carcinomas, neuroblastoma, cholangiocarcinomas, carcinosarcomas, ovarian clear cell, lung, pancreatic, prostate, renal and heptoblastoma cancers (**Table 1**). Many of these cancers have heterozygous mutations in ARID1A and express a lower level of the protein. Indeed, it has been observed that loss of ARID1A, BRG1 or SNF5 can occur without complete loss of function in some leukemias. Conversely, in ovarian clear cell carcinomas, one of the most lethal subtypes of ovarian cancer, approximately 50% of cases present with a mutation in *ARID1A* and the majority of these express a truncated form of the ARID1A protein. Interestingly, *ARID1A* mutations are more frequent in solid tumors, particularly in the gastrointestinal tract, than hematopoietic cancers.

ARID1B mutations have been identified in bladder, breast, colorectal, carcinosarcomas, lung, melanoma, cervical and gastric cancer (**Table 1**). Loss of ARID1B is detrimental to cells and mutations are known to cause a host of other diseases and syndromes relating to intellectual disability and developmental delays. A recent study has found that ARID1B expression is required in order for ARID1A-deficent tumors to survive. Cancers with a homozygous deletion of *ARID1A* can tolerate a heterozygous mutation in *ARID1B*, where only one functional copy is sufficient for tumor survival. The loss of both *ARID1A* and *ARID1B* destabilizes SWI/SNF and reduces proliferation of affected cells. This suggests that ARID1B could be a therapeutic target in ARID1A deficient cancers, providing another therapeutic angle through which to target SWI/SNF mutant cancers, and development is underway for small molecule inhibitors of ARID1A and ARID1B.

Other SWI/SNF subunit mutations

BAF57 is an accessory subunit of SWI/SNF that is only present in higher eukaryotes. BAF57 plays a direct role in determining how the SWI/SNF complex interacts with chromatin, as it contains both a high mobility group domain and DNA binding properties. There is no evidence supporting a direct role for BAF57 in the initiation of cancer but rather, in cancer progression where increased BAF57 expression correlates with tumor grade, invasion and metastasis. In breast, ovarian, prostate and endometrial carcinoma BAF57 tends to have elevated expression (**Table 1**); indeed, 38% of endometrial carcinomas are reported to exhibit increased

BAF57 expression. Mutations have only been reported in breast cancer cell lines. As part of its normal and cancer related functions, BAF57 interacts and promotes the activities of GR, AR and ER nuclear hormone receptors. In prostate cancer, BAF57 interacts with AR to increase the expression of α2-integrin, giving those cells a metastatic advantage. In breast cancer, BAF57 expression correlates with expression of PTK2, a key player in migration, motility and adhesion to promote metastasis. Loss of BAF57 decreases the rate of breast cancer metastasis to the lung and furthermore, BAF57 depletion causes a subsequent down-regulation of ER target genes. In ovarian cancer, BAF57 expression strongly correlates with sensitivities to cisplatin, doxorubicin and 5-fluorouracil, and depletion of BAF57 in ovarian cells increases the number of cells arresting in the G1 phase of the cell cycle.

BRD7 is a tumor suppressor that is down-regulated in breast, lung, gastric, colorectal, ovarian and prostate cancer (**Table 1**). While low levels of BRD7 have determined it to function as a tumor suppressor, no significant mutations have been identified. BRD7 has a bromodomain, indicating that it contributes to the localisation of SWI/SNF at loci enriched with acetylated histones. In breast cancer, BRD7 binds to the *ESR1* promoter (an ER protein) and while BRD7 depletion does not affect the recruitment of SWI/SNF and RNA polymerase II (RNA PolII) to *ESR1*, it does reduce ER expression. BRD7 is also a binding partner of BRCA1 and p53, coregulating many of the same genes. In lung cancer, when BRD7 is over-expressed it inhibits proliferation through the down-regulation of *CCND1* and *MYC*. It can also down regulate AKT to promote apoptosis.

ARID2 is only found in the PBAF form of the SWI/SNF complex, and is mutated in bladder, colorectal, esophageal, gastric, hepatocellular, lung, melanoma, NSCLC and uterine at a rate of approximately 5%–10% (**Table 1**). It is one of the most frequently homozygous deleted genes in NSCLC and has been identified as a driver mutation in melanoma. Additionally, the four major subtypes of hepatocellular carcinoma all harbor increased ARID2 mutation rates. Little is known about the mechanistic effects of ARID2 mutations or atypical expression of ARID2 in cancer, but it is proposed that ARID2 alterations disrupt the nucleotide excision repair (NER) pathway by inhibiting the recruitment of xeroderma pigmentosum complementation group G (XPG).

BAF180 has essential functions with the cell, is important for normal development and is embryonic lethal in mice. It has been suggested that BAF180 provides functional specificity via its six bromodomains that each have different targeting affinities, as well as a high-mobility group domain recognizing nucleosomes. Loss of function mutations in the BAF180 subunit have been identified in clear cell renal cell carcinoma (ccRCC) and breast cancer. It is the second most commonly mutated gene in ccRCC after *PTEN*, where it is inactivated in 40% of these cancers (**Table 1**). Loss of BAF180 promotes proliferation while re-expression of BAF180 causes G1 arrest, likely through its ability to inhibit p21 expression. BAF180 also plays a role in regulating p53 activity, suggesting that aberrations in this gene would have a general and widespread role in cancer development.

ISWI

ISWI is a large and diverse family of remodeler complexes. Each has specialized functions including DNA repair, replication and transcription. At the core of each ISWI complex is an ATPase: either SNF2H or SNF2L. The SNF2 ATPase forms the ISWI complexes; WSTF-ISWI chromatin remodeling complex (WICH) with WSTF, B-WICH complex containing WSTF and nuclear myosin I, Nucleolar remodeling complex (NoRC) with TIP5, Remodeling and spacing factor (RSF) with RSF, ATP-utilizing chromatin remodeling and assembly factor (ACF) with ACF1 and chromatin assembly complex with ACF1, CHRAC15 and CHARC17. SNF2L forms the ISWI complex Nucleosome Remodeling factor (NURF) with BPTF, RBAP46, and RBAP48 and both SNF2L and SNF2H can form CERC2-containing remodeling factor (CERF) with CECR2. Homologues of ISWI are found in a range of species, and have a highly conserved role in chromatin biology. ISWI proteins are generally tumor suppressors and display loss of function mutations in cancer. However, there are cases of ISWI-associated subunits acting as oncoproteins. ISWI has a well-studied role in DNA repair, forming part of the homologous repair (HR), nonhomologous end joining (NHEJ) and NER pathways.

SNF2H and SNF2L

SNF2H mutations have been identified in approximately 5% of colorectal and uterine cancers (**Table 2**). Alternatively, down-regulation of SNF2H in cancer can be mediated by miR-99a and miR-100 species. Loss of functional SNF2H affects the fidelity of DNA repair in cancer. Ordinarily, the ACF and WICH SNF2H complexes are both recruited to the site of DNA damage by transcription factors and histone modifications for the repair process. During DSB repair SIRT6 deacetylates histone 3 lysine 56 (H3K56), and both SIRT6 and SNF2H coordinate to incorporate γH2A.X into chromatin, then the ACF SNF2H remodeling complex relaxes chromatin at the break site. In response to UV damage, ACF and WICH SNF2H complexes respond and require a functional SLIDE domain of SNF2H to proceed with repair. After deposition at the damage site, SNF2H uses its HAND domain to reposition itself away from the center of the damage ready for the next stage of repair. SNF2H also has an important role in chromatin architecture. SNF2H, but not SNF2L is involved in the positioning and phasing of nucleosomes around CTCF binding sites. Defects and delays in DNA repair, along with loss of well-positioned nucleosomes at CTCF sites can lead to genomic instability and cancer progression in cases where SNF2H function is lost.

SNF2L is mutated at a higher rate than SNF2H, at around 4%–12% in cholangiocarcinomas, uterine, colorectal and gastric cancers (**Table 2**). However, there have been conflicting reports regarding differences in expression and function of SNF2L. In melanoma, it was found that SNF2L protein levels were undetectable in malignant cells compared to normal melanocytes. In the HeLa cervical cancer cell line, a functional study has indicated that atypical SNF2L expression causes an increase in cell proliferation and migration, through the activation of Wnt signaling pathways. Conversely, the results of another study that compared multiple cell lines indicated that SNF2L expression was highly similar between normal and cancer cells, but that cancer cells are sensitive to SNF2L knockdown. This same study found that SNF2L loss inhibits growth, down regulates p53 and p21, up regulates cell cycle

Table 2 Summary of ISWI subunits with an identified role in cancer as either a tumor suppressor and/or an oncogene

Gene	*Protein*	*Function*	*Tumor suppressor*	*Oncogene*
SMARCA5	SNF2H	ATPase, nucleosome phasing at CTCF sites, DSB damage response	Colorectal, uterine	
SMARCA1	SNF2L	ATPase, regulates transcription, DNA damage response	Cholangiocarcinomas, colorectal, gastric, uterine	
BAZ1A	ACF1	ACF ISWI complex, found at replicating pericentromeric heterochromatin, relaxes chromatin at DSB	Colorectal, esophageal, gastric, melanoma, renal cell carcinomas, squamous cell carcinoma, uterine	
BAZ1B	WSTF	WICH ISWI complex, regulates genes in EMT and proliferation pathways, involved in UV DNA damage response		Colorectal, lung
BAZ2A	TIP5	NoRC ISWI complex, shifts nucleosomes to induce DNA methylation, H4 hypoacetylation and H3K9 methylation for heterochromatin formation	Colorectal, lung, melanoma, uterine	Prostate
CECR2	CECR2	CECR2 ISWI complex, inhibits γH2A.X stability, recognizes acetylated histones, histone chaperone	Melanoma, Oligodendroglioma	Adrenal gland, breast, colorectal, esophagus, liver, lung, ovarian, uterine
BPTF	BPTF	NURF ISWI complex, recognizes residues 1–15 of H3K4, regulates HOX gene expression, up regulates EMT and pro survival pathways	Bladder, cholangiocarcinoma, colorectal, gastric, lacrimal gland adenoid cystic carcinomas, lung, melanoma, prostate	Heptacellular, lung, NSCLC, melanoma
RSF1	RSF1	RSF ISWI complex, histone chaperone, DSB repair	Colorectal, gastric, melanoma	Breast, lung

checkpoint genes, increases the phosphorylation of DNA damage response proteins as well as the level of DNA damage, and increases apoptosis. As SNF2H and SNF2L are generally not part of the same ISWI complex, the sensitivity of cancer cells to SNF2L loss could be attributed to lack of compensation from other chromatin remodeling complexes.

ACF1

ACF1 (ATP-utilizing chromatin assembly and remodeling factor 1, encoded by *BAZ1A*) is mutated in approximately 5% of all cancers (Table 2). Renal cell carcinomas have diverse copy number variants, many involving ACF1. Additionally, chromosome 14 q12-q13, where ACF1 is located is frequently amplified and over-expressed in esophageal squamous cell carcinoma. Enrichment of ACF1 is found at replicating pericentromeric heterochromatin, which is a barrier to DNA repair and correlates with somatic mutation rates in cancer. ACF1-containing ISWI complexes have a role in DNA repair, by relaxing chromatin at the DSB site. Indeed, the accumulation of DNA damage as a result of ACF1 repression further delays progression through S-phase of the cell cycle.

WSTF

WSTF (Williams syndrome transcription factor, encoded by *BAZ1B*) is partnered with SNF2H, in the WICH ISWI complex and is mutated in 5% of cancers (Table 2). A study examining any protein containing a kinase domain across colorectal cancer development, reported WSTF was significantly increased in these cancers. Moreover, over expression of WSTF in lung cancer promotes several cellular processes essential for cancer development including proliferation, colony formation, migration, invasion and EMT. Specifically, the EMT gene *fibronectin* and EMT-inducing genes *FOS*, *CEACAM6* and *N-cadherin* are up-regulated concomitant with increased WSTF. There is also up regulation of the PI3K/AKT pathway. In T-cells, it has been noted that WSTF senses L-arginine levels to promote cellular survival. Elevated L-arginine levels induce a metabolic change, which directly impacts T-cell metabolic fitness and is crucial for their antitumor response.

As is observed for many chromatin remodelers, WSTF also has a role in DNA repair. It forms a complex with RUNX2, INTS3 and γH2A.X in response to UV damage and interacts with phosphorylated γH2A.X (pTyr142) to maintain γH2A.X at sites of DNA damage. Following depletion of the oncoprotein, RUNX2, the interaction between WSTF and γH2A.X is lost, while WTSF deletion causes abnormal γH2A.X signals in early pachytene of meiosis.

TIP5

TIP5 (encoded by *BAZ2A*) is part of the ISWI NoRC complex. This complex shifts nucleosomes to induce DNA methylation, H3K9 methylation, and hypoacetylation of H4. TIP5 has a TAM (TIP5/ARBP/MBD) domain that contains a unique C-terminal extension for binding to RNA. The TAM domain binds to ncRNA, which targets the NoRC complex to the genome by recognition of histone 4 lysine 16 acetylation (H4K16ac). Binding to this mark by NoRC mediates heterochromatin formation at rRNA genes, centromeres and telomere repetitive elements to preserve the structural integrity of chromatin. Loss of a functional NoRC complex causes

centromeric and telomeric heterochromatin relaxation, abnormalities in mitotic spindle assembly, impaired chromosome segregation during mitosis and increased genome instability. Conversely, when critical sites of the TAM domain are mutated, ncRNAs can no longer bind and expression of rRNA genes increase. Silencing of rRNA genes drives cells into senescence, therefore an increase in TIP5 expression may induce senescence in cancer cells where TIP5 has been lost. TIP5 is over-expressed in prostate cancer and a potential marker for disease reoccurrence and metastasis (Table 2). TIP5 directly interacts with the PRC2 protein EZH2 and together they regulate genes involved in metastasis progression. In addition, over-expression is associated with the CpG Island methylator phenotype (CIMP) in prostate cancer.

CECR2

Cat eye syndrome chromosome region candidate 2 (CECR2) forms ISWI complexes with both SNF2H and SNF2L. In the testis, CECR2 predominately associates with SNF2H and in the neuronal cells of an embryo, with SNF2L. It contains a bromodomain, recognizing acetylated histones. CRCR2 has diverse roles in cancer and is mutated in approximately 5% of cases including in oligodendroglioma and melanomas (Table 2). It is up-regulated in cancers of the adrenal gland, breast, colorectal, liver, lung, ovarian, esophagus and the urinary tract. In leukiemas, it is a fusion partner of ASXL proteins. CECR2 is a novel DNA damage repair protein and has strong inhibitory activity on γH2A.X stability, though little else is known about the function of CECR2 in remodeling and cancer development.

BPTF

The bromodomain and PHD domain transcription factor (BPTF) is the largest subunit in the NURF ISWI complex. It recognizes residues 1–15 of H3K4, specifically binding when it is tri-methylated at active gene promoters. BPTF has a key role in development, regulating HOX gene expression and development of the mesoderm, endoderm and ectoderm. It is also essential for proper differentiation of mammary stem cells, T-cells and melanocytes.

As with CECR2, BPTF has diverse affects in different cancers. BPTF is mutated in almost 30% of lacrimal gland adenoid cystic carcinomas, involved in translocations of both prostate and lung cancer and frame shift mutations in gastric and colorectal cancer (Table 2). BPTF is over-expressed in melanoma, NSCLC, lung adenocarcinomas and HCC, where it is associated with poor prognosis and metastasis. The tumor promoting activity of BPTF has been linked to the up-regulation of EMT and pro-survival pathways, through partnership with c-MYC. It has been demonstrated that depletion of BPTF from T-cells improves antigen processing and tumor antigenicity, while in mammary cells, it results in loss of open chromatin, particularly at enhancers.

INO80-Like

The INO80-like family includes the ATPases INO80, SRCAP and p400. These complexes are involved in transcription, but have a more prominent role in DNA repair, DNA replication and exchange of histone variants. This includes the insertion of H3.3 and exchange of the canonical H2A−H2B dimer for H2A.Z-H2B at active promoters, active enhancers and at sites of DNA damage. Interestingly, while all INO80-like complexes can exchange histone variants, only INO80 and not SRCAP and p400, can translocate nucleosomes and only the p400 complex has the ability to acetylate H2A.Z through its subunit Tip60.

INO80 complexes

A significant rate of mutations in the INO80 ATPase has not been found in cancer. However, over-expression of INO80 has been reported in melanoma, NSCLC, cervical tumors and cervical cancer cell lines. In melanoma (Table 3), INO80 is required for expression of known oncogenes and tumor growth. In these cancers, INO80 occupies and increases chromatin accessibility at enhancers of tumor development genes. It is also known that recruitment of INO80 to Sox9 and MITF target genes activates genes for melanoma progression and when INO80 is silenced in melanoma cell lines there is an impairment of BRAF- and NRAS-directed tumorigenesis, as well as tumor maintenance. INO80 has a similar role in NSCLC, where it is required to facilitate chromatin de-compaction at enhancers of lung cancer associated genes and therefore, INO80 expression negatively correlates with lung cancer prognosis. In cervical cancer cells, inhibition of INO80 hinders cancer growth by initiating G1/G0 arrest, but does not affect migration and metastasis.

INO80 complexes can also contribute to cancer progression through their involvement in DNA repair, telomere stability, replication and transcription. INO80 interacts with YY1 for HR directed repair and is important for the recruitment of 53BP1, but not Rad51. This suggests that INO80 complexes have a role in the early stages of the DNA repair pathway. Deletion of either the INO80 ATPase or accessory subunit, ARP5, hampers the repair of UV-induced DNA lesions, which occurs due to the inability of DNA repair factors to assemble at the site of damage. Loss of the INO80 complex inhibits proliferation in a p21 dependent manner and creates defects in telomere structure.

INO80 has a known role in normal cells promoting progression of the replication fork through the chromatin template after stalling. This is mediated by an interaction between INO80 with CDC48, and together they evict RNA pol II from transcribed genes after a collision with the replication fork and mediate its degradation. Aberrant INO80 function preventing replication fork progression has the potential to jeopardize the completion of genomic DNA replication, and a subsequent loss of genome integrity that is likely to contribute to cancer progression.

Table 3 Summary of INO80 like complex subunits with an identified role in cancer as either a tumor suppressor and/or an oncogene

Gene	*Protein*	*Function*	*Tumor suppressor*	*Oncogene*
INO80	INO80	ATPase, loads H2A.Z-H2B dimers onto chromatin, translocates nucleosomes		Cervical, melanoma, NSCLC
SRCAP	SRCAP	ATPase, loads H2A.Z-H2B dimers onto chromatin	Bladder, colorectal, gastric, head and neck, lung, melanoma, nasopharyngeal, prostate, uterine	
EP400	p400	ATPase, loads H2A.Z-H2B dimers onto chromatin	Breast, colorectal, gastric, head and neck, lymphoma, prostate	
ERCC2	ERCC2/XPD	Role in transcription and UV damage and NER	Gastric, melanoma, uterine	
ACTR5	ARP5	Stimulates ATP hydrolysis and nucleosome sliding	Colorectal, gastric, melanoma	
KAT5	TIP60	p400 complex, acetyltransferase of H2A, H2A.Z, H2A.X, H4	Breast, head and neck squamous cell carcinoma, lymphoma, prostate	
TRRAP	TRRAP	p400 complex, required for MYC transformation, reduces proliferation gene, recruits p400 to sites of damage	Brain, melanoma	Brain
RUVBL1/2	RUVBL1/2	AAA+ ATPase, All INO80 like complexes, regulates TERT and proliferation genes		Colorectal, gastric, pancreatic

SRCAP complexes

Like INO80, Snf-2-related CREB-binding protein activator protein (SRCAP) complexes have a role in histone variant exchange. SRCAP is a known coactivator of transcription factors that interact with CBP and knockdown of SRCAP reduces the level of H2A.Z deposition within chromosomes. However, as H2A.Z is generally associated with active regions of the genome and de-condensed chromatin, it is interesting that SRCAP can be found at both active and inactive promoters. This suggests the SRCAP complex is poised at its target genes for activation upon the correct cellular signaling cascade.

Mutations in *SRCAP* have been identified in bladder, colorectal, gastric, head and neck, lung, melanoma, nasopharyngeal, prostate and uterine cancers ranging from 5% to 10% (**Table 3**) and colorectal cancers with mutated SRCAP demonstrate a high level of chromosome instability. In prostate cancer, SRCAP is a known coactivator of AR and directly targets PSA (*KLK3*). When SRCAP is lost in prostate cancer cells there is a subsequent decrease in the amount of H2A.Z enrichment at the *KLK3* promoter, a decrease in PSA gene expression and inhibited cell growth.

As with INO80 complexes, SRCAP can also contribute to cancer through the DNA damage pathway. SRCAP facilitates repair by exchanging H2A.Z-H2B dimers and is also important for the recruitment of RPA and Rad51 to break sites for DSB repair. In colorectal cancer, mutated SRCAP confers resistance to DNA damage and increases genomic instability.

p400 Complexes

p400 is a large complex with tumor suppressor activity in both primary human tumors and mouse cancer models. Mutations have been reported in head and neck, breast, prostate, colon and gastric cancers as well as lymphoma. Expression of the p400 complex is significantly down-regulated in breast cancer and in 61% of gastric cancers (**Table 3**), displaying a high correlation with invasion and metastasis in these cancers.

p400 has essential roles in transcription and DNA damage. It plays an important role in ER signaling in breast cancer, where p400 loads histone variant H2A.Z at ER responsive promoters. Inhibition of p400 and H2A.Z slows growth of breast cancer cells, and conversely over-expression of p400 increases proliferation. p400 is required for efficient DNA repair where its recruitment to sites of DNA damage is independent of ATM signaling. At DSBs p400 is required for RNF8-directed ubiquinitation of chromatin and the recruitment of repair factors BRCA1, 53BP1 and Rad51.

TIP60 is a lysine acetyl transferase (KAT), also known as KAT5, of the MYST family of acetyltransferases. TIP60 is only present in the p400 complex and not in other INO80-like complexes; its role is to acetylate H2A.Z, H2A.X, H2A and H4. TIP60 is frequently mutated in breast cancer, head and neck squamous cell carcinoma, lymphoma and prostate cancer (**Table 3**). Free H2A.Z is known to stably associate with TIP60 in the nucleus; H2A.ZK5ac strengthens this interaction and assists p400-driven deposition of H2A.Z-H2B dimers into chromatin. TIP60 is an essential part of the DNA damage response and is required to acetylate histone proteins at the site of damage, which facilitates the chromatin opening. Depletion of TIP60 reduces the rate and efficiency of DNA repair causing an accumulation of DSBs and widespread genome instability. After damage is repaired, TIP60 is required to acetylate H2A.X, which signals for H2A.X's removal from chromatin. TIP60 also has roles in transcription activation. It is a coactivator of AR and prostate cancer biopsies show TIP60 accumulates in the nucleus compared to normal prostate cells. In both xenografts and LNCaP (Lymph Node Cancer of the Prostate) cells withdrawal of androgens increases the levels of TIP60.

Accessory subunit of p400, Transformation/Transcription domain associated protein (TRRAP), has been reported as both a tumor suppressor and oncogene in brain cancer (**Table 3**). Loss of TRRAP reduces proliferation and increases sensitivity to apoptotic stimuli in brain tumors; however, TRRAP also interacts with MYC and E2F oncoproteins and is essential for MYC induced transformation. TRRAP helps recruit the p400 complex to sites of DNA damage, where TIP60 then normally acetylates histones, and

allows p400 to remodel chromatin into an open configuration allowing entry of repair machinery. TRRAP is also mutated in 4% of melanomas (Table 3).

RUVBL1 and RUVBL2

RUVBL1 and RUVBL2, also known as TIP48 and TIP49, are present in each of the INO80-like complexes. RUVBL1/2 are AAA+ ATPases with a hexamer ring structure and together they form a heterodimer. Depleting both these proteins in human cells and also in mouse models has shown they are essential for cellular proliferation. Studies have shown that cells in which RUVBL2 is knocked down are moderately more sensitive to anticancer therapies including cisplatin and 5-Aza-2′-deoxycytidine, and there is reduced cell growth after treatment.

Driver mutation events have to date not been identified in RUVBL1/2, but they are overexpressed in colorectal, gastric and pancreatic cancers contributing to cancer growth through a number of mechanisms (Table 3). Pancreatic ductal adenocarcinoma (PDAC) is known as an extremely invasive cancer that will metastasise early. Loss of RUVBL1 in PDAC reduces actin polymerization and the concentration of globular actin in the cell protrusions of migrating cells; reducing their invasiveness and motility. In NSCLC cells loss of RUVLB1/2 reduces growth and causes G2/M arrest, but there is no increase in apoptosis. There is also a role for RUVB1/2 in chromosome stability, where loss of either protein results in chromosome alignment and segregation defects, and disruption to the mitotic spindle assembly. In colon cancer, RUVBL2 regulates human telomerase reverse transcriptase (hTERT) and the increased expression of both of these is associated with advanced nodel disease. The RUVBL1/2 proteins also associate with several oncogenic factors such as c-Myc and E2F1, and several proteins in the DNA damage repair pathway to drive cancer progression.

CHD

The ATPases of this remodeling family are highly diverse, with three subfamilies found in humans. Class I contains CHD1 and CHD2, Class II is CHD3 and CHD4, which form part of the NuRD complex and CHD5, and Class III comprises of CHD6-CHD9. All CHD ATPase remodelers contain a SNF2-like ATPase domain and tandem chromodomains, but vary in their other protein domains. CHD1 and CHD2 have a DNA binding domain located towards the c-terminal; CHD3, CHD4 and CHD5 have PHD Zinc-finger-like domains, and CHD5 also contains a DEAH-box domain; CHD6 has a Pdxk domain with pyridoxal kinase function and CHD7-CHD9 have BRK domains with unknown function. As more is known about the precise role of the NuRD complex in cancer, this section will first discuss the NuRD complex and key subunits within it, followed by the remaining CHD ATPases.

NuRD complex

The Nucleosome Remodeling Deacetylase (NuRD, also known as Mi-2) complex is highly conserved from plants to animals and was first described for its role in gene repression. It is now known that it's roles in chromatin biology extend to transcriptional activation, DNA repair, replication and genome stability—all processes that must be tightly regulated to ensure proper cell function and to inhibit cancer progression. NuRD has more specific roles in development including hematopoietic stem cell maintenance and differentiation, controlling key genes in B- and T-cell development. The NuRD complex contains two catalytic subunits; an ATPase that is either one of the mutually exclusive CHD3 or CHD4, as well as either one of histone deacetylase 1 (HDAC1) or 2 (HDAC2). Other key subunits include the methyl-CpG-binding domain 2 (MBD2) and 3 (MBD3), metastasis-associated gene 1 (MTA1), 2 (MTA2) and 3 (MTA3) and retinoblastoma-binding protein 4 (RBBP4) and 7 (RBBP7).

Like SWI/SNF, the NuRD complex both promotes and represses tumor development, which is dependent on subunit composition and cell type. In addition to mutations, many subunits of this complex can contain PTMs and perturbed remodeling function may arise from disruption of this. Several lines of evidence have demonstrated that the NuRD complex associates with oncogenic transcription factors to promote the repression of tumor suppressors, as well as interacting with known fusion genes. Promyelocytic leukemia (PML)—retinoic acid receptor-α (RARα) is a fusion oncoprotein transcription factor that recruits the NuRD complex to its target loci through protein-protein interactions in order to repress tumor suppressors such as retinoic acid receptor β2. As stated above, in other cases the NuRD complex itself directly functions as a tumor suppressor.

The NuRD complex has an essential role in proliferation and is implicated in the regulation of both the G1/S and G2/M checkpoints. Knockdown of either CHD4 or MTA1 blocks G1/S transition and G2/M progression is inhibited by unrepaired DNA damage following CHD4 loss. NuRD can also prevent deacetylation of p53, which results in increased p21 levels and cell cycle arrest. In lymphocytes, NuRD foci have been observed at the pericentromeric heterochromatin of chromosomes 1, 9 and 16 during S-phase. The NuRD foci correlate with proteins associated with active replication forks such as PCNA and CAF1, suggesting a role for NuRD in assembly, stability and chromatin condensation at these regions. Interestingly, PcG complexes are usually observed at these pericentromeric heterochromatin regions, but are absent in lymphocytes with NuRD foci, suggesting a unique cell specific role for the NuRD complex.

CHD3 and CHD4

CHD3 and CHD4 are highly similar in structure and are the alternative ATPase subunits of the NuRD complex. They are both known to interact with transcription factors and cofactors in order to direct the NuRD complex to its target sites. The PHD domains of CHD3/4 recognize the tails of H3, enabling transcriptional regulation; interestingly, while H3K9me3 enhances the binding of these

remodelers to chromatin, H3K4me3 abolishes this binding. CHD4 complexes that contain HDAC1 are shown to mediate transcriptional repression via colocalisation with DNA methyltransferase 1 (DNMT1) at tumor suppressor gene promoters.

CHD3 and CHD4 are known to interact with several regulatory factors to coordinate transcriptional control in cancer. ZIP, a transcriptional corepressor for cell proliferation, survival and migration pathways, preferentially interacts with CHD3/4 from the NuRD complex in breast cancer and when ZIP is lost there is aggressive tumor growth in mouse xenografts. A similar role has been reported for CHD3/4 binding to ZGPAT in breast cancer, which also represses genes for cellular proliferation, survival and migration and when interacting with NGFI-A Binding Protein 2 (NAB2), CHD3/4 repress early growth response 1 (EGR1) activities. Although they share highly similar functions for transcriptional control, CHD3 and CHD4 have differing roles in DNA damage. At sites of DSBs, KAP1 phosphorylation disrupts the interaction with and releases CHD3 from chromatin, while CHD4 is rapidly recruitment to site of damage.

There are limited studies on the effects of CHD3 mutations in cancer. However, there are reports of mutations in colorectal, gastric and prostate cancers (**Table 4**). In ovarian cancer, CHD3 is silenced due to DNA hypermethylation of its promoter, which is correlated with tumor resistance to platinum-based therapies, more invasive tumors and an EMT phenotype. It has been proposed that CHD3 would be a useful prognostic marker for ovarian cancers, but it remains unclear whether this is likely to extend to other types of cancer.

Table 4 Summary of CHD and NuRD complex subunits with an identified role in cancer as either a tumor suppressor and/or an oncogene

Gene	*Protein*	*Function*	*Tumor suppressor*	*Oncogene*
CHD1	CHD1	ATPase, promotes HR over NHEJ	Colorectal, gastric, prostate	
CHD2	CHD2	ATPase, deposits H3.3 onto chromatin	Bladder, chronic lymphoblastic leukemia's, colorectal, gastric, monoclonal B lymphocytosis, uterine	
CHD3	CHD3	NuRD complex, ATPase, H3 tail recognition	Bladder, colorectal, gastric, lung, melanoma, ovarian, prostate, uterine	
CHD4	CHD4	NuRD complex, ATPase, H3 tail recognition, DNA damage response	Bladder, colorectal, endometrial, gastric, lung, uterine	
CHD5	CHD5	ATPase, binds unmodified H3 tails, forms NuRD like complex	Breast, colorectal, gallbladder, gastric, glioblastoma, head and neck, heptacellular, laryngeal squamous cell carcinoma, leukemia, lung, melanoma, neuroblastoma, ovarian, pancreatic, renal cell carcinoma	
CHD6	CHD6	ATPase, transcription activation	Colorectal, gastric, lung, melanoma, prostate, transitional cell bladder, prostate, uterine	
CHD7	CHD7	ATPase, transcription activation	Colorectal, gastric, lung, medulloblastoma, melanoma	Breast, pancreatic
CHD8	CHD8	ATPase, establishes heterochromatin to euchromatin boundaries	Breast, colorectal, esophagus, gastric, lung, prostate, uterine	Endometrial, ovarian, prostate
CHD9	CHD9	ATPase	Colorectal, gastric, lung, melanoma, neuroblastoma, uterine	
MBD2	MBD2	NuRD complex, binds methylated DNA, mutually exclusive with MBD3	Gastric, colorectal, prostate	Brain, breast, colorectal
MBD3	MBD3	NuRD complex, binds unmethylated DNA, mutually exclusive with MBD2	Colorectal, endometrial, gastric, pancreatic	Brain
MTA1	MTA1	NuRD complex, mutually exclusive with MTA2 and MTA3, binds transcription factors		Breast, endometrial, esophageal, hepatocellular, lung, lymphoma, ovarian, pancreatic, prostate
MTA2	MTA2	NuRD complex, mutually exclusive with MTA1 and MTA3, binds transcription factors		Breast
MTA3	MTA3	NuRD complex, mutually exclusive with MTA1 and MTA2, binds transcription factors	B-cell lymphomas, breast, ovarian, prostate	
HDAC1/2	HDAC1/2	NuRD complex, Histone deacetylase		Breast, cervical, colorectal, endometrial, gastric, hepatocellular, lung, pancreatic, prostate, thyroid
RBBP4	RBBP4	NuRD complex, provides structural support	Lung, prostate	
RBBP4	RBBP4	NuRD complex, provides structural support	Cholangiocarcinomas	

CHD4 mutations have been found in a variety of cancers. It is mutated in 17% of endometrial cancer and 23.7% uterine carcinomas with these mutations predicted to impair function (Table 4). Loss of CHD4 also occurs in gastric and colorectal cancers that have microsatellite instability. The contribution of CHD4 to cancer progression is intriguingly diverse. Following DSB breaks, CHD4 is rapidly recruited to sites of damage, and its activity is dependent on binding to the PARP complex. Phosphorylation of CHD4 is then catalyzed by ATM enabling CHD4 to interact with RNF8 to mediated chromatin unfolding. Mutations in CHD4 at S1349—the site of phosphorylation—reduces its association with chromatin at break sites. Similarly, depletion of CHD4 results in hypersensitivity to DNA damage and accumulation of unrepaired breaks. CHD4 is also important for the cell cycle, where its loss results in cell cycle delay through the G2/M checkpoint.

MBD2 and MBD3

MBD proteins recognize methylated or unmethylated DNA and provide a mechanism for crosstalk to occur between the DNA sequence and chromatin. DNA methylation primarily follows a CpG context and can occur either in response to chromatin compaction or provide the signal that chromatin compaction is required. CpG sites are not distributed evenly throughout the genome; instead, they tend to be clustered into regions of high CpG density, called CpG Islands. CpG Islands are generally found at the promoters of ubiquitously expressed genes and are unmethylated in normal somatic cells. DNA methylation is frequently and ubiquitously disrupted in cancers and is characterized by genome-wide DNA hypomethylation, and site-specific DNA hypermethylation. Promoter DNA hypermethylation, particularly at CpG Islands, is associated with tumor suppressor transcriptional silencing.

MBD2 and MBD3 are mutually exclusive subunits of the NuRD complex. They share 71% sequence homology; however, MBD2 is capable of binding to methylated DNA, while MBD3 is not. This difference is due to two amino acid changes in the methyl-binding domain; K30 to H30, and Y34 to F34. Interestingly, MBD2 knockout causes only mild defects in cells, while MBD3 loss is embryonically lethal. Mutations in these proteins have been found in neurological disorders and tend to be truncating missense mutations.

MBD2 has a prominent role in suppressing transcription and over-expression induces gene silencing across the genome. Overlapping with the methyl-binding domain (MBD) is a transcriptional repressor domain (TRD)—that is absent from MBD3—suggesting that the methyl- binding and repressive functions of MBD2 are tightly connected. MBD2 is up-regulated in colorectal cancer cell lines, breast cancer and in brain cancer, but down-regulated in prostate and gastrointestinal cancers (Table 4). MBD2 contributes to tumor development by silencing the tumor suppressor genes: *GSTP1* in prostate cancer, *CDKN2A* in colorectal cancer, and *hTERT* in the HeLa cell line by promoter DNA hypermethylation. It has been demonstrated in cell lines that the knockdown of MBD2 allows for the re-expression of tumor suppressors p16INK4A and p14ARF. Furthermore, MBD2 depletion within the NuRD complex increases chromatin accessibility.

MBD3 is normally enriched at unmethylated promoters and enhancers, containing the active marks H3K4me3 and histone 3 lysine 27 acetylation (H3K27ac) respectively. It is also reported to bind to 5-hydroxymethylated regions in brain tissue and in ES cells. MBD3 has inactivating mutations in cancer and is down-regulated in gastrointestinal, endometrial and pancreatic cancer. However, there are reports that it is up-regulated in brain tumors. MBD3 can function as a tumor suppressor, demonstrated by it binding to the un-phosphorylated JUN oncoprotein to repress JUN's transcriptional activity. When MBD3 is inactivated in mice, the expression of JUN target genes increases, leading to hyper-proliferation and susceptibility to tumor development. Functional studies to understand the oncogenic roles of MBD3 found it recruits HDACs to silence tumor suppressor promoters including *CDKN1A*, and interactions with the proto-oncogene, *FBI-1* in transformed cell lines.

MTA1, MTA2 and MTA3

The functions of the MTA subunits are very well studied in cancer development. Each subunit is associated with transcription factor binding but mutually exclusive within the NuRD complex. Within the MTA family there are three alternate subunits: MTA1, MTA2 and MTA3. MTA1 and MTA2 promote cancer development, while MTA3 is a tumor suppressor (Table 4).

Increased MTA1 levels have been detected in cancers of breast, esophageal, endometrial, pancreatic, ovarian, lunge, prostate, liver and in lymphoma and is positively correlated with higher tumor grade, invasion and poor prognosis. This is in part due to activation of MTA1 by the MYC oncoprotein, which requires MTA1 for transformation of cells. In breast cancer, up-regulation of the HER2 pathway increases MTA1 expression, which then interacts with ER to suppress transcription, including the BRCA1 gene. MTA1 has also been linked to breast cancer through tumor hypoxia, since hypoxia induces MTA1 expression to turn on hypoxia-inducible factor 1α (HIF1α) promoting angiogenesis. MTA1 can also inactivate p53 inhibiting growth arrest and apoptosis.

Like MTA1, MTA2 is known to promote breast cancer development (Table 4). In these cancers, MTA2-containing NuRD complexes associate with TWIST, a key player in EMT and metastasis, and it is this association that causes subsequent repression of *CDH1*, promoting EMT. MTA2 also interacts with the ER receptor and MYC to promote breast cancer development.

MTA3 has opposing roles to both MTA1 and MTA2, acting as a tumor suppressor. Mutations have been reported in breast, ovarian and prostate cancers. MTA3 inhibits EMT through the suppression of SNAIL, a factor known to decrease cell adhesion and increase motility. MTA3 is also required for BCL-6 repression of genes involved in plasma cell differentiation in B-cell lymphomas.

HDAC1 and HDAC2

HDAC1 and HDAC2 are the only two histone deacetylases to form a part of the NuRD complex and share 80% sequence homology. As they can also form part of the CoREST and SIN3 complexes that are both associated with transcriptional repression, it can make

their role within the NuRD complex difficult to determine. It is unknown whether NuRD complexes containing HDAC1 or HDAC2 work synergistically or on different regions of the genome. However, it is clear that they have differing roles in development where HDAC1 is essential for stem cell survival and is embryonically lethal, while HDAC2 is not.

HDAC1/2 have a low frequency of mutations, but have a tendency to be over-expressed in cancers of the colon, breast, prostate, thyroid, cervical, gastric, hepatocellular carcinoma, pancreatic, endometrial and lung cancers (Table 4). A negative correlation with overall survival has been reported in colorectal cancer, hepatocellular carcinoma, pancreatic, endometrial, lung cancer. Additionally, an association with advanced disease, metastasis and aggressive disease has been found in hepatocellular carcinoma, colorectal cancer, prostate, NSCLC, breast cancer. Furthermore, HDAC2 truncating mutations have been reported in cancers with microsatellite instability, with these cells more resistant to the antiproliferative and pro-apoptotic effects of HDAC inhibitors.

As with most chromatin remodeling complexes, HDAC1/2 has roles in transcription and promotion of cancer development pathways. HDAC1/2 over-expression results in increased cell proliferation, migration, angiogenesis and invasion, and decreased apoptosis. HDAC1 is known to promote proliferation of breast cancers by repressing ER transcription. Loss of HDAC1/2 causes arrest in G1 or in G2/M and reduces proliferation in breast cancer.

RBBP4 and RBBP7

The RBBP proteins directly associate with histone tails. Like HDACs, they can also be subunits within other complexes making their role complicated to understand. It is assumed that these subunits provide structural support and promote protein-protein interactions within the NuRD complex. RBBP4 is mutated in approximately 5% of lung and prostate cancers, and RBBP7 is mutated in 12.5% of cholangiocarcinomas (Table 4). Functional studies in LNCaP prostate cancer cells have shown that over-expression of either RBBP4 or RBBP7 results in reduced proliferation.

CHD5

More recently, CHD5—also a Class II CHD ATPase—has been demonstrated to form NuRD-like complexes containing each of the main subunits detailed above. CHD5 preferentially binds to unmodified H3K4 tails via it's dual PHD domains, but can also bind H3K27me3 through its tandem chromodomains. CHD5 is predominately expressed in neurons and the testis; however, low expression has been associated with poorer survival and advanced tumors in a variety of cancer tissue types. Mutations in key residues of CHD5 prevent it binding to H3, and in turn, prevent it from inhibiting tumor growth. Several tumors including breast, lung, colorectal cancer, ovarian, gastric, laryngeal squamous cell carcinoma, gall bladder, hepatocellular carcinoma, pancreatic, leukemia and renal cell carcinoma (Table 4), have demonstrated significant hypermethylation at the CHD5 promoter, concomitant with a decrease in expression. Several of these studies demonstrate that CHD5 expression can be restored with 5-Aza-2′-deoxycytidine treatment, which reduced cellular proliferation. Deletion of the 1p36 chromosome locus containing CHD5 has also been found in glioblastoma, neuroblastoma, renal cell carcinoma and ovarian cancer.

CHD5 suppresses genes involved in motility, proliferation, apoptosis, EMT markers and epigenetic regulators. CHD5 also has a role in the DNA damage response with an increase in γH2A.X associated with CHD5 down-regulation in pancreatic cancer. CHD5 controls proliferation through activation of *CDKN2A* to mediate Rb and p53 pathways.

Class I and class III CHD ATPases

CHD1 is a tumor suppressor and plays a significant role in the development of prostate cancer. CHD1 is located at 5q21, and deletions within this region are one of the most frequent to occur in prostate cancer, second only to PTEN. Approximately 17% of prostate cancers contain a CHD1 mutation or deletion (Table 4). CHD1 has a synthetic-lethal relationship with PTEN, where in PTEN-deficient prostate cancer, CHD1 is stabilized. Loss of CHD1 in these cancers reduces proliferation and survival of cells supporting the hypothesis that CHD1 is important in the early stages of prostate cancer development. Deletion of CHD1 in mouse prostate epithelial cells results in morphological changes and increased invasiveness, but not transformation. CHD1 loss has also been linked to PSA failure and ERG-fusion absence and is required for androgen receptor (AR) transcription and regulates tumor suppressor genes including NKX3–1, FOXO1 and PPARγ. Prostate cancer patients with CHD1 depletion are more sensitive to PARP inhibitors that target tumors with HR DSB defects. It has been suggested that CHD1 is a prognostic marker for stratification of prostate cancer patients for PARP inhibitor therapeutics. CHD1 is also mutated in 5%–10% of cancers of the digestive tract. (Table 4).

CHD1 deletion affects HR DSB repair but not NHEJ. CHD1 is required to open chromatin around the break site, in order to facilitate HR repair protein entry. When CHD1 is depleted in cancer, the tumors switch from utilizing HR to NHEJ mechanisms, leading to increased error prone repair. CHD1 depleted cancers tend to have increased genome instability, concomitant with increased chromosome deletions, particularly on chromosomes 2q, 5q and 6q.

Like CHD1, CHD2 is also a tumor suppressor, but less information about its role in cancer exists. Mutations in CHD2 have been reported in cancers of the digestive tract ranging from 5% to 10%, 5.3% in chronic lymphoblastic leukemia's (CLL) and 7% of monoclonal B lymphocytosis that can evolve to CLL (Table 4). The majority of known mutations are truncating, or occurring in a functional domain. Functional studies following CHD2 depletion in mice have shown heterozygous mutations increase their susceptibility to lymphoma due to the abnormal differentiation of stem cells. Loss of CHD2 also decreases the level of H3.3 deposition in chromatin at promoters and at sites of DNA damage. Cells containing CHD2 mutations also have a higher level of γH2A.X remaining after damage and an aberrant DNA damage response after x-ray radiation. Furthermore, loss of functional CHD2 results in growth and viability defects.

CHD6 was discovered in 2002 and studies of the role of CHD6 in cancer are also limited. Mutations have been reported in 19.5% of colorectal cancers and 7% in transitional cell bladder cancer (Table 4). CHD6 has a high affinity for short linker DNA, down to 20 bp and disrupts nucleosomes in a nonsliding mechanism. CHD6 colocalizes with hypo- and hyper- phosphorylated forms of RNA PolII and is present at the sites of RNA synthesis, suggesting that it positively regulates transcription.

CHD7 has been reported as both a tumor suppressor and an oncogene. CHD7 is enriched at sites marked by H3K4me1/2, located near the TSS and near DNase hypersensitive sites, suggesting a pro-transcriptional role. Mutations have been reported in 11.1% of gastric cancers and 42% of colorectal cancer cases (Table 4). CHD7 mutations have been linked with lung cancer in heavy smokers and a number of lung cancer cell lines display PVT1-CHD7 fusion genes. CHD7 has been described as an oncogene and associated with more aggressive subtypes in breast cancer. Deletion of CHD7 in breast cancer cell lines inhibits proliferation and is associated with down-regulation of known oncogenes, such as NRAS. In pancreatic cancer, patients with low CHD7 expression are more sensitized to gemcitabine, the primary treatment for these cancers, and have increased overall survival. CHD7 could therefore be used to stratify patients predicted to have a positive response to gemcitabine in pancreatic cancer.

CHD8 plays a role in both transcription and at the boundaries between heterochromatin and euchromatin. CHD8 binds to active promoters in the G1/S transition and is required for the loading of E2F1 and E2F3 onto genes expressed exclusively during S-phase; MLL recruitment to these genes is impaired in the absence of CHD8. CHD8 is a marker of aggressive gastric cancer, with 15.9% of cases containing mutations and 35.75% exhibiting with a loss of expression. In these cancers, CHD8 regulates b-catenin/Wnt pathways and low expression of CHD6 is considered to be a prognostic marker for poor outcome. CHD8 has a bimodal role in prostate cancer; the CHD8 promoter is hypermethylated in 45% of cases, but high CHD8 expression results in poor clinical outcomes and metastasis. Conversely, CHD8 is a coregulator of androgen dependent transcription and loss of CHD8 is sufficient to stimulate proliferation of prostate cancer. CHD8 mutations are associated with increased risk of cancer development in those carrying familial BRCA1 mutations. In ovarian cancer, amplification of the NSD3-CHD8-BRD4 pathway is associated with worse survival rates. This amplification also occurs in 9% of serous endometrial cancer and again, is associated with worse overall survival. In AML, these proteins colocalize across the genome and are released from chromatin by BET inhibitor treatment.

Little is known about the role of CHD9 in cancer, though a study of neuroblastoma found mutations in approximately 4% of patients. Down-regulation of CHD9 in these cancers was correlated with increased bone metastasis and decreased patient survival (Table 4). The mutation sites identified were close to a phosphorylation site for CHD9, potentially inhibiting the ability of this remodeler to be phosphorylated and therefore, for CHD9 to be activated. CHD9 is also mutated in cancers of the digestive tract, including 11.1% of gastric cancers. To date, growing evidence suggests that CHD9 is a tumor suppressor.

ATRX

ATRX an ATP-dependent chromatin remodeler with high homology to SWI/SNF and with roles in genome stability, DNA damage and heterochromatin formation. ATRX forms a complex with DAXX, a chaperone of histone H3.3. Somatic mutations in ATRX have been identified in brain tumors and osteosarcomas, and are positively correlated with increased rates of single nucleotide variation in cancer. ATRX binds to the 3′ exons of zinc finger genes to maintain genomic stability and also has a major role in the formation of heterochromatin. ATRX deposits the histone variant H3.3 at pericentric heterochromatin and at telomeres to maintain H3K9me3 enrichment. Reduced ATRX is associated with loss of H3K9me3 and telomere lengthening—a feature of many cancers. ATRX also maintains the silencing of imprinted genes and retrotransposons through the same mechanism.

Concluding Remarks

Chromatin modifying proteins have an integral role within a cell to maintain proper chromatin integrity and genome stability (Fig. 4). Chromatin remodelers and modifiers control access to the genes needed for development and progression of cancer. In normal cells, there is tight regulation around these genes and disruption to chromatin remodelers in cancer either by way of mutation and/or changes in expression driven by epigenetic mechanisms can lead to the altered chromatin structure. DNA is therefore extremely vulnerable to further mutations from damaging agents, either intra- (e.g., DNA methylation) or extra- cellular (e.g., UV damage). Particularly, the unrestricted access to genes due to global chromatin instability and "loosening" is all that cancer cells need to gain a simple proliferative and survival advantage. Aberrant function of chromatin remodelers is known to directly result in tumor development, and many cancer types contain mutations in at least one chromatin remodeler.

Prospective Vision

Moving forward, it will be particularly important to characterize the role of epigenetic mechanisms in driving atypical changes in chromatin remodeler expression. Moreover, it will be essential to determine whether epigenetic reprogramming of the cancer genomes causes additional mutations affecting chromatin remodeler activity. Future study into the role of ncRNA targeting chromatin remodellers to the genome is an exciting area of development. ncRNA is ideal for this purpose due to the sequence specificity and the dynamic secondary structure. DNA methylation and activity of the APOBEC family of cytidine deaminases involved in C to U mRNA repair may be of particular interest as the field seeks to characterize the evolution of chromatin remodeller mutations over

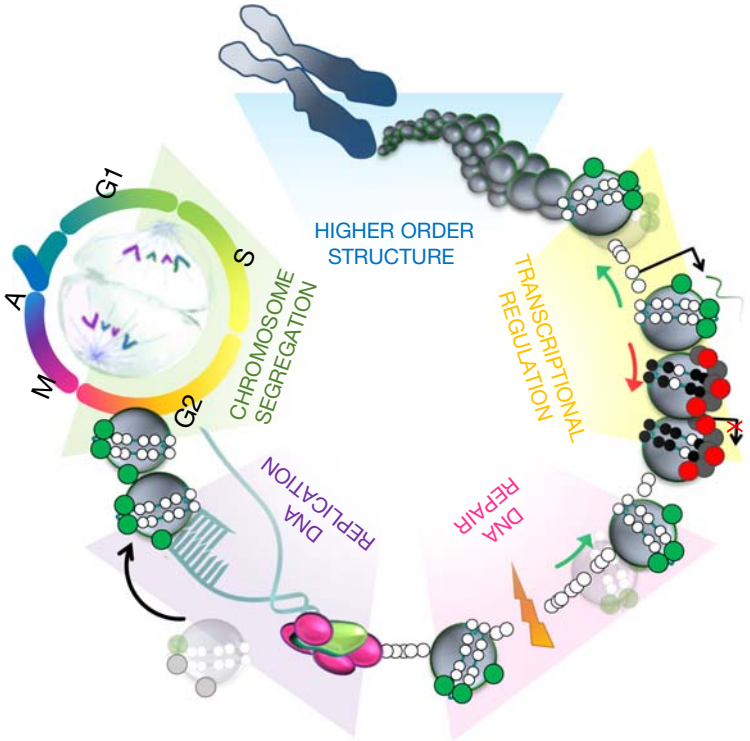

Fig. 4 *The mutation or disruption of chromatin remodellers has the potential to reduce genome integrity in cancer.* Atypical expression or activity of chromatin remodelers by mutation or epigenetic reprogramming has the potential to alter higher order chromatin structures, including enhancer-promoter interactions by DNA looping; transcriptional regulation (abnormal gene expression patterns); DNA repair (misrepair or incomplete repair), DNA replication (unfaithful) and chromosome segregation (impaired) from the mitotic spindle during the cell cycle. Nucleosomes, *large gray circles*; CpG sites, *small white circles*; active histone posttranslational modifications, *green circles*; repressive posttranslational histone modifications, *red circles*; transcriptional start site, *arrow*.

the course of cancer progression. The interplay between chromatin remodellers and the epigenome is likely to be complex, and bi-directional. The influence of somatic chromatin remodeller mutations on the epigenome are likely to hold key clues to cancer etiology and identification of the most promising epigenetic therapies.

See also: Mutations in DNA Methyltransferases and Demethylases. Mutations in Histone Lysine Methyltransferases and Demethylases.

Further Reading

Brachmann, R.K., Davis, P.K., 2003. Chromatin remodeling and cancer. Cancer Biology & Therapy 2 (1), 23–30.
Chen, J., Herlong, F.H., Stroehlein, J.R., Mishra, L., 2016. Mutations of chromatin structure regulating genes in human malignancies. Current Protein and Peptide Science 17, 411–437.
Clapier, C.R., Iwasa, J., Cairns, B.R., Peterson, C.L., 2017. Mechanisms of action and regulation of ATP-dependent chromatin-remodeling complexes. Nature Reviews Molecular Cell Biology 18, 407–422.
Deindl, S., Hwang, W.L., Hota, S.K., et al., 2013. ISWI remodelers slide nucleosomes with coordinated multi-base-pair entry steps and single-base-pair exit steps. Cell 152 (3), 442–452.
Lai, A.Y., Wade, P.A., 2011. Cancer biology and NuRD: A multifaceted chromatin remodeling complex. Nature Reviews Cancer 11, 588–596.
Nair, S.S., Kumar, R., 2012. Chromatin remodeling in cancer: A gateway to regulate gene transcription. Molecular Oncology 6 (6), 611–619.
Mayers, K., Qiu, Z., Alhazmi, A., Landry, J.W., 2014. ATP-dependent chromatin remodeling complexes as novel targets for cancer therapy. Advanced Cancer Research 121, 183–233.
Marfella, C.G.A., Imbalzano, A.N., 2007. The CHD family of chromatin remodelers. Mutation Research 618 (1–2), 30–40.
Skulte, K.A., Phan, L., Clark, S.J., Taberlay, P.C., 2014. Chromatin remodeler mutations in human cancers: Epigenetic implications. Epigenomics 6 (4), 397–414.
Watanabe, R., Kanno, S., Roushandeh, A.M., Ui, A., Yasui, A., 2017. Nucleosome remodeling, DNA repair and transcriptional regulation build negative feedback loops in cancer and cellular aging. Philosophical Transactions of the Royal Society B 371, 1731.
Wilson, B.G., Roberts, C.W.M., 2011. SWI/SNF nucleosome remodelers and cancer. Nature Reviews Cancer 11, 481–492.

Mutations in DNA Methyltransferases and Demethylases

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Glossary

CpG islands Genomic regions with a high frequency of CpG sites. In mammalian genomes, CpG islands are typically 200–3000 base pairs in length, less methylated than other CpG sites cross the genome, and many of them are associated with gene promoters and play a role in gene transcriptional regulation.

Epigenetics Stable heritable traits that cannot be explained by changes in DNA sequence. The epigenetic inheritance is maintained through cell divisions and passed from one cell generation to the next even though they do not involve changes in the DNA sequence of the organism. The known mechanisms of epigenetic inheritance involve regulation of gene activity and expression by covalent modifications of chromatin (DNA and histones), nucleosome positioning, RNA transcripts, and prions, etc.

Hematopoiesis The process of generating all types of blood cellular components from hematopoietic stem cells (HSCs). This process involves the maintenance of the HSCs by a self-renewal mechanism and step-wise differentiation from HSCs into different lineages of mature blood cells. The cell fate determination during hematopoiesis is controlled by cellular autonomous mechanisms, which include epigenetic regulation, transcription factors and signaling pathways, as well as extracellular signals induced by cytokines and cell–cell interactions.

Leukemia A group of hematopoietic cancers that usually begin in the bone marrow and result in high numbers of abnormal white blood cells which are not fully differentiated. The major types of leukemia include acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL).

Myelodysplastic syndromes (MDS) A heterogeneous group of hematopoietic malignancies characterized by impaired peripheral blood cell production and most commonly a hypercellular, dysplastic-appearing bone marrow.

Tricarboxylic acid (TCA) cycle A looped metabolic pathway that consists of series of enzyme-catalyzed chemical reactions occurred in living cells to release energy through oxidation of acetate, in the form of acetyl-CoA, into carbon dioxide and water. It is a key metabolic pathway that connects carbohydrate, fat, and protein metabolism, as it provides precursors of certain amino acids, as well as the reducing agent NADH that are used in numerous other biochemical reactions. It is also known as citric acid cycle (CAC) and the Krebs cycle.

Introduction

Recent cancer genomic studies have led to the discovery of many previously unrecognized cancer-causing genes. Functional classification of these genes facilitates us to pinpoint essential cellular regulatory processes that contribute to tumorigenesis. DNA methylation is one of such processes, since several newly identified mutated genes in cancers encode enzymes responsible for "writing" and "erasing" an important epigenetic mark of the genome—5-methylcytosine (5mC; i.e., methylation of the DNA base cytosine at the 5th atom in the pyrimidine ring). DNA methylation plays important roles in many biological processes in mammalians, including transcriptional repression, genomic imprinting and X chromosome inactivation, as well as cell fate decision. As illustrated in Fig. 1, multiple members of three families of enzymes that control the key steps of DNA methylation and demethylation have been found being recurrently mutated in cancer: (1) DNMT3A belongs to a family of DNA methyltransferases (DNMTs) that transfer the methyl group from *S*-adenosyl methionine (SAM) to cytosine. (2) TET2 (ten-eleven translocation 2) belongs to a family of TET dioxygenases that convert the 5mC to 5-hydroxymethylcytosine (5hmC) through a hydroxylation reaction, followed by further processing to complete the demethylation. (3) IDH1 and IDH2 belong to a family of isocitrate dehydrogenases (IDHs) that convert isocitrate to α-ketoglutarate, which is an essential co-factor for the TETs. Thus, DNMTs and TETs act as "writers" and "erasers" of DNA methylation, respectively, whereas IDHs are important co-regulators; mutations in these enzymes cause aberrant DNA methylation patterns in cancer. Accumulating studies seek to explore the mechanism of how the mutations in these enzymes contribute to tumorigenesis and to identify potential new therapeutics to treat cancer. In this article, we will introduce the basic concepts and newly acquired knowledge regarding the functions of the DNA methyltransferases and demethylases in development and cancer. An adequate understanding of the underlying mechanisms should lead to identification of new therapeutic strategies for cancer treatment.

Location and Biological Functions of DNA Methylation

Location of DNA Methylation in Human Genome

In mammals, the majority of 5mCs occurs on the cytosine nucleotide followed by a guanine nucleotide (CpG site), although few non-CpG 5mCs have also been observed in specific cell types such as embryonic stem cells, some neural cells, and hematopoietic

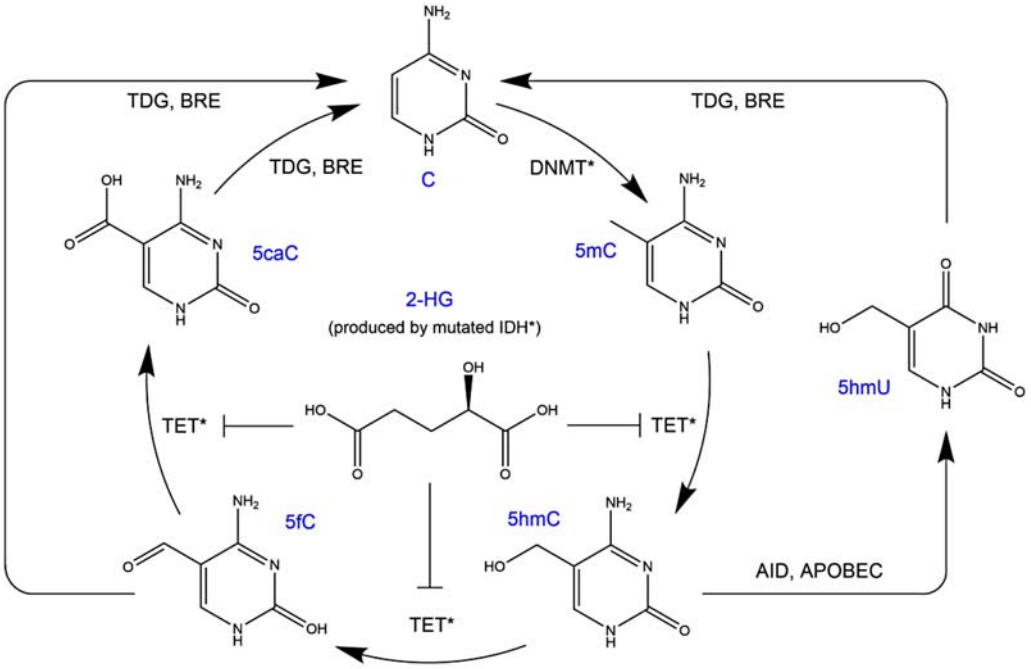

Fig. 1 Mutated enzymes in the pathway of DNA methylation and demethylation. Asterisks denote the enzymes known to be affected by somatic mutations in cancer. The DNA methyltransferases (DNMTs) catalyze DNA methylation by transferring a methyl group to cytosine (C) to form 5-methylcytosine (5mC). The TET methylcytosine dioxygenases oxidize 5mC to 5-hydroxymethylcytosine (5hmC), and further oxidation reactions results in the successive conversion of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). The 5hmC can also be deaminated to 5-hydroxymethyluracil (5hmU) by AID/APOBEC (activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex). These oxidation products can be removed by the TDG (thymine DNA glycosylase) and BRE (repaired by the base excision repair) enzymes, returning to an unmodified cytosine. Activities of the TETs are dependent on α-ketoglutarate (α-KG), the natural product of isocitrate dehydrogenases (IDHs), but are inhibited by 2-hydroxyglutarate (2-HG), the product of cancer-associated IDH mutants.

progenitors. Mammalian genomes are depleted of CpG sites, probably because 5mC can be spontaneously deaminated to thymine and therefore is vulnerable to mutagenic potentials. Indeed, the total number of CpG sites in humans is approximately 28 million, which is much lower than what is expected according to the average GC content of human genome. The CpG sites in the genome are overall heavily methylated, except for some specific genomic regions such as CpG islands. In different genomic regions, the localization pattern, regulatory mechanism and biological function of the methylated CpG sites could be different (Fig. 2).

In the specific genomic regions named CpG islands, which have a higher CpG density than the rest of the genome, the CpG sites are much less methylated. As many mammalian genes have CpG islands associated with their promoters, the 5mCs in CpG islands play an important role in gene transcriptional regulation. It has been shown that the presence of multiple methylated CpG sites in the CpG islands results in silencing of gene expression. Given the relative stability of 5mC, the gene silencing mediated by methylation of CpG islands is usually stable. This mechanism is particularly important for genomic imprinting, an epigenetic phenomenon in which certain genes are expressed from only one of the two parental chromosomes and their expression is determined in a parent-of-origin specific manner. Besides the imprinted genes, 5mCs also regulate gene expression in development and tumorigenesis. Notably, however, the CpG islands associated with gene promoters rarely show tissue-specific methylation pattern. Instead, the differences of CpG methylation between tissues, or between normal and cancer samples, are observed in the regions called CpG island shores, located a short distance from the CpG islands.

The CpG methylation also locates in the gene body, and its enrichment in these regions is positively correlated with gene expression levels. In mouse embryonic stem cells, this distribution pattern has been shown to be established and maintained through a direct recognition of histone lysine 36 trimethylation (H3K36me3) by the Pro-Trp-Trp-Pro (PWWP) domain of the DNMT3B. Given the high similarity between DNMT3A and DNMT3B, DNMT3A should be able to recognize H3K36me3 as well, though probably in different cell types and/or genome sites. The intragenic DNA methylation has been suggested to play a role in repression of cryptic transcriptional initiation and regulation of splicing, although the detailed mechanisms remain to be further clarified.

DNA methylation plays an important role in permanent silencing of transposable and viral elements, which comprise approximately 45% of the human genome. If expressed, these elements are potentially harmful to the cell. However, it is interesting to note that these elements may also provide a resource for stimulating immune responses. Indeed, recent studies have shown that some

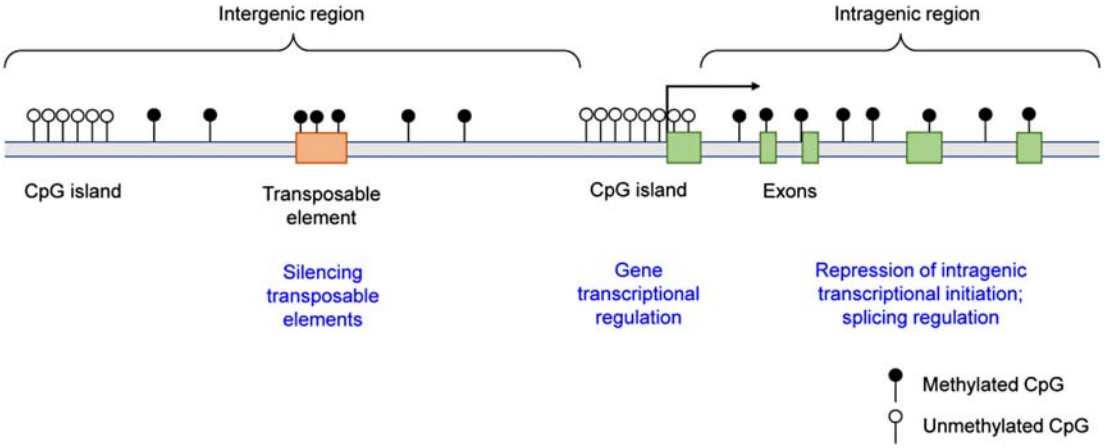

Fig. 2 Localization and basic functions of DNA methylation in human genome. The majority of DNA methylation (5mC) occurs on the CpG sites, and most CpG sites tend to be methylated, except for the CpG islands, which are less methylated. The CpG islands associated with gene promoters play an important role in transcriptional regulation, whereas those located in the gene body may regulate cryptic intragenic transcriptional initiation and splicing. The methylated CpG sites located in the intergenic regions are important for silencing the transposable elements. Modified from Wikipedia (https://en.wikipedia.org/wiki/File:DNAme_landscape.png).

demethylating agents can induce a cell autonomous immune activation response by stimulating expression of the endogenous retroviruses. This response probably explains some of the anti-tumor activities of these drugs.

DNA Methylation Dynamics in Development and Cancer

Despite its stability, the patterns of DNA methylation can be substantially altered in development and cancer. In mammals, there are two waves of genome-wide DNA demethylation and re-methylation during the development. The first wave occurs in early embryogenesis. DNA methylations on both paternal and maternal genomes are largely erased shortly after fertilization, and a de novo methylation then takes place during the implantation stage of the embryo. The second wave of genome-wide DNA demethylation occurs during the specification of primordial germ cells (PGCs), the first germline cells established during embryogenesis and the precursors for both the oocytes and sperms. At the beginning of their specification and migration, PGCs are shown to have the same DNA methylation patterns as the epiblast cells from which the PGCs are generated. However, by the time they arrive at the genital ridge, their DNA methylations are almost completely erased. This DNA methylation reprogramming is possibly required for totipotency of the germ cells in the newly formed embryos.

Even before the discovery of the mutations of the DNA methyltransferase and demethylases in cancer, altered DNA methylation patterns have been heavily implicated in various cancers. As a stable gene silencing mechanism, DNA hypermethylation has been commonly observed in cancers to silence tumor-suppressor genes. Particularly, in some familial cancers, when one allele of the key tumor-suppressor gene has mutated in germ line, there is a fair chance that the second allele is silenced by DNA methylation. Besides the individual cancer-related genes that are directly targeted by DNA methylation, alterations of DNA methylation patterns in broad genomic regions, such as repetitive elements, low-density CpG regions, and lamin-associated domains, have also been observed in cancer cells, although their functional roles in cancer remain to be defined.

It has been widely accepted that a genome-wide profile of DNA methylation (methylome) of cancer is a useful method not only for understanding the molecular mechanism of epigenetic regulation of cancer, but also for precise diagnosis and treatment of cancer. Differentially methylated regions (DMRs) are commonly used to describe potentially functionally relevant genomic regions that are differentially methylated between different cells/tissues. In another way, the concept of CpG island methylator phenotypes (CIMPs) has been invented to identify concordant CpG methylations of a group of genes in a certain type of cancer and thereby to classify a subtype of cancer from others based on their specific methylation patterns.

Mutations in *DNMT3A*

DNMT3A is one of the most frequently mutated genes in adult hematopoietic malignancies. It belongs to an evolutionarily conserved family of methyltransferases. Except for *DNMT3A*, none of other members of the family have been found being recurrently mutated in cancer, though *DNMT3B*, the closest homologue of *DNMT3A*, is mutated in patients with a rare autosomal recessive immune disorder termed immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome. It is also unanswered why *DNMT3A* mutation is only heavily implicated in hematopoietic malignancies, but not other types of cancer. Understanding the basic function of *DNMT3A* in DNA methylation regulation and normal hematopoiesis would be helpful to clarify the functions and mechanisms of *DNMT3A* in leukemogenesis.

The DNMT Family

The DNMT family in humans contains five members: DNMT1, DNMT2 (also known as TRDMT1), DNMT3A, DNMT3B and DNMT3L (Fig. 3). They are evolutionarily conserved as each of them has homologue(s) found in vertebrates and they likely share a common ancestor in invertebrates such as fruit fly (Fig. 3A). They share a homologous catalytic domain in their C-terminuses, and some of them have acquired additional domains that may mediate substrate recognition, protein interaction and other regulatory activities (Fig. 3B). Indeed, the biological functions of the DNMT family members are highly specified. Although DNMT2 is the most conserved member in the family, it has been shown not being able to methylate DNA, instead it can methylate the transfer RNA (tRNA) Asp-GTC on cytosine 38 in the anticodon loop. The other DNMTs possess DNA methylation activity but they are functionally classified as two types: de novo and maintenance methyltransferases. DNMT3A, DNMT3B and DNMT3L are de novo DNMTs, as they create hemimethylated CpG sites in double-strand DNA, and are responsible for setting up the pattern of methylation. The high similarity between DNMT3A and DNMT3B implies that they may exert redundant functions, but they may also play different roles in gene- and cell type-specific manners. While DNMT3L is catalytically inactive, it can facilitate the activities of DNMT3A and DNMT3B. DNMT1 is the maintenance DNMT that adds methyl group to the cytosine when one strand has already been methylated, and is responsible for maintaining the methylation pattern that had been established by the de novo DNMTs.

Structure and Function of DNMT3A

DNMT3A contains three conserved domains (Fig. 3B). The PWWP domain can bind both DNA and histone marks such as H3K36me3, through which DNMT3A is recruited to the H3K36me3 enriched genomic regions, including gene body region and

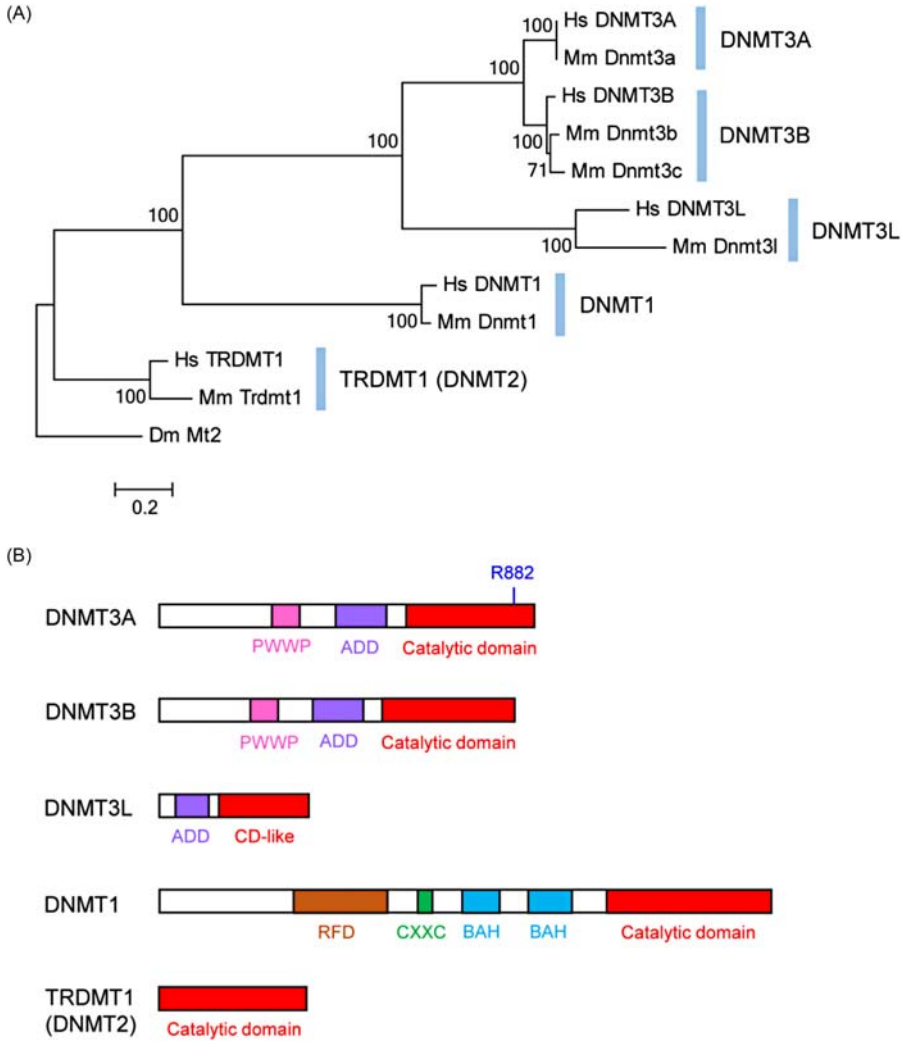

Fig. 3 The DNMT family. (A) Phylogenic tree of human (*Homo sapiens*, *HS*) and mouse (*Mus musculus*, *Mm*) DNMT family members. Construction of the tree is based on an alignment of the amino acid sequences of the catalytic domains. The methyltransferase 2 (Mt2) of fruit fly (*Drosophila melanogaster*, *Dm*) represents the common ancestor of the mammalian DNMTs. (B) Domain architectures of the human DNMT family members. The DNMT3A mutational hotspot R882 is localized within the catalytic domain.

pericentromic heterochromatic foci. The ATRX-DNMT3-DNMT3L (ADD) domain binds H3 tail peptides with or without lysine 4 (H3K4) methylation, and, in the presence of H3K4me3, acts as an autoinhibitor for the enzymatic activity of DNMT3A. The C-terminal catalytic domain is a C5-type *S*-adenosyl methionine (SAM)-dependent methyltransferase domain. It has been shown that the catalytic domain preferentially binds unmethylated DNA. DNMT3A can form oligomers, including dimer and tetramer, through two distinct binding interfaces in the catalytic domain. The oligomeric states of DNMT3A, as well as its hetero-oligomerization with DNMT3B and DNMT3L, are important for their catalytic activities. Regulation of the oligomerization thus greatly contribute to the function of DNMT3A in both hematopoiesis and tumorigenesis.

DNMT3A in Hematopoiesis

Deletion of *Dnmt3a* in mouse models leads to enhanced self-renewal of hematopoietic stem cells (HSCs). Although the *Dnmt3a*-null HSCs are phenotypically indistinguishable from wild-type HSCs, with identical cell surface markers, intact abilities to differentiate to all hematopoietic lineages, and similar levels of proliferation and apoptosis, serial bone marrow transplantation assays indicate a gradual expansion of the *Dnmt3a*-null long-term hematopoietic stem cells (LT-HSCs). Transplantation of the *Dnmt3a*-null HSCs lead to development of myeloid and lymphoid malignancies, which recapitulate many clinical features of human malignancies caused by *DNMT3A* mutations.

Dnmt3a-null HSCs display significant genome-wide hypomethylation with focal areas of hypermethylation. The DMRs identified in *Dnmt3a*-null HSCs are significantly enriched at the edges of large hypomethylated regions known as methylation canyons. Many methylation canyon-associated genes involved in HSC self-renewal are up-regulated in *Dnmt3a*-null HSCs and remain inappropriately activated in differentiated cells, suggesting that the *Dnmt3a* deficiency results in an inability of the cells to turn off the genes that regulate differentiation.

DNMT3A Mutations in Hematopoietic Malignancy

DNMT3A mutations are presented in approximately 20% of patients with de novo and secondary acute myeloid leukemia (AML), especially in the French–American–British (FAB) classified M4 and M5 subtypes. *DNMT3A* mutated AMLs frequently harbor *NPM1* (*nucleophosmin 1*) and *FLT3* (*fms related tyrosine kinase 3*) mutations. Although *DNMT3A* mutations have been defined as an earliest driver event in tumorigenesis, the impact of *DNMT3A* mutations in AML prognosis is under debate. Besides AML, *DNMT3A* mutations have also been reported in other myeloid malignancies, including myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN) and myelodysplastic/myeloproliferative overlapping disease (MDS/MPN), although their prevalence is much lower than in AML. Furthermore, *DNMT3A* mutations have also been recurrently detected in lymphoid malignancies. Notably, in T-cell acute lymphoblastic leukemia (T-ALL), the prevalence of *DNMT3A* biallelic mutation is higher than in myeloid malignancies, implying potentially different mechanism for *DNMT3A*-driven lymphoid and myeloid neoplastic transformation.

The majority of *DNMT3A* mutations detected in hematopoietic malignancies occur within the C-terminal catalytic domain, with a significant enrichment for mutations at amino acid arginine 882 (R882), although non-R882 missense and truncating mutations are found with much lower frequency in other domains. In myeloid malignancies, R882 mutations are usually heterozygous, whereas in T-ALL, there is a high frequency of non-R882 biallelic mutations. The DNMT3A R882H mutant has been shown to exert a dominant-negative effect against the wild-type protein. Mechanistically, the R882H mutant can dimerize with wild-type DNMT3A, but prevent the formation of a tetramer, which represents a stronger enzymatic activity. Thus, the existence of the R882H mutant can reduce the DNA methyltransferase activity in the cells, explaining the genome-wide hypomethylation observed in patients with *DNMT3A* R882 mutations. In addition, DNMT3A R882 mutants may also exert its function through affecting other proteins/pathways. For example, DNMT3A R882 mutants can interact with the polycomb repressive complex 1 (PRC1) to down-regulate specific target genes involved in hematopoietic differentiation. It can also activate the mechanistic target of rapamycin (mTOR) pathway and increase the protein level of cyclin-dependent kinase 1 (CDK1), which are associated with a dysregulation of cell cycle of HSCs. Indeed, the mTOR inhibitor rapamycin elicited a significant therapeutic response in the knock-in and bone marrow transplantation mouse models of the *DNMT3A* R882H mutation.

Mutations in *TET2*

TET2 mutations have been found to be associated with multiple hematopoietic malignancies, as well as melanoma and few cases of glioma. *TET2* somatic mutations and loss of heterozygosity (LOH) occur in patients with MDS, MPN and AML. *TET2* mutations have also been reported in T-cell lymphomas. Unlike *DNMT3A*, *IDH1* and *IDH2* (see below), *TET2* does not display a dominant hotspot mutation site, instead nonsense and missense mutations, as well as deletions, are found across multiple exons.

The TET Family

The founding member of the TET protein family was first identified as a fusion partner of *MLL* (*mixed-lineage leukemia*; also known as *lysine methyltransferase 2A*, *KMT2A*) in patients with t(10;11)(q22;q23) AML, and therefore this gene was named as *ten-eleven translocation 1* (*TET1*). The TET family contains three members, TET1, TET2 and TET3 (**Fig. 4**A), all of which have been identified as

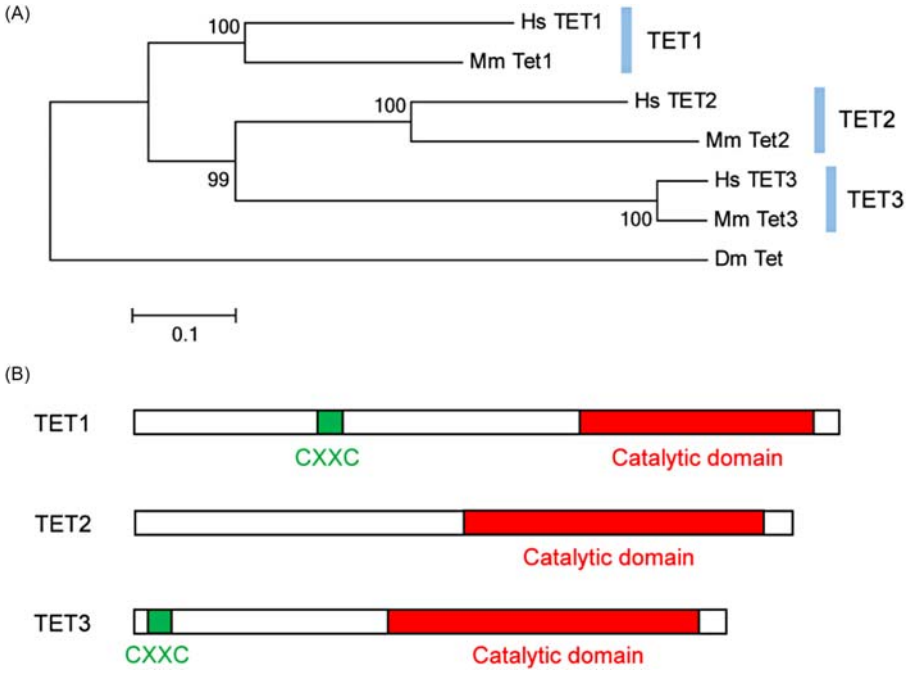

Fig. 4 The TET family. (A) Phylogenic tree of human (*HS*) and mouse (*Mm*) TET family members. Construction of the tree is based on an alignment of the amino acid sequences of the catalytic domains. The fruit fly (*Dm*) Tet protein represents the common ancestor of the mammalian TETs. (B) Domain architectures of the human TET family members.

methylcytosine dioxygenases that catalyze the conversion of 5mC to 5hmC. The three TET family members share a catalytic domain in their C-terminuses; TET1 and TET2 also contain a N-terminal CXXC domain, whereas TET2 does not (Fig. 4B).

Structure and Function of TET2

The dioxygenase activity of the TET family members is mediated by the double-stranded beta helix (DSBH) fold domain termed base J-binding protein (JBP) domain. This JBP domain virtually defines a much larger superfamily of 2-oxoglutarate (2-OG)- and ferrous iron (Fe(II))-dependent dioxygenases (2OGFeDO), which are found in various eukaryotes, bacteria and bacteriophages. Notably, the co-factor of TETs, 2-OG (also known as α-ketoglutarate (α-KG)), is an important metabolite in the tricarboxylic acid (TCA) cycle (also known as citric acid cycle or Krebs cycle), and it is converted from isocitrate by the IDHs. Thus, the discovery of TETs as key enzymes for DNA demethylation makes a direct connection between epigenetic regulation and cellular metabolism. Alterations of both aspects had already been considered as hallmarks of cancer but the mechanistic link between them has otherwise been lacking.

Besides the catalytic JBP domain, TET1 and TET3, but not TET2, contain a conserved CXXC domain that is shown to bind unmethylated CpG sites. Interestingly, although TET2 does not contain the CXXC domain, a CXXC domain-containing gene, *IDAX* (also known as *CXXC4*), locates upstream of the *TET2* genomic locus, suggesting that an ancient CXXC-containing *TET2* gene was split into two separate genes through evolution. Nevertheless, biochemical studies revealed that the IDAX protein is still able to interact with TET2, and that this interaction is associated with caspase-mediated TET2 degradation, providing a flexible regulatory mechanism for TET2 stabilization.

TET2 in Hematopoiesis

Deletion of *TET2* in hematopoietic cells in mice leads to an expansion of hematopoietic stem/progenitor cells (HSPCs) in vivo. These cells show increased re-plating potential and colony formation ability in vitro. In competitive bone marrow transplantation assays, *TET2*-null HSPCs are capable of outcompeting wild-type cells. These observations suggest that *TET2* deficiency results in increased self-renewal of HSCs, which may lead to neoplastic transformation. Indeed, aged *TET2* knockout mice have been reported to develop myeloid malignancies, which are mostly characterized as a chronic myelomonocytic leukemia (CMML)-like disease. In addition, other hematopoietic malignant phenotypes, such as a MDS-like disease and increased immature thymic T cells and mature splenic B cells, were observed in other *Tet2* knockout mouse models. These diverse phenotypes of the *Tet2*-deleted mice may attribute to different approaches to target *Tet2* and/or different genetic background. It is also conceivable that, because there was always a considerable latency before the mice developed full-blown hematopoietic malignancies, secondary genetic events acquired through time could contribute to tumorigenesis and specify disease phenotypes.

TET2 Mutations in Hematopoietic Malignancy

Somatic deletions and mutations in *TET2* were first found in patients with MDS, MPN and AML based on mapping of loss-of-heterozygosity and microdeletions within a minimal region of chromosome 4q24. It turned out that *TET2* mutations are among the most common genetic abnormalities observed in patients with MDS (6%–26%) and CMML (20%–58%). *TET2* mutations are also frequently observed in primary and secondary AMLs (12%–32%). In addition, *TET2* mutations are detected in B-cell and T-cell lymphomas. This broad spectrum of hematopoietic malignancies associated with *TET2* mutations in humans is consistent with the above described diverse phenotypes of *Tet2*-knockout mice. *TET2* mutations can be observed at both early and late stages of the development of hematopoietic malignancies, and they may exert different function at different stages. *TET2* mutations are mostly heterozygous, in which the wild-type allele of *TET2* in the patients remains expressing, whereas biallelic *TET2* inactivation occurs in less than 10% of patients with leukemia. Furthermore, there is not a prominent hotspot mutation site in *TET2*. These observations suggest that *TET2* can function as a haploinsufficient tumor suppressor in most patients.

By a curious paradox, mutations of the two enzymes, *DNMT3A* and *TET2*, which apparently take opposite biochemical actions on DNA methylation (i.e., writing versus erasing), result in hematopoietic malignancies similarly. Furthermore, *DNMT3A* and *TET2* have been found to be co-mutated in some patients with hematopoietic malignancies, and, as described above, loss of *Dnmt3a* or *Tet2* in mice similarly results in expansion of HSCs. These observations suggest that *DNMT3A* and *TET2* may work in parallel and/or cooperatively in hematopoiesis and malignancies. Consistent with this notion, it has been shown that Dnmt3a and Tet2 can cooperatively repress the erythroid transcription factor *Klf1* (*Kruppel like factor 1*), and that the double knockout of *Dnmt3a* and *Tet2* synergistically up-regulate *Klf1* and related pathway, which may contribute to malignant transformation.

Mutations in *IDH1* and *IDH2*

Interference with cellular energy metabolism has recently been defined as one of the emerging hallmarks of cancer. *IDH1* and *IDH2* are key enzymes involved in the TCA cycle; frequent mutations of both enzymes in cancer provide a prominent example to support this notion. *IDH1* mutations were first identified in glioblastoma through whole-exome sequencing, and *IDH2* mutations were identified in AML through a candidate gene approach. Mutations of *IDH1* and *IDH2* have now been detected in a broad spectrum of cancer types including astrocytoma, glioblastoma and AML.

The IDH Family

The IDHs catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate, which is an essential step in the TCA cycle. There are five IDHs in human genome and they belong to two distinct subclasses: IDH1 and IDH2 utilize NADP(+) as the electron acceptor, whereas IDH3 α, β and γ use NAD(+). They also show different subcellular localization: while IDH2, IDH3 α, β and γ localize to mitochondria, IDH1 predominantly functions in the cytoplasm. The IDH family is extremely conserved through evolution, as both of the NADP(+)- and NAD(+)-dependent IDHs have homologues in all eukaryotes ranging from yeasts to humans. Unlike the DNMT and TET families in which the different members in mammals share a common ancestor in invertebrates, the homologues of both IDH1 and IDH2 are found in invertebrates such as the nematode *Caenorhabditis elegans* (**Fig. 5**A), suggesting that their different roles were specified much earlier through evolution than that of the DNMT and TET family members.

Gain-of-Function Mutations in *IDH1* and *IDH2*

Mutations of *IDH1* and *IHD2* identified in cancer are heterogenous, and, as same as *DNMT3A*, they clearly show mutational hotspots—most of the mutations occur at one of three highly conserved arginine residues: R132 of IDH1 and R140 and R172 of IDH2 (**Fig. 5**B). Based on the evolution analysis and sequence alignment, the R132 in IDH1 is exactly the homologous residue of R172 in IDH2 (**Fig. 5**C). As there has not been evidence for haploinsufficiency of *IHD1* and *IDH2* in cancer, their mutational pattern suggests a possibility that these mutants might be gain-of-function. Supportive of this possibility, the mutant IDH1 and IDH2 have been found to acquire a neomorphic enzymatic activity that converts α-KG to 2-hydroxyglutarate (2-HG). It has also been shown that patients with AML harboring *IDH1* or *IDH2* mutations have significant higher level of 2-HG in their serum, suggesting that 2-HG could be considered as a biomarker of *IDH*-mutated AMLs. Furthermore, 2-HG has been shown to be sufficient to promote malignant transformation of hematopoietic cells, and this effect is reversible upon removal of 2-HG. Thus, 2-HG represent an "oncometabolite," which is produced specifically in cancer cells and exert a cancer-inducing activity.

Mutual Exclusivity Between *IDH1/2* and *TET2* Mutations

The structural similarity between α-KG and 2-HG suggests that the 2-HG could act as an inhibitor of the enzymes that naturally require α-KG as a co-factor. Indeed, members of several subfamilies of α-KG-dependent dioxygenases have been shown to be inhibited by 2-HG. These dioxygenases include the TETs, which, as described above, are responsible for erasing the DNA methylation and are also frequently mutated in hematopoietic malignancies. Interestingly, genomic and epigenomic studies of a large cohort of patients with AML have demonstrated that *IDH* mutations and *TET2* mutations are mutually exclusive, and that patients

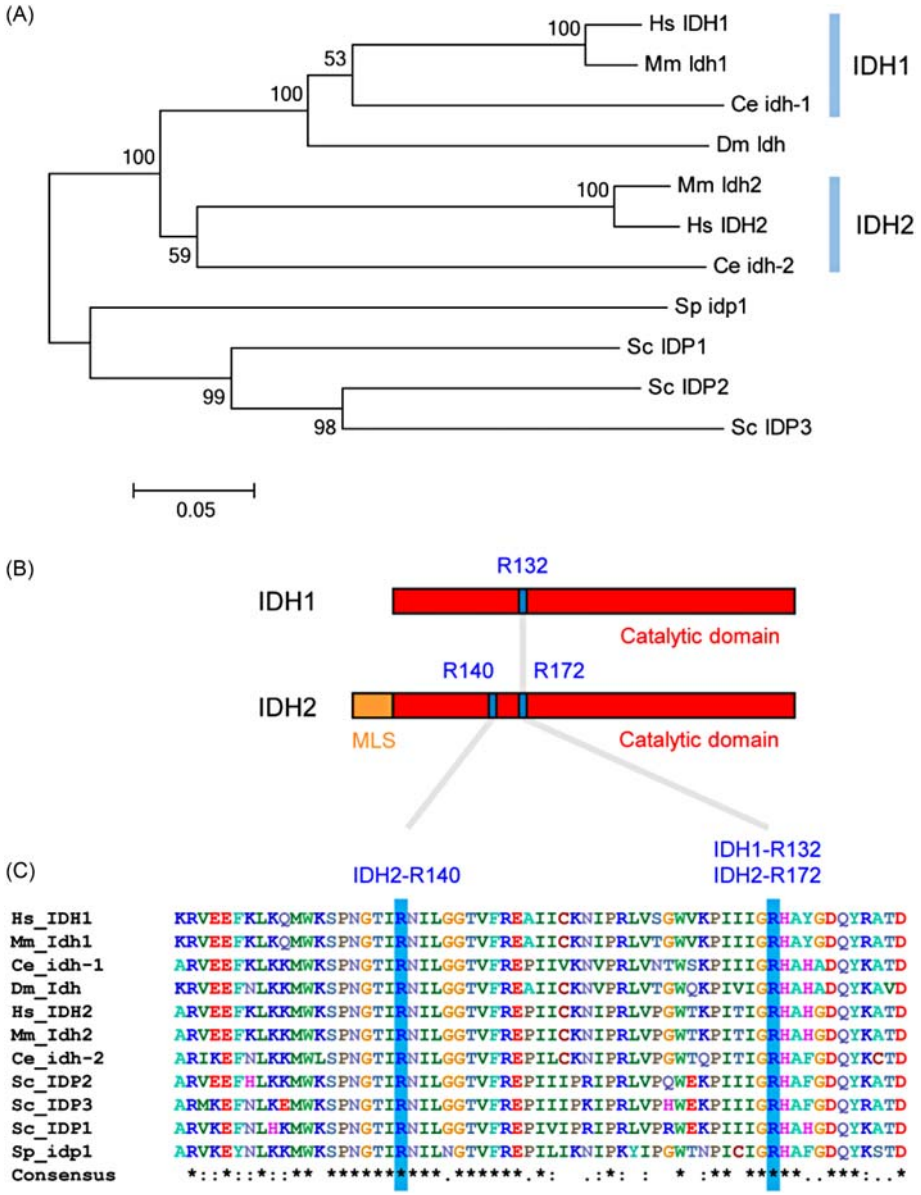

Fig. 5 The IDH1/2 family and the mutational hotspots. (A) Phylogenic tree of the IDH1/2 family members in human (*HS*), mouse (*Mm*), fruit fly (*Dm*), nematode (*Caenorhabditis elegans*, *Ce*), fission yeast (*Schizosaccharomyces pombe*, *Sp*) and baking yeast (*Saccharomyces cerevisiae*, *Sc*). Note that the *Caenorhabditis elegans* contains two IDHs (idh-1 and idh-2) related to the mammalian IDH1 and IDH2, respectively. (B) Domain architectures of human IDH1 and IDH2. A mitochondrial localization signal (MLS) is on the N-terminus of IDH2. The three mutational hotspots are localized in the catalytic domains of IDH1 and IDH2; the R132 of IDH1 is corresponding to the R172 of IDH2. (C) Alignment of the amino acid sequence context of the mutational hotspots in the IDH1/2 family members, showing the high conservation of these two R residues as well as their sequence context.

harboring mutations in *TET2* or *IDH1/2* share similar genome-wide methylation profiles, thus suggesting that the *TET2* and *IDH1/2* mutations affect the same pathway.

2-HG Is Involved in Many Cellular Processes Through Various α-KG-Dependent Dioxygenases

Besides the TETs, many other α-KG-dependent dioxygenases can also be inhibited by 2-HG. Given that these enzymes play important roles in different signaling pathways, the cancer-associated *IDH1* and *IDH2* mutations and their product 2-HG are involved in many cellular processes and thereby can regulate different aspects of development and cancer.

First, the Jumonji C domain-containing histone lysine demethylases are α-KG dependent dioxygenases sensitive to 2-HG. Ectopic expression of *IDH1/2* mutants leads to dramatic accumulation of lysine-methylated histones. Compared to the wild-type cells, IDH1 mutated oligodendroglioma shows elevated H3K9me3 and H3K27me3.

Second, a pair of α-KG-dependent dioxygenases, the fat mass and obesity-associated protein (FTO) and the alkB homologue 5 (ALKBH5) RNA demethylases, are responsible for removing the RNA N^6-adenosine methylation (m6A); the m6A modulates several aspects of RNA functions including splicing, nuclear export, stability and translation. There has been evidence suggesting that the IDH mutants affects mRNA m6A levels in leukemia cells likely through 2-HG inhibition of FTO activity.

Third, in the hypoxia signaling pathway, the stability of the hypoxia-inducible factors (HIFs) is mainly regulated through HIF hydroxylation mediate by two families of enzymes, namely the three prolyl hydroxylase domain proteins (PHD1, PHD2 and PHD3) and an asparaginyl hydroxylase factor inhibiting HIF (FIH). These hydroxylases require α-KG as a co-factor and potentially can be inhibited by 2-HG. The correlations between IDH mutation, DNA methylation and hypoxia signaling turn out to be cellular context dependent; the detailed underlying mechanisms require further clarification.

Lastly, the type IV collagen can be hydroxylated at their proline and lysine residues by the α-KG-dependent prolyl 4-hydroxylases 1, 2 and 3 (P4HA1, 2 and 3) and procollagen-lysine 2-oxoglutarate 5-dioxygenases 1, 2 and 3 (PLOD1, 2 and 3), respectively. Through 2-HG inhibition of type IV collagen hydroxylation, the *IDH* mutations may trigger collagen maturation defects which possibly contribute to tumor behaviors such as angiogenesis.

Targeted Therapy

The discovery of frequent mutations in epigenetic modifiers provide an enormous potential for targeted therapy of cancer. In contrast to the transcription factors, which also play an important role in gene regulation and heavily implicated in cancer but difficult to be targeted, inhibition of the enzymes responsible for DNA methylation and other epigenetic modifications is a much easier strategy. As many of the mutations in DNA methyltransferases and demethylases have been shown to be driver mutations occurred in cancer stem cells, inhibition of these mutated enzymes provides an opportunity to eliminate the cancer stem cells which otherwise are barely targeted by chemotherapy. Furthermore, the cross-talks between DNA methylation and cellular metabolism, as well as other signaling pathways, suggest a potential for combination therapy with DNA methylation agents and other available therapeutics, such as receptor tyrosine kinase inhibitors, DNA damaging agents, mTOR inhibitors, and immune therapy.

Two DNA methyltransferase inhibitors, 5-azacytidine (azacytidine, AZA) and decitabine (5-aza-2′-deoxycytidine, DAC) have already obtained approval for treatment of MDS and leukemia by the US Food and Drug Administration. Both drugs are cytidine analogues that can incorporate into DNA, block the catalytic activities of DNMTs and trigger their degradation, leading to a global DNA demethylation in the cells. Many studies have suggested that the promoter demethylation and derepression of the aberrantly silenced tumor suppressor genes underlies the anti-tumor effects of these DNMT inhibitors. However, recent studies have provided another mechanism that the DNMT inhibitors can induce the expression of double strand RNAs derived at least in part from endogenous retroviral elements, leading to an activation of interferon signaling pathway and an immune response. These studies open a new perspective on application of DNMT inhibitors in cancer immunomodulation.

Many pharmacological inhibitors of mutated IDH1 and IDH2, which are expected to diminish 2-HG production in IDH1 and IDH2 mutated cells, have been developed and are currently under clinical investigation. Among these inhibitors, for example, AGI-6780 is capable of binding in an allosteric manner at the dimer interface of the mutated IDH2 (IDH2-R140Q) but is less potent against wild-type IDH2. Interestingly, treatment with AGI-6780 induced differentiation of human acute myelogenous leukemia cells, providing a great potential for a differentiation therapy of cancer.

In summary, since the initial identification of recurrent *IDH* mutations in glioblastoma and *TET2* and *DNMT3A* mutations in myeloid malignancies, encouraging and rapid progress has been made to frame new concepts, clarify underlying mechanisms and develop targeted therapeutic strategies. While the detailed molecular mechanisms of epigenetic regulation of development and cancer need to be more thoroughly investigated, the development of drugs by mechanism-based design and functional screening approaches, as well as the translational and clinical studies, are of great importance for grasping the favorable opportunity to target these enzymes and metabolites, thereby completing the bench-to-bedside transition efficiently.

Acknowledgments

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See also: Acute Myelogeneous Leukemia: Diagnosis and Treatment. Mutations in Chromatin Remodeling Factors. Mutations in Histone Lysine Methyltransferases and Demethylases.

Further Reading

Bowman, R.L., Levine, R.L., 2017. TET2 in normal and malignant hematopoiesis. Cold Spring Harbor Perspectives in Medicine 7, a026518. https://doi.org/10.1101/cshperspect.a026518.

Brunetti, L., Gundry, M.C., Goodell, M.A., 2017. DNMT3A in Leukemia. Cold Spring Harbor Perspectives in Medicine 7, a030320.
Dai, Y.J., Wang, Y.Y., Huang, J.Y., Xia, L., Shi, X.D., Xu, J., et al., 2017. Conditional knockin of Dnmt3a R878H initiates acute myeloid leukemia with mTOR pathway involvement. Proceedings of the National Academy of Sciences of the United States of America 114, 5237–5242.
Dang, L., Su, S.M., 2017. Isocitrate dehydrogenase mutation and (*R*)-2-hydroxyglutarate: from basic discovery to therapeutics development. Annual Review of Biochemistry 86, 305–331.
Dang, L., White, D.W., Gross, S., Bennett, B.D., Bittinger, M.A., et al., 2009. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462, 739–744.
Delhommeau, F., Dupont, S., Della Valle, V., James, C., Trannoy, S., et al., 2009. Mutation in TET2 in myeloid cancers. New England Journal of Medicine 360, 2289–2301.
Jones, P.A., Baylin, S.B., 2002. The fundamental role of epigenetic events in cancer. Nature Reviews Genetics 3, 415–428.
Ko, M., An, J., Pastor, W.A., Koralov, S.B., Rajewsky, K., Rao, A., 2015. TET proteins and 5-methylcytosine oxidation in hematological cancers. Immunological Reviews 263, 6–21.
Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., et al., 2010. DNMT3A mutations in acute myeloid leukemia. New England Journal of Medicine 363, 2424–2433.
M Gagné L, Boulay, K., Topisirovic, I., Huot, M.É., Mallette, F.A., 2017. Oncogenic activities of IDH1/2 mutations: from epigenetics to cellular signaling. Trends in Cell Biology 27, 738–752. https://doi.org/10.1016/j.tcb.2017.06.002.
Moore, L.D., Le, T., Fan, G., 2013. DNA methylation and its basic function. Neuropsychopharmacology 38, 23–38.
Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., et al., 2008. An integrated genomic analysis of human glioblastoma multiforme. Science 321, 1807–1812.
Rasmussen, K.D., Helin, K., 2016. Role of TET enzymes in DNA methylation, development, and cancer. Genes & Development 30, 733–750.
Shih, A.H., Abdel-Wahab, O., Patel, J.P., Levine, R.L., 2012. The role of mutations in epigenetic regulators in myeloid malignancies. Nature Reviews Cancer 12, 599–612.
Wang, J.H., Chen, W.L., Li, J.M., Wu, S.F., Chen, T.L., et al., 2013. Prognostic significance of 2-hydroxyglutarate levels in acute myeloid leukemia in China. Proceedings of the National Academy of Sciences of the United States of America 110, 17017–17022.
Weisenberger, D.J., 2014. Characterizing DNA methylation alterations from The Cancer Genome Atlas. Journal of Clinical Investigation 124, 17–23.
Wu, S.C., Zhang, Y., 2010. Active DNA demethylation: many roads lead to Rome. Nature Reviews Molecular Cell Biology 11, 607–620.
Yan, X.J., Xu, J., Gu, Z.H., Pan, C.M., Lu, G., et al., 2011. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nature Genetics 43, 309–315.

Mutations in Histone Lysine Methyltransferases and Demethylases

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Glossary

Compound heterozygous mutations Compound heterozygous mutations consist of two different mutations in the paternal and maternal alleles of a gene.

Dominant negative A dominant negative variant acts to reduce the activity of the wild-type gene.

Gain-of-function mutation A mutation that confers a new molecular function on the mutated gene.

Genetic dominance One allele in a heterozygous genotype masks the effect of the other.

Haploinsufficiency A single functional copy of a gene cannot provide sufficient gene product to preserve the wild-type phenotype leading to an altered or diseased state.

Heterozygous mutation A mutation of only one allele of a gene.

Homozygous mutation An identical mutation of both, the paternal and maternal alleles, of a gene.

Loss-of-function mutation A mutation that results in the gene product having less or no function (being partially or completely inactivated).

Epigenetic modification Covalent modification of the DNA or chromatin that encodes epigenetic information.

Epigenetics Stable heritable traits (or "phenotypes") that are not caused by changes in DNA sequence but often are not fully stable.

SET domain A 130 to 140 amino acid protein domain initially characterized in the Drosophila proteins Su(var)3–9, Enhancer-of-zeste and Trithorax that contains the active center of one family of PKMTs.

JmjC domain A protein domain that adopts the cupin fold and contains the active center of one family of KDMs.

PKMT Enzymes that methylate lysine residues in proteins.

KDM Enzymes that demethylate methyllysine residues in proteins.

Introduction

Setting and Removing of Histone Lysine Methylation

All human cells contain the same genetic information, yet they follow different developmental pathways and differentiate into the numerous different cells types found in the human body. The cellular fate and phenotype are determined by epigenetic regulatory mechanisms, which modulate the chromatin structure and control gene expression. The basic functional unit of chromatin is the nucleosome, which contains 147 base pairs of DNA wrapped around a core histone octamer comprising of two subunits of each of the histones H2A, H2B, H3, and H4. Epigenetic signals include DNA methylation, noncoding RNAs, and modifications of the histone proteins mainly within the N-terminal tails of H3 and H4, which are subject to a large variety of posttranslational modifications like acetylation, methylation, phosphorylation, or ubiquitylation. Methylation of histone tails can occur on Lys and Arg residues.

Lysine methylation is a dynamic process and methylation levels change during biological processes such as cellular differentiation or carcinogenesis. Histone lysine methylation is introduced by protein lysine methyltransferases (PKMTs, also abbreviated as KMTs) which use S-adenosyl-L-methionine (AdoMet) as methyl group donor and belong to two distinct families of proteins (see **Table 1** for a compilation of all human PKMTs and KDMs). Most histone methylating PKMTs contain a SET domain of approximately 130–140 amino acids, while currently known examples of seven-ß-strand PKMTs with histone substrates are limited to DOT1L. For the removal of lysine methylation, two families of lysine demethylase enzymes (KDMs) are responsible, the LSD family and enzymes containing Jumonji C (JmjC) domains. LSD1 and LSD2 are members of the monoamine oxidase superfamily, which are using flavin adenine dinucleotide and molecular oxygen as cofactors for the oxidation of C–N single bonds to an unstable imine intermediate generating hydrogen peroxide as byproduct. Subsequently, the imine is hydrolyzed and the methyl group is released as formaldehyde. This catalytic mechanism permits demethylation of secondary and tertiary but not of quaternary amines, limiting the substrate to mono- and dimethylated lysines. The second and much larger family of JmjC domain containing KDMs comprises about 20 human enzymes subgrouped into five subfamilies. These enzymes utilize oxygen, α-ketoglutarate, and Fe(II) ions as cofactors in the hydroxylation of lysine-bound methyl groups which is followed by the elimination of the hydroxymethyl group as formaldehyde. Enzymes of this family catalyze the removal of methyl groups from mono-, di-, and trimethylated lysine residues at various sites of the histone tails. PKMTs and KDMs are involved in several diseases including cancer.

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Table 1 List of human PKMTs and KDMs with their different abbreviations, main histone substrates, and the corresponding UniProtKB entry

Enzyme name	*Full name*	*Abbreviation and abbreviation of selected synonyms*	*Main histone substrate(s)*	*KB#*
DOT1L	DOT1-like protein	DOT1L, KMT4	H3K79	Q8TEK3
EZH1	Enhancer of zeste homolog 1	ENX-2	H3K27	Q92800
EZH2	Enhancer of zeste homolog 2	EZH2, KMT6	H3K27	Q15910
G9a	Euchromatic histone-lysine *N*-methyltransferase 2	BAT8, C6orf30, G9A, KMT1C, NG36	H3K9	Q96KQ7
GLP (EuHMT1)	G9a-like protein 1	EUHMTASE1, GLP, KIAA1876, KMT1D	H3K9	Q9H9B1
MLL1 (KMT2A)	Myeloid/lymphoid or mixed-lineage leukemia protein 1	MLL1, KMT2A	H3K4	Q03164
MLL2 (KMT2B)	Myeloid/lymphoid or mixed-lineage leukemia protein 4	HRX2, KIAA0304, MLL2, MLL4, TRX2, WBP7	H3K4	Q9UMN6
MLL3 (KMT2C)	Myeloid/lymphoid or mixed-lineage leukemia protein 3	MLL3, KMT2C	H3K4	Q8NEZ4
MLL4 (KMT2D)	Myeloid/lymphoid or mixed-lineage leukemia protein 2	ALR, MLL2, MLL4	H3K4	O14686
SETD1A	SET domain-containing protein 1A	KMT2F, SET1, SET1A	H3K4	O15047
SETD1B	SET domain-containing protein 1B	KIAA1076, KMT2G, SET1B	H3K4	Q9UPS6
NSD1	Nuclear receptor-binding SET domain-containing protein 1	NSD1, KMT3B	H3K36	Q96L73
NSD2	Histone-lysine *N*-methyltransferase NSD2	NSD2, MMSET, WHSC1	H3K36	O96028
NSD3	Nuclear SET domain-containing protein 3	WHSC1L1		Q9BZ95
SET7/9 (SETD7)	SET domain containing protein 7	KIAA1717, KMT7, SET7, SET9	H3K4	Q8WTS6
SETD2	SET domain-containing protein 2	SETD2, KMT3A	H3K36	Q9BYW2
SETD8 (PR-SET7)	Histone-lysine *N*-methyltransferase KMT5A	PRSET7, SET07, SET8, SETD8	H4K20	Q9NQR1
SETDB1	SET domain bifurcated 1	KIAA0067, KMT1E	H3K9	Q15047
SETDB2	Histone H3-K9 methyltransferase	C13orf4, CLLD8, KMT1F	H3K9	Q96T68
SMYD1	SET and MYND domain-containing protein 1	/	H3K4	Q8NB12
SMYD2	SET and MYND domain-containing protein 2	KMT3C	H3K4 and H3K36	Q9NRG4
SMYD3	SET and MYND domain-containing protein 3	ZMYND1, ZNFN3A1	H3K4 and H3K5	Q9H7B4
SMYD4	SET and MYND domain-containing protein 4	KIAA1936		Q8IYR2
SMYD5	SET and MYND domain-containing protein 5	RAI15		Q6GMV2
SUV39H1	Suppressor of variegation 3–9 homolog 1	KMT1A, SUV39H	H3K9	O43463
SUV39H2	Suppressor of variegation 3–9 homolog 2	KMT1B	H3K9	Q9H5I1
SUV420H1	Suppressor of variegation 4–20 homolog 1	KMT5B	H4K20	Q4FZB7
SUV420H2	Suppressor of variegation 4–20 homolog 2	KMT5C	H4K20	Q86Y97
KDM1A	Lysine-specific histone demethylase 1A	AOF2, KDM1, KIAA0601, LSD1	Methylated H3K4 and H3K9	O60341
KDM1B	Lysine-specific histone demethylase 1B	AOF1, C6orf193, LSD2	H3K4	Q8NB78
KDM2A	Lysine-specific demethylase 2A	CXXC8, FBL7, FBXL11, JHDM1A, KIAA1004	Methylated H3K36	Q9Y2K7
KDM2B	Lysine-specific demethylase 2B	CXXC2, FBL10, FBXL10, JHDM1B, PCCX2	Methylated H3K4 and H3K36	Q8NHM5
KDM3A	Lysine-specific demethylase 3A	JHDM2A, JMJD1, JMJD1A, KIAA0742, TSGA	Methylated H3K9	Q9Y4C1
KDM3B	Lysine-specific demethylase 3B	C5orf7, JHDM2B, JMJD1B, KIAA1082	Methylated H3K9	Q7LBC6
KDM4A	Lysine-specific demethylase 4A	JHDM3A, JMJD2, JMJD2A, KIAA0677	Methylated H3K9 and H3K36	O75164
KDM4B	Lysine-specific demethylase 4B	JHDM3B, JMJD2B, KIAA0876	Methylated H3K9	O94953
KDM4C	Lysine-specific demethylase 4C	GASC1, JHDM3C, JMJD2C, KIAA0780	Methylated H3K9 and H3K36	Q9H3R0
KDM4D	Lysine-specific demethylase 4D	JHDM3D, JMJD2D	Methylated H3K9	Q6B0I6
KDM4E	Lysine-specific demethylase 4E	KDM4DL	Methylated H3K9	B2RXH2
KDM5A	Lysine-specific demethylase 5A	JARID1A, RBBP2, RBP2	Methylated H3K4	P29375
KDM5B	Lysine-specific demethylase 5B	JARID1B, PLU1, RBBP2H1	Methylated H3K4	Q9UGL1
KDM5C	Lysine-specific demethylase 5C	DXS1272E, JARID1C1, SMCX1, XE169	Methylated H3K4	P41229
KDM5D	Lysine-specific demethylase 5D	HY, HYA, JARID1D, KIAA0234, SMCY	Methylated H3K4	Q9BY66
KDM6A	Lysine-specific demethylase 6A	UTX (Ubiquitously transcribed X-chromosome tetratricopeptide repeat protein)	Methylated H3K27	O15550
KDM6B	Lysine-specific demethylase 6B	JMJD3, KIAA0346	Methylated H3K27	O15054

Critical Enzymatic Properties of PKMTs and KDMs

PKMTs were initially identified as histone lysine methyltransferases and shown to have very important roles in the regulation of gene expression and chromatin biology. Later, they were also shown to methylate several nonhistone substrates. Similarly, KDMs show activity on histone and nonhistone proteins, which both have roles in cancer. Locus-specific histone lysine methylation can signal either gene activation or repression, depending on the site of modification. For example, H3K4me3 has an activating function, while H3K9me3 and H3K27me3 are repressive marks. For the biological outcome of the lysine methylation it is also important to consider that a lysine side chain can be mono-, di-, or trimethylated and different methylated forms in general cause different effects. For example, H3K4me1 is associated with enhancers, while H3K4me3 is associated with promoters of active genes. The biological effects of lysine methylation are mediated by reading domains, which specifically interact with methylated lysine residues and trigger downstream signaling. Lysine methylation of nonhistone proteins can influence their stability and interaction with other proteins. However, the biological effects of nonhistone lysine methylation are not well understood in most cases.

The substrate spectrum of PKMTs and KDMs is determined by the recognition of the target peptide in the active site, which includes readout of the peptide sequence. Moreover, the access of PKMTs and KDMs to target lysine residues is controlled by the interaction of the enzyme with substrate proteins. In the case of histone methylation and demethylation, the locus-specific targeting of the PKMTs and KDMs by either endogenous binding domains or (more often) other proteins and RNAs is essential for the biological activity. In the cell, PKMTs and KDMs form large complexes with a number of additional proteins, which have roles in their regulation and targeting. These complexes often contain other epigenetic writers or erasers and they regularly contain several DNA binding and epigenetic reading domains for locus-specific targeting. For example, while isolated EZH2 is weakly active, the PRC2 complex consisting of EZH2 and six protein partners, which has a molecular weight of approximately 500 kDa, is a highly active H3K27 trimethyltransferase. Regulation of PKMTs and KDMs often includes conformational changes between active and auto-inhibited states that prevent the aberrant activity at nontarget sites. Moreover, the ability of PKMTs and KDMs to generate different methylation states is an important property of these enzymes. More to that, PKMTs and KDMs differ in their ability to use differentially methylated lysine residues as substrates. For example, the SUV420H1 enzyme introduces H4K20 trimethylation. However, it is only weakly active on unmethylated H4, but needs monomethylated H4K20 as substrate, which is generated by the SET8 PKMT. Hence SUV420H1 is dependent on lysine methylation introduced before by SET8, which restricts its biological effects. Similarly, LSD family KDMs are not able to remove methylation from trimethylated lysine residues, which limits their potential effects.

In summary, the critical enzymatic properties of PKMTs and KDMs are their amino acid sequence recognition, the preference for particular methylation states of the target lysine, their ability to generate mono-, di-, or trimethyllysine, protein–protein interaction, and their regulation and targeting. All these properties can be potentially altered by mutations in cancer cells, which in turn can cause massive changes in the signaling potential of the mutated enzymes.

Somatic Cancer Mutations in Epigenetic Enzymes

Carcinogenesis is driven by the acquisition of a series of mutations and epigenetic changes, many of which ultimately confer a growth advantage upon the cells in which they have occurred. Epigenetic enzymes that modify histone proteins and DNA are involved in several diseases including cancer. Various genome-wide exome sequencing studies revealed several genes that are recurrently mutated in tumor tissues. Among them are mutations in genes that are involved in epigenetic functions either directly or indirectly, including many PKMTs and some KDMs, which will be described in this article in detail.

The prevalence of somatic mutations in chromatin-modifying genes highlights the central role of transcriptional dysregulation in tumorigenesis. Somatic mutations in these enzymes might lead to either loss- or gain-of-function similar to mutations in other genes. Loss-of-function refers to mutations that reduce or disrupt the enzymatic activity, while gain-of-function refers to mutations, which confer novel activities on the mutated enzymes. Loss-of-function mutations include not only nonsense mutations, frameshift mutations, and deletion, which all directly disrupt protein synthesis, but also some missense mutations, which affect amino acid residues essential for catalysis or protein folding, for example. Somatic mutations can occur on one allele only (heterozygous) or on both alleles. If both alleles are affected, usually they carry different mutations (compound heterozygous). Gain-of-function effects are caused by missense mutations leading to amino acid exchanges, which alter critical enzymatic properties. They are almost always dominant yielding biological effects in heterozygous state. Loss-of-function mutations have biological effects if they occur as compound heterozygous mutation such that both alleles are affected. Heterozygous loss-of-function mutations (still combined with one wild-type allele) can have biological effects if the mutations act in a dominant negative or haploinsufficient manner meaning that the mutated gene reduces the activity of the remaining wild-type gene or the reduced amount of the wild-type gene product is not sufficient to fulfill its biological task.

The physiological effects of loss-of-function mutations are often predictable because they directly relate to the function of the corresponding gene product. In contrast, understanding the pathogenic mechanism of gain-of-function mutations is complex and it requires an in-depth experimental investigation of each specific mutation of the corresponding protein. On the other hand, understanding the mechanisms of gain-of-function mutations provides unique information about carcinogenic pathways and it may aid in the development of individualized cancer therapies.

Somatic Cancer Mutations in PKMTs and KDMs

This review will focus on the description of somatic cancer mutations in PKMTs and KDMs (Fig. 1). The strongest enrichment of somatic cancer mutations among PKMTs is observed in EZH2, but MLL3, MLL4, SETD2, SYMD1, SMYD3, and SUV420H1 also show relatively high frequencies of somatic cancer mutations when compared to the size of the proteins. Among KDMs, the highest frequency of mutations is observed in KDM6A followed by KDM4D. Of note, this distribution may be influenced by sample bias, because the initial observation of mutations in one enzyme will always stimulate targeted follow-up work that may lead to the discovery of additional mutations. In addition, the mutational spectrum depends on the tumor type, but currently databases are dominated by blood cancers (due to the relative ease of preparing pure tumor cells for analysis). Therefore, the picture may change in future when more data for various other cancers will become available.

As described above, somatic cancer mutations in PKMTs and KDMs could have loss-of-function or gain-of-function effects. While frame-shifts and deletion mutations will mainly have loss-of-function effect, the situation is less clear for missense mutations, which cause an amino acid exchange in the protein. Such point mutations could inactivate the enzyme, if they disrupt cofactor binding, folding, or the binding of the substrate peptide. However, point mutations can also have gain-of-function effects, like the alteration of the peptide interaction potentially leading to the methylation or demethylation of novel substrates, the change of the product methylation state or preferred substrate methylation state, or an alteration of the interaction with other proteins leading to changes in the regulation or targeting of the PKMT (Fig. 2). Depending on the original role of the mutated PKMT or KDM in the regulation of cancer-related genes by histone methylation, somatic mutations in PKMTs or KDMs can lead to the overexpression of oncogenes or loss of expression of tumor suppressor genes via different pathways. If effects are based on the altered methylation of nonhistone proteins, oncogenic proteins can be stabilized or activated or proteins with tumor suppressor activity can be destabilized or inactivated.

To allow an initial prediction of the main effect of somatic cancer mutations in enzymes, one can calculate the ratio of the missense mutations divided by the sum of frame-shift and nonsense mutations (Sense Score) (Fig. 3). This number corresponds to the probability of a widespread gain-of-function mechanism, because a missense mutation has the potential to cause gain-of-function effects, while frame-shift or nonsense mutations will lead to loss-of-function. Thus, a high Sense Score as seen in DOT1L, GLP, SETD1A, SMYD1, SMYD4, SMYD5, SUV39H1, KDM1B, KDM4A, and KDM4B proposes a gain-of-function effect of many mutations in these enzymes. These effects are often dominant and the mutated enzymes act as oncogenes. In contrast, low Sense Scores as in MLL4, SETD2, or KDM6A predict loss-of-function effects as main mechanism suggesting that the corresponding wild-type proteins have a tumor suppressor activity. However, the Sense Score can only provide a rough approximation, since each mutation obviously has its own effects on the enzyme that may differ from the "average" effects of all other mutations. Moreover, epigenetic enzymes can have different roles in different tumors acting as oncogenes in some cases and tumor suppressors in others. In the following sections, we will introduce several important families of PKMTs and KDMs, briefly summarize their role in cancer, and describe the effects of somatic cancer mutations in the respective enzymes.

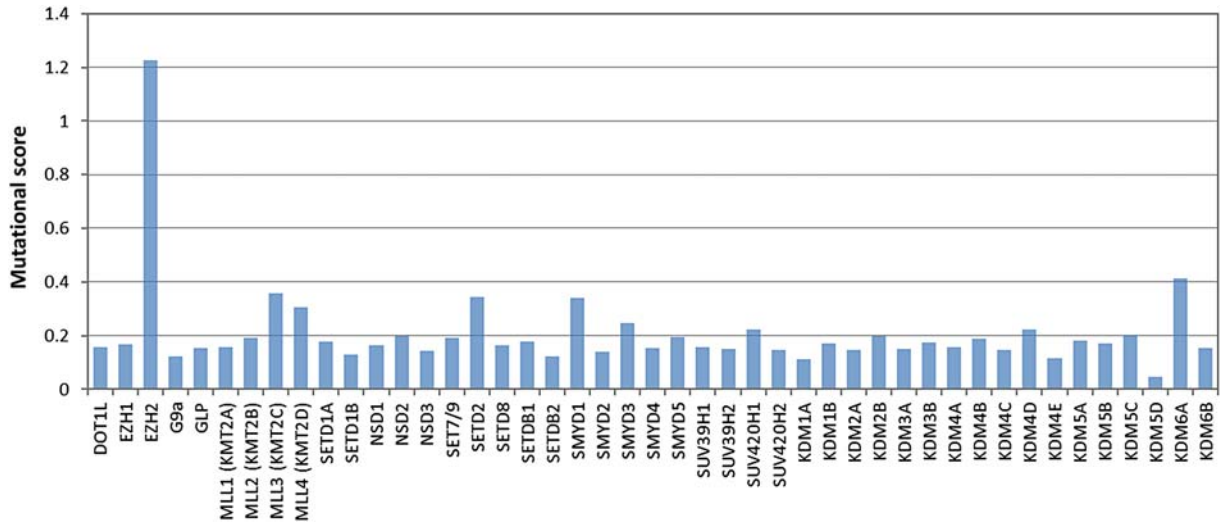

Fig. 1 Graphical representation of the Mutational Scores of PKMTs and KDMs. Data were retrieved in September 2017 from the Cosmic database. The Mutational Score is defined by the ratio of the total number of somatic tumor mutations in the corresponding enzyme divided by the length of the protein in amino acids.

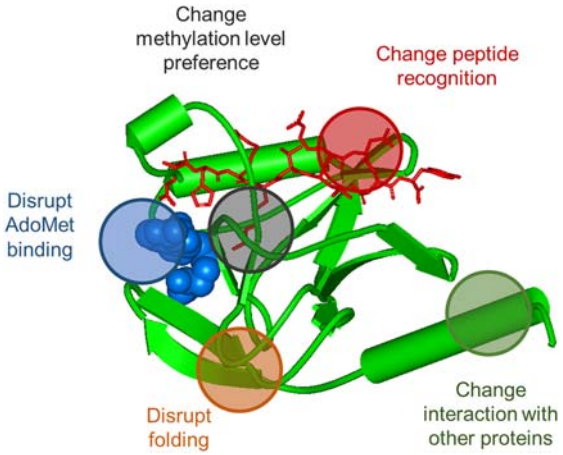

Fig. 2 Potential effects of somatic cancer mutations in PKMTs and KDMs. Mutations may interfere with AdoMet binding or protein folding, which will lead to loss of activity. Mutations may alter the methylation level preference of the PKMTs and KDMs with respect to either the product or the substrates representing a gain-of-function effect. In addition, mutations may change the interaction with other proteins and alter the regulation or targeting of the PKMT. Finally, mutations may change the recognition of the target peptide or binding of the substrate protein leading to either loss of activity on the original substrate or eventual activity on novel substrates. From Kudithipudi, S. and Jeltsch, A. (2014). Biochimica et biophysica acta (BBA)—review on cancer 1846, 366–379 with permission.

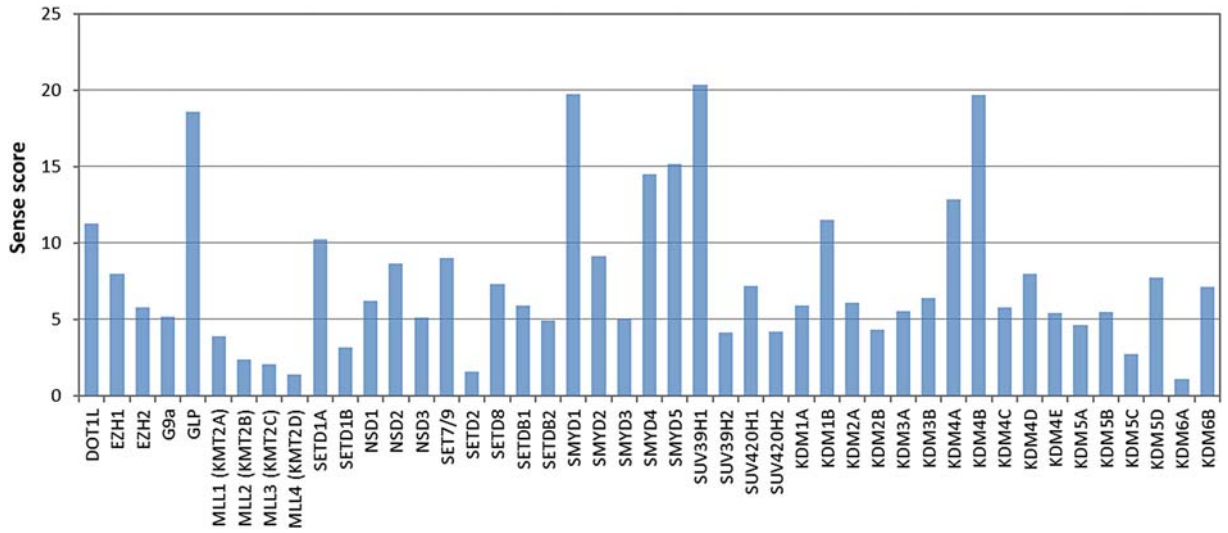

Fig. 3 Graphical representation of the Sense Scores of somatic cancer mutations in PKMTs and KDMs. Data were retrieved in September 2017 from the Cosmic database. The Sense Score is defined by the ratio of the missense mutations (which depending on the exchange can cause loss-of-function or gain-of-function effects) divided by the number of frame-shift and nonsense mutations (which will usually cause loss-of-function effects).

Specific Roles of PKMTs and Their Known Somatic Mutations in Cancer

The EZH2 H3K27 PKMT

The Polycomb repressive complex 2 (PRC2) introduces H3K27me3, which is an important repressive chromatin mark. In mammals, the PRC2 core complex consists of EED, SUZ12, NURF55, Rbap46/48, and the catalytic subunits EZH1 or EZH2. It also has some variable additional subunits including PHF1, JARID2, and AEBP2, which further regulate the PRC2 activity. The SET domain containing EZH1 and EZH2 subunits represent the catalytically active components that introduce up to three methyl groups on H3K27. The interaction between EZH1/2 and the embryonic ectoderm development (EED) protein in the PRC2 complex is particularly essential for activity because EED stimulates the catalytic activity of EZH2 as a scaffolding protein. Moreover, EED contains a seven-bladed β-propeller WD-40 domain forming an aromatic cage that binds trimethylated H3K27 and mediates the association of PRC2 with H3K27me3 containing chromatin thereby supporting the propagation of H3K27me3. Genes repressed by PRC2 include HOXC8, HOXA9, MYT1, CDKN2A, and retinoic acid target genes, which all have important roles in development

and differentiation. In addition, Polycomb proteins play an important role in epigenetic gene silencing in X-chromosome inactivation and imprinting. Furthermore, Polycomb target genes include transcription factors (TFs), signaling genes that play a main role in cell fate determination, and tumor suppressor genes, which function to prevent uncontrolled cell proliferation. The PRC2 complex may also serve as a recruiting platform for DNA methyltransferases, thereby linking these two epigenetic repression systems.

The Role of EZH2 and Its Somatic Mutations in Cancer

Mutations in Polycomb proteins cause developmental defects due to the deregulation of gene expression of key TFs, such as the Homeobox proteins. Polycomb-mediated silencing of those genes prevents the cell from undergoing senescence, which might ultimately lead to the occurrence of cancer. EZH2 overexpression is observed in many tumors and it correlates with a poor prognosis in several tumor types. EZH2 overexpression can lead to the hyper-silencing of differentiating genes or tumor suppressor genes, which can be reverted pharmacologically with EZH2 inhibitors. However, other studies reported that EZH2 has both oncogenic and tumor suppressive roles. The oncogenic histone H3K27M missense mutation identified in pediatric glioblastoma inhibits the PRC2 complex by binding to the catalytic active site of EZH2 and blocking its activity. Moreover, EZH2 methylates nonhistone proteins including the TF GATA4 and retinoic acid receptor alpha, which also could have an effect in carcinogenesis.

Next-generation sequencing of follicular lymphoma and diffuse-large B-cell lymphoma has revealed recurrent somatic mutations within the catalytic SET domain of EZH2 very frequently at Y641 (mainly to F, N, S, and H) and less frequently at A677 (mainly to G). Heterozygosity and the presence of equal quantities of both mutant and normal mRNA as well as expressed protein suggest a dominant gain-of-function mode of action. The Y641 residue is part of the so-called aromatic cage in the active center of EZH2 that surrounds the target lysine ε-amino group. For several PKMTs it was reported that mutations of aromatic cage residues affect the substrate preference and product pattern. The structure of the wild-type and mutant SET domain of EZH2 showed that the hydroxyl group of Y641 (supported by the carbonyl of A677) forms a hydrogen bond to one proton of the lysine ε-amino group, which prevents trimethylation of the target lysine. As a consequence, wild-type EZH2 introduces mono- and dimethylation of H3K27 preferentially using the unmethylated peptide as substrate, while the Y641 mutants prefer mono- and dimethylated peptide substrates to introduce trimethylation. Therefore, these mutations change the methylation level of the product of the EZH2 reaction. EZH2 overexpression or activating mutations likely have a common carcinogenic mechanism based on the hyper-silencing of differentiating genes or tumor suppressor genes. The dominant mode of action of these hyperactive variants suggests EZH2 inhibitors as therapeutic strategy for cancers containing this mutation or overexpressed EZH2. Indeed, several efficient EZH2 inhibitors were developed showing promising effects in animal models and first clinical studies. This development illustrates that understanding of the mechanism of cancer mutations in PKMTs is an important step for targeted and individualized cancer treatment.

The MLL1 and MLL3 H3K4 PKMTs

The mixed lineage leukemia (MLL) family of PKMTs are composed of six members: MLL1–4, SET1A, and SET1B. Each enzyme has H3K4 methyltransferase activity, but they are not redundant and have specific cellular functions. H3K4 can be mono-, di-, and trimethylated and specific MLL proteins introduce defined methylation states of H3K4 at distinct genomic regions. MLL1/2 (KMT2A/B) are trimethyltransferases at promotor regions of genes involved in early development and hematopoiesis, MLL3/4 (KMT2C/D) introduce monomethylated H3K4 at enhancer elements, and the SET1A and SET1B proteins contribute to the bulk of H3K4 trimethylation in mammals. To achieve full methyltransferase activity, MLL proteins require the formation of a core complex containing tryptophan-aspartate repeat protein-5 (WDR5), retinoblastoma-binding protein-5 (RBBP5), and absent small homeotic-2-like (ASH2L) (WRA complex). Interaction with complex partners reorients the SET-I helix in the SET domain switching the enzyme from an inactive autoinhibited state into an active conformation. This effect is mainly caused by the interaction with the RBBP5/ASH2L heterodimer, leading to efficient activation of MLL3 in the presence of these two factors. The interaction of MLL1 with the RBBP5/ASH2L heterodimer is weaker compared to the other MLL enzymes. Therefore, MLL1 requires WDR5 for efficient binding of the RBBP5/ASH2L heterodimer such that MLL1 only exhibits full methyltransferase activity in the presence of all three complex partners. The isolated SET domains of MLL1 and MLL2 also show activity and monomethylate H3K4. In complex with WDR5, ASH2L, and RBBP5 they trimethylate the substrate lysine residue. Although the MLL1 protein contributes to only about 5% of the global H3K4 trimethylation, it is majorly responsible for the regulation of HOX genes and loss of WDR5 expression in cells reduced the H3K4 methylation of HOX gene promoters and decreased Hox gene expression. In addition to the core complex partners, different MLL complexes contain variable additional subunits involved in more specific targeting and regulation.

The Role of MLL1 and Its Somatic Mutations in Cancer

MLL1 undergoes numerous chromosomal translocations with more than 70 different partner genes that are found in several human acute leukemia. AF4, AF9, AF10, and ENL are reported to form fusion partners in more than 75% of MLL associated leukemia; these fusion genes are sufficient to transform normal cells to leukemic cells. In these chromosome translocations, the N-terminus of MLL1 is fused in frame with the partner proteins such that the fusion proteins retain the DNA binding and targeting domains of MLL1, but they lose the C-terminal catalytic SET domain. This leads to dysregulation of developmental genes, like HoxA9 and Meis1.

Aggressive leukemia in children and adults with MLL1 gene translocations show high expression of HOX genes. MLL1 knockdown affects cell cycle-regulating genes especially cyclin A, cyclin B, and p57, which results in a cell cycle arrest in the G2/M phase and apoptosis in cultured cells. Malignant cells are more sensitive to MLL1 knockdown than the normal cells, which might be due to the essential requirement of the MLL1 gene for the high proliferation of malignant cells. Consequently, knockdown of MLL1 in a xenograft mouse tumor model was shown to suppress tumor growth. In summary, MLL1 is an oncogenic protein; it is overexpressed in tumors or it forms fusion partners with other genes finally enhancing the expression of HOX genes. Similarly, MLL2, the closest paralog of MLL1, functions as oncogene and its deletion led to a reduction in the survival of MLL–AF9 leukemia cells. In addition, the combined loss of MLL1 and MLL2 further reduced AML growth. These discoveries indicate that addressing MLL1 and/or MLL2 could be valuable therapeutic strategies.

Several somatic cancer mutations were found in MLL1 leading to nonsense, missense, and frame-shift mutations (Fig. 1), but only few of them have been studied mechanistically. The catalytic activity of four MLL1 cancer mutants found in different tumors was determined in absence and presence of the complex partners WDR5, RBBP5, and ASH2L. Two mutants, R3903H and S3865F, showed partial or complete loss of activity. This result was in agreement with the relatively low Sense Score of MLL1 mutants. However, two mutants, R3841W and R3864C, were more active than wild-type MLL1 and both were not stimulated by the WRA complex. Because of their hyperactivity and the lost control of activity by the WRA complex, these mutants are likely to cause gain-of-function effects. Mechanistically, all these effects were triggered by conformational changes of the enzyme into more stable active or inactive states. These data show that different somatic cancer mutations of MLL1 found in different tumors can lead to inhibition or stimulation of MLL1 combined with a loss of the regulation of its methyltransferase activity by complex partner proteins. This finding highlights the multifaceted roles of MLL1 in cell fate determination and gene regulation. Furthermore, the inhibition of the cancer mutants by the MM-102 inhibitor was tested, which disrupts the interaction between MLL1 and WDR5 leading to growth inhibition of MLL-AF9 leukemia cells. In line with the enzymatic results described above, the activity of R3841W and R3864C, which did not depend on WDR5 binding for activity, was not reduced by MM-102, indicating that this compound would not be a therapeutic option for patients with these mutations in MLL1. This observation highlights the importance of genetic analysis of tumor samples combined with specific functional investigation of the effect of somatic tumor mutations in the design of individualized therapies.

The Role of MLL3 and Its Somatic Mutations in Cancer

MLL3 is often deleted in myeloid leukemia and inactivation of MLL3 in mice leads to epithelial tumor formation, which suggests that the methyltransferase activity of MLL3 suppresses tumorigenesis. MLL3 expression was also decreased by at least twofold in almost half of the breast cancer patients when compared to the matched normal tissue, but the expression changes did not correlate with the tumor progression and the pathological effects of changes in the MLL3 expression in breast cancer are still ambiguous. In addition, in colorectal cancer cell lines the MLL3 promoter is hypermethylated, which results in the repression of MLL3 protein expression. Taken together, these findings indicate that MLL3 has a tumor suppressor role in several cancers.

MLL3 and MLL4 are frequently mutated in cancers (Fig. 1) but their pathological effects are not known in most cases. MLL3 shows a high frequency of loss-of-function mutations (Fig. 3), which are spread over the entire protein including the PHD fingers and SET domain. Three mutations located in the catalytic SET domain of MLL3 (S4757C, N4848S, and Y4884C) were investigated regarding their effects on critical enzymatic properties. One mutation, N4848S, led to a loss of catalytic activity, which is in agreement with the location of N4848 in the AdoMet binding site of MLL3. This result is in line with the evidence for a tumor suppressive function of MLL3 described above. However, another mutation, Y4884C, located in the aromatic pocket of the SET domain, showed a striking gain-of-function effect. Kinetic data demonstrate that MLL3 wild-type preferentially transfers one single methyl group to unmethylated H3K4 generating H3K4me1. In contrast, in vitro methylation studies showed that the Y4884C mutation converts MLL3 into a trimethyltransferase with H3K4me1 as preferred substrate. This result was further confirmed by global histone H3K4me3 methylation analysis from human cell lines. Hence, the mutagenic phenotype and pathogenic mechanism of Y4884C mirrors that of Y641 mutations in EZH2. By generation of abnormal H3K4me3 instead of H3K4me1, enhancer regions could be reprogrammed to function as promoters leading to abnormal gene expression. Interestingly, like in MLL1, these results suggest that each MLL3 mutant has individual roles during tumorigenesis, which may be related to the corresponding tumor types and tumorigenic pathways in different cell types. MLL3 inhibitors might be beneficial in patients carrying the Y4884C mutation, while they are unlikely to be effective in cases with inactive MLL3.

MLL4 is a H3K4 monomethyltransferase belonging to the same subfamily as MLL3. It is often found mutated in cancers and a large fraction of the MLL4 cancer mutations are nonsense or frame-shift mutations which inactivate the enzyme (Fig. 3), indicating a tumor suppressor role of MLL4 at least in some cell types. Somatic loss-of-function mutations of MLL4 were observed on a single copy of the gene suggesting that MLL4 acts as a haploinsufficient tumor suppressor.

The DOT1L H3K79 PKMT

DOT1L was initially identified in a genetic screen aiming to identify proteins involved in telomeric silencing in yeast. The yeast and human homolog was later demonstrated to methylate K79 of histone H3, which is located in the core structure of histone H3. DOT1L is a unique histone lysine methyltransferase because it does not contain a SET domain. Instead, its active site is located in a seven-ß-strand Rossmann-fold domain, also found in other class I methyltransferases, like protein arginine and DNA

methyltransferases and other PKMTs, which methylate nonhistone substrates. H3K79 methylation is associated with active gene transcription and it is also involved in cell cycle regulation. H3K79me3 and me2 are majorly localized in the coding regions of active genes, whereas H3K79me1 is broadly distributed across the coding region. DOT1L-mediated H3K79 methylation is important for embryonic development and a germline knockout of the mouse DOT1L homolog causes embryonic lethality. DOT1L is the sole enzyme that methylates H3K79 indicating that it represents the main target protein in diseases related to the aberrant H3K79 methylation.

The Role of DOT1L and Its Somatic Mutations in Cancer

During the last years, several reports have demonstrated that DOT1L is linked to MLL-mediated leukemia. As described above, 70% of the infant leukemia patients were found to have MLL translocations, in which the N-terminal part of MLL containing its DNA recognition motifs is expressed as a fusion protein with several partner proteins like AF10, AF9, AF4, or ENL. These fusion proteins retain the MLL DNA binding and chromatin targeting domains, which results in an altered MLL target gene regulation depending on the fusion partner. Interestingly, it was found that all of these fusion proteins physically interact with DOT1L either directly or indirectly. This leads to a recruitment of DOT1L to MLL target loci. The resulting increased H3K79 methylation is a positive transcription mark that, bypassing the normal transcription regulation, causes the expression of proleukemogenic genes (like HOXA9 and MEIS1) and by this stimulates the development of leukemia. H3K79 trimethylation plays a crucial role in the regulation of HOX genes such as Hoxa9, Hoxa7, and Meis1, which are overexpressed after aberrant H3K79 methylation in leukemic cells. Thus, DOT1L is an example in which the mistargeting of a PKMT causes methylation at aberrant genomic loci.

By rational drug design and genetic approaches, it was shown that either inhibition of the activity of DOT1L or loss of the DOT1L protein has dramatic effects on the expression of HOX genes. A DOT1L-specific competitive inhibitor of AdoMet binding selectively inhibited H3K79 methylation and blocked the expression of leukemogenic genes. This compound was efficient in killing leukemic cells with MLL translocations with relatively small effect on non-MLL leukemic cells. This result shows that the methyltransferase activity of DOT1L plays a central role in the MLL fusion-mediated cellular transformation and maintenance of the transformed state and it is a very important drug target. In addition to its pathological function in MLL-mediated leukemia, H3K79 hypermethylation was observed in lung cancer cell lines and tissues. Downregulation of DOT1L in lung cancer cell lines blocked the proliferation of cells, which further supports the oncogenic role of DOT1L. Several somatic cancer mutations in DOT1L have been identified (**Fig. 1**), but their mechanism has not yet been described. Based on the known role of DOT1L in cancer, gain-of-function or hyperactivation effects of the mutations are likely, which is also in agreement with the high Sense Score of DOT1L somatic tumor mutations (**Fig. 3**).

The SETD2 H3K36 PKMT

SETD2 was first described as Huntingtin-interacting protein B (HYPB). Later, studies have uncovered that it can trimethylate H3K36 via its SET domain, leading to its renaming to SETD2. The SETD2 protein interacts with the Ser2/Ser5 hyperphosphorylated RNA polymerase 2 during transcriptional elongation via its SRI (Set2 Rpb1 interacting) domain, which explains why H3K36 trimethylation is enriched in the body of actively transcribed genes. In humans, H3K36 mono- and dimethylation is introduced by several PKMTs (including the NSD enzymes), while H3K36me3 is only generated by SETD2.

The Role of SETD2 and Its Somatic Mutations in Cancer

The SETD2 protein interacts with p53 via its C-terminal SET and WW domains and stimulates p53 activity. Knockdown of SETD2 lowered the expression of different p53 target genes such as Puma, Noxa, Huntingtin, and p21. In addition, SETD2 also increases the stability of the p53 protein by lowering the expression of the HDMT2 Ring finger-type E3 ubiquitin ligase, which triggers degradation of p53 by ubiquitination. SETD2 mRNA expression was lost in human breast malignant tissue and the SETD2 expression levels were negatively correlated with the tumor stage. These data suggest that SETD2 can be used as a biomarker for the breast cancer and SETD2 acts as a tumor suppressor gene.

SETD2 is among the PKMTs with the highest frequency of somatic cancer mutations (**Fig. 1**). Somatic mutations of SETD2 are often observed in high grade gliomas, clear cell renal cell carcinoma, and leukemias and the majority of them are nonsense and frame-shift mutations, which lead to loss-of-function (**Fig. 3**). Gliomas with disruption of SETD2 showed a considerable decrease in H3K36 trimethylation, indicating that the SETD2 mutants negatively affect the activity of the enzyme. SETD2 truncating mutations are majorly found in the C-terminus, which either leads to the loss of the SET or SRI domains; the former leads to loss of PKMT activity and the latter disrupts the interaction with the phosphorylated RNA polymerase II. The SETD2 D1616N mutation that is located in the SET domain of the protein has been identified in the cRCC cell lines, which show a considerable loss of H3K36 trimethylation, similarly as other cRCC tumor cell lines containing mutations in the SETD2 protein. Finally, K36M mutations in histone H3, found in chondroblastomas at frequencies of up to 96%, inhibit SETD2 and lead to genome wide loss of K36me3 accompanied by gains in K27me3 and changes in the expression of cancer-associated genes. All these results document that the loss of SETD2 function plays a pivotal role in the initiation and progression of cancer, and somatic cancer mutations of SETD2 are examples of loss-of-function.

The NSD Family of H3K36 PKMTs

The nuclear receptor-binding SET domain containing protein (NSD) family consists of three members: NSD1, NSD2, and NSD3. They differ in protein size and domain organization and have specific biological functions in normal development and pathophysiology. All NSD enzymes contain a catalytic SET domain, in addition to several PHD domains and two PWWP domains. The SET domain introduces H3K36 mono- and dimethylation using unmethylated H3K36 as substrate in vivo and in vitro. The other domains mediate the interaction with chromatin and other proteins. NSD1 and NSD2 were reported to methylate several other substrates including H1.5 K168, which is efficiently methylated by NSD1, and H4K44, which is methylated by NSD1 and NSD2. Defects in the NSD family proteins are implicated in several diseases. Mutations and deletions in NSD1 lead to SOTOS syndrome, a developmental disease associated with fetal overgrowth. Loss of NSD2 activity causes Wolf-Hirschhorn syndrome (WHS) which is characterized by several developmental defects, growth retardation, and brain anomalies leading to mental retardation.

The Role of NSD Enzymes and Their Somatic Mutations in Cancer

An increasing number of reports document defects in NSD family members in several cancers: NSD1 is associated with acute myeloid leukemia and multiple leukemia, NSD2 is linked to prostate cancer and myeloid leukemia, and NSD3 is associated with breast cancer and lung cancer. Protein expression studies with 3000 different tumor samples revealed higher NSD2 expression when compared to normal tissue. Moreover, NSD2 expression positively correlated with the progression and advanced tumor stage. The cells with high levels of NSD2 contained enriched H3K36me2 and lower levels of H3K27 trimethylation and they showed a change in the distribution of H3K36me2, which is enriched in gene bodies in normal cells, but dispersed in the NSD2 overexpressing cells. Depletion of NSD2 in a xenograft mouse tumor model showed a decrease of H3K36me2 and also a reduction in tumor growth. Moreover, NSD proteins are involved in chromosomal translocations forming different fusion proteins in several cancers including fusions to the nucleoporin gene (NUP98) in NUP98-NSD1 or NUP98-NSD3, which are found in acute myeloid leukemia. The fusions enhance HOXA5-A10 gene expression via increasing H3K36 dimethylation. In summary, the deregulation of NSD proteins plays a causative role in cancers and the histone methyltransferase activity of these proteins is critical for promoting malignant growth.

Recent genomic sequence data of cancer cell lines revealed somatic mutations in NSD enzymes (Fig. 1), which especially in the case of NSD2 suggest a gain-of-function mechanism (Fig. 3). The E1099K mutation was found at very high frequency in NSD2 mostly in pediatric lymphoid malignancies, such as hypodiploid acute lymphoid leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma, and lung or stomach adenocarcinoma. E1099 is located in the SET domain in a loop next to the histone binding groove and in vitro methylation assays have shown higher activity of NSD2 E1099K on nucleosomes. In addition, increased H3K36 dimethylation and decreased H3K27me3 were observed in cell lines harboring the E1099K mutant, also suggesting an increased H3K36 methyltransferase activity. However, different from the EZH2 mutations described above, the E1099K mutation enhances the methyltransferase activity of NSD2 without apparent changes of the product specificity. In addition, ALL tumors frequently contain a D1125N mutation in NSD2; this variant is also associated with higher H3K36me2 methyltransferase activity. Hence, the somatic cancer mutations in NSD2 are examples of a gain-of-function similarly as the overexpression of NSD2. These observations indicate that NSD2 has a vital role as oncoprotein and is a very promising drug target in particular for tumors with overexpressed or hyperactive NSD2.

The SMYD PKMT Family

The SMYD PKMT protein family consists of five members named SMYD1–5. SMYD proteins are not well characterized; among them SMYD2 and SMYD3 are best studied. They were grouped based on their similar domain architecture containing a split SET domain, which carries an inserted MYND (Myeloid, Nervy, and DEAF-1) domain. The MYND domain is responsible for protein–protein interactions and the split SET domain contains the catalytic elements as in other SET domain enzymes. SMYD2 has been reported to monomethylate several lysine residues on histone and nonhistone proteins. It was initially shown to methylate H3K36 but later it was reported that the interaction with HSP90α changes its specificity toward H3K4. Additional studies showed that SMYD2 also methylates K266 of estrogen receptor alpha, K370 of p53, and K810 and K860 of the retinoblastoma (RB) protein. SMYD3 was initially identified as H3K4 di- and trimethyltransferase. Later reports showed that SMYD3 can also methylate H4K20 with a preference for the dimethylated lysine as a substrate, and that it trimethylates histone H4 at K5. Apart from that, SMYD3 also methylates nonhistone proteins, like vascular endothelial growth factor receptor 1 (VEGFRI) at K831, which enhances its kinase activity. Overall, the methylation site(s) of SMYD3 are still ambiguous and the full spectrum of substrate proteins is not well known.

The Role of SMYD Family Enzymes and Their Somatic Mutations in Cancer

Methylation of nonhistone targets by SMYD2 has clear connections to cancer and overexpression of SMYD2 has a tumor-promoting effect. For example, SMYD2 methylation of p53 at K370 impairs the tumor suppressor activity of p53. Moreover, methylation of the Rb protein at K810 enhances its phosphorylation at S807 and S811 leading to the dissociation of Rb from E2F, which enhances its transcriptional activity and promotes cell cycle progression. SMYD2 is significantly overexpressed in several cancers. For example,

the SMYD2 mRNA levels were reported to be almost 8-fold increased in leukemic cells, when compared with normal bone marrow controls and patients with high SMYD2 expression showed lower survival rate. Similarly, SMYD2 protein overexpression was identified in esophageal squamous cell carcinoma (ESCC), bladder, and gastric cancer. Knockdown of SMYD2 in ESCC cell lines with overexpression of SMYD2 led to the suppression of proliferation due to a G_0–G_1 arrest, suggesting that SMYD2 inhibitors might be good cancer drugs in these cases. In summary overexpression of SMYD2 is frequently observed in several cancers and it is correlated with lower survival rate indicating that SMYD2 acts as an oncoprotein.

Similarly as SMYD2, SMYD3 acts as an oncogenic protein. Elevated levels of SMYD3 expression were observed in colorectal carcinoma (CRC), hepatocellular carcinoma (HRC), breast, pancreatic, gastric, and lung cancer cells, whereas weak or no expression was observed in noncancerous cells of other tissues. In agreement with this, suppression of SMYD3 significantly reduced the growth of CRC and HRC cells. In breast cancer cell lines, a truncated SMYD3 protein lacking the first 34 amino acids was observed as well, which was shown to have a higher activity than the full-length SMYD3. Moreover, it has been shown that SMYD3 upregulates the expression of the oncogene matrix metalloproteinase MMP-9, which is involved in tumor progression and metastasis by stimulating cell migration, tumor invasion, and angiogenesis. Suppression of SMYD3 reduces MMP9 gene expression supporting its oncogenic property. Furthermore, SMYD3 methylation of additional nonhistone substrates is a key event in Ras-driven carcinomas. SMYD3 methylates the MAP3K2 kinase at K260, which increases MAP kinase signaling and leads to the formation of Ras-driven carcinomas. In addition, MAP3K2-K260 methylation inhibits binding of PPP2 phosphatase complex, which is a cellular phosphatase and a key negative regulator of MAP kinase signaling pathway.

Low SMYD4 gene expression has been reported in breast cancer cells where it was correlated to higher expression of the platelet-derived growth factor receptor α (Pdgfr-α). In several mammalian cancers, Pdgfr-α is an oncogene, which is involved in the proliferation and survival of tumors. Re-expression of SMYD4 in breast cancer cell lines repressed the Pdgfr-α gene, suggesting that SMYD4 acts as potential tumor suppressor at least in this cellular model system. Altogether these data suggest that the SMYD proteins play vital roles in regulating the expression of oncogenes in several cancers with SMYD4 acting as tumor suppressor while SMYD3 and SMYD2 function as oncogenes.

Recent genomic sequencing in cancer patients revealed several somatic mutations in SMYD proteins with SMYD1 and SMYD3 showing the highest proportion of mutations (**Fig. 1**). The somatic mutations in SMYD PKMTs include missense and nonsense mutations as well as insertions and deletions. Interestingly, the mutational patterns are indicative of a gain-of-function effect in particular for SMYD1, but also SMYD4 and SMYD5 (**Fig. 3**). This finding is not in agreement with the observation that SMYD4 acts as tumor suppressor in breast cancer cells as described above, suggesting that the role of SMYD4 is complex and tumor type specific. Since SMYD proteins methylate several histone and nonhistone substrates they may play roles in gene expression regulation and tumor formation. It will be important to determine the full substrate spectrum of all SMYD proteins and understand the pathogenic mechanism of the somatic missense mutations on the methylation of different substrate proteins. Hyperactive somatic mutations could have the same effect as the overexpression of SMYD2 or SMYD3 in cancer cells and they may, for example, aberrantly increase the lysine methylation of tumor suppressor p53 and decrease its tumor suppressor function. Additionally, it will be interesting to see whether SMYD protein mutations may change the product specificity of the PKMT, although such an effect still awaits experimental validation.

Roles of KDMs and Their Known Somatic Mutations in Cancer

The UTX H3K27 KDM Family

The Ubiquitously Transcribed Tetratricopeptide repeat on Chromosome X (UTX) demethylase, also known as KDM6A, is a member of the Jumonji-containing lysine demethylase family. UTX catalyzes the demethylation of H3K27me2/3 in an Fe(II)-dependent dioxygenase reaction using α-ketoglutarate and oxygen as cosubstrates with the production of formaldehyde and succinate. UTX was found as a specific complex partner of the MLL3/4, which catalyze the H3K4 monomethylation at enhancers of active genes. H3K4 and H3K27 have antagonistic roles, as H3K27me2/3 leads to the repression of the same target genes, which are activated by H3K4 methylation. By demethylation of H3K27, UTX supports the maintenance of the H3K4 activation mark set by MLL3/4. UTX knockdown studies have documented its essential role in embryonic development, tissue-specific differentiation, and its requirement for correct reprogramming.

The Role of UTX and Its Somatic Mutations in Cancer

Deletion of UTX and point mutations in UTX cause the Kabuki Syndrome (KS) characterized by distinctive facial features, developmental delay, mild to moderate intellectual disability, postnatal growth retardation, and additional features such as skeletal anomalies. Previously, mutations in MLL4 were found in about half of all KS patients indicating that the switching from H3K27 methylated silenced to H3K4 methylated active chromatin states is disturbed in this disease. Moreover, UTX was the first demethylase that was found somatically mutated and associated with human cancer. Cancer mutations in the UTX gene include homozygous (in females) or hemizygous (in males) large deletions, nonsense mutations, small frame-shifting insertion/deletions, and consensus splice site mutations that lead to aberrant splicing and premature termination codons. Inactivating UTX mutations and deletion were found in several cancer types, including multiple myeloma, leukemia, esophageal, and renal cancer (**Figs. 1 and 3**). These data document that UTX acts as a tumor suppressor. Still, it was found that GSKJ4, an inhibitor of UTX and

JMJD3, is a promising suppressor of breast cancer stem cells (BCSCs) due to its efficient repression of the expansion and self-renewal capacity of BCSCs. In addition, xenograft models confirmed a significant inhibition of tumor progression in vivo. This study suggested that targeting UTX is a therapeutic option for cancer treatment, despite of its tumor suppressive role in some cancers, indicating that PKMTs and KDMs (and their somatic mutations) have tumor-specific effects.

The KDM1 Family

The human KDM1 family of histone demethylases, LSD1 (KDM1A) and LSD2 (KDM1B), catalyze the demethylation of mono- and dimethyl marks of H3K4 and H3K9. The enzymes contain a SWIRM and Amine oxidase domain, LSD2 also an N-terminal Zinc finger domain. In LSD1 the Amine oxidase domain is split by an inserted Tower domain. The substrate specificity of LSD1 is influenced by its association with different partners. LSD1 generally demethylates H3K4me1/2, but when LSD1 interacts with the androgen receptor (AR) its enzymatic specificity switches to H3K9me1/2. This dual activity enables LSD1 to regulate both the repression and activation of genes. LSD1 is a component of large transcription regulatory complexes including the nucleosome remodeling and deacetylating (NuRD) complex, RCOR1 complex, and the CtBP corepressor complex. The interaction with RCOR1 is especially relevant, since it stabilizes the protein and strongly promotes its H3K4me1/2 demethylation activity. In embryonic stem cells, KDM1A has been identified at enhancers, in association with the NuRD complex. When associated with these complexes, LSD1 promotes gene silencing by removing activating methyl marks from H3K4. KDM1A-containing complexes are recruited to their respective target genes in the genome by a series of TFs that act in specific cell types during normal development or, potentially aberrantly, in human disease. In contrast, when LSD1 interacts with the androgen or estrogen receptor, it promotes transcriptional activation by demethylating the repressive H3K9me2 (dimethylation of histone H3 at K9) histone modification. In addition, the enzyme can work on nonhistone substrates including p53, DNMT1, E2F1, MYPT1, STAT3, HIV Tat, HSP90, and MTA1. LSD2 has been described to demethylate H3K4me1/2 but the biological roles of LSD2 (KDM1B) are less known.

The Role of KDM1 Enzymes and Their Somatic Mutations in Cancer

LSD1 and its downstream targets are involved in a wide range of normal physiological processes, including embryonic development, stem cell maintenance, and differentiation, but it is also a key player in oncogenic processes, including tumor-cell growth and metastasis, compromised differentiation, enhanced cell motility, and metabolic reprogramming. LSD1 has been reported to be overexpressed in a variety of tumors and inactivating LSD1 or downregulating its expression inhibits cancer-cell development. The function of somatic tumor mutations in LSD1 and LSD2 has not yet been described, but the mutational spectrum of LSD2 (**Fig. 3**) suggests the occurrence of gain-of-function mutations.

The Role of KDM4 Enzymes and Their Somatic Mutations in Cancer

The KDM4 family comprises five members (KDM4A-E). The KDM4A, B, and C proteins are very similar to each other and contain a JmjN and JmjC domain, two PHD-, and two Tudor domains. KDM4D and KDM4E lack the C-terminal region including the PHD and Tudor domains. All KDM4 enzyme demethylate H3K9, KDM4A-C also H3K36, and some of them also H3K27. KDM4 enzymes have roles in steroid receptor regulation and they can activate the expression of oncogenes. KDM4 genes were found amplified and overexpressed in various tumor types. The effects of somatic cancer mutations have not yet been described, but a very high Sense Score of KDM4B and KDM4A mutations (**Fig. 3**) is in agreement with an oncogenic role of these enzymes.

Somatic Mutations in Related Proteins

Examples of other frequently mutated epigenetic enzymes not described in detail in this article include the DNA methyltransferase 3A (DNMT3A), the Ten-eleven-translocation 2 methylcytosine hydroxymethyltransferase (TET2), and SWI/SNF chromatin remodeling complexes. DNMT3A is an essential DNA methyltransferase frequently containing an R882H mutation in blood cancer, which has been reported to reduce its activity and changes its oligomerization in a dominant negative way and by this it may alter the DNA methylation pattern. Somatic TET2 mutations are frequently observed in various cancers. These include nonsense, deletion, and missense mutations at very crucial residues for the enzymatic activity thus predicted to inactivate the enzyme. Finally, mutations that inactivate SWI/SNF chromatin remodeling complexes subunits are found in nearly 20% of human cancers. These complexes catalyze the opening or condensation of chromatin and by this help to execute the epigenetic program.

In addition, recurrent somatic mutations were found in genes indirectly related to PKMTs and KDMs. Different isocitrate dehydrogenase (IDH) isoforms catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) in the TCA cycle, while reducing NAD^+ or $NADP^+$ depending on the isoform catalyzing the reaction. Unexpectedly, frequent mutations were found in IDH enzymes and shown to be involved in the progression of tumors. All discovered IDH mutations are missense mutations in the active site of the enzymes, often affecting R132 in IDH1, and either the analogous arginine residue (R172) or R140 in IDH2. The mutations occur heterozygously, suggesting that the alterations lead to a dominant gain-of-function effect. Unexpectedly, the mutant IDH enzymes were found to catalyze the NADPH-dependent reduction of α-KG to 2-hydroxyglutarate (2-HG). This oncometabolite has a similar structure as α-KG and acts as competitive inhibitor for enzymes that employ α-KG as cofactor, like

the TET enzymes and the Jumonji family of histone lysine demethylases. In agreement with this observation, hypermethylation of many histone methylation marks was observed following introduction of IDH1 mutations into cells and DNA hypermethylation by IDH mutations has been observed as well. Another example of somatic cancer mutations with indirect epigenetic effects are mutations in the histone proteins themselves. As already mentioned, different cancers frequently contain missense mutations in genes encoding histone H3.3 (H3F3A) and H3.1 (HIST3H1B), which alter methylated lysine residues to methionine, like K27M or K36M. When incorporated into chromatin, the histone mutants were shown to inhibit the corresponding PKMTs leading to massive disturbances of chromatin modification patterns.

Prospective Vision

Proteomic and genomic approaches have allowed addressing several mechanisms of carcinogenesis, but cancer is continuously confronting us with new challenges. It is well established that cancers arise as a result of gene mutations and clonal proliferation of cells containing growth-promoting alterations. The specific mutations identified in defined cancer subtypes have important diagnostic significance. For instance, Janus kinase 2 mutation (JAK V2617F) is present in many myoproliferative neoplasma and polycythemia vera and now this mutation is used as diagnostic marker for this condition. In addition, epigenetic alterations were recognized to play important roles in tumor formation. The recent discovery and characterization of somatic tumor mutations in epigenetic enzymes connect these two processes, because in these cases the genetic changes drive the appearance of epigenetic alterations. Two large classes of epigenetic enzymes are PKMTs and KDMs and the investigation of the role of somatic mutations in these enzymes in carcinogenesis is an emerging approach to the mechanistic understanding of tumor formation and progression.

While the mechanism of loss-of-function mutations in PKMTs and KDMs can be deduced from the function of the corresponding gene, this is not necessarily true for gain-of-function mutations, which might have diverse pathogenic effects: they might enhance the enzymatic activity, increase the stability of the protein, or alter the cellular targeting. Somatic mutations in PKMTs and KDMs might also alter their association with interacting partners leading to changes in the substrate profile, or recruit the enzymes to specific chromatin loci resulting in the altered expression of particular oncogenes or tumor suppressor genes. The higher activity of a mutated PKMT or KDM might lead to abnormal methylation of histones at particular loci, which either enhance or inhibit the transcription of oncogenes or tumor suppressor genes. Mutations in the catalytic SET domain of PKMTs and JmjC domain of KDMs might change their peptide sequence specificity leading to the methylation or demethylation of novel targets. Mutations may also change the product specificity leading to a change in the final methylation state of the target lysine residues. In most cases, the mechanistic consequences of somatic mutations in PKMTs and KDMs are not yet known and careful biochemical studies will be needed for their elucidation. The study of the pathomechanism of somatic cancer mutations in PKMTs and KDMs holds great promises for several reasons:

(1) Different studies have demonstrated that depending on the tumor type, inhibition of a PKMT or KDM or its hyperactivity and loss of regulation can promote tumor formation illustrating the complex and multifaceted role of these epigenetic enzymes in cell fate determination and gene regulation. Therefore, dedicated biochemical investigations are needed for each somatic tumor mutation of important PKMTs and KDMs in order to decipher its pathological role.
(2) It will be important in future to investigate the effects of somatic mutations in order to develop individualized cancer treatments. For example, inhibitors developed against specific PKMTs or KDMs might assist treatment of cancers with hyperactive enzyme mutants, but these inhibitors cannot be used to treat the cancers caused by the loss of enzyme activity. This makes the investigation of the pathomechanism of somatic cancer mutations very relevant for the development of novel more causative and individualized tumor therapies.
(3) The investigation of somatic cancer mutations in PKMTs and KDMs is also imperative for the general understanding of the role of these enzymes in carcinogenesis. Different processes such as expression changes, abnormal posttranslational modifications (PTM), or somatic mutation can alter the activity of PKMTs or KDMs in cells. However, expression levels and PTM patterns of proteins are very difficult to measure in tumor tissues and these changes may occur only transiently during a critical phase of cellular transformation. In contrast, somatic cancer mutations are stable, and they can be studied in tumor samples, cell lines, or in vitro much more easily. Therefore, studying and understanding of the mechanism of somatic cancer mutations is a powerful tool to unravel the role of the corresponding PKMT and KDM even in cases not involving mutations.
(4) The screening for somatic cancer mutations holds great promises in clinical practice, since the detection of mutations occurs at DNA level, which can be done from small samples of cancer tissue. In contrast, the detection of expression changes or aberrant PTMs needs RNA and proteomic methods, which are much more demanding with respect to sample size and sample treatment. Furthermore, simple patient blood samples can be used as normal controls in mutational screenings, while the acquisition of matching "normal" tissue for comparison is sometimes very difficult in expression studies and proteomic studies.

In summary, the investigation of the pathomechanism of somatic cancer mutations in PKMTs and KDMs is an important and urgent challenge in biomedical research. For this, relevant assay systems need to be established to investigate the properties of the wild-type enzymes and their cancer mutants in vitro and in cellular settings. These studies will also pave the way toward the generation of novel and more potent and specific PKMT and KDM inhibitors and guide their rational application in clinical studies.

See also: Mutations in DNA Methyltransferases and Demethylases. Mutations in Chromatin Remodeling Factors.

Further Reading

Bannister, A.J., Kouzarides, T., 2011. Regulation of chromatin by histone modifications. Cell Research 21, 381–395.
Chi, P., Allis, C.D., Wang, G.G., 2010. Covalent histone modifications–miswritten, misinterpreted and mis-erased in human cancers. Nature Reviews Cancer 10, 457–469.
Dawson, M.A., Kouzarides, T., 2012. Cancer epigenetics: From mechanism to therapy. Cell 150, 12–27.
Forbes, S.A., Beare, D., Boutselakis, H., Bamford, S., Bindal, N., Tate, J., Cole, C.G., Ward, S., Dawson, E., Ponting, L., Stefancsik, R., Harsha, B., Kok, C.Y., Jia, M., Jubb, H., Sondka, Z., Thompson, S., De, T., Campbell, P.J., 2017. COSMIC: Somatic cancer genetics at high-resolution. Nucleic Acids Research 45, D777–D783.
Greer, E.L., Shi, Y., 2012. Histone methylation: A dynamic mark in health, disease and inheritance. Nature Reviews Genetics 13, 343–357.
Hamamoto, R., Saloura, V., Nakamura, Y., 2015. Critical roles of non-histone protein lysine methylation in human tumorigenesis. Nature Reviews Cancer 15, 110–124.
Huether, R., Dong, L., Chen, X., Wu, G., Parker, M., Wei, L., Ma, J., Edmonson, M.N., Hedlund, E.K., Rusch, M.C., Shurtleff, S.A., Mulder, H.L., Boggs, K., Vadordaria, B., Cheng, J., Yergeau, D., Song, G., Becksfort, J., Lemmon, G., Weber, C., Cai, Z., Dang, J., Walsh, M., Gedman, A.L., Faber, Z., Easton, J., Gruber, T., Kriwacki, R.W., Partridge, J.F., Ding, L., Wilson, R.K., Mardis, E.R., Mullighan, C.G., Gilbertson, R.J., Baker, S.J., Zambetti, G., Ellison, D.W., Zhang, J., Downing, J.R., 2014. The landscape of somatic mutations in epigenetic regulators across 1,000 pediatric cancer genomes. Nature Communications 5, 3630.
Kudithipudi, S., Jeltsch, A., 2014. Role of somatic cancer mutations in human protein lysine methyltransferases. Biochimica et biophysica acta (BBA)—review on cancer 1846, 366–379.
Morera, L., Lübbert, M., Jung, M., 2016. Targeting histone methyltransferases and demethylases in clinical trials for cancer therapy. Clinical Epigenetics 8, 57.
Suva, M.L., Riggi, N., Bernstein, B.E., 2013. Epigenetic reprogramming in cancer. Science 339, 1567–1570.
Tian, X., Zhang, S., Liu, H.M., Zhang, Y.B., Blair, C.A., Mercola, D., Sassone-Corsi, P., Zi, X., 2013. Histone lysine-specific methyltransferases and demethylases in carcinogenesis: New targets for cancer therapy and prevention. Current Cancer Drug Targets 13, 558–579.
Watson, I.R., Takahashi, K., Futreal, P.A., Chin, L., 2013. Emerging patterns of somatic mutations in cancer. Nature Reviews Genetics 14, 703–718.
Weirich, S., Kudithipudi, S., Jeltsch, A., 2017. Somatic cancer mutations in the MLL1 histone methyltransferase modulate its enzymatic activity and dependence on the WDR5/RBBP5/ASH2L complex. Molecular Oncology 11, 373–387.
Weirich, S., Kudithipudi, S., Kycia, I., Jeltsch, A., 2015. Somatic cancer mutations in the MLL3-SET domain alter the catalytic properties of the enzyme. Clinical Epigenetics 7, 36.
You, J.S., Jones, P.A., 2012. Cancer genetics and epigenetics: Two sides of the same coin? Cancer Cell 22, 9–20.

Relevant Websites

UniProtKB: http://www.uniprot.org/uniprot/—Description: The UniProt Knowledgebase (UniProtKB) is the central hub for the collection of annotated, functional information on proteins.
Cosmic: http://cancer.sanger.ac.uk/cosmic—Description: COSMIC is designed to store and display somatic mutation information and contains information relating to human cancers.
HIstome: The Histone Infobase: http://www.actrec.gov.in/histome/—Description: A database of human histones, their posttranslational modifications and modifying enzymes.
dbEM A Database of Epigenetic Modifiers: http://crdd.osdd.net/raghava/dbem/—Description: dbEM is a database of epigenetic modifiers curated from cancerous and normal genomes. It is created to study role of epigenetic proteins in oncogenesis and cancer drug resistance.
EpiFactors http://epifactors.autosome.ru/—Description: EpiFactors is a database for epigenetic factors, corresponding genes and products.

Mutations: Driver Versus Passenger

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Glossary

Driver mutation A driver mutation is causally implicated in tumor development. It has been positively selected for and has contributed to the survival and proliferation of a tumor.

Genomic mutation rate This refers to the number of mutations in a genome. In cancer genomic mutations rates are heterogeneous can vary considerably both between individuals and within tumor genomes.

Mutational signature A mutational signature is a distinctive profile that has been inferred from mutations in tumor genomes. These signatures are the consequence of exposure to multiple mutational processes.

Passenger mutation A passenger mutation is a mutation that has not contributed to tumor development.

Proto-oncogene Proto-oncogenes normally require a gain-of-function mutation or chromosomal gain to drive tumorigenesis, and when mutated in cancer they promote proliferation.

Tumor heterogeneity This refers to genomic or epigenomic differences between different patients, different tumors from the same patient, or different regions in an individual tumor.

Tumor suppressor gene Tumor suppressor genes are generally antiproliferative and in general require inactivation of both alleles to contribute to tumor development.

Introduction

In the 1950s Armitage and Doll provided evidence, based on cancer incidence, that tumorigenesis is the end-result of several successive events (e.g., mutations) in a cell. Since then molecular studies have described the transformation of a normal cell into a cancer cell as a multistep process, with each intermediate stage conferring a selective advantage on the cell. For example, in colorectal cancer, the canonical pathway of tumorigenesis involving the gradual accumulation of mutations in *APC*, *KRAS*, and then genes such as *PIK3CA*, *SMAD4*, and *TP53* has been well described.

These alterations to specific genes or regions of the genome are a result of aberrations in the DNA sequence or structure (e.g., translocations, mutations, and copy-number alterations). One of the consequences of the inherent genomic instability underlying the tumorigenic process is the collateral accumulation of passenger mutations in cancer genomes that are unlikely to contribute to tumor formation. Only a subset of the mutations present in a tumor cell actually contribute to the survival and proliferation of that tumor—in fact, a significant majority of the mutations observed in a tumor cell are "passenger mutations," which have a neutral or even negative effect on the cell's survival. In contrast, a small number of mutations in cancer cells are "driver mutations" which actively benefit the survival of the tumor. A driver mutation is not necessarily required for the survival of the tumor at every stage in its life, but by definition must have been actively selected for at some point in a tumor's evolutionary history. Genes with the potential to carry driver mutations are called cancer genes. The identification of cancer genes and the specific mutations in these genes that act as drivers is a key focus of cancer research.

Driver genes may be classified according to the cellular pathway that a gene affects—genes that operate in the same pathway tend to be mutated in a mutually exclusive manner in an individual tumor, as a mutation that has the same effect as an already existing mutation will not be selected for. Some cancer genes are mutated more frequently than others—for most cancers, the genomic "landscape" of mutations features a small number of "mountains" (genes that are very frequently mutated in that cancer) and a far greater number of "hills" (genes that are only rarely mutated in that cancer). The process of tumorigenesis is driven by alterations to specific genes.

At a rudimentary level, these cancer-driving genes can be classified as either proto-oncogenes (e.g., *BRAF*, *KRAS*, and *MYC*) or tumor suppressor genes, for example *TP53*, *PTEN*, and *CDKN2A*. Proto-oncogenes normally require a gain-of-function mutation or chromosomal gain to drive oncogenesis, and when mutated or dysregulated in cancer, they promote proliferation. On the other hand, tumor suppressor genes are generally antiproliferative and in general require inactivation of both alleles to contribute to tumor development. This can occur in many ways, such as loss of function mutations, deletions, or epigenetic silencing. In addition to proto-oncogenes and tumor suppressor genes, genes directly involved in DNA damage response pathways (e.g., base excision repair and mismatch repair genes), which repair various types of damage to DNA, have been proposed as an additional type of cancer gene. More recently, genes with novel roles in tumorigenesis, such as splicing and chromatin remodeling genes, have been implicated in cancer.

Driver mutations may activate oncogenes or inactivate tumor suppressor genes. Because there are far more ways to inactivate a protein than to activate it, oncogene driver mutations tend to be focal amplifications or missense mutations at specific codons, whereas tumor suppressor mutations tend to be deletions, frameshifts, splice-site mutations, and nonsense mutations found in

a wider range of sites across the gene. This has led to a "20/20" rule of thumb for predicting whether a known cancer gene is more likely to be an oncogene or a tumor suppressor—if over 20% of the mutations in a cancer gene are missense and in consistent locations, that gene is likely to be an oncogene, whereas if over 20% of the mutations in a cancer gene are nonsense or otherwise inactivate the protein then the gene is likely to be a tumor suppressor. It is important to note the distinction between a driver gene and a driver mutation. A driver cancer gene is a gene for which there is very strong evidence of a role in tumorigenesis, and which may be affected by driver mutations. A list of cancer driver genes is curated and updated in the Sanger Institute Cancer Gene Census (http://cancer.sanger.ac.uk/census). However a driver gene may also contain passenger mutations, which do not confer a gain or loss of function at the protein level.

Since driver mutations are far rarer than passenger mutations, it would not be practical to functionally test all mutations identified in cancer genomes. Instead, researchers examining cancer genome data predict which mutations are most likely to be drivers. The main methods for doing this are examining the frequency of the mutation across different tumors and predicting the likely functional impact of the observed mutation. Frequency based approaches are based on the fact that driver mutations are by definition actively selected for. This means that a driver mutation should appear more frequently across many samples of a given cancer type than would be expected given the background mutation rate. Function based approaches examine the likely biological impact of a mutation and try to identify putative drivers by looking more closely at mutations likely to inactivate a tumor suppressor or activate an oncogene. Analyzing the likely impact of a mutation may involve predicting the impact of that mutation on the structure of the protein, the evolutionary conservation of the region mutated, the biochemical similarity of the original amino acid and its replacement, the protein domain affected or the protein family of the identified cancer gene.

The recent advances in sequencing technologies have revolutionized cancer genome analysis and have created an unprecedented amount of genomic data from tumors. Interpretation of these data has required the development of new analytical and statistical approaches for the identification of cancer driver genes. Furthermore the progress in the field in the last decade has unearthed previously unknown mechanisms of tumor development and has implicated a host of novel driver genes. Strides have been made in understanding factors affecting genomic mutation rates, the mutational processes impacting tumor genomes, and intratumor heterogeneity. Whole genome sequencing data have facilitated the search for noncoding driver mutations in cancer. In the following sections we discuss coding and noncoding cancer driver identification and highlight the progress that has been made, the issues and considerations in driver and passenger mutation identification, discuss how passenger mutations can provide clinically relevant information, and summarize the main cancer genome analysis initiatives worldwide.

Cancer Gene Identification

Chromosomal Alterations

Aneuploidy is observed frequently in tumor cells. Cytogenetic techniques such as spectral karyotyping (SKY), fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), and SNP arrays have been employed in the past to identify small and large-scale chromosomal alterations (e.g., amplifications, gains, losses, and deletions) in cancer. In addition, chromosomal translocations and gene fusion events have been shown to play an important role in tumor development in some cancer types, particularly in leukemias and lymphomas. Perhaps the most well-known chromosomal translocation in cancer is the Philadelphia chromosome, which results in the chimeric, constitutively active tyrosine kinase *BCR–ABL* fusion protein in chronic myeloid leukemia (CML). This driver translocation event has been the focus of much attention due to the effectiveness of the tyrosine kinase inhibitor Gleevec (imatinib) in treating CML. Numerous other chromosomal translocations in cancer have been described and are catalogued in the Mitelman Database of Chromosome Aberrations in Cancer (https://cgap.nci.nih.gov/Chromosomes/Mitelman). This database is manually curated from published literature and relates the aberrations to tumor characteristics.

Coding Sequence Mutations and Mutational Screens

Somatic mutations in the coding sequence of a gene, for example single nucleotide variants (SNVs) and small insertions or deletions (indels) can have varying consequences (**Fig. 1**). SNVs can be classified as (1) synonymous (no amino acid change), missense (amino acid change), or nonsense (change of the amino acid to a stop codon); and (2) loss of function or gain of function. Indels can result in frameshift mutations (change of the reading frame) or in-frame mutations (no change to the reading frame). Different types of mutations affect genes altered in cancers. For example, oncogenes usually sustain gain-of-function mutations, such as the *BRAF* V600E mutation in melanoma and other cancers. This mutation leads to the constitutive activation of the protein even in the absence of an activating signal resulting in sustained signaling of the *MEK/ERK* pathway. Conversely, tumor suppressor genes can be inactivated by loss-of-function mutations. For example, a host of loss of function missense and nonsense mutations has been found in the cell cycle gene *CDKN2A* in multiple cancer types.

To identify novel cancer genes mutational screens in the early 21st century used capillary sequencing to focus on previously implicated signaling pathways and gene families such as the tyrosine kinases, lipid kinases, tyrosine phosphatases, and tyrosine kinase receptors. These studies highlighted the significance of members of these gene families in cancer by identifying recurrent mutations in genes such as *BRAF*, *PI3KCA*, and *EGFR* in multiple cancer types. In 2006, the first genomic mutational screen of the somatic landscape of cancers was conducted in human breast and colorectal tumors. In an initial screen, the authors sequenced the coding sequences of $>13{,}000$ genes in 11 breast and 11 colorectal tumors, removing any silent changes, and known germline

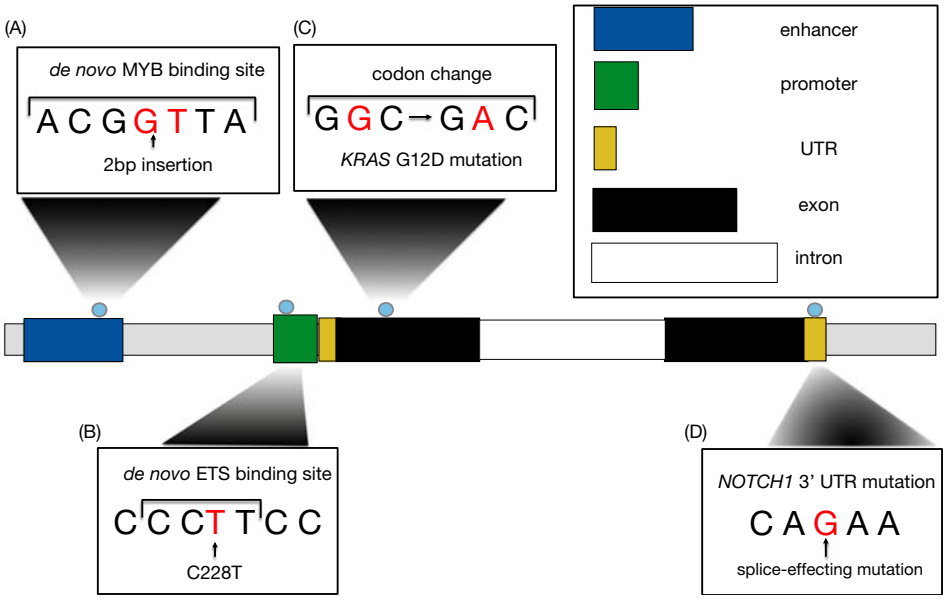

Fig. 1 Driver mutations can operate by a variety of functional mechanisms. Mutations in enhancers (A) or promoters (B) can alter the regulation of driver genes, effecting their expression or response to transcriptional signals. Coding mutations (C) change the amino acid sequence of a gene and can alter the function of the encoded protein. UTR mutations (D) can have a variety of effects, including altering the splicing of the encoded driver gene. From Piraino, S. W., Furney, S. J. (2016). Beyond the exome: The role of non-coding somatic mutations in cancer. *Annals of Oncology* **27**(2), 240–248.

events or SNPs. Candidate mutations were then sequenced in 24 additional breast and colorectal tumors. They developed a statistical score (cancer mutation prevalence score—CaMP) to discriminate driver mutations from passenger events. The CaMP score adjusts for the background mutation rate and takes into account the type of the base mutated, the resulting base change, the 5′ and 3′ neighbors, and the codon usage. Using this approach the study identified 122 and 69 candidate genes for the breast and colorectal tumors, respectively. Among the lessons learnt from this landmark study, and an expanded follow-up in 2007, were that the majority of the genes identified had not been previously implicated in cancer, and that genes showed different biases in the type of nucleotide changes. Furthermore, it highlighted that different genes were mutated in the two cancer types and that samples of the same cancer type showed heterogeneous mutational landscapes. This first large-scale sequencing effort identified important principles and issues in the identification of candidate driver genes and mutations and laid the foundation for subsequent cancer genome profiling initiatives, such as The Cancer Genome Atlas (TCGA), which were to take advantage of the development of new sequencing technologies.

Advances in Sequencing Technologies

Rapid technological advances in sequencing technologies over the last decade have paved the way for massively parallel sequencing (MPS) or next-generation sequencing (NGS) enabled cancer genomics. The first cancer genome sequence was reported by the Genome Center at Washington University in 2008. Using MPS, the study sequenced DNA from tumor and normal skin cells from a patient with acute myeloid leukemia (AML) at a coverage of 33 times for the tumor genome and 14 times for the normal genome, finding only eight heterozygous, nonsynonymous somatic single nucleotide variants in the tumor genome. In 2010, the Cancer Genome Project at the Sanger Institute published the whole genome sequences of a malignant melanoma cell line and a small-cell lung cancer cell line. They compared each cell line to the respective normal genomes to identify somatic variants, alterations and distinctive mutational signatures corresponding to DNA damage due to ultraviolet light in the melanoma genome and tobacco smoke in the lung cancer genome. Building on these foundations, the enormous NGS-driven activity in the field of cancer genomics in the last decade has unearthed previously unknown mechanisms of tumor development and has implicated a host of novel driver genes. One of the most prominent examples is the discovery of recurrent somatic mutations in genes that encode spliceosome components in multiple cancer types, including chronic lymphocytic leukemia, uveal melanoma, and breast cancer. The majority of spliceosome-related mutations are found in splicing factor 3B subunit 1 (*SF3B1*), serine/arginine-rich splicing factor 2 (*SRSF2*), U2 small nuclear RNA auxiliary factor 1 (*U2AF1*), and zinc finger RNA-binding motif and serine/arginine-rich 2 (*ZRSR2*). In addition, mutations in genes implicated in epigenetic regulation and chromatin remodeling processes have been identified in a large number of studies. The mutated genes are involved in a range of processes, including DNA methylation (e.g., *DNMT3A*, *DNMT1*), DNA demethylation (e.g., *TET1*, *TET2*), histone methylation (e.g., the lysine methyltransferases *EZH2*, *SETD2*, and *MLL* family genes), histone acetylation (e.g., the histone acetyl transferases *CREBBP* and *EP300*, and histone deacetylase (HDAC) family genes), the SWI/SNF complex chromatin remodeling complex (e.g., *ARID1A*, *ARID1B*, *ARID2*, SWI/SNF-related

matrix-associated actin-dependent regulator of chromatin (SMARC) genes, and *PBRM1*), and alternative lengthening of telomeres (e.g., *ATRX* and *DAXX*).

Cancer Gene Census

In 2004, the Cancer Genome Project published a census of cancer genes, curated from the literature. This database (http://cancer.sanger.ac.uk/census), which is updated regularly, is a list of genes in which mutations have been causally implicated in cancer and contains relevant information including the tumor types in which mutations are found and the type of mutation/alteration affecting each gene. Currently, there are >560 cancer genes in this database, of which ~100 are mutated in the germline setting and >500 are mutated somatically in cancer. Translocations are the commonest mechanism of altering a gene (55%), followed by missense mutations (43%), nonsense mutations (27%), frameshift mutations (27%), splicing mutations (13%), large deletions (7%), and amplifications (4%).

In addition to the discovery of new driver mutations and genes, whole genome sequencing and pan-cancer genome analyses have identified a variety of factors and mutational processes that affect mutation rates in cancer genomes, and confound the differentiation of driver and passenger mutations. For instance, there is considerable variation in mutation rates both within and between cancer types (**Fig. 2**). In the next two sections, we explore the factors that affect mutation rates and discuss the mutational processes that have been identified in cancer genomes. In this light, it is important to acknowledge the importance of analyzing both driver and passenger mutations to elucidate the mechanisms and variables influencing the acquisition of somatic mutations in cancer genomes. Accounting for these factors can then assist in the refinement of approaches for driver mutation identification.

Factors Impacting Genomic Mutation Rates

Somatic mutation rates vary considerably across regions of the genome. Understanding the factors that contribute to this variation is essential for methods for the detection of driver genes, and is also independently of interest in terms of understanding cancer biology (**Fig. 3**). One of the first genomic features to be associated with genomic mutation rate is gene expression level. Highly transcribed genes generally show lower mutation rates compared to weakly expressed genes. It has also been noted that the transcribed strand of genes shows lower mutations rates compared to the nontranscribed strand, which has been interpreted as suggesting that the impact of gene expression on mutation rate may be due to transcription-coupled nucleotide excision repair.

DNA repair has emerged as a central factor in mutation rate variation in cancer genomes. Early efforts to characterize the variability of mutation rate across the genome identified chromatin markers associated with chromatin state as being particularly associated with cancer genome mutation rate. Markers of closed chromatin have generally been associated with higher mutation rate, while open chromatin states have been associated with lower mutation rate. This observed association was given a mechanistic interpretation when chromatin state was linked to access of DNA repair machinery to the underlying DNA in the context of mutation rates. It has been observed that while mismatch repair proficient tumors demonstrate substantial mutation rate variability across the genome, this variability is considerably reduced in mismatch repair deficient tumors. Combined with the observed association of mutation rate with chromatin state, these results suggest a model in which open chromatin state makes DNA more available for repair, resulting in lower mutation rates in these regions when DNA repair is proficient.

Other studies have suggested a plausible role for the interaction of DNA repair and chromatin state in more specific chromatin interactions. Reduced mutation rates have been observed in DNase I hypersensitive sites, while this hypomutation is absent in tumors with deficient nucleotide excision repair, again suggesting easier access of repair machinery in open chromatin as a factor influencing mutation rates. While DNase I hypersensitivity sites show reduced mutation rates, increased mutation rates have been

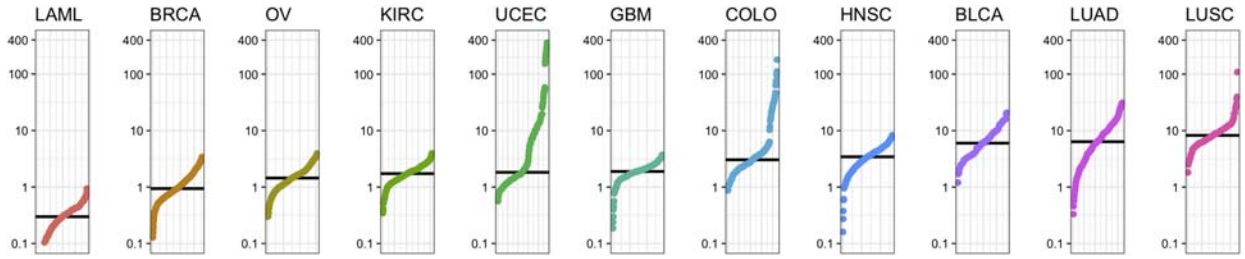

Fig. 2 Mutation rates per Mb across different cancer types. The *black bar* is the median mutation rate of each cancer type. There is considerable variation in mutation rates both within and between cancer types. Some of the top cancer in terms of median mutation rate (LUSC and LUAD) have a known environmental exposure that can causes large numbers of mutation (tobacco smoke). UCEC and COLO have moderate median mutation rates and long tail of samples with very high mutation rates, possibly as a result of hypermutator tumors within these cancer types. *BRCA*, breast adenocarcinoma; *LUAD*, lung adenocarcinoma; *LUSC*, lung squamous cell carcinoma; *UCEC*, uterine corpus endometrial carcinoma; *GBM*, glioblastoma multiforme; *HNSC*, head and neck squamous cell carcinoma; *COLO*, colon and rectal carcinoma; *BLCA*, bladder urothelial carcinoma; *KIRC*, kidney renal clear cell carcinoma; *OV*, ovarian serous carcinoma; *LAML*, acute myeloid leukemia. Data was obtained from the supplemental materials of https://www.nature.com/nature/journal/v502/n7471/full/nature12634.html.

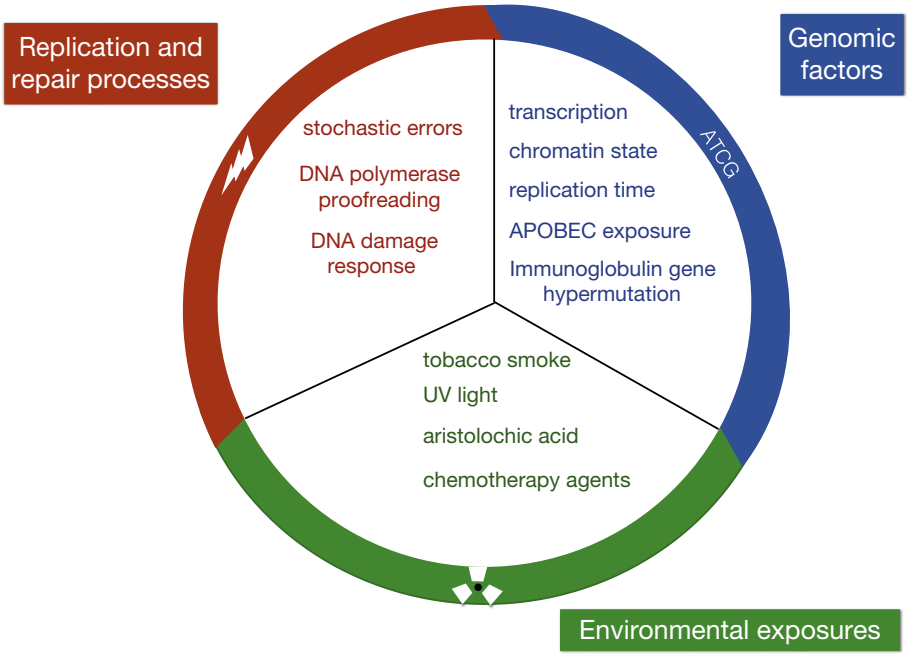

Fig. 3 Many factors influence mutations rates both within individual cancer genomes and across individuals. Here we give several examples of factors that influence mutation rates from three categories: environmental exposures, factors that impact DNA replication and repair, and features that vary across the genome. From Piraino, S. W., Furney, S. J. (2016). Beyond the exome: The role of non-coding somatic mutations in cancer. *Annals of Oncology* **27**(2), 240–248.

noted at the actual site of protein binding. Transcription factor binding sites coincide with decreased excision repair activity as measured by excision repair sequencing, and the excess mutation rate at these sites relative to flanking regions is disrupted in XPC-deficient skin cancers, suggesting that this mutation rate variability is again caused by access to DNA repair factors. In colorectal cancer, CTCF binding sites have an excess of mutations, while colorectal tumors harboring mutations that disrupt the proofreading activity of POLE actually have fewer mutations at these sites compared to background mutations, again implicating an interaction between protein binding and DNA repair.

Understanding mutation rate variability both within and between cancer genomes has important implications for the detection of driver genes. The number of mutations available for driver gene discover influences the power to detect driver genes. Mutation rate variability can also lead to "false positives" in analyses, genes that have high mutation rates but are not driver genes. Accounting for factors that influence mutation rate can help improve the performance of statistical methods and decrease the likelihood of false positives. Recently, ratiometric methods which use the ratio of different types of mutations as opposed to raw rates have been suggest as a method that can improve performance in the presence of un-modeled variation in mutation rates.

In addition to varying across the genome, mutation rates also vary substantially across individuals. Environmental exposures such as tobacco smoke, UV light, and aristolochic acid can result in increased mutation rates in cancer genomes. Mutation rates across individuals are also impacted by variability in the activity of certain cellular processes. In the next section we discuss some of the environmental and endogenous-driven mutational processes that contribute to the heterogeneity of mutation rates.

Mutational Signatures

DNA is exposed to constant attacks from a variety of exogenous and endogenous sources. These exposures often trigger mutational processes and result in DNA damage, which results in the accumulation of somatic mutations. Each mutational process affects specific nucleotides and generates patterns of base substitutions, indels or structural variations, thus leaving recognizable imprints on the cancer genome, known as mutational signatures. The recent deluge of data produced by global sequencing initiatives, in tandem with mathematical modeling approaches, has allowed the identification of a growing number of mutational signatures in human cancer and the completion of mutational catalogues. At least 30 distinct mutational signatures have been identified so far, and classified into signatures associated with endogenous mutational processes, signatures associated with exogenous mutational processes and signatures of mutational processes with unknown origins. Mutational signatures offer evidence of the biological history of a cancer, and pan-cancer analyses of next generation sequencing data have revealed that tumor types are characterized by discrete genome-wide mutation profiles, which are a consequence of different exposure to mutational processes. Such mutational records therefore provide the opportunity to identify and examine new mechanisms of mutagenesis.

Current efforts have identified distinct mutational signatures from the analysis of mutations from thousands of cancer genomes (http://cancer.sanger.ac.uk/cosmic/signatures). About half of these have been attributed to known exogenous carcinogenic exposure or endogenous mutational processes (Fig. 4). Experimental exposure to exogenous mutagens such as tobacco smoke and ultraviolet (UV) radiation has been shown to produce specific classes of mutations (mainly G • C → T • A transversions in the former, and C • G → T • A transitions in the latter) that matched those emerging from analyses of mutated genes in lung cancers and melanomas, respectively. Other mutations, however, occur as a result of disruptions in endogenous processes, such as mismatch repair (MMR) deficiency and DNA replication errors that result in discrete mutational signatures. For example, Signature 1 is the result of an endogenous mutational process characterized by spontaneous deamination of 5-methylcytosine and is correlated with age of cancer diagnosis. The etiologies of several of the mutational signatures remain unelucidated.

Mutational Signatures Linked to Specific Driver Genes

A number of the mutational signatures have been linked to defects in or the actions of specific genes. The *APOBEC* gene family of deaminases has a major role in viral restriction and suppression of retrotransposition, and aberrant activity of *APOBEC* in human cancer is reported to produce mutational signature 2 and 13. Genes involved in DNA damage response pathways are known to contribute to tumorigenesis in many cancer types. Defects in gene involved in homologous recombination, in particular *BRCA1* and *BRCA2*, result in a range of mutations, which correspond to particular mutational signatures. Defects in genes involved in mismatch repair, which results in microsatellite instability and an increased mutation rate, are associated with four specific mutational signatures. Altered activity of the *POLE* gene, which encodes the catalytic subunit of an enzyme involved in DNA proofreading, is proposed as the mutational process underlying another mutational signature and has been associated especially with colorectal cancers. Therefore the combination of driver and passenger mutations can provide a powerful insight into the processes underlying tumor development. In addition, recent studies have highlighted how signatures and mutational burdens can be relevant clinically. For example, whole genome sequencing of pancreatic cancers revealed that the *BRCA*-associated mutation signature

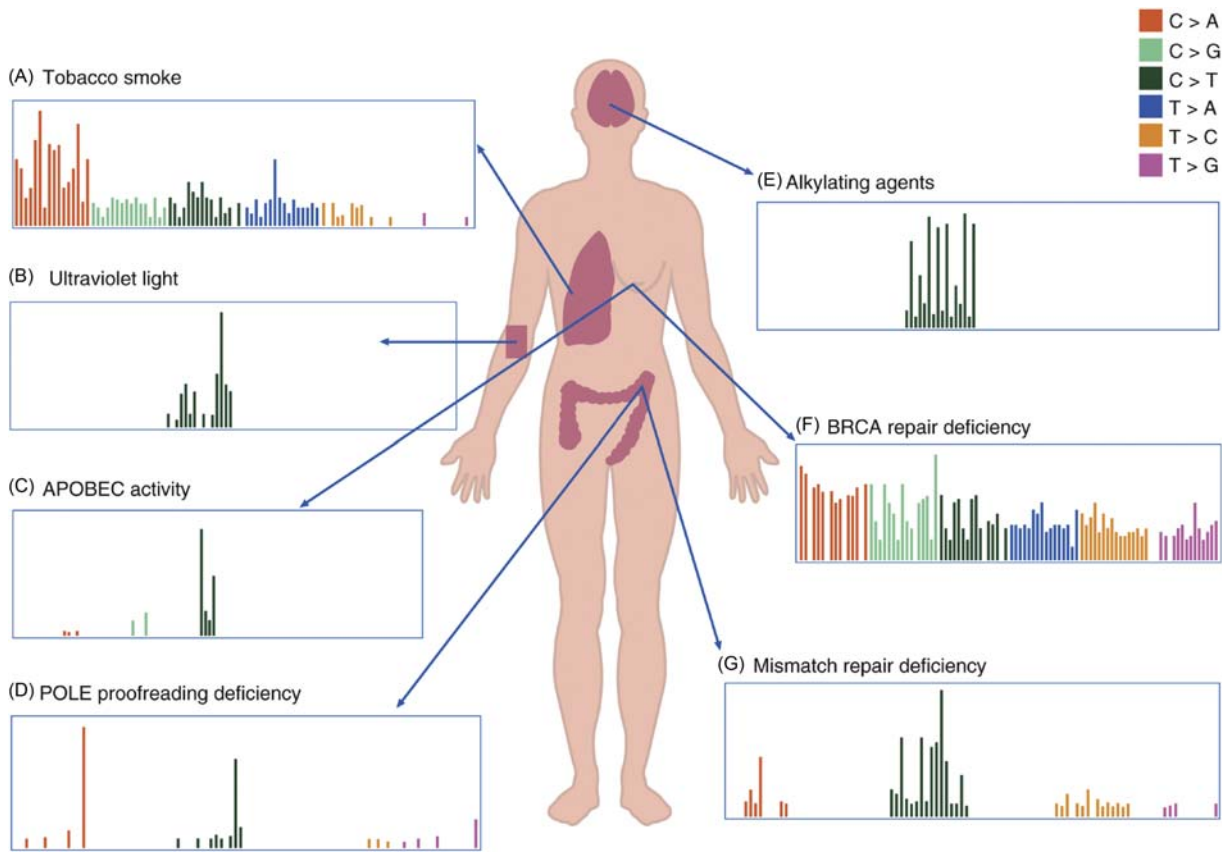

Fig. 4 Some of the mutational signatures identified to date have significant clinical or epidemiological implications. Signatures such as those associated with tobacco smoke (A), ultraviolet light (B) and alkylating agents (E), can reveal previous mutagenic exposure. Signatures associated with altered DNA damage response including deficiency in *POLE* (D), *BRCA* (F) and mismatch repair genes (G) may serve as markers for prognosis and response to certain types of therapy. Further signatures have been attributed to the activity of the AID/APOBEC family of cytidine deaminases (C). From Piraino, S. W., Furney, S. J. (2016). Beyond the exome: The role of non-coding somatic mutations in cancer. *Annals of Oncology* **27**(2), 240–248.

was correlated with genomic instability as well as sensitivity to platinum-based therapies, suggesting that some of the mutational signatures may be potential biomarkers for therapeutic response. Furthermore, a number of studies have suggested that sensitivity to immune checkpoint inhibition is associated with the mutational landscape of tumors, with factors such as mutation burden, neo-antigen burden, and DNA repair deficiency playing a role in response to therapy.

Methods of Driver Identification

Driver mutations confer a selective advantage to the cancer cells in which they occur, and as a result are under positive selection within the tumor. In cancer sequencing data, this positive selection is expected to result in properties that distinguish driver mutations from passenger mutations. Several classes of methods have been developed which exploit these properties in order to use sequencing data to identify putative driver mutations (Fig. 5). The previous two sections have outlined some of the mutational processes and factors that can confound the systematic identification of driver genes. In this section, we discuss the properties that driver mutations display and methods that have been developed to detect driver mutations based on these properties. It is fundamental to note that the foundation of all the approaches we discuss is the identification of *somatic* variants in cancer genomes, a task in itself that is not trivial. A number of prominent somatic mutation calling algorithms have been developed including

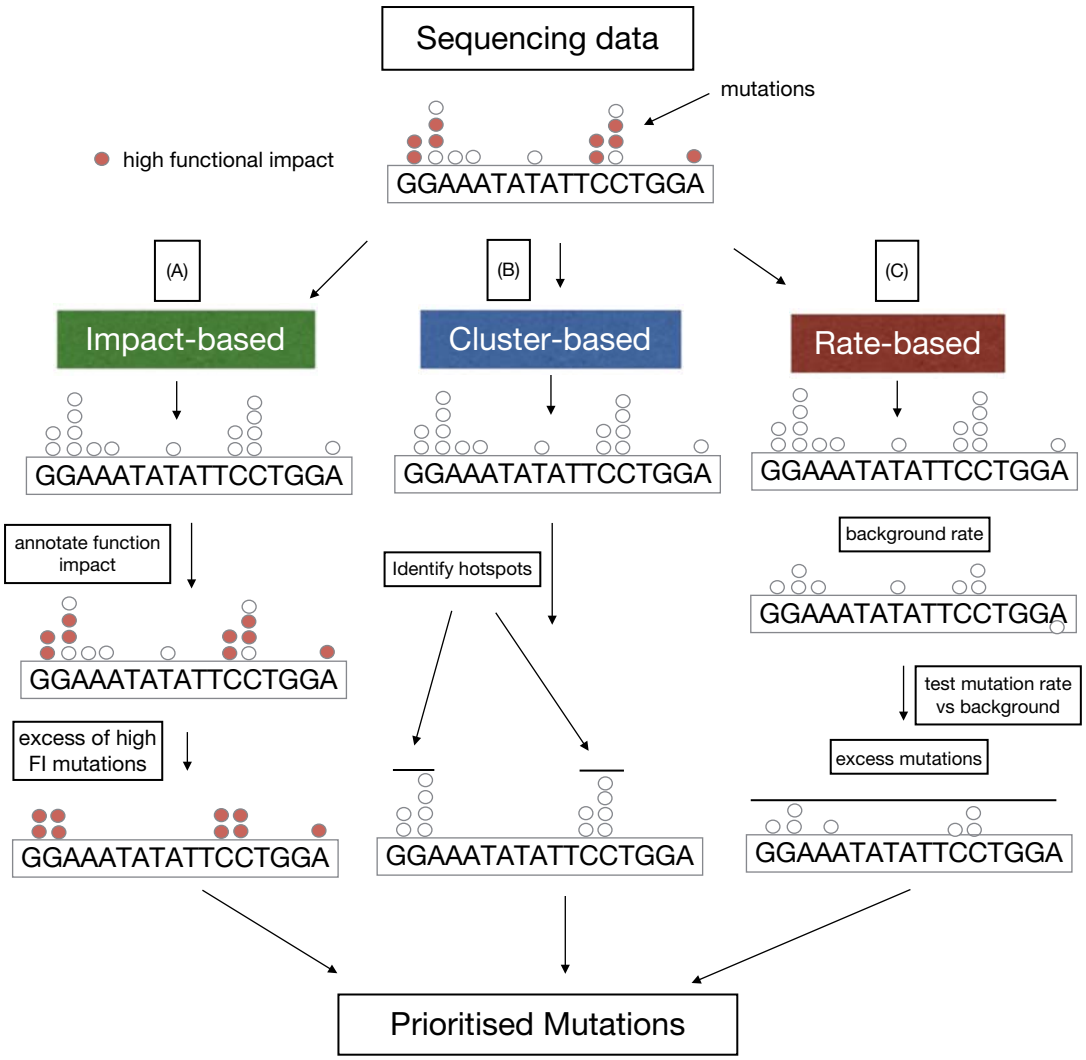

Fig. 5 Examples of DNA sequences that might be identified by different driver detection methods. In (A), *color* indicates the functional impact of a mutation, and the sequence shows a large number of highly functionally impactful mutations, and therefore could be identified by functional impact-based methods. In (B) the sequence has highly localized hotspot regions, and could be identified by clustering-based methods. In (C) the sequence shows a large, evenly distributed mutation rate, and could be identified by rate-based methods. From Piraino, S. W., Furney, S. J. (2016). Beyond the exome: The role of non-coding somatic mutations in cancer. *Annals of Oncology* **27**(2), 240–248.

Varscan (http://dkoboldt.github.io/varscan/), SomaticSniper (http://gmt.genome.wustl.edu/packages/somatic-sniper/), Mutect (https://software.broadinstitute.org/gatk/documentation/tooldocs/current/org_broadinstitute_gatk_tools_walkers_cancer_m2_MuTect2.php), CaVEMan (https://github.com/cancerit/CaVEMan), and Strelka (https://github.com/Illumina/strelka). Each of these approaches for somatic single nucleotide variant identification has its own approach, biases, and limitations. As such, users must remain cognizant that any list of somatic variants output by an algorithm is a combination of true and false positives, and may omit false negative mutations (those that are present in the tumor but were not identified by the algorithm). Furthermore, the computational identification of short insertions and deletions has proved to be a far more difficult task than point mutations to date.

Although driver mutations mechanistically impact cancer cells at the level of the individual mutation, mutations within the same functional elements may have similar biological effects. As a result, mutations are often considered in terms of their impact on functional elements. In particular, much cancer genomic research has focused on the identification of driver genes, protein-coding genes that contain driver mutations. We focus on the identification of driver genes, although the concepts discussed here are applicable to other functional elements as well.

One of the most prominent properties expected of driver mutations in their increased occurrence in tumor genomes. Driver mutations are not simply generated by mutational processes in cancer genomes and remain unselected for like passenger mutations, but are instead causally related to the development of the tumor. As a result, driver mutations are expected to appear more frequently across multiple independent tumors in cancer sequencing data relative to the base rate that these mutations would occur at due to mutational processes alone. This concept has given rise to a class of methods that seek to identify regions of the genome that display an excess of mutations relative to an expected base rate that is due to mutational processes. Under this model, an excess of mutations is a signature of positive selection, and genes that are mutated more frequently than expected are possible driver genes.

The baseline or background model of mutation rates is a critical component of these methods. Seminal work by multiple groups has demonstrated that the mutation rate within cancer cells is not constant across the genome, but instead varies along with genomic features such as gene expression, chromatic state, epigenetic markers, and base composition. As a result, rate-based methods for the identification of driver genes typically use a model for the neutral mutation rate of a gene and statistically compare the observed mutation counts to their expectation under the background model. Examples of rate-based methods include MutSigCV (http://software.broadinstitute.org/cancer/software/genepattern/modules/docs/MutSigCV), and Genome MuSiC (http://gmt.genome.wustl.edu/packages/genome-music/).

In additional to methods based on mutation rate, another class of methods focuses on the functional impact of mutations that occur within a gene. Genes under positive selection are expected to display a "functional impact bias," an excess of mutations that have functional effects compared to mutations that are not as impactful in terms of biological function. Functional impact based methods start with a method of identifying the degree of functional impact that individual mutations have. For example, a simple method could be based on the idea that nonsynonymous mutations are more likely to have large functional consequences compared to synonymous mutations. The functional bias method then looks for genes that tend to have large numbers of high functional impact mutations compared to lower impact mutations. Driver mutations have functional effects that contribute to tumorigenesis, and as a result, functional impact methods identify driver genes as genes that display an excess of functionally impactful mutations. These methods can be extended in novels ways by incorporating different measures of functional impact and by including a model of the expected functional impact score for a gene or genomic element that accounts for potential confounding factors, such as mutational processes. Examples of functional impact based methods include OncodriveFM and its extension OncodriveFML. Appropriately chosen measures of functional impact may also assist in the identification of interesting categories of driver genes. For example, looking for genes with an excess of truncating or other deleterious mutations can help in the identification of tumor suppressor genes, while identification of genes with an excess of mutations targeting phosphorylation sites can be used to identify genes involved in aberrant phosphorylation signaling.

A critical component of functional impact methods is variant annotation. Many software tools, databases, and other resources aim to facilitate this task, and some resources are available that bring together multiple existing resources to address this challenge. Within the wider field of genomics and analysis of genetic variants, a host of tools exist to annotate genetic variations, including the Variant Effect Predictor (VEP, http://www.ensembl.org/info/docs/tools/vep/index.html), and ANNOVAR (http://annovar.openbioinformatics.org/en/latest/), which provide general annotation of genetic variants. These general annotations also give access to more specific functional annotations, including tools such as SIFT (http://sift.jcvi.org), PolyPhen (http://genetics.bwh.harvard.edu/pph2/), MutationAssessor (http://mutationassessor.org/r3/), and many others aimed at predicting the functional impact of mutations. Cancer-specific mutation annotation such as CHASM (http://wiki.chasmsoftware.org/index.php/Main_Page), and Oncotator (https://portals.broadinstitute.org/oncotator/) also exist.

A third method for the identification of driver genes is based on the observation that driver mutations which target a specific functional element within a protein (e.g., a phosphorylation site) will often be clustered near to each other in the linear sequence of the gene. Genes can contain domains and elements that make up a single functional protein component or serve a common function. If a functional element can be impacted by multiple different mutations along its length, then driver mutations targeting that element may cluster within the region of the gene containing the functional element. As a result, genes that display significant spatial clustering of mutations as opposed to mutations evenly distributed throughout the gene can be classified as putative driver genes.

Noncoding Driver Mutations

In addition to coding driver mutations, recent efforts directed at the noncoding regions of the genome have revealed the presence of noncoding driver mutations. Major efforts to characterize the functional properties of noncoding DNA, particularly regulatory regions, have created the opportunity to discover important insights about the role of noncoding variation in disease, including cancer. We review important examples of noncoding driver mutations and the role they play in cancer biology.

Among the most important and well-known noncoding driver mutations are *TERT* promoter mutations (Fig. 1). *TERT* mutations occur in a mutually exclusive manner and result in the creation of an *ETS* transcription factor binding site, resulting in increased expression of the *TERT* transcript. *TERT* promoter mutations are extremely frequent across multiple cancer types and have been associated with clinical features in multiple cancer types.

TERT promoter mutations are an example of noncoding driver variants caused by point mutations. Another noncoding driver mutation that has been identified is generated by a small indel in T-cell acute lymphoblastic leukemia. It has been observed that insertions create a *MYB* binding site upstream of the *TAL1* oncogene, resulting in increased *TAL1* expression.

There have also been several attempts to systemically identify putative noncoding driver mutations in cancer. These efforts have employed a variety of methods for the identification of putative noncoding driver regions, including rate and clustering-based methods similar to those used to examine coding driver genes, methods based on annotations of noncoding mutations such as the impact of the mutation on regulatory motifs, and methods based on correlating the presence of mutations with gene expression across tumors. In addition to identifying prominent noncoding drivers such as *TERT*, these efforts have also revealed other putative noncoding driver regions such as regions upstream of *PLEKHS1*, *WDR74*, *SDHD*, and *MED16*.

In addition to pan-cancer studies of potential noncoding driver mutations, several large genomic studies in individual cancer types have identified recurrent noncoding mutations. Analysis of breast tumors identified recurrent promoter mutations upstream of one protein-coding gene (*FOXA1*), and two noncoding RNA genes (*RMRP* and *NEAT1*). An analysis in chronic lymphocytic leukemia revealed mutations in the 3′-untranslated region of the gene *NOTCH*1, resulting in aberrant splicing of the *NOTCH1* transcript, as well as mutations in an enhancer putatively targeting the gene *PAX5*.

Notwithstanding the discoveries made by these studies to date, the interrogation of the somatic noncoding genome is complex and encumbered by our lack of knowledge about the function of noncoding DNA relative to coding sequences. The generation of a greater number of whole genome sequences of tumors, through initiatives such as the pan-cancer analysis of whole genomes, in tandem with efforts to understand functional elements in the genome, for example the Encyclopedia of DNA Elements (ENCODE) Project, should elucidate the importance of non-coding elements in tumor development further.

Tumor Heterogeneity

Tumor heterogeneity is thought to play a significant role in treatment resistance and failure. Tumor heterogeneity can be classified as:

(i) Intertumor heterogeneity, which is differences between tumors in different patients (Fig. 2). This is the focus of many cancer studies, contributes to differential patient responses to therapy and is the basis for precision medicine approaches.
(ii) Intersite heterogeneity, which describes differences between distinct tumors within an individual patient (e.g., between primary and metastatic tumors, or between multiple metastatic sites).
(iii) Intratumor heterogeneity, which refers to differences between cellular populations in a distinct tumor, the extent of which has been demonstrated through recent multiregion next generation sequencing analyses.

In this section, we discuss intersite and intratumor genetic heterogeneity, which are based on the view of cancer as an evolutionary process that can give rise to genetically divergent subclonal populations within tumors. Morphological differences in tumors have been described in numerous pathology studies and cytogenetic studies have revealed intratumor heterogeneity in copy number alterations. Intratumor heterogeneity refers to the fact that cells within a tumor do not share identical genomes—different cells acquire different mutations as the tumor grows. In addition to the clonal mutations that are shared by all cells in the tumor and that were presumably present in the tumor's ancestral cell, tumor cells may also harbor subclonal mutations, which are unique to a cell or subset of cells in the tumor. These genomic differences can take the form of point mutations, copy number variations or differences in chromosomal structure or number. With the use of next generation sequencing, the extent of intratumor heterogeneity is beginning to be understood more clearly, as is the fact that some tumor types show a greater degree of heterogeneity than others. Furthermore, some driver mutations are more likely to be subclonal than others—for example, across several cancer types it has been suggested that mutations in genes involved in the *PIK3-AKT-mTOR* pathway are more likely than genes involved in the *RAS-MAPK* pathway to be subclonal. The likelihood of a mutation being clonal or subclonal can depend on the cancer type—for example, mutations in *TP53* are almost always clonal in ovarian cancer and nonsmall cell lung cancer, but are often subclonal in chronic lymphocytic leukemia and clear cell renal cell carcinoma.

Since intratumor heterogeneity is only detectable in sequenced samples, detecting subclonal mutations and working out whether apparently clonal mutations are truly clonal is dependent on how many tumor regions are sequenced. Sampling bias in methods used to select regions to sequence may lead to subclonal mutations appearing to be clonal, or to subclonal clinically

relevant driver mutations being missed in sequencing. For example, the TRACERx lung cancer study has shown that significantly more driver events are identified with the use of multiregion sequencing than by analysis of a single sample. In fact, >75% of the tumors in this study harbored a subclonal driver alteration, with genes such as *TP53*, *PIK3CA*, *KRAS*, and *NF1* being affected.

As well as directly impacting the biology and clinical course of a cancer, intratumor heterogeneity allows researchers to analyze the evolutionary history of a tumor by extrapolating the temporal sequence in which the driver mutations occurred. Understanding of intratumor heterogeneity of driver mutations and tumor evolution may facilitate more refined management of cancer therapy by using known evolutionary constraints to contain the cancer to slow its progress. However, the existence of intratumor heterogeneity poses a major challenge for targeted therapies of driver mutations—a given therapy may successfully disrupt the major clonal driver mutation, but if cells in the targeted tumor harbor subclonal driver mutations that the therapy does not eradicate, the cancer may recur.

The majority of sequencing studies have focused on the cancer genomic landscape of primary tumors. However, several studies have identified intersite heterogeneity between matched primary and metastatic tumors. This is of particular importance if different driver mutations are required for tumor development and for metastatic spread. Furthermore, a metastatic site may be seeded by a cell from the original tumor that harbors subclonal driver mutations. Initial large-scale studies of metastatic tumors, such as the MSK-IMPACT and the Michigan Oncology Sequencing (MI-ONCOSEQ) Program, have begun assess the metastatic genomic landscape. The MSK-IMPACT results were similar when compared to the primary tumors from the TCGA. However, important differences between the two patient cohorts were identified. For example, *TP53* was significantly enriched for mutations in several tumor types. However, these studies did not profile matched primary and metastatic tumors from individual patients. Sequencing of matched samples will elucidate further intersite heterogeneity of driver mutations and tumor evolution.

Number of Driver Mutations

Cancer is driven by the acquisition of driver mutations that disrupt normal biological processes and allow somatic cells to form detectable lesions. This raises the question, how many driver mutations are typically required for cancer to develop? Early work on this problem used the relationship between cancer incidence and age to attempt to estimate the number of "rate limiting" events that are required before the formation of various cancers, estimating this number in the range of six to seven events. More recent work has attempted to estimate the number of driver mutations required in lung and colorectal cancer by comparing the increased incidence in cancer due to certain risk factors (smoking in lung and genetic predisposition in colorectal) with the increased mutational burden caused by those risk factors. This method estimates that approximately three driver mutations may be required in these cancers.

It remains unclear exactly how many driver mutations are required in different cancer types, though cancers of the blood require fewer drivers than solid tumors. Despite this uncertainty, it appears likely that most if not all cancers require more than one driver mutation to proliferate. Additional drivers may be required for metastasis and for relapse after therapy, although subclonal mutations could also be selected for in response to treatment. In addition to their number, there is considerable interest in when mutations occur during the evolution of a tumor. Driver mutations may occur throughout the development of the tumor. The time during tumorigenesis at which certain drivers tend to emerge can give clues about the role the driver plays in tumor development (e.g., the stepwise accumulation of mutations in *APC*, *KRAS*, and subsequently genes including *PIK3CA*, *SMAD4*, and *TP53* in certain colorectal tumors). In this way some drivers may only be selected once other driver mutations have already occurred or during a particular phase of tumor development. The presence and frequency of driver mutations may also change throughout tumor development as certain subclones are selected and increase in frequency within the tumor. The analysis of whole genome sequences from projects such as the pan-cancer analysis of whole genomes and the application of phylogenetic methods to sequencing data from multiple regions of tumors is beginning to elucidate some of the early and late driver mutations that occur during tumor evolution. Indeed, initial analysis of the pan-cancer analysis of whole genomes dataset has suggested that each tumor carries on average 4.6 driver events.

Oncogenomic Resources

Interest in cancer genomics in recent years has driven the creation of many publically available resources aimed at facilitating better understanding of the cancer genome.

International Cancer Genome Consortium

The International Cancer Genome Consortium (ICGC, http://icgc.org) is a large collaborative project which has produced substantial amounts of genomic data from many countries and cancer types. ICGC provides access to genomic data such as somatic mutations, RNA expression, methylation, and clinical data from large numbers of cancer patients. ICGC provides several ways of accessing data easily and conveniently, including an interactive data portal.

The Cancer Genome Atlas

The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov) is a large collaboration initiated by the National Institutes of Health similar to ICGC which hosts a large set of -omics data. Like ICGC, TCGA also allows access to a vast array of data through the TCGA data portal.

Catalogue of Somatic Mutations in Cancer

The Catalogue of Somatic Mutations in Cancer (COSMIC, http://cancer.sanger.ac.uk/cosmic) is a database of cancer genomic information organized by the Wellcome Trust's Sanger Institute. It includes databases of cancer somatic mutations, as well as several other components, such as curated lists of cancer genes and cancer mutations (e.g., the Cancer Gene Census) and the cancer cell lines project, which provides data on cancer cell lines. COSMIC also provides resources on mutational signatures in cancer (http://cancer.sanger.ac.uk/cosmic/signatures).

Pan-Cancer Analysis of Whole Genomes

The pan-cancer analysis of whole genomes (PCAWG, http://docs.icgc.org/pcawg/) is a large collaborative effort to assemble a set of uniformly processed cancer whole genomes from multiple cancer types available in an easily accessible manner. The data from PCAWG are available through the ICGC as well as several other data portals and analysis utilities. PCAWG provides the research community with access to an international sample of over 2800 donors and vast numbers of somatic mutations from a wide array of cancer types.

cBioPortal for Cancer Genomics

The cBioPortal for Cancer Genomics (http://www.cbioportal.org/) provides a visualization and analysis platform for cancer genomics data sets, including the TCGA and many smaller studies that have used a variety of sequencing strategies to profile tumors. Results from DNA and/or RNA sequencing of $>45{,}000$ samples can be queried through a user-friendly web interface.

Cancer Genomics Software

Analysis of cancer genomic data often makes use of specialized software. Publically available software exists for many important tasks in cancer genomics, including identification of driver genes, mutation annotation, and analysis of intratumor heterogeneity. Several research groups and institutions provide central locations for multiple different cancer genomic software applications, including the McDonnell Genome Institute at Washington University in St. Louis (http://gmt.genome.wustl.edu/index.html), The Broad Institute (https://www.broadinstitute.org/data-software-and-tools), and the Sanger Institute (http://www.sanger.ac.uk/science/tools). Many other publically available software tools also exist outside these institutional repositories.

Variant Interpretation

Several resources also seek to link cancer variants to information that may give insights into the clinical or therapeutic relevance of mutations, such as the Cancer Genome Interpreter (https://www.cancergenomeinterpreter.org/home), OncoKB (http://oncokb.org), and CIViC (https://civic.genome.wustl.edu/about). OncoKB is a precision oncology database, curated by a team of clinicians and investigators at Memorial Sloan Kettering Cancer Center, containing information about the effects of and treatment options for driver mutations or alterations. The database provides information about alterations in >400 cancer genes and classifies treatment options according to clinical actionability. Clinical Interpretation of Variants in Cancer (CIViC) is an open access, open source database providing information on the prognostic, diagnostic and predisposition of inherited and somatic variants. CIViC has been designed to enable precision medicine and address many of the issues inherent in the clinical interpretation of cancer genome alterations. CIViC currently contains several thousand curated interpretations of clinical relevance for >1700 variants in 330 cancer genes.

Prospective Vision

We have witnessed a genomics revolution as advances in next generation sequencing technologies have resulted in dramatic decreases in sequencing costs. As a consequence, NGS has moved from research and development spheres into the clinical arena to enhance precision medicine for patients. Indeed, over the last decade oncology has been at the forefront of the application of clinical genomics to diagnosis and treatment. Genomic profiling has increasingly become common in many cancer types and clinical trials have been instigated to match patients to targeted therapies based on shared genomic features. With the significant decrease in sequencing costs it has been predicted that millions of cancer patients will have their tumors sequenced over the next decade. One of most the significant and immediate challenges in the field of cancer genomics is the clinical interpretation

of mutational data. The refinement of methods to identify driver mutations and genes, to interpret the clinical significance of specific mutations, and to match patients to therapies based on these mutations and on their genomic profiles, is imperative over the coming years. Finally, the identification of driver mutations promoting recurrence and resistance to therapy will be of significant interest for the foreseeable future.

See also: Chromosome Rearrangements and Translocations. Copy Number Variations in Tumors. Mutational Signatures and the Etiology of Human Cancers.

Further Reading

Alexandrov, L.B., et al., 2013. Signatures of mutational processes in human cancer. Nature 500 (7463), 415–421.
Kandoth, C., et al., 2013. Mutational landscape and significance across 12 major cancer types. Nature 502 (7471), 333–339.
McGranahan, N., Swanton, C., 2017. Clonal heterogeneity and tumor evolution: Past, present, and the future. Cell 168 (4), 613–628.
Piraino, S.W., Furney, S.J., 2016. Beyond the exome: The role of non-coding somatic mutations in cancer. Annals of Oncology 27 (2), 240–248.
Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz Jr., L.A., Kinzler, K.W., 2013. Cancer genome landscapes. Science 339 (6127), 1546–1558.

Relevant Websites

cBioPortal, n.d. http://www.cbioportal.org/—cBioPortal.
CIViC, n.d. https://civic.genome.wustl.edu—CIViC.
COSMIC, n.d. http://cancer.sanger.ac.uk/cosmic—COSMIC.
ICGC, n.d. http://icgc.org—International Cancer Genome Consortium (ICGC).
Mitelman Database, n.d. https://cgap.nci.nih.gov/Chromosomes/Mitelman—Mitelman Database.
TCGA, n.d. https://cancergenome.nih.gov—The Cancer Genome Atlas (TCGA).

Myelodysplastic Syndromes: Mechanisms, Diagnosis, and Treatment

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William Vainchenker, Gustave Roussy, Villejuif, France

Glossary

Myelodysplastic syndrome (MDS) Clonal hematopoietic stem-cell disorders that associate cell dysplasia and ineffective hematopoiesis.
Myelodysplastic syndrome/myeloproliferative neoplasm (MDS/MPN) Independent category of diseases that associate MDS feature with overproduction of at least one category of myeloid cells.
Spliceosome Intracellular multiprotein complex involved in the maturation of messenger RNAs.

Nomenclature

ARCH Age-related clonal hematopoiesis
ASCT Allogeneic stem cell transplantation
CHIP Clonal hematopoiesis of indeterminate potential
CMML Chronic myelomonocytic leukemia
EPO Erythropoietin
ESA Erythropoiesis supporting agents
HMA Hypomethylating agents
IPSS International Prognostic Scoring System
MDS Myelodysplastic syndrome
MDS/MPN Myelodysplastic syndrome/myeloproliferative neoplasm
MDS-RS Myelodysplastic syndrome with ring sideroblasts
sAML Secondary acute myeloid leukemia

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic stem-cell disorders characterized by cell dysplasia and ineffective hematopoiesis. These acquired bone marrow failures lead to blood cytopenias with a variable propensity to evolve into acute myeloid leukemia (AML). They most commonly develop in older adults (median age at diagnosis is 65–70 years and less than 10% MDS patients are younger than 50) with a slight male predominance, except for MDS with isolated 5q deletion in which women predominate. The age-adjusted annual incidence rate of these malignancies is estimated to be 4 per 100,000 individuals (reaching at least 75 cases per 100,000 and possibly more after 65 years of age). Risk factors include rare but increasingly recognized inherited genetic predisposition, to be explored in pediatric and young adult cases as well as families with other cases of MDS. Environmental factors include exposure to chemotherapy, radiation, and other toxic insults such as benzene and derivatives and tobacco smoking, but 85% of patients do not have any well-identifiable cause. The pathophysiology of MDS combines sequentially acquired chromosome aberrations and somatic mutations with microenvironmental factors. Disease diagnosis remains mostly based on blood and bone marrow cytological examination. The number and extent of cytopenias, percentage of blast cells in the marrow, and nature of genetic alterations provide prognostic information. Treatment varies from careful monitoring to allogeneic stem cell transplantation. Two therapies, hypomethylating agents and lenalidomide, were approved for these patients. Median overall survival ranges from a few months to almost a decade, depending on age, degree and number of cytopenias, blast percentage, and cytogenetic and genetic aberrations.

MDS Diagnosis

An MDS diagnosis is suspected when peripheral blood cytopenias are identified on a routine analysis or a blood exam performed because of nonspecific clinical symptoms: fatigue and destabilization of a preexisting cardiovascular disease because of anemia, repeated infections because of neutropenia and neutrophil dysplasia, bleeding because of thrombocytopenia and platelet dysfunction. Cytopenias can be detected also while exploring an immune disorder including vasculitis, polychondritis,

and polyarthritis, which are commonly associated with MDS and often diagnosed simultaneously. Anemia, which is the most frequent cytopenia (90% of MDS), is usually nonregenerative and typically macrocytic. Neutropenia and thrombocytopenia are less frequent (30%), and usually combined with anemia. The presence of circulating blast cells over 1% is used for disease classification while the presence of immature granulocytes is rare. A monocyte count higher than $1000/mm^3$ and 10% of white blood cell count reclassifies the disease as chronic myelomonocytic leukemia (CMML), which belongs to a distinct category of myeloid malignancies according to the World Health Organization (WHO) and associates myelodysplastic and myeloproliferative features (MDS/MPN). Of note, a fraction of patients diagnosed with an MDS secondarily develop a monocytosis, joining the MDS/MPN category.

MDS diagnosis requires morphologic inspection of bone marrow aspiration and sometimes biopsy. The aspirate allows for detailed evaluation of dysplastic features, including ring sideroblasts (erythroblasts with abnormal accumulation of iron in perinuclear mitochondria), and for precise evaluation of the percentage of marrow blasts (to be assessed on 500 nucleated cells). The bone marrow trephine biopsy, whose utility is more controversial, allows for a more accurate determination of bone marrow cellularity and detection of marrow fibrosis. Because morphological assessment of dysplasia can be subjective, especially in the absence of blast excess, interobserved diagnostic discrepancies were observed in up to 20% of patients, especially those with a low grade MDS. Therefore, additional techniques are used to support MDS diagnosis.

The most important additional test remains the analysis of bone marrow cell karyotype, as a cytogenetic abnormality is found in 40%–50% of MDS, with partial or complete loss or gain of chromosomes (5q-, 7q-, −7, +8, 20q-, 17p-) being the most frequent findings. These heterogeneous abnormalities, whose identification supports the diagnosis when morphologically uncertain, have a strong prognostic significance. For example, complex karyotype is usually associated with an excess of blast cells and a poor outcome. Cytogenetics can also guide the therapeutic choice, for example, lenalidomide is used preferentially to treat MDS with isolated 5q-. The routine 20 metaphase cytogenetic analysis can be completed by fluorescence in situ hybridization (FISH) with probes targeting the most-frequently altered chromosomes when fewer than 20 mitoses have been obtained by routine cytogenetics. The occurrence of additional chromosomal aberrations with evolution can precede transformation to AML.

Flow cytometry with increasingly standardized combinations of markers helps recognizing minimal dysplasia by identifying abnormal phenotypic patterns, thus could enter the standard workup in the next future. Specifically, identification of an accumulation of classical monocyte subset in the peripheral blood is a strong argument to predict evolution of an MDS into a CMML.

Finally, DNA sequencing has established that MDS arises through the sequential acquisition of somatic mutations in a set of recurrently involved genes. New generation sequencing (NGS) analyses are increasingly included in the routine work-up of patients with MDS as they inform on diagnosis, prognostic, and potential therapeutic targets.

In patients with minimal or no diagnostic evidence of dysplasia and no blast excess, additional tests exclude other causes of cytopenia that include aplastic anemia, paroxysmal nocturnal haemoglobinuria clone, toxic exposure, vitamin or iron deficiency, hypersplenism, auto-immune cytopenias, viral infection, hereditary context, and others. When another cause of cytopenia has been excluded, patients with a cytopenia but no dysplasia are considered in the subset of idiopathic cytopenia of unknown significance (ICUS). A fraction of them may have clonal somatic mutations and cytogenetic abnormalities, and their natural history is still poorly known.

The 2016 WHO classification of myeloid malignancies still defines MDS subtypes on the basis of the number of dysplastic and cytopenic lineages, the prevalence of blasts, the percentage of ring sideroblasts (RS), and the presence of specific cytogenetic abnormalities.

A limited role is given to genetics as the mere presence of recurrent somatic mutations cannot be considered as definitive evidence of MDS diagnosis, for example, can be detected in patients with ICUS. In certain context however, genetic data provide diagnostic utility in MDS, for example, mutations in the spliceosome gene *SF3B1* define a subgroup of patients with ring sideroblasts and favorable outcome. Detection of an *SF3B1* mutation therefore establishes a diagnosis of MDS-RS in the presence of cytopenias and dysplasia, even when RSs make up as few as 5% of all nucleated erythroid cells (the traditional cutoff is 15%).

MDS Pathophysiology

Haploinsufficiency Through Chromosomal Deletion

A characteristic event is the occurrence, in a hematopoietic stem cell, of an interstitial deletion in the long arm of chromosome 5, known as del(5)(q31q33), leading to gene haploinsufficiency. The size of the deletion varies but, in MDS patients with an isolated 5q-, a common deleted region that encompasses about 40 genes has been identified, defining the 5q- syndrome. This deletion induces haploinsufficiency for the ribosomal subunit *RPS14*, which triggers a p53-dependent block in erythroid proliferation and differentiation, and haploinsufficiency of *CSNK1A1* (*CSK1*) and two micro-RNAs, mir-145 and mir-146a, which may account for dysmegakaryopoiesis and thrombocytosis. MDS with complex karyotypes have larger 5q deletions with haploinsufficiency of tumor suppressor genes such as *CTTNA1* or *EGR1*. The selective clonal suppression of del(5q) cells by lenalidomide preserves del(5q) hematopoietic stem cells. A similar haploinsufficiency model might apply also for MDS with del(7q), del(20q), and other recurrent interstitial deletions.

Recurrently Mutated Genes

A comprehensive picture of the mutational landscape in MDS has emerged over the past 10 years. With an approach focusing on a set of ~50 genes recurrently mutated in myeloid malignancies, a somatic mutation in at least one gene is identified in 90% of patients. These genes encode RNA splicing factors, epigenetic regulators, cohesin components, transcription factors, DNA damage response and signal transduction molecules. These mutations are sequentially acquired, suggesting a disease initiation step with founder mutations driving asymptomatic clonal expansion within the hematopoietic compartment (the so-called age-related clonal hematopoiesis—ARC—or clonal hematopoiesis of indeterminate potential—CHIP), followed by acquisition of mutations that both synergize with the founder mutation on hematopoietic stem cells/progenitors and impair later stages of hematopoiesis and alter blood counts to generate an overt MDS, leading, in 30%–40% of patients, to eventual secondary AML (sAML).

The spliceosome is a multiprotein complex that mediates intron excision and exon ligation in the generation of mature messenger RNA. Genes encoding components of the spliceosome, most commonly *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*, are the most frequently mutated genes detected in MDS (60% of patients). Mutant splicing factors result in altered patterns of premessenger RNA splicing. The splicing factor mutations are generally heterozygous and mutually exclusive, suggesting that either cells cannot tolerate two mutations or these changes have a redundant role in disease pathogenesis. *SF3B1* mutation, the most frequent one, is strongly associated with ringed sideroblasts.

A second common class of mutations are those affecting genes involved in DNA methylation and histone modification. Recurrent mutations in *DNMT3a*, a DNA methyltransferase, *TET2*, an enzyme that hydroxylates methylated cytosines to initiate the process of DNA demethylation, and isocitrate dehydrogenase gene 1 (IDH1) and IDH2 alter the DNA methylation pattern. *IDH* gene mutations result in the generation of 2-hydroxyglutarate, an oncometabolite that inhibits the activity of numerous targets, including TET2. Components of histone modification complexes are also recurrently mutated in MDS, most commonly *ASXL1* and *EZH2*, which are affected by loss-of-function mutations in approximately 20% and 5% of cases, respectively.

Another commonly altered multiprotein complex is cohesin, which is composed of STAG1, STAG2, SMC1A, SMC3, and RAD21. Mutually exclusive loss of function mutations in these components are found in ~10% and ~20% of low- and high-risk MDS, respectively. These mutations may disrupt DNA loops involved in enhancer-promoter interactions, thus driving aberrant transcriptional programs and complementing loss-of-function mutations in core transcription factors.

Mutations in transcription factors such as GATA2 and RUNX1 usually occur somatically. However, they can also be inherited in the germline and cause familial bone marrow failure syndromes with a propensity to evolve into myeloid malignancies.

Missense mutations in the tumor suppressor *TP53* are highly prevalent among patients with MDS who have undergone chemotherapy. They are frequently associated with the loss of the second *TP53* allele via deletion of the short arm of chromosome 17 and their presence correlates with a poor outcome, even with most aggressive treatment regimens.

Pre-MDS Clonal Mutations

Recurrent somatic mutations in myeloid malignancy-associated genes, such as *DNMT3a*, *TET2*, and *ASXL1* can be identified in the peripheral blood of healthy individuals with normal blood counts. These CHIP or ARCH increase the risk of malignant transformation (0.5%–1% per year) through the acquisition of additional mutations (even though the principal cause of morbidity is vascular diseases). Accordingly, mutations in the above-mentioned epigenetic regulators are founder events in MDS pathogenesis. Germline polymorphisms and cell extrinsic factors may promote the rare progression of CHIP into overt MDS or AML. For example, treatment with chemotherapy could enable the preferential expansion of clones carrying mutations in *TP53*.

Post-MDS Clonal Mutations

Evolution to sAML can be considered the final stage of disease progression through the acquisition of characteristic genetic changes, such as activating mutations in signaling molecules such as *FLT3* and *N-RAS*, as well as inactivating mutations in *CEBPA*. At the time of leukemic transformation, the antecedent MDS clone is outcompeted by aggressive subclones. Of note, mutations in *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, and *STAG2* identify a subset of patients with AML who have no history of preceding MDS and who share the aggressive clinical behavior of sAML.

Epigenetic Changes

Genetic alterations are frequently associated with epigenetic changes arising in hemopoietic stem cells and including aberrant DNA methylation, which was most studied in the promoter region of the tumor suppressor gene P15 *INK4B*. These alterations are only partly related to gene mutations in epigenetic regulators. Hypomethylating drugs appear to modulate the methylation of DNA without eradicating the mutated cells, which can be sufficient to get a more balanced hematopoiesis.

Ineffective Hematopoiesis

The occurrence of cytopenias despite a generally hypercellular marrow indicates ineffective hematopoiesis, which was shown to result from an increased susceptibility of clonal myeloid progenitors to apoptosis. This excessive apoptosis can result from intrinsic

stresses due to the above-described genetic and epigenetic alterations. For example, iron retention in SF3B1 mutated erythroblasts induce mitochondrial depolarization and ribosomal defects in 5q- syndrome lead to reticulum endoplasmic stress. One of the hallmarks of MDS is activation of the NLRP3 inflammasome, which drives clonal expansion and pyroptotic cell death, independently of genotype, Pyroptosis is triggered by the alarmin S100A9 that is found in excess in MDS HSPCs and bone marrow plasma excessive apoptosis can also result from external insults triggered by the microenvironment such as death receptor ligands, including Fas-L and tumor necrosis factor, and myelosuppressive cytokines, such as transforming growth factor β. Importantly, a shift of from apoptosis to proliferation that emerges in a subclonal progenitor, for example, as a consequence of NFkB pathway activation, leads to disease progression to acute myeloid leukemia.

Microenvironment and Immune Cells

The hematopoietic stem-cell niche contributes to the pathogenesis of MDS. In the recent years, several mouse models have shown that genetic alterations in specific cells of this microenvironment could promote the emergence of a myelodysplastic clone. Clonal T-cell expansion is commonly detected, especially in patients with a hypoplastic syndrome, inflammatory Th17 cells and myeloid-derived suppressive cell may contribute to ineffective hematopoiesis whereas regulatory T cells contribute to evasion from antitumoral immunity in higher-risk disease. Several of the drugs commonly used in MDS may have an effect on this pathological environment and immune reaction, beyond their specific effect on the malignant cells. It remains unknown if these abnormalities are secondary events that modify the environment of the leukemic clone and facilitate its dominance on polyclonal hematopoiesis or they are primary events that favor the emergence of mutated clones able to escape this altered environment. Analysis of the genetic predisposition to MDS and other myeloid malignancies may help to distinguish the egg and the chicken: if the environment defect is the first to appear, it is expected that some hereditary cases will be the consequence of mutations altering the environment.

Among myeloid malignancies, MDS are at the crossroad of several disorders: (1) CHIP and ICUS that we have previously described (2) aplastic anemia, including those of auto immune origin and (3) *BCR-ABL*-negative MPNs in which primary myelofibrosis could be considered as an MDS. One interesting hypothesis is that somatic mutations identified in these diseases have been selected as they provide HSCs an advantage on aging or immunologically altered HSCs. These mutations provide some advantage to early stages of hematopoiesis but some of them are deleterious for terminal hematopoiesis, leading to cell dysplasia. These mutations can induce a genetic instability that favors secondary mutations and clonal evolution, or behave as rescue mutations such as JAK2V617F mutation whose acquisition in the context of SF3B1-driven refractory anemia with ring sideroblasts, induce some correction of anemia and a thrombocytosis, improving the disease outcome.

Risk Stratification

Because the prognosis of patients with MDS is highly heterogeneous, accurate prognostic systems are used to guide the therapeutic choices and timing. A number of scoring systems have been developed, the most widely used being the International Prognostic Scoring System (IPSS) and the Revised IPSS (IPSS-R).

The original IPSS, established in 1997, assigned to each patient a risk score that is based on bone marrow blast percentage, cytogenetics, and number of cytopenias (**Table 1**). Patients with low or intermediate-1 risks are considered to have low-risk disease, those with intermediate-2 or high scores to have high-risk MDS. Median OS ranges from approximately 5 months in untreated

Table 1 International prognostic scoring system for myelodysplastic syndromes (IPSS-R)

Define first the cytogenetic risk as follows					
Good	Normal, -Y, del(5q), del(20q)				
Intermediate	All other situations				
Poor	Complex (3 or more abnormalities) or chromosome 7 abnormalities				
Then, include the cytogenetic risk in the scoring system					
Points	0	0.5	1	1.5	2
Cytogenetic	Good	Intermediate	Poor		
BM blast	<5%	5%–10%		11%–20%	20%–29%
Cytopenias[a]	0–1	2 or 3			
Finally, calculate the prognostic risk score					
0					Low risk
0.5–1					Intermediate risk 1
1.5–2.0					Intermediate risk 2
2.5–3.5					High risk

[a]Cytopenias defined as either hemoglobin <100 g/L or absolute neutrophil count <1800/μL or platelet count <100,000/μL.

Table 2 Revised international prognostic scoring system for myelodysplastic syndromes (IPSS-R)

Define first the cytogenetic risk as follows							
Very good	-Y, del(11q)						
Good	Normal, del(5q), del(12p), del(20q), double including del(5q)						
Intermediate	del(7q), +8, +19, i(17q), any other single or double independent clones						
Poor	Complex (>3 abnormalities)						
Then, include the cytogenetic risk in the scoring system							
Points	0	0.5	1	1.5	2	3	4
Cytogenetic	Very good		Good		Intermediate	Poor	Very poor
BM blast	Up to 2%		2%–5%		5%–10%	>10%	
Hemoglobin	>10		8–10	<8			
Platelets	>100	50–100	<50				
ANC	>0.8	<0.8					
Finally, calculate the prognostic risk score							
Lower or equal to 1.5 points							Very low risk
>1.5–3							Low risk
>3–4.5							Intermediate risk
>4.5–6							High risk
>6							Very high risk

patients with high risk to almost 6 years in patients with low-risk disease. This very simple and easy to use system has limitations, especially in patients with low risk disease.

The IPSS-R, which was developed from a data set of over 7000 patients, uses different cut-off points of cytopenias, places a higher weight on cytogenetics, and refines the bone marrow blast categories (Table 2). Again, this score separates MDS patients with lower risk (very low or low risk scores) from those with higher risk (high or very high risk scores). The main limitation of IPSS-R is that no drug therapy has been approved yet using this scoring system.

A new molecular IPSS system is now expected as somatic mutations may impact the therapeutic choice. It is now clear that patient outcomes progressively worsen as the number of oncogenic mutations increases. In addition, specific somatic mutations reproducibly predict patient outcomes in univariate analysis, including the better clinical outcome of MDS with SF3B1 mutations and the worse outcome of those with *TP53*, *EZH2*, *ETV6*, *RUNX1*, *ASXL1*, and *SRSF2* mutations. However, genetics only modestly improves scoring systems such as R-IPSS and their precise role in clinical practice has still to be better delineated. Specific mutations can help in specific settings, for example, *TP53* and *DNMT3A* mutations are associated with a poor outcome after allogeneic stem cell transplantation, patients with *TET2* mutations better respond to azacitidine and those with *IDH1* or *IDH2* mutations may deserve targeted drugs.

Nevertheless, morphologic and clinical criteria still play a central role in evaluating MDS prognosis and these systems could be further refined by introducing comorbidities that affect the natural history of MDS patients while specific scoring systems could improve the prognostication in hypocellular and therapy related MDS.

Treatment of MDS

Therapeutic decision-making is guided by individual risk assessment performed using the IPSS, R-IPSS or similar tools. MDS patients with an indolent disease course and a relatively long life expectancy do not necessarily warrant therapy, for example, those without significant cytopenias can be monitored closely without treatment. Therapy is introduced to reduce transfusion needs and improve quality of life. A common practice is to start with growth factor support and consider lenalidomide or an azanucleoside secondarily. Patients that fail are candidates for clinical trials or allogeneic stem cell transplantation (ASCT). Ideally, treatment may delay transformation to AML and improve survival. Earlier therapeutic intervention is proposed to lower risk patients with a less favorable prognosis, with several innovative therapeutics currently tested, including transforming growth factor β superfamily ligand traps to treat anemia, oral azanucleosides, proteasome inhibitors, and antagonists of toll-like receptor signaling. Because higher-risk MDS patients have a higher rate of progression to AML and shorter survival, the goal of treatment is to alter the natural history of these diseases, prevent progression to AML and improve overall survival. Current available therapies include hypomethylating agents, intensive chemotherapy, and ASCT. Additional supportive care measures include the use of prophylactic antibiotics and iron chelation. There are no approved interventions for patients with progressive or refractory disease after these treatments. The management of patients with an intermediate score must be individualized, taking into consideration age, presence of bone marrow fibrosis and other relevant prognostic data such as gene mutations.

Supportive Care

Prophylactic antibiotics are usually used in the context of cytotoxic or immunosuppressive therapy and not in untreated patients as there is not major increase in the risk of infection in those with isolated neutropenia. Iron accumulation, as a consequence of red cell

transfusions, could increase the risk of infection as well as that of AML transformation, and induce liver cirrhosis or cardiomyopathy in lower risk, long living patients, but the use of chelation therapy is currently limited to patients with very high ferritin levels, above 2500 ng/mL.

Growth Factor Support

The vast majority of MDS patients develop some degree of anemia during the course of the disease, approximately 40% become transfusion-dependent, and severe anemia decreases health-related quality-of-life and increases cardiovascular risk. There is no consensus on the optimal hemoglobin level threshold for transfusions. Erythroid stimulating agents (ESA) are commonly used in these patients to prevent or correct anemia. A 3-month treatment is needed before evaluating the response. Recommended doses are higher than those used for patients with anemia due to chronic renal insufficiency. Treatment is generally well tolerated, and the incidence of cardiac and thrombotic events is low. Response rates range from 30% to 50% with a median response duration of 18–24 months. Algorithms have been developed, for example, by the Nordic MDS group, to predict response to ESAs, which is much higher in patients with a low endogenous serum erythropoietin (EPO) level EPO levels of <500 U/L and with low transfusion requirement (<2 units of packed red blood cells each month). Substantial improvements in well-being correlate with erythroid response. ESA are discontinued when the transfusion effect is lost.

The addition of granulocyte-colony-stimulating-factor (G-CSF) has been proposed in patients failing to respond to ESAs alone. ESAs may improve the natural history of the disease but questions on their potential tumorigenic effect have resulted in increased scrutiny of their use. Pooled analyses and large retrospective studies suggest a small OS benefit with these agents in the MDS population responding to treatment. In patients with thrombocytopenia, the use of eltrombopag remains cautious as there is a fear that, like romiplostim, the use of the MPL agonists expose to disease transformation and marrow fibrosis.

Immunomodulatory Drugs (IMiDs)

Thalidomide initially demonstrated some activity in reducing red blood cell transfusion needs in patients with MDS that had previously failed ESAs. Then, lenalidomide was developed as an orally bioavailable analog of thalidomide with immunomodulatory, antiangiogenic, and antiproliferative properties. In patients with anemia and del(5q), lenalidomide (10 mg for 21 days every 4 weeks) has become the standard of care. del(5q) is the most common cytogenetic abnormality in MDS (10%–15%). The so-called "5q-syndrome" includes refractory anemia, isolated del(5q), female predominance, normal to increased monolobulated megakaryocytes, $<5\%$ bone marrow myeloblasts and relatively indolent course. In patients with a 5q-syndrome and a good platelet count, lenalidomide results in lower transfusion requirements and, in 50% of them, cytogenetic complete remission. Response is fast (median time 5 weeks). The median rise of hemoglobin is 5 g/dL. The most common adverse events are early neutropenia and thrombocytopenia, which generally occur within the first two cycles. Their occurrence is associated with a higher probability of response as they correspond to suppression of the del(5q) clone. While deep venous thrombosis is a well-known adverse event associated with the use of lenalidomide in multiple myeloma, it was reported in only 3% of MDS patients. This treatment does no increase the incidence of AML transformation and increases survival for responding patients, indicating that the drug can change the natural history of this specific MDS.

A mechanism of action of lenalidomide has been deciphered. This drug binds to an E3 ubiquitin ligase, alters its substrate affinity and induces the selective degradation of a kinase named casein kinase 1A1. This kinase is encoded by a gene in the commonly deleted region of chromosome 5. Complete loss of this kinase triggers p53-mediated apoptosis. This explains why mutations in *TP53* are associated with poor response to lenalidomide and *TP53*-mutant subclones expand in patients treated with this drug.

Lenalidomide has been used also in transfusion-dependent MDS patients without chromosome 5 alterations: 25% achieved transfusion independence after a median of 5 weeks of therapy, with median response duration of 41 weeks. The median hemoglobin increase was less robust than that observed in patients with isolated del(5q), cytogenetic responses were infrequent, and the median duration of transfusion independence was shorter than in patients with 5q-syndrome. The drug may not be used in patients with thrombocytopenia.

DNA Methyltransferase Inhibitors

Aberrant DNA methylation may be an important component of MDS pathogenesis. DNA methyltransferases (mainly DNMT3A/B) are the main enzymes that catalyze the addition of methyl groups to the 5′ position of cytosine nucleotides into DNA. Two drugs that inhibit DNMTs are the standard of care for most patients with high risk MDS, namely 5-azacitidine and 5-aza-2′-deoxycitidine (decitabine). These azanucleosides are also known as hypomethylating agents (HMAs).

Azacitidine is a ribonucleoside that is phosphorylated by uridine cytidine kinase to be incorporated into RNA. Decitabine is a deoxyribonucleoside that is directly incorporated into DNA. About 10% of azacitidine is also incorporated into DNA through enzymatic conversation by ribonucleotide reductase. Decitabine requires phosphorylation by a deoxycytidine kinase to become biologically active. Both drugs incorporate into nucleic acids during the S-phase and irreversibly bind to and deplete DNMTs. Demethylation of DNA was associated with clinical response in chronic myelomonocytic leukemia.

Although response rates to these two drugs are similar, only 5-azacitidine (75 mg/m^2 subcutaneously daily for 7 days every 4 weeks) has been demonstrated to improve survival in higher risk MDS patients, thus is the standard front line treatment in these patients. Patients respond slowly, in 90% of cases by six cycles, suggesting that therapy should not be abandoned prior to six cycles unless there is documented disease progression. Fifty to sixty percentage of these patients experience hematologic improvement after six cycles, which improves their quality of life compared to best supportive care only. The median duration of response is 15 months. Therapy should be given indefinitely in the absence of severe toxicity or disease progression. The most common toxicities are hematologic, grade 3 or 4 neutropenia is most common and may be more prominent with the initial cycles whereas nonhematologic toxicity is dominated by injection-site reactions to subcutaneous injections. Loss of response after discontinuation can be rapid and retreatment results in inferior quality and duration of responses compared to initial treatment.

Achievement of CR is not a prerequisite for improved survival. Power of mutational data to predict HMA response is modest. Nevertheless, early clonal mutations in *TET2* gene, encoding a protein involved in the demethylation of 5-methylcytosine, have been associated with an increased response rate. A set of differentially methylated regions, mostly localized in nonpromoter regions, also predicts response to HMAs.

Low dose HMAs can be considered in patients with lower risk disease that are transfusion dependent and have failed or are not candidates for growth factor support or lenalidomide. An oral formulation of 5-azacitidine is being studied for patients with lower risk MDS.

AML-Like Induction Chemotherapy

The classical anthracycline- and cytarabine-based chemotherapy used to treat AML is less efficient in treating high-risk MDS. In addition to being hard to use because of the advanced age of MDS patient, this treatment results in lower CR rates (40%–60%), higher treatment-related mortality and morbidity, shorter CR duration ($\sim$12 months) and longer periods of aplasia. In particular, patients with a complex karyotype or an alteration of chromosome 7 have a low CR rate and a short duration of response. Therefore, AML-like therapy is now restricted to younger patients with favorable karyotype, usually as a bridge to allogeneic stem cell transplantation. A large retrospective study comparing azacitidine to induction chemotherapy or both prior to allogeneic stem-cell transplant found that OS, event-free survival, relapse rate and nonrelapse mortality are similar between azacitidine and induction chemotherapy.

Immune Therapy

Immune-modulatory agents can be used in hypocellular MDS patients, which are a difficult group of patients. A deregulation of both cellular (abnormal CD8$^+$ cytotoxic T-lymphocytes) and innate immunity is identified in a subset of MDS patients, autoimmunity is commonly associated to these diseases, and there is overlap between MDS and immune-mediated bone marrow failure syndromes such as aplastic anemia. Equine antithymocyte globulin (ATG, 15 mg/kg intravenously for 5 days) combined with oral cyclosporine for 6 months, but also growth factors and steroids, which are all used in aplastic anemia, are considered in younger MDS patients with severe hypocellular bone marrow and short transfusion dependency when allogeneic stem cell transplantation is not feasible. Responses are slow as they may take up to 6 months to manifest. Response rates are around 30%. The impact of ATG based therapy on transformation-free and overall survival is still questioned. Among the new agents, the activity of alemtuzumab, an antibody against CD52, and eltrombopag, which was tested as salvage therapy, is currently investigated.

Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation is the only curative therapeutic strategy for MDS. Timing of the procedure, optimal conditioning regimen and maintenance therapies are still debated issues.

Because of its high morbidity and mortality, allogeneic stem cell transplantation is usually not recommended in patients with lower risk MDS at initial presentation, even if they are young. Because of the time required for donor identification, it is important, nevertheless, to search rapidly for an appropriate donor while evaluating the risk of this therapeutic approach.

Young patients with higher risk disease or hypoplastic MDS at diagnosis and MDS patients who have been exposed to multiple therapies should be considered for transplantation. Early transplantation is associated with a prolonged survival obtained in 30%–50% of the patients.

It is important to identify which patients will benefit the most from transplant through accurate pretransplant risk evaluation. Risk factors include cytogenetics (monosomy 7 predicts a poor outcome), elevated serum ferritin levels and some genetic alterations as mutations in *TP53*, *DNMT3A*, *RUNX1*, and *ASXL1* mutations are potential markers of poor outcome.

Different transplant modalities and donor sources are currently evaluated, which may allow considering older patients for transplantation in the future. Retrospective studies suggest that MDS patients between ages 60 and 70 with a suitable donor can benefit from transplant following a reduced intensity conditioning as long as they are medically fit.

Questions include whether or not disease debulking with chemotherapy or hypomethylating agents is recommended prior to transplant when marrow blasts $>10\%$ in order to decrease the very high relapse risk posttransplant. Most of these patients receive azanucleosides as a donor search is initiated and pretransplant testing is completed. Posttransplant hypomethylating use could also

improve outcome, especially in patients who showed decreased donor chimerism (< 80%) while still in morphologic and hematologic complete response.

Investigational New Therapeutic Options

All the currently available agents for the treatment of MDS have considerable shortcomings and every patient with a relapsed or refractory disease should be considered for an investigational clinical trial if allogeneic stem cell transplantation is not feasible.

Novel therapeutic targets have emerged from recent advances in our understanding of the molecular pathophysiology of these diseases. Targeted molecules include small-molecule inhibitors of either mutant isocitrate dehydrogenase enzymes, or spliceosome, which are currently evaluated in early phase clinical trials. Kinases inhibitors, including those targeting Flt3 and the Ras pathway could also take place in the precision medicine approach.

Another approach, whose development has been limited by toxicity, is the combination of azanucleoside with other drugs such as lenalidomide, anthracyclines or histone deacetylase inhibitors. An oral formulation of azacitidine and second-generation azanucleosides such as SGI-110, a dinucleotide form of decitabine and deoxyguanosine that protects it from deamination, are also tested.

Since thrombocytopenia can be difficult to manage in MDS patients, thrombopoietin receptor agonists, including romiplostim and eltrombopag, have been tested. Concerns regarding an increase in the rate of progression to AML have led to stop the development of romiplostim. Preliminary results indicated that eltrombopag increases platelet count in lower risk MDS patients.

Agents targeting a variety of pathways are currently under clinical investigation for the treatment of anemia, including inhibitors of p38 MAPK and Tie2 (ARRY-614), PI3K and PLK1 (rigosertib), and the TGF-β superfamily (LY2157299, sotatercept). Rigosertib, clofarabine and immune checkpoint inhibitors are also tested in MDS. Finally, high doses of vitamin C could partially revert the phenotypic effects of *TET2* mutations but the potential interest of these observations deserve to be tested in clinical trials.

Prospective Vision

A number of genetic alterations that drive MDS have been identified but how these somatic aberrations occur and the conditions in which they generate the disease phenotype remain open questions: the role of germline factors, microenvironment alterations and host factors such as immune response and microbiota composition in disease emergence and progression may be clarified in the coming years. Lenalidomide and hypomethylating agents have a major place in the current armamentarium against MDS, as targeted therapies have demonstrated limited efficacy so far, but coming years may define the potential interest of IDH and spliceosome inhibitors and, hopefully, alternative strategies will emerge from ongoing pathophysiological studies.

Further Reading

Arber, D.A., Orazi, A., Hasserjian, R., et al., 2016. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127, 2391–2405.
Canaani, J., Nagler, A., 2016. Established and emerging targeted therapies in the myelodysplastic syndromes. Expert Review of Hematology 9, 997–1005.
de Witte, T., Bowen, D., Robin, M., et al., 2017. Allogeneic hematopoietic stem cell transplantation for MDS and CMML: Recommendations from an international expert panel. Blood 129, 1753–1762.
Deininger, M.W.N., Tyner, J.W., Solary, E., 2017. Turning the tide in myelodysplastic/myeloproliferative neoplasms. Nature Reviews. Cancer 17, 425–440.
Delhommeau, F., Dupont, S., Della Valle, V., et al., 2009. Mutation in TET2 in myeloid cancers. The New England Journal of Medicine 360, 2289–2301.
Greenberg, P., Cox, C., LeBeau, M.M., et al., 1997. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 89, 2079–2088.
Greenberg, P.L., Tuechler, H., Schanz, J., et al., 2012. Revised international prognostic scoring system for myelodysplastic syndromes. Blood 120, 2454–2465.
Hellström-Lindberg, E., van de Loosdrecht, A., 2013. Erythropoiesis stimulating agents and other growth factors in low-risk MDS. Best Practice & Research. Clinical Haematology 26, 401–410.
Inoue, D., Bradley, R.K., Abdel-Wahab, O., 2016. Spliceosomal gene mutations in myelodysplasia: Molecular links to clonal abnormalities of hematopoiesis. Genes & Development 30, 989–1001.
Malcovati, L., Cazzola, M., 2015. The shadowlands of MDS: Idiopathic cytopenias of undetermined significance (ICUS) and clonal hematopoiesis of indeterminate potential (CHIP). Hematology. American Society of Hematology. Education Program 2015, 299–307.
Olnes, M.J., Sloand, E.M., 2011. Targeting immune dysregulation in myelodysplastic syndromes. Journal of the American Medical Association 305, 814–819.
Raaijmakers, M.H., 2014. Disease progression in myelodysplastic syndromes: Do mesenchymal cells pave the way? Cell Stem Cell 14, 695–697.
Sallman, D.A., Davila, M.L., 2017. Is disease-specific immunotherapy a potential reality for MDS? Clinical Lymphoma, Myeloma & Leukemia 17S, S26–S30.
Sperling, A.S., Gibson, C.J., Ebert, B.L., 2017. The genetics of myelodyplastic syndrome: From clonal haematopoiesis to secondary leukaemia. Nature Reviews. Cancer 17, 5–19.
Talati, C., Sallman, D., List, A., 2017. Lenalidomide: Myelodysplastic syndromes with del(5q) and beyond. Seminars in Hematology 54, 159–166.
Wong, T.N., Ramsingh, G., Young, A.L., et al., 2015. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. Nature 2518, 552–555.

Relevant Websites

https://www.mds-foundation.org/.
https://www.cancer.gov/types/myeloproliferative/patient/myelodysplastic-treatment-pdq.

Nasopharyngeal Carcinoma: Diagnosis and Treatment

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Abbreviations

^{18}F-FDG PET/CT 2-Deoxy-2-[fluorine-18]fluoro-D-glucose PET integrated with CT
5-FU 5-Fluorouracil
CT Computed tomography
EA Early antigen (of the Epstein-Barr virus)
EBV Epstein-Barr virus
EGFR Epidermal growth factor receptor
FNA Fine-needle aspirates
HDACi Histone deacetylase inhibitors
HLA Human leukocyte antigen
IARC International Agency for Research on Cancer (WHO)
ICD-O International Classification of Diseases for Oncology
IMRT Intensity-modulated radiotherapy
LMP Latent membrane protein (of the Epstein-Barr virus)
MRI Magnetic resonance imaging
NPC Nasopharyngeal carcinoma
PD-L1 programmed death ligand 1
PET Positron emission tomography
RT Radiotherapy
RT-PCR Real-time polymerase chain reaction
VCA Virus capsid antigen (of the Epstein-Barr virus)
VEGF Vascular endothelial growth
WHO World Health Organization

Definition and Classification

Nasopharyngeal carcinoma (NPC) is a carcinoma which arises in the nasopharyngeal mucosa and shows light microscopic or ultrastructural evidence of squamous differentiation. The term encompasses three distinct entities: nonkeratinizing, keratinizing, and basaloid squamous cell carcinoma.

Nonkeratinizing squamous cell carcinoma (ICD-O code: 8072/3) is composed of large atypical cells, without morphological evidence of keratin production, whereas keratinizing squamous cell carcinoma (8071/3; also called large cell squamous cell carcinoma, keratinizing squamous cell carcinoma, and keratinizing epidermoid carcinoma) is a squamous cell carcinoma with prominent production of keratin. Basaloid squamous cell carcinoma (8083/3) is histologically characterized by the presence of cells with hyperchromatic nuclei, scant amount of cytoplasm, and peripheral nuclear palisading.

Presentation and Diagnosis

NPCs most commonly originate in the pharyngeal recess (fossa of Rosenmüller), or—less frequently—in the superior posterior wall of the nasopharynx. The tumor can present as a smooth bulge in the mucosa, a discrete raised nodule (in some cases with surface ulcerations), or an infiltrative fungating mass. In some cases, the lesions are not grossly visible. NPC is highly malignant, with extensive locoregional infiltration and lymphatic spread occurring early in the course of the disease.

Most patients present with locoregionally advanced disease, commonly with cervical lymph node metastases. The presenting symptoms are related to the presence of a mass in the nasopharynx (e.g., epistaxis, obstruction, and blood-stained postnasal drip), Eustachian tube dysfunction (such as hearing impairment, tinnitus, and serous otitis media), and skull base involvement with impairment of the cranial nerves, most frequently the fifth and the sixth (e.g., headache, diplopia, facial pain, numbness, and paresthesia). Painless neck mass appears due to lymph node metastases. About 10% of patients are asymptomatic.

Fiberoptic endoscopic examination of the nasopharynx following complete physical examination including palpation of the head and neck for adenopathy is routinely used to elicit the tumor. The jugulodigastric node is the most commonly palpable

node at presentation. Computed tomography (CT), positron emission tomography (PET)-CT scan, or magnetic resonance imaging (MRI) is used to evaluate the extent of primary tumor and adenopathy as well as the skull base invasion. MRI is often more helpful than CT as it provides a better resolution for assessing parapharyngeal spaces, marrow infiltration of the skull base, intracranial disease, and deep cervical nodes. Neuro-ophthalmological and audiological evaluations allow to evaluate the degree of impairment of the cranial nerve function.

Diagnosis is made based on the pathological evaluation of the nasopharyngeal mass from the primary tumor site. Determining the Epstein-Barr virus (EBV) infection status helps to confirm nonkeratinizing NPC diagnosis. Detecting Epstein-Barr virus (EBV) by hybridization in situ for the Epstein-Barr encoding region (EBER) is particularly helpful in the evaluation of the cervical lymph nodes harboring undifferentiated or poorly differentiated squamous cell carcinoma, with the positive result being strongly suggestive of the NPC diagnosis (see "**Biomarkers**" section). A cytological evaluation of fine-needle aspirates (FNA) of the neck followed by immunohistochemistry is useful for diagnosing metastatic keratinizing and nonkeratinizing NPC.

Epidemiology and Risk Factors

Burden

Even though NPC is the most common neoplasm of the nasopharynx, it is relatively uncommon compared to other cancer types. However, it has a very unique geographical distribution overall, with remarkable variations in incidence. About 70% of cases are diagnosed in east and south-east Asia, and those occurring in south-central Asia, and east and north Africa roughly account for the remaining 30%, while the annual incidence in North America is only 0.3–0.7 cases per 100,000 population. In 2012, almost 86,700 new NPC cases were reported worldwide (about 0.6% of all cancers), of which over 21,200 occurred in the WHO South-East Asia region. The highest incidence rates were reported for Malaysia, followed by Singapore before Indonesia and Vietnam (**Fig. 1**). The peak incidence is between 40 and 60 years of age.

The age-standardized incidence of NPC has declined over the past decades, particularly among Hong Kong Chinese, and the NPC-related mortality rates are also going down. Still, 120,000 new NPC cases and over 20,000 NPC-related deaths are predicted for the year 2030.

Etiology and Risk Factors

Men are two to three times more likely to develop NPC than women. In contrast to Caucasians in whom NPC is rare, NPC is quite common in some specific ethic groups, including the Inuits in the North Pole, the Bidayuh in Borneo, and the Nagas in northern India, suggesting that they may have a genetic predisposition to develop the disease.

The etiology of NPC seems to be multifactorial, with viral infections playing an important but not exclusive role in the tumorigenesis. Epstein-Barr virus (EBV), a very common herpesvirus, is strongly associated with the development of nonkeratinizing NPC, in particular in endemic NPC regions, but not with that of the keratinizing type. Conversely, infection with high-risk types of the human papilloma virus (HPV) has been shown to be associated with NPC from nonendemic regions and in particular with the keratinizing type, even though the evidence is not yet so strong as for EBV.

Consumption of salted and fermented food has been implicated in the etiology of nonkeratinizing NPC in populations where this type is endemic. In particular, consumption of traditional salted fish in China has been shown to be a risk factor. These foods have a high content of N-nitrosamine. The carcinogenicity of N-nitrosamine compounds has been clearly shown for several other cancer sites and it is also hypothesized to be the carcinogen associated with the NPC risk.

Tobacco smoking and heavy alcohol consumption have also been proposed as putative risk factors, however the evidence for the latter remains controversial. Among other environmental factors, occupational exposure to wood dust, heat, smoke, formaldehyde, and chemical fumes have been identified as potentially contributing to the risk of NPC, and two of these exposures have been classified as carcinogenic for the nasopharynx by the IARC monographs (**Table 1**).

The risk of developing NPC is also associated with genes coding for certain tissue antigens (human leukocyte antigen (HLA) complex). For example, high-resolution genotyping studies have consistently shown an association between NPC and the *HLA-A*0207* allele which is common in the Chinese population. Genetic polymorphisms in genes coding for metabolic and DNA repair enzymes have also been associated with an increased risk of NPC. Familial clustering of NPC is well documented. However, the clinical characteristics of familial and sporadic NPCs do not differ.

Pathology and Genetics

Deletions on chromosomes 3p and 9p (spanning the *CDKN2A* gene on chromosome 9p21) have been identified to be early events in NPC carcinogenesis. Most frequent chromosome gains are on chromosome 12. Gene fusions have been reported in about 10% of cases. Somatic mutations in the *TP53* gene are common (15%–20%).

The most remarkable characteristic of the NPC genome is the presence of multiple alterations targeting chromatin remodeling pathway, with three genes having been identified as significantly mutated by whole-exome sequencing: *BAP1*, *MLL2*, and *TSHZ3*. The most frequently altered gene in this category is *ARID1A*. Alterations in the chromatin remodeling pathway have been shown to

be associated with both EBV burden and poor overall survival in univariate but not in multivariate analysis. Interestingly, suppressing endogenous *ARID1A* in vitro results in an increased anchorage-independent colony formation, cell migration and xenograft growth. Another altered pathway includes receptor tyrosine kinases (*ERBB2*, *ERBB3*) and their downstream effectors (*PI3KCA*, *KRAS*). Patients with alterations in this pathway seem to have shorter survival and more advanced stage disease. Finally, a number of alterations are observed in genes regulating autophagy (Table 2).

Biomarkers

Epstein-Barr Virus (EBV)

EBV is prevalent in NPC, with almost all cases of nonkeratinizing NPC being associated with EBV infection. EBV testing has been shown to be useful for early detection and differential diagnosis of NPC as well as for prognostication and monitoring response to treatment (Table 3).

The most reliable method to detect EBV in tumor tissue is in situ hybridization for EBV-encoded RNA (EBER) or immunohistochemical staining for latent membrane protein (LMP), even though the reliability and specificity of this method is questioned by some sources. EBV DNA load in blood serum or plasma may be quantified by real-time polymerase chain reaction (RT-PCR)

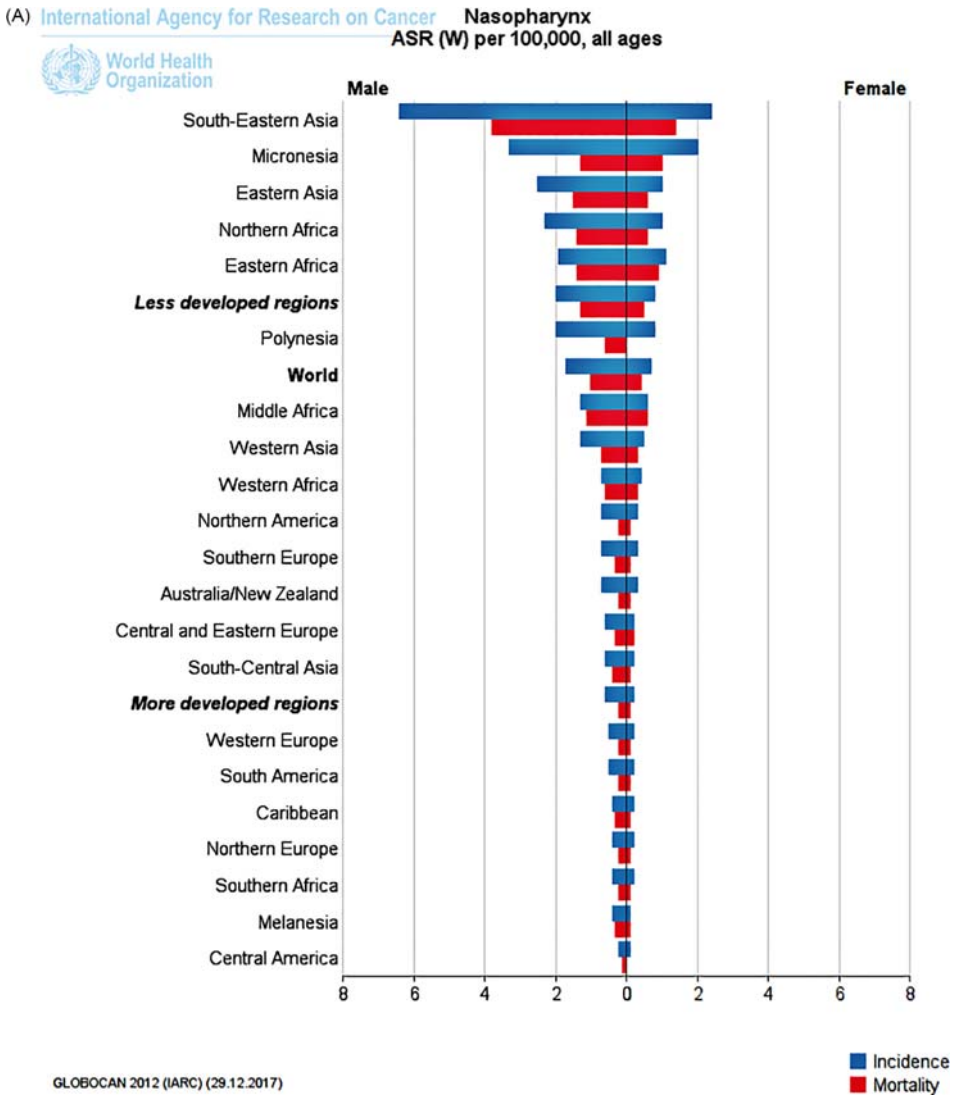

Fig. 1 Incidence and mortality of nasopharyngeal carcinoma (NPC) worldwide. (A) Age-standardized incidence and mortality rates (ASR) by gender and geographical area. (B) Incidence distribution worldwide. From Ferlay, J. et al. (2013). *GLOBOCAN 2012 v1.0, Cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet].* Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr (accessed February 20, 2018).

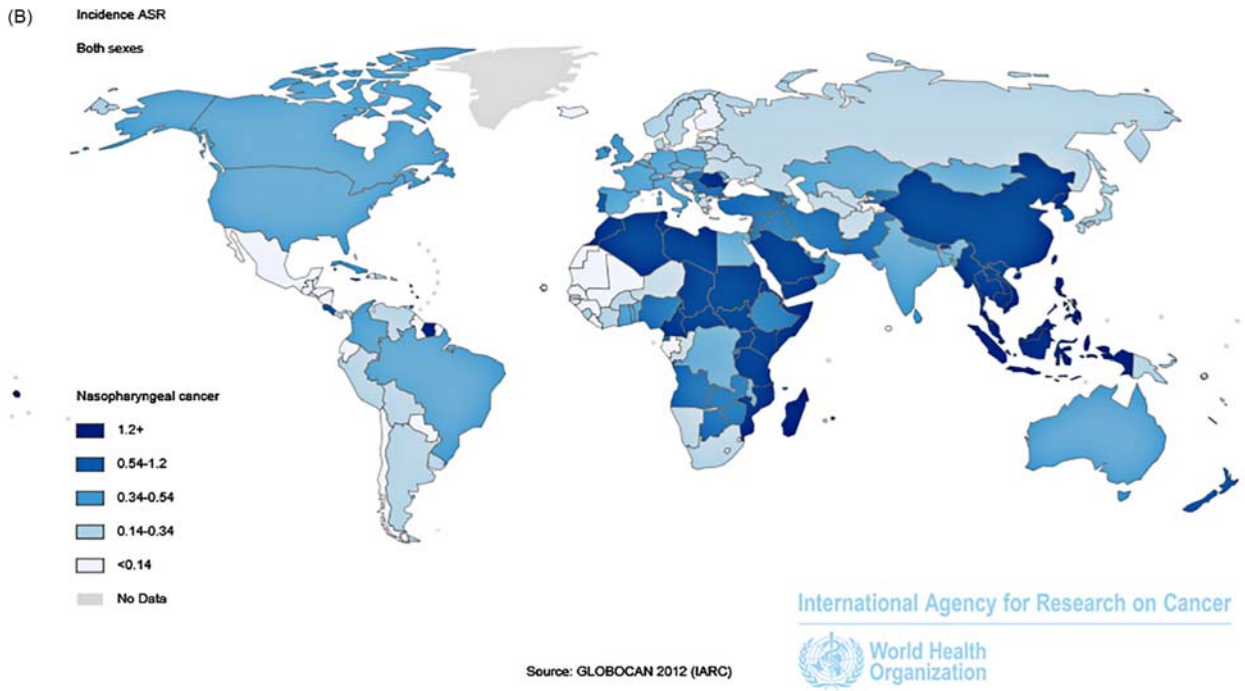

Fig. 1 (*continued*).

Table 1 Risk factors for nasopharyngeal carcinoma (NPC)

Carcinogenic agents classified by IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (volumes 1–120)
Agents of sufficient evidence for nasopharynx carcinogenicity in humans
Epstein-Barr virus (EBV; association shown for NK-NPC)
Formaldehyde
Chinese-style salted fish consumption (NK-NPC)
Tobacco smoking (K-NPC)
Wood dust
Other carcinogenic agents and lifestyle risk factors
High-risk human papillomaviruses (HPV; K-NPC)
Heavy alcohol consumption (putative risk factor)
Occupational exposure to heat, smoke, or chemical fumes (putative)
Phenotypic and genetic risk factors
Male sex
Ethnicity
Family history of NPC

K-NPC, keratinizing NPC; *NK-NPC*, nonkeratinizing NPC.

targeting such genomic EBV sequences as *BamHI-W*, *EBNA*, or *LMP*. However, the sensitivity and specificity of PCR-based assays varies and given the plethora of commercially available assays used in clinical settings (e.g., from Viracor Eurofins and Roche Molecular Diagnostics), there is an urgent need to harmonize and validate reliable EBV detection and quantification protocols.

In patients who present solely with cervical adenopathy, finding EBV in tissue is strongly suggestive of a primary NPC and indicates a necessity of a more in-depth diagnostics to this effect. Testing for EBV also helps to make a differential diagnosis in case of nonkeratinizing or undifferentiated histology, the latter of which for example being sometimes difficult to distinguish from lymphoma at a histological level. Several studies have also shown a diagnostic value of EBV detection in transoral brushing specimens and in nasopharyngeal swabs for detecting both primary and recurrent NPC.

A number of studies have shown elevated titers of IgA antibodies against EBV antigens (virus capsid antigen (VCA) and EBV early antigen (EA)) in NPC patients compared to healthy individuals, supporting the use of EBV serology as a screening method. High titers persisting after therapy have also been associated with a worse prognosis. Other studies suggested that EBV DNA load in blood serum or plasma is a more reliable diagnostic, monitoring, and prognostic marker. In particular, it has been validated as an

Table 2 Gene loci that are most frequently altered in nasopharyngeal carcinoma

Pathway	*Gene (locus)*	*Alteration type*
–	Deletions on chromosomes 3p and 9p (spanning *CDKN2A* on 9p21)	
Chromatin remodeling	*ARID1A* (1p36.11)	Deletions and point mutations; likely associated with EBV and poor survival
	BAP1 (3p21.1)	Point mutations
ERBB/P13K signaling (cell metabolism)	*PIK3CA* (3q26.32)	Activation by hot-spot mutations and amplifications
	ERBB2 (17q12) and *ERBB3* (12q13.2)	Point mutations
	KRAS (12p12.1)	Amplifications and point mutation
	AKT2 (19q13.2)	Amplifications
	PTEN (10q23.31)	Deletions
Autophagy regulation	*ATG2A* (11q13.1), *ATG7* (3p25.3), and *ATG13* (11p11.2)	Deletions and point mutations
G1/S transition (cell-cycle) control	*CDKN2A* (9p21.3)	Deletions and point mutations
	TP53 (17p13.1)	Deletions and point mutations

Table 3 Biomarkers for the detection and management of nasopharyngeal carcinoma (NPC)

Biomarker	*Characteristics and clinical utility*
Diagnostic and monitoring biomarkers	
EBV in tissue	Detected in tumor tissue, strongly suggestive of primary nonkeratinizing NPC; useful for differential diagnosis in case of nonkeratinizing or undifferentiated histology, and for early detection in patients with cervical adenopathy; detected in brushing biopsies/swabs, strongly specific for detecting primary or recurrent NPC
EBV serology	Elevated anti-EBV IgA titers in NPC patients compared to healthy individuals
EBV DNA load in blood serum/plasma	Elevated EBV DNA levels in NPC patients compared to healthy individuals; also a potential monitoring biomarker allowing early detection of local and distant treatment failures; variable detection sensitivity issues remain to be solved
Prognostic biomarkers	
Tumor stage at presentation	Inversely correlated with prognosis
Tumor size/increasing tumor volume	Increasing tumor volume associated with an increased risk of local treatment failure
EBV serology	High titers persisting after treatment associated with poor prognosis
EBV DNA load in blood serum/plasma	EBV DNA levels inversely correlated with overall survival in early-stage NPC patients; persistent EBV detection after definitive radiotherapy associated with an increased risk of tumor recurrence and with adverse prognosis; detection methods require standardization and validation (cut-offs, timing, sensitivity)
Predictive biomarkers	
EBV DNA load in blood serum/plasma	Persistent high posttreatment levels may be a criterion to select patients most likely to benefit from adjuvant chemotherapy after chemoradiation (currently being tested)

EBV, Epstein-Barr virus.

independent prognostic marker which inversely correlates with overall survival in early-stage NPC patients. Several studies have confirmed that persistent EBV detection after definitive radiotherapy, with or without chemotherapy, is associated with an increased risk of tumor recurrence and with adverse prognosis. It has also been associated with distant metastases. Monitoring EBV DNA levels can therefore complement traditional imaging techniques in surveillance for local and distant treatment failures. However, the detection sensitivity of EBV DNA in plasma remains an issue and standardized cut-off values for prognostication have to be defined and validated. Moreover, the detection periods or time-points are not clearly defined (e.g., what is the period of posttreatment recurrence predicted by elevated EBV DNA levels, or else at what time points and for how long these levels should be monitored).

The use of EBV DNA load as a biomarker to select patients that are most likely to benefit from adjuvant therapy following chemoradiation has also been suggested. The effectiveness of posttreatment patient stratification based on EBV DNA load into low-risk (undetectable posttreatment plasma EBV DNA) and high-risk as a strategy to select patients for adjuvant therapy is currently being tested in phase II and III clinical trials. The goal of this approach is to create a treatment decision tree allowing to eliminate unnecessary adjuvant chemotherapy for low-risk patients and maximize progression-free survival for those of the high-risk patients who are most likely to benefit from such a regimen. Conversely, monitoring EBV DNA levels during the initial chemoradiation (mid-treatment monitoring) has been suggested as a potential tool to identify cases with a good response to treatment and a favorable prognosis. It has been hypothesized that it may also allow to personalize treatment regimens, with decreasing EBV DNA levels indicating a possibility to de-intensify the treatment. However, this approach has not been validated and remains controversial.

Other Prognostic and Predictive Biomarkers

The most powerful prognostic biomarker for NPC is tumor stage at presentation, with the overall survival decreasing with increasing tumor stage (Table 3). Tumor size, and in particular increasing tumor size, is a negative prognostic factor associated with an adverse patient outcome, with an estimated 1% increase in the risk of local treatment failure per 1 cm^3 increase in tumor volume. Also the involvement of neck lymph nodes adversely affects the outcome.

Other factors that have been linked to diminished survival by some (but not all) studies include patient age, male sex, cranial nerve palsy and ear symptoms at presentation, and a long interval between biopsy and the beginning of radiotherapy. The relationship between NPC histological type (keratinizing versus nonkeratinizing) and prognosis remains unclear, with conflicting reports.

The significance of high-risk HPV types is not yet elucidated. EBV and HPV infections are usually mutually exclusive. Several studies have suggested that HPV-related tumors are associated with worse prognosis than those related to EBV but may have a better outcome than tumors in patients negative for both EBV and HPV.

Many putative molecular and immunohistochemical prognostic markers have been studied but so far only serum/plasma EBV DNA load tests have been applied in routine clinical practice.

Developing reliable screening biomarkers for early detection of NPC in high-risk patients, predictive biomarkers which would allow to predict response to treatment and apply personalized treatment regimens, as well as monitoring biomarkers for early detection of recurrence would be invaluable in the management of NPC.

Management and Therapy

NPC is usually not curable by surgical resection due to the tumor localization as tumor-free margins cannot be obtained at the base of the skull. Therefore, radiotherapy (RT) with high-energy x-rays, often combined with chemotherapy is the treatment of choice, with different modalities depending on the tumor clinicopathological features.

Small (T1) early-stage tumors with no lymph node involvement and no distant metastases are highly curable by RT alone (definitive RT), while a combination of RT and chemotherapy is used for more advanced tumors. Induction chemotherapy prior to RT has been shown to give high response rates but the overall survival benefit is not clear, even though some studies have suggested that using a combination of cisplatin, 5-fluorouracil (5-FU), and a taxane (usually docetaxel) for the induction therapy may significantly improve survival. So far, RT with concurrent cisplatin-based chemotherapy is the standard treatment in Western countries. It may be followed by adjuvant chemotherapy (either cisplatin with 5-FU, or carboplatin with 5-FU). However, studies on the associated clinical benefits give conflicting results. In particular, it is worth to bear in mind that adjuvant therapy after RT is often poorly tolerated. Validating biomarkers which would allow to select patients most likely to benefit from adjuvant therapy would add much value to the current treatment schemes. EBV DNA levels in blood plasma offer some hope to this effect (see "**Biomarkers**" section).

The RT with concurrent chemotherapy regimen appears to increase the 5-year survival rates up to two-fold. However, many patients do not complete the planned therapy because of its toxicity. Given the NPC localization and high total radiation doses, RT alone may result in severe sequelae including local ulceration, occasionally with necrosis, retinopathy, fibrosis of soft tissues in the neck, and middle ear changes. Chemoradiotherapy is associated with an even higher rate of hematological and nonhematological acute toxic effects. Late adverse effects from RT may be limited by using modern radiation technology, for example intensity-modulated radiotherapy (IMRT) which is the recommended option for NPC RT wherever available. It allows to minimize irradiation of normal tissue adjacent to the tumor, thus substantially limiting the radiation-induced adverse effects.

A comprehensive assessment of the primary tumor response to treatment includes clinical examination, endoscopic examination of the nasopharynx (with or without biopsy) to assess superficial lesions, radiological imaging for the assessment of deeper lesions within the skull base, and EBV DNA titer measurement. Of note, differentiating between postradiation changes and residual disease on radiological imaging may be challenging. To this effect, PET with 2-deoxy-2-[fluorine-18]fluoro-D-glucose PET integrated with CT (^{18}F-FDG PET/CT) has emerged as the most sensitive and specific method. However, some characteristics captured by MRI are also useful to assess early treatment response.

In case of primary treatment failure (local recurrence), re-treatment is the most frequent choice and it has higher success rates than re-treatment of other head and neck cancers. Still, high total dose irradiation of the primary site is needed. This may be done by IMRT or brachytherapy. Limited post-RT failures in the neck may be controlled by surgery (neck dissection).

Patients with metastatic NPC have heterogeneous outcomes and long-term survivorship is possible. Cisplatin-containing doublet regimens remain the standard for the first-line systemic treatment of metastatic disease, with the most "classic" choice being cisplatin with 5-FU (response rates of 70%–80%). Other cisplatin-containing combinations for doublet and even triplet regimens, for example with gemcitabine, capecitabine, paclitaxel, and docetaxel, have also been tested. However, the reports on their efficacy (measured by tumor response rates, progression-free survival, and/or overall survival) compared to the conventional treatment remain contradictory. In case of platinum-resistant disease, a monotherapy with gemcitabine, capecitabine, or docetaxel is used, with reported response rates of approximately 30%–40%. Several doublet combinations have been tested to this effect but none of them has been shown to yield better response rates.

In the era of personalized medicine, targeted molecular therapies also hold promise for treatment of recurrent and metastatic NPC. In particular, inhibitors of epidermal growth factor receptor (EGFR), such as cetuximab, and of vascular endothelial growth factor (VEGF) have shown clinical efficacy in patients with platinum-refractory disease. Of note, VEGF-targeting drugs increase the

risk of bleeding and are therefore not suitable for patients who have a recurrent tumor within a previously irradiated field or a tumor invading major vascular structure.

The high prevalence of EBV in NPC patients and its relation to patient outcome opens the door to EBV-specific T-cell-based immunotherapies. Several vaccines targeting EBV in recurrent and metastatic NPC patients are currently under clinical trials, with promising results. Moreover, EBV-associated tumors express programmed death ligand 1 (PD-L1). A phase II trial testing nivolumab, a PD-L1 inhibitor, in recurrent and metastatic NPC is currently ongoing. Positive results would support the strategy to combine non-antigen-specific immune checkpoint inhibitors with immunogenic vaccination against tumor-specific antigens, which could become standard for the treatment of metastatic NPC in the future.

Prospective Vision

Many cancer genomic studies have focused on prognostic biomarkers. However, while identifying patients at higher risk of recurrence and unfavorable prognosis is important to define optimal treatment regimens, identifying new targets for treatment of metastatic NPC remains the major urgent unmet need. Indeed, metastases are frequent in NPC patients and systemic therapy is key for a successful treatment, with personalized approach being the choice of the future. Among others, mutations in the genes of the chromatin remodeling pathway, linked to EBV burden, seem to be potentially interesting surrogate targets as tumors with these mutations may be sensitive to histone deacetylase inhibitors (HDACi). Several clinical trials testing the safety, tolerability and efficacy of different HDACi in combination with other drugs are currently underway. Moreover, given its apparently frequent involvement in NPC, targeting the autophagy pathway may represent another, yet untested, opportunity.

See also: Oral and Oropharyngeal Cancer: Pathology and Genetics.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer.

Chua, M.L.K., et al., 2016. Nasopharyngeal carcinoma. The Lancet 387 (10022), 1012–1024.

International Agency for Research on Cancer, 2017. In: El Naggar, A.K., Chan, J.K.C., Grandis, J.R., Takata, T., Slootweg, P.J. (Eds.), WHO classification of head and neck tumors, 4th edn. IARC, Lyon, France.

Kim, K.Y., et al., 2017. Current state of PCR-based Epstein-Barr virus DNA testing for nasopharyngeal Cancer. Journal of the National Cancer Institute 109 (4).

Kim, K.Y., et al., 2017. Clinical utility of Epstein-Barr virus DNA testing in the treatment of nasopharyngeal carcinoma patients. International Journal of Radiation Oncology, Biology, Physics 98 (5), 996–1001.

National Comprehensive Cancer Guidelines (NCCN). NCCN clinical practice guidelines in oncology. Head and neck cancers. Version 1.2018 – February 15, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Lee, S.C., et al., 2014. The genomic landscape of nasopharyngeal carcinoma. Nature Genetics 46 (8), 866–871.

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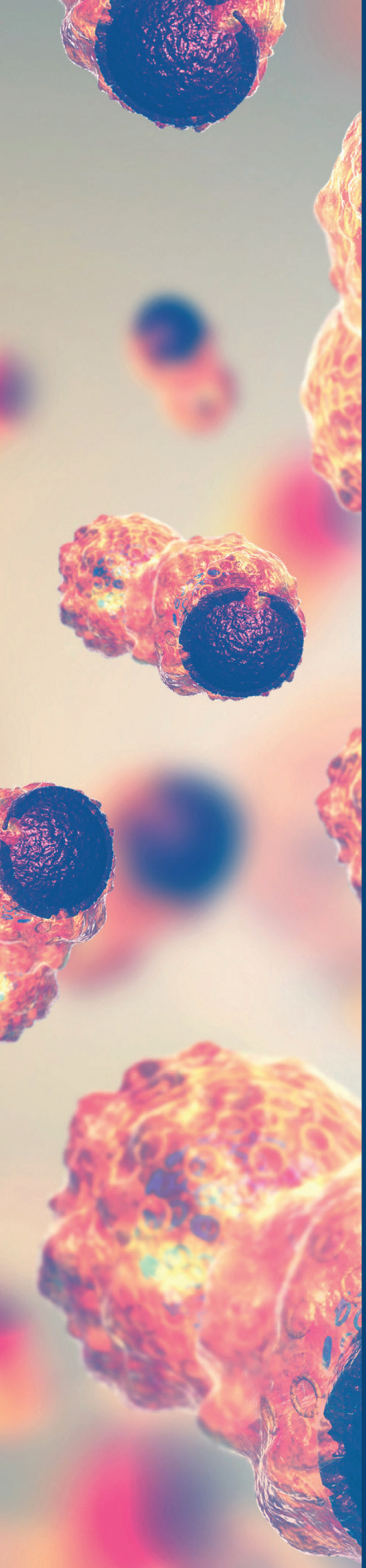

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His research focuses on *TP53* mutations and p53 protein regulation in cancer and chronic diseases. From 1994 to 2011, he has led the development of the international IARC TP53 database, a source of information on the causes and consequences of mutations affecting the *TP53* suppressor gene in cancer. His work addresses the mechanisms of *TP53* mutagenesis as well as the prognostic and predictive significance of *TP53* mutations in lung, liver, and oesophageal cancers. His studies on p53 regulation have focused on the role of environmental mutagens in *TP53* mutagenesis, on the biochemical mechanisms of p53 control by oxidation-reduction and by metabolism, and on the identification of p53 isoforms as factors acting as dominant inhibitors of p53 functions in cancers without *TP53* mutations. His current activities focus on germline *TP53* mutation and on the diversity of genetic and nongenetic factors that modulate the penetrance of the Li–Fraumeni Syndrome, as well as on the mechanisms that maintain optimal p53 protein balance in cells and tissues over lifetime. He is the author of over 450 publications and 50 book chapters. He is Editor of the Cancer Biology section of *Current Opinion in Oncology*. He has co-edited books on p53 (*25 Years of p53 Research*, 2005, 2007, *p53 in the Clinics*, Springer), a textbook on molecular epidemiology (*Molecular Epidemiology: Principle and Practice*, IARC Press, 2011) and two-volume textbook on human biobanking (*Human Biobanking, Principle and Practice*, 2017, 2018).

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Fred Bosman's research activities (combining diagnostic and experimental pathology) focused on the biology of digestive tract cancer, notably Barrett's esophagus and colorectal cancer, with a strong emphasis on the development of molecular diagnostics. He has written over 350 original publications and over 50 book chapters. Fred Bosman was Series co-editor of the 4th edition of the WHO Series *Classification of Human Tumours*, the international standard for tumor classification, and co-editor of the Volume on *Tumours of the Digestive Tract*.

Graham A. Colditz, MD, DrPH, FAFPHM, is an internationally recognized leader in cancer prevention. As an epidemiologist and public health expert, he has a longstanding interest in the preventable causes of chronic disease, particularly among women. He focuses his research on early life and adolescent lifestyle, growth, and breast cancer risk. He is also interested in approaches to speed translation of research findings into prevention strategies that work. Dr. Colditz developed the award-winning Your Disease Risk website (www.yourdiseaserisk.wustl.edu) which communicates tailored prevention messages to the public. He has published over 1100 peer-reviewed publications, six books and six reports for the Institute of Medicine, National Academy of Sciences. His h-index is over 220.

In October 2006, on the basis of professional achievement and commitment to public health, Dr. Colditz was elected to membership of the National Academy of Medicine, an independent body that advises the US government on issues affecting public health. In 2011, he was awarded the American Cancer Society Medal of Honor for cancer control research. In 2012 he received the AACR-American Cancer Society Award for Research Excellence in Cancer Epidemiology and Prevention. He also received awards in 2014 for cancer prevention research from ASCO and from AACR. During 2016 he served on the Implementation Science Work Group of the Blue-Ribbon Panel to advise the National Cancer Moonshot. He received the 2018 Daniel P. Schuster Award for Distinguished Work in Clinical and Translational Science, Washington University School of Medicine. He was also elected as a Fellow, American Association for the Advancement of Science

Carlo La Vecchia received his MD from the University of Milan and a Master of Science degree in Medicine (epidemiology) from Oxford University. Presently, he is Professor of Medical Statistics and Epidemiology at the Faculty of Medicine at the University of Milan. Dr. La Vecchia serves as an editor for numerous clinical and epidemiologic journals. He is among the most renowned and productive epidemiologists in the field with over 2040 peer-reviewed papers in the literature and is among the most highly cited medical researchers in the world, according to ISI HighlyCited.com, the developer and publisher of the Science Citation Index (2003, 2017, H index 153, H10 index 1543, second Italian in Clinical Medicine). Dr. La Vecchia is Adjunct Professor of Medicine at Vanderbilt Medical Center and the Vanderbilt-Ingram Cancer Center (2002-18).

Gerd. P. Pfeifer received a PhD degree in biochemistry from Goethe University in Frankfurt, Germany. After postdoctoral training, he became a faculty member at the Beckman Research Institute of the City of Hope in Duarte, California, where he spent much of his career working on cancer research. In 2014, Dr. Pfeifer joined the new Center for Epigenetics at the Van Andel Research Institute in Grand Rapids, MI, United States, as a Professor of Epigenetics. Dr. Pfeifer has authored more than 300 publications, has held an NIH MERIT award, and was elected Fellow of the American Association for the Advancement of Science in 2015. Research in Dr. Pfeifer's laboratory has been concerned with genetic and epigenetic mechanisms of human carcinogenesis, with emphasis on DNA methylation and genetic toxicology.

Marco Alessandro Pierotti graduated in 1973 in Biological Sciences at the University of Milan, Italy, and started working at the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) in Milan. From 1978 to 1980 he was Visiting Investigator at the Laboratory of Chemical Cancerogenesis of the NCI-NIH Bethesda (MD, United States) and Postdoctoral Research Fellow at the Laboratory of Viral Oncology of the Memorial Sloan-Kettering Institute in New York. In 2006, Dr. Pierotti was appointed Scientific Director of the Fondazione IRCCS Istituto Nazionale dei Tumori in Milan, where, since 1970, he had already held various positions, including Director of the Department of Experimental Oncology.

Since 1988, he has been Professor of Molecular Genetics of Cancer at the Postgraduate School of Oncology, University of Milan Medical School and co-director of the Laboratory of Molecular Diagnosis at the INT. In September 2014 he resigned from his position at INT to take the position of President and CEO of Nerviano Medical Sciences (NMS) srl, one of the biggest oncological pharma companies in Europe. In April 2015 he left the company and took the position of Scientific Coordinator of the Institute of Pediatric Researches (IRP) in Padua, Italy, devoted to study molecular aspects of the main pediatric diseases with particular focus on pediatric onco-hematology. The Institute was created by a private Charity, The City of Hope Foundation of Monte Malo (Vi).

Since 2000 he is Senior Group leader of the Molecular Genetics of Cancer group at the Institute FIRC of Molecular Oncology (IFOM, Milan). Past President (2006–08) of the Italian Cancer Society, Dr. Pierotti is a member of the American Association for Cancer Research and of its Advisory Board and the Laboratory Research Awards Selection Committee. He has also been President (2006–08) of the European Association for Cancer Research (EACR) and in this role was among the founders of the European CanCer Organisation (ECCO) where he was appointed as member of the Policy Committee.

In recent years, he was the Italian Representative at the Scientific Committee of the International Agency for Research on Cancer (IARC), Lyon. From 2006 to 2014 he was Scientific Secretary of Alleanza Contro il Cancro (ACC), promoted by the Italian Ministry of Health. In 2008 he was Member of the Evaluation of the Research Program Functional and Structural Genomics for DKFZ. He was an expert for the Oncology Research Projects of the European Community and was a consultant in oncology research for the Ministries of Research of different Countries. From 2006 to 2014 he was Chair of the regional Project, The Region Lombardy Oncological Network (ROL), selected in 2014 by the EC as one of the best examples of oncological network. His appointments in the Organisation of European Cancer Institutes (OECI) started in 2007 when he took the position of Vice-President. From 2008 he was the President-elect and then the President of the Organization. Finally, in 2014 he was appointed OECI Executive Secretary.

Over the years, Dr. Pierotti has been Principal Investigator or Head of several national and international research grants, funded by both private and public bodies. His authorship includes over 470 publications that deal with various aspect of experimental oncology including studies on immunology, biochemistry, and molecular biology using both experimental and human tumors.
In addition, since its fifth edition he is the first author of the chapter on "Oncogenes" in the most reputed textbook *Cancer Medicine* (Holland-Frei). The metrics of his scientific activity is summarized by an H index of 96 and total citations of 37.256 (Google scholar June 2018).

Professor **Thomas Tursz**, born in Kraków, Poland, in 1946, died in Paris, France, on April 27 2018. He was Professor of Oncology at the Faculty of Medicine Paris-Sud since 1986 and General Director of the Institut Gustave Roussy (1994–2010). He was the leader of the French Doctoral School of Oncology which he founded in 1999, and President of the French Federation of Comprehensive Anticancer Centres (FNCLCC) from 2004 to 2010. He was highly involved in the European Organization for the Research and Treatment of Cancer (EORTC) as both Chairman of the Scientific Advisory Committee (2003–06) and Vice President of the Board (2006–09). His experience as President of the FNCLCC was crucial for the Organization of European Cancer Institutes (OECI) when he acted as President from 2002 to 2005.

His scientific interests included the biology of virus-induced tumors, as well as immunological responses including the role of thioredoxin in lymphocytes infected by Epstein–Barr virus. In the clinical research area, he conducted a number of important clinical trials in breast cancer, lung cancer, and soft-tissue sarcoma. He had a particular interest in cytokines and gene therapy, and his clinical research activities were further disseminated to the European level when he was the Chairman of the Sarcoma Group of the EORTC (1993–96).

Prof. Tursz received several prestigious awards, such as the Prix de Cancérologie from the French National League Against Cancer (1979), the Bernard Halpern Immunology Award (1983), the Rosen Oncology Award (1989), the Grand Prix in Oncology from the Academy of Medicine (1992), the Hamilton Fairley Award for clinical research (1998), and the Prix de Rayonnement Français (2001). He was the author of 350 international scientific publications. He was also an esteemed member of the Editorial Board of *Molecular Oncology* ever since its creation in 2007.

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HOW TO USE THE ENCYCLOPEDIA

Structure of the Encyclopedia

All articles in the encyclopedia are arranged alphabetically as a series of entries.

There are four features to help you easily find the topic you are interested in: an alphabetical contents list, cross references, a full subject index, and contributors.

1. Alphabetical contents list: The alphabetical contents list, which appears at the front of each volume, lists the entries in the order that they appear in the encyclopedia. So that they can be easily located, entry titles generally begin with the key word or phrase indicating the topic, with any generic terms following. For example, "Multiple Myeloma: Pathology and Genetics" is the entry title rather than "Pathology and Genetics of Multiple Myeloma".
2. Cross references: Virtually all the entries in the encyclopedia have been extensively cross-referenced. The cross references which appear at the end of an entry, serve three different functions:
   i. To draw the reader's attention to related material on other entries
   ii. To indicate material that broadens and extends the scope of the article
   iii. To indicate material that covers a topic in more depth

Example
The following list of cross-references appears at the end of the entry "Carcinogen—DNA Adducts".

> ***See also:*** Cancer Risk Reduction Through Lifestyle Changes. Cell Responses to DNA Damage. Genetic Instability. Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2. Molecular Epidemiology and Cancer Risk. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics.

3. Index: The index appears at the end of volume 3 and includes page numbers for quick reference to the information you are looking for. The index entries differentiate between references to a whole entry, a part of an entry, and a table or figure.
4. Contributors: At the start of each volume there is a list of the authors who contributed to all volumes.

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PREFACE

Cancer holds a special status in biology, medicine, and society. It offers formidable challenges to basic, clinical, and population science; it ranks at the top of medical research priorities and healthcare costs in most countries. The general public is continuously exposed to news of discoveries promising to defeat the disease in the near future. Indeed, ground-breaking advances are being made, from preventive vaccines to genome-guided personalized medicine, sophisticated imaging and surgical technologies, and systemic therapies aimed at awakening natural immune responses against cancer. These novel therapeutic approaches make cure a real possibility for a growing number of patients. They also launch cancer care into a new era of maintaining the disease under control for an indefinite period of time, turning it into a form of chronic disease. At the same time, evidence-based prevention and early detection strategies have opened a new window on the natural history of the disease, enabling effective intervention well ahead of diagnosis. As a result, the first two decades of this millennium have witnessed a marked decrease in the mortality and, in some instances, the incidence of cancers that have dominated the death toll in more developed countries during the second half of the 20th century.

A turning point in our understanding of cancer is the deciphering of the human genome and its spin-off endeavors aimed at exploring the genomic landscape and architecture of human cancers. These discoveries are causing a major overhaul of our vision of cancer as a dynamic, rapidly evolving, and heterogeneous disease at the individual level. Harnessing this complexity requires mastering increasingly complex sources of data at molecular, cellular, systemic, personal, environmental, and societal level, heralding the emergence of big-data science in cancer diagnosis and treatment. However, this exceptional acceleration in knowledge and solutions cannot hide the fact that cancer remains a global scourge that exerts a massive burden on humankind and societies worldwide, in particular in societies in transition and in low-resource contexts.

Today, cancer crystallizes many of the major societal challenges pertaining to lifestyles, sustainable development and environmental policies, demography and population aging, access to education and healthcare, sharing of resources and knowledge, and protection of persons and personal information. The information on cancer available at a fingertip is overwhelming in volume, complexity, veracity, and velocity. We worked on the Third Edition of Elsevier's *Encyclopedia of Cancer* with this rapidly changing background. Rather than aiming at developing a comprehensive framework encompassing all aspects, we attempted to address the literal meaning of the greek terms ἐγκύκλιος παιδεία, which means "general education". While we retained some articles from the previous edition, which, at the time of the publication, represented an exceptional achievement of Dr. J. Bertino, we largely modified the structure and the list of chapters, and the possibility of continuous update of the articles has been a great incentive for us and for the authors of the chapters. This new edition of the Encyclopedia consists of six major parts: (i) mechanisms of cancer, (ii) hallmarks of cancer, (iii) causes of cancer, (iv) cancer prevention and control, (v) diagnosis and treatment of specific cancers, and (vi) pathology and genetics of specific cancers. This repartition is necessarily artificial and is complemented by the extensive cross-references between articles. The repartition, however, reflects our effort to identify discrete topics that would best address the needs of a wide community of readers.

The primary target readership of the Encyclopedia comprises medical and other health science students, as well as non-specialized physicians and other health practitioners. Cancer researchers, oncologists, and other cancer professionals may find the articles pertaining to their specific field to be too short, over-simplistic, and perhaps obsolete; they too, however, may benefit from articles on topics other than their own. The Encyclopedia also offers an easy way to navigate across concepts and topics that should be appealing to readers from other communities, including social sciences or stakeholders in public decision-making.

We were fortunate to work with a formidable team of section editors, including Fred Bosman, Graham Colditz, Carlo La Vecchia, Gerd Pfeifer, and Marco Pierotti. An additional section editor was Professor Thomas Tursz, who passed away prematurely during the preparation of the Encyclopedia. Thomas was a great colleague and mentor, and a major figure in oncology in France and internationally. We had the privilege to involve him in the last project of his long career, and we want to dedicate this work to him. We wish to thank the many article authors, who agreed to contribute to the success of this international endeavor, and in particular Dr. Katarzyna Szymańska, who drafted several cancer-specific articles. Finally, we want to thank the staff at Elsevier, whose patience and perseverance helped us bringing the project to the final stage. All these individuals are responsible for the many strengths of the new edition of the *Encyclopedia of Cancer*, while weaknesses are mainly ours.

Paolo Boffetta
Pierre Hainaut

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Neuroblastoma: Diagnosis and Treatment

Gudrun Schleiermacher and Thierry Philip, Curie Institute, Paris, France

Glossary

Image-defined risk factors in neuroblastoma (IDRF) Guidelines for imaging and staging of neuroblastic tumors as defined per consensus in the International Neuroblastoma Risk Group Project.

International neuroblastoma pathology classification (INPC) Guidelines for the pathological characterization and staging of peripheral neuroblastic tumors.

International neuroblastoma risk group—staging system (INRG) The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pretreatment risk stratification for patients with peripheral neuroblastic tumors, taking into account clinical, radiological, pathological, and biological features. By defining homogenous pretreatment patient cohorts, the INRG classification system will greatly facilitate the comparison of risk-based clinical trials conducted in different regions of the world and the development of international collaborative studies.

International neuroblastoma staging system (INSS) International consensus for the diagnosis and staging of neuroblastoma.

Iodine-123-metaiodobenzylguanidine scintigraphy (MIBG scintigraphy) A scintigraphy method using a radiolabeled molecule, Iodine-123-metaiodobenzylguanidine, which is similar to norepinephrine; based on its uptake by neuroblastic cells through the norepinephrine transporter this scintigraphy method is useful for the diagnosis and metastatic work up of peripheral neuroblastic tumors.

Introduction

Neuroblastoma is the most common extracranial solid tumor of early childhood. It is an embryonic tumor derived from the developing sympathetic nervous system corresponding to the cells of the neural crest that form the adrenal medulla and the sympathetic ganglia. The large clinical heterogeneity, with a spectrum ranging from the possibility of spontaneous regression to threatening progression despite all treatment, as well as the important biological heterogeneity, has led to intense research on clinical and biological prognostic factors with an aim of improving the definition of risk defined patient subgroups and of developing new therapeutic approaches.

Epidemiology of Neuroblastoma

Neuroblastoma represents 8%–10% of all pediatric cancers, with a mean annual incidence of 7–12 cases per million children in Western countries, and a prevalence of 1 case per 8000–10,000 births. Neuroblastoma is a disease of early childhood, with a median age at diagnosis of 18 months, and 90% of patients diagnosed before the age of 5 years. Age at diagnosis is an important prognostic factor, as patients under 18 months usually have a better prognosis, whereas in adolescents and adults is very rare it generally shows a more indolent clinical course with de novo resistance to chemotherapy treatments. Neuroblastoma is slightly more frequent in males than in females (M/F ratio 1.1–1.2). As neuroblastoma can be detected following an increase of urinary catecholamine metabolites (see below), screening programs were put in place in several countries including Japan, Germany, France, and Canada. An increase in the prevalence of neuroblastoma in infants was observed in these countries; however, there was no decrease neither in the prevalence or nor in the mortality of neuroblastoma in patients older than 1 year. This suggests that spontaneous regression of symptomless neuroblastoma is at least as frequent as neuroblastoma leading to clinical symptoms.

Etiology of Neuroblastoma

Developmental Origin

During embryonal development, the neural crest arises from the neural tube after its closure. Neural crest development involves epithelial to mesenchymal transition, and cells will migrate from the neural tube for the further development of numerous anatomic structures, giving rise to various cell types, including neural, pigmented, craniofacial, and conotruncal cardiac cells, during embryogenesis. This complex process is regulated by various epigenetic programs based on histone modification and DNA methylation, and transcriptional programs involving multiple transcription factors which further activate downstream transcriptional regulators of proliferation and differentiation. The neural crest of the trunk contributes to the development of the peripheral nervous system, including sympathetic ganglia and the adrenal gland. It is thought that neuroblastoma arises from these embryonal sympathoadrenal progenitors.

To date, the precise etiology of neuroblastoma is unknown, and unlike many adult malignancies, environmental factors are not thought to play a major role, although predisposing effects of prenatal exposures to potentially toxic substances warrant further investigation. However, genetic factors, at both a constitutional and a somatic level, are thought to play a major role in neuroblastoma development.

Hereditary Genetic Factors

Several observations support the hypothesis of a major role of underlying hereditary genetic factors in the etiology of neuroblastoma.

First, rare familial cases have been described, accounting for $< 1\%$ of all neuroblastoma cases. Gain-of-function mutations in the tyrosine kinase domain of the anaplastic lymphoma kinase gene *ALK* have been detected in the majority of familial cases associated with an autosomal dominant pattern of inheritance with incomplete penetrance.

Second, neuroblastoma can arise in a context of syndromic associations. Neural crest-derived developmental disorders associated with an increased risk to develop neuroblastoma have been linked to inactivating mutations of the *PHOX2B* gene, a master regulator of neural crest development, which was identified as the first predisposition mutation in neuroblastoma. Whereas expansions of the second polyalanine stretch of PHOX2B are mainly observed in patients presenting with Ondine's curse (also called CCHS, for Congenital Central Hypoventilation Syndrome) associated with a low risk of peripheral neuroblastic tumors, nonpolyalanine repeat expansion mutations occur preferentially in patients with Hirschsprung's disease which is associated with a higher risk to develop neuroblastoma. Further associations between neuroblastic tumors and cancer predisposition syndromes include neurofibromatosis type 1 (*NF1*), characterized by constitutive activation of the RAS–MAPK pathway, as well as Noonan syndrome with involvement of the *PTPN11* gene.

Third, recent powerful genome-wide association studies have demonstrated that neuroblastoma is a disease of complex underlying genetic factors as at least a dozen common polymorphic alleles, occurring at various loci throughout the genome, have been shown to influence neuroblastoma oncogenesis. Of low individual impact on disease initiation (relative risks of 1.5–2.0), these polymorphic alleles can cooperate in an individual patient to promote malignant transformation during neurodevelopment, and some genes targeted by these polymorphic alleles play potential roles in the pathogenesis of neuroblastoma, including BARD1, LMO1, DUSP12, DDX4, HACE1, and LIN28B. The described polymorphic alleles show a correlation with either high-risk or low-risk disease, indicating that favorable and unfavorable forms of neuroblastoma may represent distinct entities in terms of the genetic events that initiate tumorigenesis.

Furthermore, rare cases with various abnormal constitutional karyotypes have been described in neuroblastoma patients, including constitutional copy number anomalies, balanced and unbalanced translocations, as well as specific chromosome deletions. Altogether, there are probably as yet undiscovered additional genes that predispose to neuroblastoma when altered in the germline. However, to date there are no clinically validated guidelines for who should be screened for germline mutations, nor how to perform surveillance of patients or families with known predisposition alleles.

Somatic Genetic Alterations

With only 1%–2% of neuroblastoma occurring in a familial or predisposition context, over 98% of all cases occur sporadically. A large number of recurrent somatic genetic alterations have been described in neuroblastoma, the most frequent of which concern quantitative genomic alterations with gains or losses of genetic material. These genetic abnormalities are closely linked to the distinct biological and clinical subgroups of the disease.

Amplification of the *MYCN* oncogene, mapping to 2p24.1, occurs in approximately 25% of all neuroblastomas and 40% of high-risk tumors. It remains one of the most important genetic alterations associated with advanced stages of disease, an aggressive phenotype, and poor outcome, and is the first genetic marker to be used in clinical practice for risk stratification and adaptation of treatment intensity. Closely associated with poor survival in localized disease and in infants, its prognostic impact in metastatic disease of older children with an overall poor outcome is less pronounced. At a cytogenetic level, amplification of the MYCN oncogene occurs either as double-minute chromosomes or homogeneously staining regions, which contain between 10 to over 100 ectopic copies of the *MYCN* oncogene. The oncogenic role of MYCN has been clearly demonstrated as its ectopic expression in the neural crest is sufficient to drive neuroblastoma tumorigenesis in zebrafish and mice models. Its oncogenic role is based on an enhancement of the expression of genes involved in cell proliferation, and on the repression of genes involved in differentiation and apoptosis.

Other recurrent amplifications concern the *ALK* gene on chromosome 2p23, as well as amplicons of chromosome 12q13–14 encompassing, among others, the MDM2 and CDK4 genes. Preliminary data indicate that NB with focal amplifications other than MYCN might present with atypical clinical features and a poorer outcome.

Other recurrent structural alterations recurrently observed in neuroblastoma concern segmental chromosome alterations (SCA), including deletions of chromosome arms 1p, 3p, 4p, and 11q, and gains of 1q, 2p, or 17q. Deletion of 1p36 is observed in 20%–35% of cases, predicts survival in multivariate analyses, and is significantly associated with aggressive disease markers. Deletions of 11q in a consensus region at 11q23 occur in approximately 40% of cases and are inversely correlated with *MYCN* amplification, identifying a molecularly distinct high-risk patient subset, characterized by advanced stage, older age, and a higher genomic instability with a higher number of chromosome breakpoints. Gains of chromosome 17q21-qter represent the most frequent genetic

alteration in neuroblastoma, occurring in 70% of tumors. Numerous studies have reported that 17q gain is significantly associated with advanced stage of disease, increased patient age, *MYCN* amplification, and other unfavorable genetic parameters.

The overall genomic profile has been shown to be of prognostic impact in neuroblastoma. Whereas numerical chromosome alterations, consisting of gains or losses of whole chromosomes, are associated with a favorable outcome, segmental chromosome alterations of any chromosome region, without or with numerical chromosome alterations, are associated with a higher risk of relapse. Although intense decade-long research has focused on the identification of hypothetical tumor suppressor genes or oncogenes in recurrently altered regions of chromosome loss or gain, the smallest regions of overlap remain quite extensive, and to date do not point to single gene candidates as tumor suppressors or oncogenes. This suggests that an overall imbalance of copy number regions is of importance in neuroblastoma oncogenesis.

Recent next generation sequencing approaches have indicated that most neuroblastoma harbor only few mutations, with an average of 10–20 predicted nonsynonymous variations in coding regions per genome, indicating an exonic mutation frequency of 0.2–0.4 per Mb. The frequency of somatic events strongly correlates with tumor stage, lower-stage tumors harboring a lower number of mutations.

The most frequent recurrent somatic mutation in neuroblastoma concerns the gene *ALK* (anaplastic lymphoma kinase), with mutations activating the tyrosine kinase domain in approximately 10% of all cases at diagnosis. The somatic ALK-1174 mutation appears to contribute to a more aggressive phenotype, but unlike ALK-1275 mutations, these specific mutations are not found in familial neuroblastoma and are not tolerated in the germline. The oncogenic role of activating ALk mutations in neuroblastoma has been demonstrated in vitro and in vivo in both zebrafish and mouse models, with co-expression of ALK-F1174L and MYCN producing a synergistic effect for neuroblastoma tumorigenesis in mice. Small molecule inhibitors targeting the activated kinase domain of ALK are now available, making this a promising target for molecular therapy but still requiring more specific development.

Other recurrent mutations in neuroblastoma target distinct cellular pathways and include PTPN11 mutations (in 3% of cases), as well as genes involved in cytoskeleton maintenance, neuritogenesis, and other regulators of the RAC/Rho pathway.

Interestingly, genes involved in chromatin remodeling have been found to be targeted in a significant number of cases, either by mutations or by structural variations, including mutations in the ARID1A/ARID1B genes. Somatic alterations of ATRX are associated with an increase in telomere length, and with an absence of the ATRX protein in the nucleus. ATRX alterations appear to be more frequent in older children and occur in mutually exclusive fashion with *MYCN* amplifications. ATRX mutations are associated with activation of a telomere maintenance mechanism termed alternate lengthening of telomeres (ALT), which may be associated with primary chemotherapy resistance. Recurrent genomic rearrangements of the promotor region of the *telomerase reverse transcriptase* (*TERT*) gene on chromosome 5p15.33 have been described in $>10\%$ of neuroblastoma cases, defining a further subgroup of high-risk disease and occurring in mutually exclusive fashion with *MYCN* amplification and *ATRX* mutations.

Thus, a large number of high-risk neuroblastomas are affected by genetic alterations of either *MYCN*, *TERT*, or *ATRX*, all of which converge to an activation of telomere lengthening mechanisms either by direct activation or by ALT, leading to a capacity of near-infinite cell proliferation. Advances in the development of inhibitors of these pathways and their evaluation in clinical trials will lead to new treatment opportunities.

Studies in relapsed neuroblastoma have suggested that genetic alterations can evolve over time and that clonal evolution is common, resulting in the acquisition of somatic alterations in known oncogenic pathways, some of which are targetable. Early evidence suggests that activation of the MAPK pathway and other signaling pathways of epithelial-mesenchymal transition processes might emerge at relapse and might represent promising targets for molecular targeted treatment approaches. Altogether, both spatial and temporal genetic heterogeneity plays an important role in neuroblastoma (**Fig. 1**).

In addition to genetic changes, neuroblastoma can also be characterized by specific expression profiles. A large number of studies have focused on the analysis of differential expression patterns, seeking to identify expression patterns that might enable to distinguish patients with different clinical courses and thus define different prognostic subgroups in high-risk disease, and potentially to identify new therapeutic targets. However, these approaches have not yet found a routine application in a clinical setting.

The paucity of recurrent genetic mutations as compared to adult tumors in adults indicates that additional mechanisms such as epigenetic alterations may play an important role in the molecular pathogenesis of these developmental tumors. Alterations in DNA methylation represent one of the most common molecular events in neoplasia, and CpG-island hypermethylation of gene promoters is a frequent mechanism for functional inactivation of relevant tumor-associated genes in neuroblastoma.

Cellular Identity

In neuroblastoma, two distinct cellular identities corresponding to either a sympathetic noradrenergic identity, or to a neural crest cell-like, mesenchymal identity can be evidenced. These cellular identities are governed by distinct core regulatory circuits corresponding to different super-enhancer profiles, including the transcription factors PHOX2B, HAND2, and GATA3 for the sympathetic noradrenergic and the AP-1 transcription factors for the neural crest cell-like identity. The committed adrenergic cells and undifferentiated mesenchymal cells can coexist in a tumor and, importantly, can interconvert. This represents an additional important aspect of tumor heterogeneity with an important role with regards to differential sensitivity to chemotherapies.

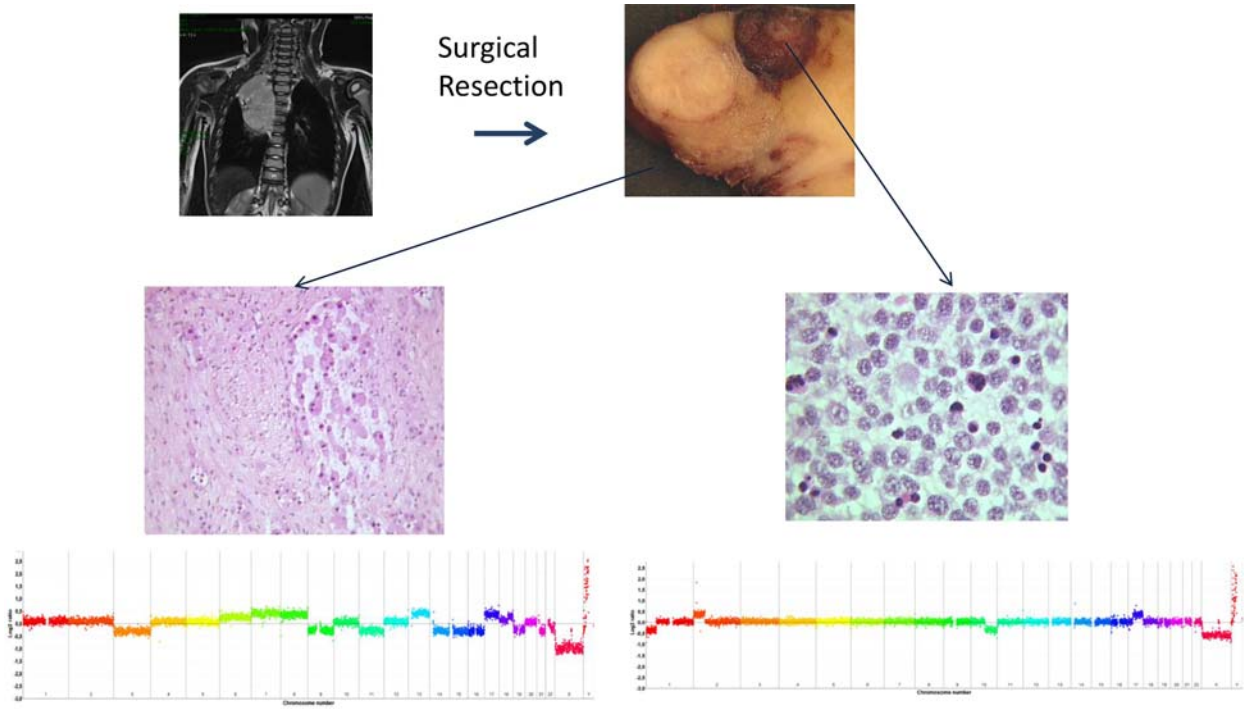

Fig. 1 Macroscopical and microscopical heterogeneity of neuroblastoma. This tumor was histologically composed of a stroma-rich component, and of several stroma-poor nodules corresponding to neuroblastoma, stroma-poor, poorly differentiated. Different copy number profiles can be observed: numerical chromosome alterations (NCA) only, versus segmental chromosome alterations (SCA) with (in this case) chromosome 1p and 11q deletion, and chromosome 2p and 17q gain.

Biological Pathways

A number of biological pathways corresponding to important hallmarks of cancer appear to be affected in neuroblastoma. These include abnormal patterns of gene or protein expression involved in tumor differentiation, apoptosis, drug resistance, angiogenesis, invasion, and metastasis.

Neurotrophin signaling plays a central role in normal neuronal development and may be involved in both differentiation and regression of neuroblastoma. Differential expression of tropomyosin receptor kinase (Trk) receptors has been shown to influence both the biological characteristics and clinical behavior of neuroblastoma, with TrkA expression (with the ligand NGF) observed in favorable neuroblastoma, and TrkB expression (with the ligand BDNF) observed in unfavorable neuroblastoma.

Delayed activation or disruption of normal apoptotic pathways may also be an important phenomenon involved in spontaneous regression, as well as therapy resistance of neuroblastoma. Major elements of the apoptotic signaling cascade with abnormal expression or activation patterns include the BCL2 family, survivin, and caspase-8. Acquired resistance to chemotherapeutic agents may also be conferred by enhanced drug efflux via overexpression of classical multidrug resistance proteins, including the multidrug resistance gene 1 (*MDR1*) and the gene for the multidrug resistance-related protein (*MRP*).

Increased tumor angiogenesis and expression of pro-angiogenic factors, such as vascular endothelial growth factor, are both correlated with an aggressive phenotype in neuroblastoma. Angiogenesis inhibitors represent a potential treatment option that is currently being evaluated in clinical trials.

Immunological Aspects

Immune factors and the tumor microenvironment contribute to the variable natural history of neuroblastoma. As age at diagnosis is of strong prognostic impact in patients with neuroblastoma, it is likely that age-related differences in the host immune reaction to a developing tumor might influence outcome.

Further evidence of the possible role of disease control by the immune system derives from observations of the neuroblastoma-associated paraneoplastic syndrome OMS, in which lymphoid infiltrates are frequently observed in the tumor; these patients typically have an excellent oncological prognosis.

In metastatic neuroblastoma, higher levels of CD163+ M2 macrophages have been demonstrated, a finding associated with adverse outcomes in several other cancers. Age-dependent differential expression of inflammatory genes associated with tumor associated macrophages has also been observed in neuroblastoma. Cancer-associated fibroblasts in the neuroblastoma microenvironment have been shown to play a role in the immunosuppression which might favor tumor development in neuroblastoma.

Altogether, in high-risk neuroblastoma infiltration with suppressive myeloid cells can decrease antitumor immune responses and favor tumor growth. Both active and passive immunotherapies, including antibody-based therapies, are now being explored in neuroblastoma.

Further advances in the understanding of the biology of neuroblastoma are currently based on the use of deep-sequencing approaches for comprehensive molecular characterization of the tumor cells and the host, also at a single cell level to account for tumor heterogeneity, with the goal not only to define precise patient risk groups, but also to identify key therapeutic targets.

Diagnosis

Clinical Presentation

Clinical signs and symptoms of neuroblastoma depend on the localization of the primary tumor and its metastatic sites. Localized disease often presents as an incidental finding, whereas metastatic disease is linked to systemic symptoms.

Primary tumors can arise anywhere along the sympathetic nervous system, but the distribution varies across the sites with age. Cervical neuroblastomas, observed in 4%–5% of cases, may involve the cervical ganglion (ganglion stellatum), causing Horner's syndrome (ptosis, miosis, enophthalmus, and anhidrosis). Thoracic neuroblastomas, localized in the upper, middle, or lower mediastinum and representing 15% of all cases, may be asymptomatic or more rarely cause respiratory distress. Over half of all primary tumors have their origin in the medulla of the adrenal gland, a localization associated with poorer survival. Bilateral primary adrenal tumors occur in $<1\%$ of all cases, possibly occurring in a context of genetic predisposition. Altogether abdominal localization is observed in 80% of cases, and large abdominal tumors can cause hypertension, abdominal distention, and pain. Pelvic tumors are uncommon and either present as an asymptomatic mass, or cause constipation or urinary symptoms. Tumors arising in any paraspinal sympathetic ganglia can grow along the nerve roots, penetrate into the intervertebral foramina, and lead to spinal cord compression, resulting in neurological symptoms. In very rare cases, no primary tumor can be identified.

Metastatic disease is present in 50% of patients at diagnosis, with regional or distant lymph nodes, bone marrow, and bone involved most frequently, whereas liver and skin metastasis can be observed especially in young infants with favorable outcome. Metastasis to other sites such lung or CNS occurs more rarely. Metastatic involvement can cause systemic symptoms such as fever, weight loss, bone pain, or signs of bone marrow involvement such as anemia and thrombocytopenia. Specific metastatic sites can lead to localized bone pain and limping, while periorbital metastasis can cause periorbital ecchymoses (Hutchinson's syndrome). Other clinical symptoms such as respiratory distress linked to compression by a large mass, coagulation disorders due to liver involvement, or lower body edema due to vena cava compression occur more rarely.

Associated paraneoplastic syndromes can be observed in approximately 5% of all patients with neuroblastoma. A hypersecretion of the vaso-intestinal peptide (VIP) can lead to abundant intractable watery diarrhea, requiring treatment with somatostatine or octreotide in rare cases if the symptoms persist after resection of the tumor.

Opsoclonus-myoclonus syndrome (OMS, also called Kinsbourne Syndrome or Dancing Eye Syndrome) is a rare neurological syndrome characterized by the association of opsoclonus (rapid, multidirectional eye movements), myoclonus, and cerebellar ataxia, most likely as a result of autoimmune process. This syndrome is observed in 2%–3% of children with neuroblastoma, while a neuroblastoma can be detected in 50%–80% of all children with OMS. These symptoms may precede the detection of a neuroblastoma, and may improve following surgical resection, but in many cases patients require specific immunosuppressive treatment for the neurological symptoms. Symptoms may reappear during infectious episodes, and although the oncological outcome of these patients is favorable, the majority of these children have long-term neurological sequelae.

Laboratory Parameters and Tumor Markers

Elevation of serum markers such as LDH, NSE, or ferritine are associated with unfavorable prognosis, but these markers are not specific to neuroblastoma.

An increase in urinary secretion of catecholamines or their metabolites, including dopamine, homovanillic acid (HVA), and/or vanillylmandelic acid (VMA), can be detected in 90% of patients. The profile of urinary catecholamines reflects the degree of cellular maturation with a high dopamine level or HVA/VMA ratio associated with an unfavorable prognosis. An elevation of norepinephrine or epinephrine occurs more rarely and can lead to arterial hypertension. With catecholamine metabolites routinely measured in urine samples at diagnosis and during treatment and follow-up, plasma levels of normetanephrine, metanephrine, and methoxytyramine might represent a convenient alternative to urine markers.

Radiological Local and Metastatic Assessment

Disease evaluation at diagnosis requires radiologic assessment of the primary tumor as well as an assessment of possible osteomedullary, soft tissue, or other metastatic sites.

Although ultrasound is often the first imaging exam because of its high availability and non-invasive nature, local assessment should include an exploration either by CT or MRI, with a high preference now given to MRI due to a higher contrast resolution and an absence of exposure to ionizing radiation, despite the longer acquisition times and sedation requirements in young children (Fig. 2). Neuroblastic tumors are often heterogeneous, and might present calcifications and involvement of regional lymph nodes.

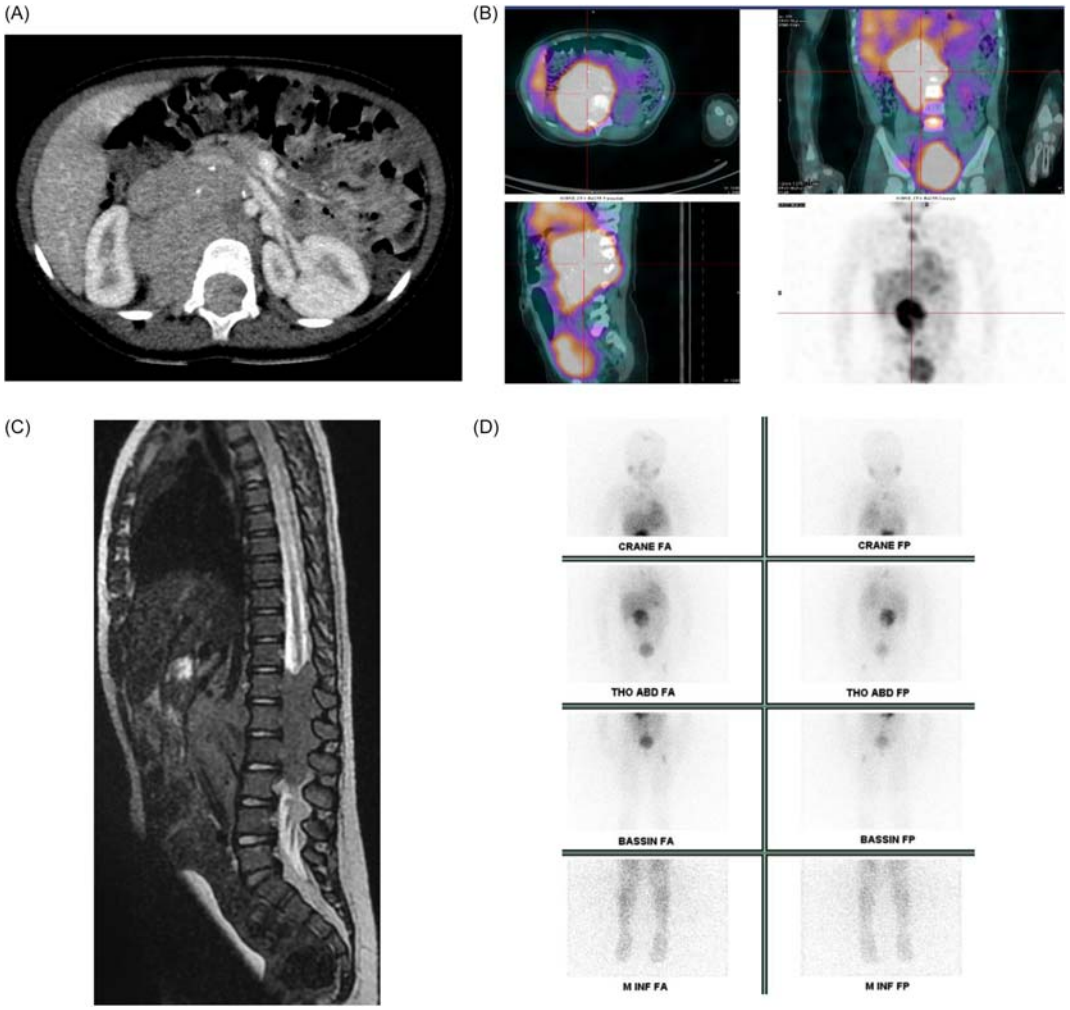

Fig. 2 Imaging of a neuroblastoma at diagnosis in a 2-year-old girl. An extensive retroperitoneal latero-aortic mass with microcalcifications shows intraforaminal invasion between D12 and L2 with spinal cord compression. The mass encases the aorta. MRI imaging reveals mediastinal lymph nodes confirmed my MYBG SPECT, which furthermore shows multiple osteomedullary lesions.

The precise analysis of local extension and involvement of adjacent organs includes the identification of image defined risk factors (IDRFs) in relation to possible surgical excision, taking into account perivascular disease, infiltration of tissues and adjacent organs, intraforaminal infiltration, and the epidural space of the spinal canal.

The extent of local disease is classified according to the absence or presence of IDRFs. The classification currently proposed by the INRG staging system categorizes tumors as L1 (limited tumor to a body compartment and the absence of any IDRF) or L2 (presence of IDRFs).

In addition to an evaluation of local disease extent, metastatic soft tissue, and bone marrow disease are evaluated by a meta-dibenzylguanidine scintigraphy (mIBG), preferably using iodine 123 (^{123}I), rather than ^{131}I due to lower exposure to ionizing radiation, improved quality of images, and lower thyroid toxicity. mIBG uptake depends on expression of the norepinephrine transporter, and about 90% of neuroblastomas express this transporter and thus are mIBG-avid. mIBG imaging has an estimated sensitivity and specificity of 90% and 99%, respectively. New imaging modalities using multimodal camera systems and integration of single photon emission computed tomography (SPECT) with CT combine the contrast provided by tumor-avid radioactive drugs with the anatomic precision of CT. Different scores (SIOPEN score, Curie score) have been developed to quantify metastatic involvement based on mIBG imaging, also enabling comparisons across different clinical trials in different cooperative groups. For mIBG non-avid neuroblastoma, disease extent should be evaluated by an additional technique, either with a technetium-99 bone scan, or ^{18}F-fluorodeoxyglucose positron emission tomography–computerized tomography (PET-CT). Recent PET techniques using ^{18}F-DOPA or ^{68}Ga-DOTATATE are now also being evaluated.

Comprehensive metastatic assessment also requires an assessment of bone marrow involvement. This investigation requires bilateral bone marrow aspirates and trephine bone marrow biopsies, obtained from bilateral iliac crest sites, with histological examination and immunohistochemistry for a quantitative approach to detect metastatic disease. Approaches measuring neuroblastoma

specific gene transcripts such as *PHOX2B* or tyrosine hydroxylase (*TH*) with qRTPCR can provide additional prognostic information, but are not yet incorporated into routine clinical evaluation. New approaches for detection and monitoring of disease might also rely on study of liquid biopsies such as analysis of cell-free circulating tumor DNA (Table 1).

Pathology of Neuroblastic Tumors

A pathological analysis of a tumor sample obtained either by surgical resection at diagnosis, or by surgical or percutaneous needle biopsy, allows confirmation of the histological diagnosis and a more precise classification according to the criteria of the International Neuroblastoma Pathology Committee (INPC).

Peripheral neuroblastic tumors show different degrees of differentiation, enabling one to distinguish ganglioneuroma tumor cells show maturation with terminal differentiation, ganglioneuroblastoma (some tumor cells show neuronal differentiation, others correspond to ganglion cells), and neuroblastoma (mostly immature small round tumor cells) with differentiating, poorly differentiated or undifferentiated features. A new histopathological classification of neuroblastoma combined biological characteristics including patient's age and a cellular index of mitosis and karyorrexis (MKI). The International Neuroblastoma Pathology Committee (INPC) defined a modification of the initial classification, taking into account the component of stromal Schwannian cells to classify neuroblastic tumors into four categories: neuroblastoma (Schwannian stroma-poor = SP); ganglioneuroblastoma, intermixed (Schwannian stroma-rich = SR); ganglioneuroblastoma, nodular (composite SR/SD and SP); and ganglioneuroma (Schwannian stroma-dominant = SD). The INPC defines a histoprognostic classification taking into account these categories, the index of mitosis and karyorrexis (MKI), and the age at diagnosis.

Staging and Prognostic Classification

In neuroblastoma, disease staging based on the extent of the disease is crucial in order to define prognosis and to inform treatment intensity. The previously used INSS definitions are based on the local and distant tumor extension and the resectability of the tumor (Table 2). However, this staging system is based on peroperative findings, and cannot easily be used in a context of unresectable disease.

The International Neuroblastoma Risk Group (INRG) classification system was then designed to identify homogeneous risk groups prior to any treatment to permit uniform therapeutic stratification and a comparison of patients groups in clinical trials conducted by different cooperative groups.

Table 1 Analyses for diagnostic confirmation and risk stratification of neuroblastoma

Tumor biopsy: pathology and biology	Pathological, immunohistochemical analysis and INPC classification
	FISH for *MYCN*, *aCGH*, SNPa or other techniques for genomic copy number profile
	DNA index (Ploidy)
	Optional: *ALK* mutations, NGS sequencing panel, expression profile
	Storage of tumor tissue for further translational studies recommended
Bilateral bone marrow aspirate and biopsy	Cytology, pathology, IHC
Imaging	Ultrasound, CT and/or MRI of primary site (neck, chest, abdomen, pelvis)
	Cranial CT or MRI (if clinically involved)
	^{123}I-mIBG scan; if tumor is not mIBG avid, ^{18}FDG-PET scan
Laboratory	Full blood count, blood coagulation studies
	Blood electrolytes, creatinine, uric acid, liver function tests
	Ferritin, LDH
	Urine VMA, HVA, dopamine

Table 2 INSS staging system

Stage	*Description*
1	Localized tumor with complete gross excision, with or without microscopic residual disease. Representative ipsilateral lymph node microscopically negative for tumor
2A	Localized tumor with incomplete gross excision. Representative ipsilateral non-adherent lymph nodes microscopically negative for tumor
2B	Localized tumor with or without complete gross excision, with ipsilateral non-adherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be microscopically negative for tumor
3	Unresectable unilateral tumor, infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral lymph node involvement; or midline tumor with bilateral extension by infiltration or lymph node involvement
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver and/or other organs (except as defined for Stage 4S)
4S	Localized primary tumor (as defined for Stage 1, 2A, or 2B), with dissemination limited to liver, skin, and/or bone marrow ($<$10%). Limited to infants $<$12 months of age

Table 3 International neuroblastoma risk group staging system (INRGSS)

Stage	*Description*
L1	Localized tumor confined to one body compartment, no involvement of vital structures; image defined risk factors (IDRFs) absent
L2	Locoregional tumor with presence of one or more image-defined risk factors (IDRFs)
M	Involvement of distant metastatic sites (unless stage MS)
MS	Metastatic disease in children <18 months with metastases confined to the skin, liver, and/or bone marrow (bone marrow involvement should be limited to $<10\%$)

In this system, the extent of the disease is determined by the presence or absence of IDRFs and/or the presence of metastases at diagnosis, prior to any treatment (**Table 3**). This staging system based on preoperative, diagnostic images will be more robust than one based on operative findings and enable classification of tumors that are not resected upfront. Since the surgical risk factors are based on radiological findings, the term *image-defined risk factors* (IDRFs) was chosen and a consensus was reached for the definition of IDRFs.

The INRG Task force developed the INRG Consensus Pretreatment Classification Schema to establish a consensus approach for current pretreatment risk stratification (**Table 4** and **Fig. 3**). This is important in order to facilitate the comparison of risk-based patient populations in different clinical trials conducted by cooperative groups, by defining homogeneous pretreatment patient cohorts. In addition to the INRG stage, other prognostic factors are included in this classification, including age at diagnosis (age >18 months being associated with unfavorable outcome), pathology and genetic characteristics, including MYCN amplification status (MYCN amplification is associated with unfavorable prognosis), and the overall genomic copy number

Table 4 Modified International Neuroblastoma Risk Groups (INRG), taking into account the genomic profile

INRG stage	*IDRFs of primary tumor*	*Distant metastases*	*Age at diagnosis (months)*	*Histological category*	*Grade of differentiation*	*MYCN status*	*Genomic profile*	*Ploidy*	*Risk group for treatment strategy*
L1/ L2	Any	Absent	Any	GN, GNB intermixed	Any	NA	Any	Any	Very low
L1	Absent	Absent	Any	GNB nodular, NB	Any	NA	Any	Any	Very low
L1	Absent	Absent	Any	GNB nodular, NB	Any	A	Any	Any	High
L2	Present	Absent	<18	GNB nodular, NB	Any	NA	Favorable	Any	Low
L2	Present	Absent	<18	GNB nodular, NB	Any	NA	Unfavorable	Any	Intermediate
L2	Present	Absent	≥18	GNB nodular, NB	Differentiating	NA	Favorable	Any	Low
L2	Present	Absent	≥18	GNB nodular, NB	Differentiating	NA	Unfavorable	Any	Intermediate
L2	Present	Absent	≥18	GNB nodular, NB	Poorly differentiated, undifferentiated	NA	Any	Any	Intermediate or high
L2	Present	Absent	≥18	GNB nodular, NB	Poorly differentiated, undifferentiated	A	Any	Any	High
M	Any	Present	<18	Any	Any	NA	Any	Hyperdiploid	Intermediate
M	Any	Present	<12	Any	Any	NA	Unfavorable and/or diploid		Intermediate
M	Any	Present	12–18	Any	Any	NA	Unfavorable and/or diploid		High
M	Any	Present	<18	Any	Any	A	Any	Any	High
M	Any	Present	≥18	Any	Any	Any	Any	Any	High
Ms	Any	Present	<12	Any	Any	NA	Favorable	Any	Low
Ms	Any	Present	12–18	Any	Any	NA	Favorable	Any	Intermediate
Ms	Any	Present	<12	Any	Any	NA	Unfavorable	Any	Intermediate
Ms	Any	Present	12–18	Any	Any	NA	Unfavorable	Any	High
Ms	Any	Present	<18	Any	Any	A	Any	Any	High

Notes: Definition of risk groups according to data from iINRG; http://inrgdb.org/neuroblastoma-information/staging-system.

L1, localized disease without image-defined risk factors; *L2*, localized disease with image-defined risk factors; *M*, metastatic disease, *Ms*, metastatic disease in infants (<18 months) restricted to liver, skin, and bone marrow; *GN*, ganglioneuroma; *GNB*, ganglioneuroblastoma; *NB*, neuroblastoma; *NA*, non-amplified; *A*, amplified. Favorable genomic profile corresponds to absence and unfavorable to presence of segmental chromosome alterations (SCA).

Cohn, S.L., Pearson, A.D., London, W.B., Monclair, T., INRG Task Force, et al. (2009). The International Neuroblastoma Risk Group (INRG) classification system: An INRG Task Force report. *Journal of Clinical Oncology* **27**, 289–297.

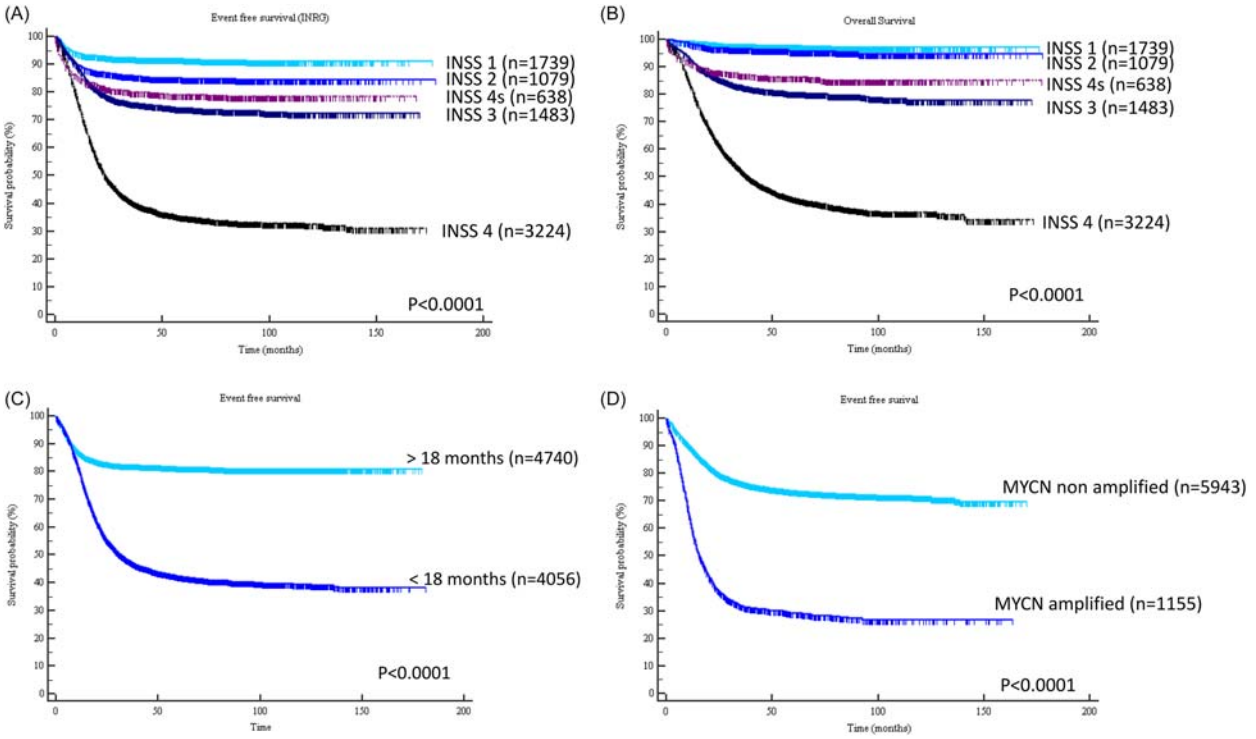

Fig. 3 Event-free survival (EFS) and overall survival (OS) of neuroblastoma patients (8800 patients with data recorded in the iINRG). EFS and OS were calculated according to the Kaplan Meier method, and compared using the log rank test. Clinical, pathological, and biological features of 8800 patients included in the iINRG database were analyzed to determine EFS and OS according to tumor stage (INSS), MYCN amplification status, and age at diagnosis. (A and B) EFS and OS according to INSS. Patients with INSS stage 4 have a poorer outcome. (C) EFS according to age at diagnosis, using a cut-off of 18 months at diagnosis. Patients aged >18 months at diagnosis have a poorer outcome. (D) EFS according to MYCN amplification. Patients whose tumors present MYCN amplification have a poorer outcome. Data from iINRG; http://inrgdb.org/neuroblastoma-information/staging-system; Cohn, S.L., Pearson, A.D., London, W.B., Monclair, T., INRG Task Force, et al. (2009). The International Neuroblastoma Risk Group (INRG) classification system: An INRG Task Force report. *Journal of Clinical Oncology* **27**, 289–297.

profile (the presence of segmental chromosomal alterations being of unfavorable prognosis). With the evolution of molecular biology techniques allowing even more detailed tumor biological characterization, it is likely that the risk groups will be further refined.

Early Detection and Screening

With the possibility of screening methods based on the detection of urinary catecholamines, several cooperative groups in France, Japan, the United States, Canada, and Germany have investigated the feasibility and impact of large-scale population-based screening programs. However, initial programs based on screening at an early age did not change the incidence of advanced disease after 1 year of age, and mass screening-negative children who presented later with neuroblastoma showed unfavorable genomic indicators like *MYCN* amplification. In an attempt to be more effective in preventing advanced disease, screening was then postponed to older infants (>6 months). However, although screening of 7–12-month-old infants resulted in more than one-third of neuroblastoma patients being identified by screening with unfavorable tumor genetic markers, it did not significantly reduce mortality. In a large German study based on urine screening for neuroblastoma at approximately 1 year of age, a similar incidence of stage 4 neuroblastoma was detected in the screened and the control group, and in a follow-up study, similar mortality rates between the two groups were observed. Thus, these results did not support a general screening for neuroblastoma at 1 year of age, and the screening program was abandoned.

Treatment of Neuroblastoma

The therapeutic management of patients with neuroblastoma depends on the risk groups and reflects the heterogeneity of this pathology, with a very broad spectrum of possible management, from simple observation to intensive multimodal treatment. Upfront, first-line treatment modalities currently include chemotherapy, surgery, radiotherapy, and immunotherapy.

Chemotherapy

As the majority of patients present with localized unresectable disease or metastasis at diagnosis and thus require systemic treatment, chemotherapy plays a pivotal role in the treatment of neuroblastoma. Major chemotherapeutic agents which have been used over the past decades include alkylating agents (cyclophosphamide, busulfan, melphalan), vinca alkaloids (vincristine), platinum derivatives (cisplatin and carboplatin), epipodophyllotoxins (etoposide), and anthracyclines (doxorubicin) with well-established activities against neuroblastoma, and are considered standard options.

Only few new drugs have been introduced into first-line therapy in recent years. Topotecan, irinotecan, and temozolomide are expected to improve the response rate slightly in high-risk neuroblastoma patients. High-dose chemotherapy with various combinations of busulfan, melphalan, carboplatin, and etoposide, followed by autologous stem cell transplantation (ASCT) rescue, is currently used as consolidation treatment in high-risk patients.

Surgery

Surgery plays a key role in both diagnosis and treatment. A tumor sample, either from the primary or in some situations from a metastatic site, is required at the time of diagnosis to confirm the pathological diagnosis and to establish biological features for risk classification. This can be obtained by surgical resection, surgical biopsy, or in many instances by percutaneous needle biopsies. With the rising importance of both pathological and biological investigations of tumor tissue, it is important to ascertain a sufficient sample size to enable all required analyses at the time of diagnosis.

For patients with localized disease, surgery is the treatment of choice in the absence of IDRFs and if surgical resection is deemed feasible. For these patients, in the absence of unfavorable biology such as MYCN amplification, surgery is the only treatment necessary to cure the majority of patients with localized disease, even if macroscopic remnants of the tumor are left behind. If surgical risk factors are detected in patients with localized disease, pre-surgical chemotherapy is necessary to induce tumor decrease prior to resection. However, IDRFs frequently persist after neoadjuvant chemotherapy and decisions of surgical resection should be taken following careful multidisciplinary discussions.

In contrast to its role in localized disease, in patients with metastatic disease, the place of aggressive surgery is still controversial. Meta-analyses in large patient cohorts are hampered by fragmented and inconsistent documentation of surgical approaches and a lack of immediate post-operative imaging results to distinguish complete from partial macroscopic resection. Due to the character of neuroblastoma, microscopically complete resection is not attainable in a majority of cases, and not of relevance. As local relapse can occur also in initially metastatic disease, the current recommendation in most high-risk treatment protocols is to perform resection of the primary tumor after inducing remission of metastases. In delayed primary or second-look surgery, the surgeon evaluates the response to therapy, and removes the residual tumor to a maximum extent whenever possible.

With the development of precision medicine programs based on detailed molecular characterization and search for targetable genetic alterations to orient toward early phase clinical trials, the role of a new tumor biopsy in case of relapse is now also widely acknowledged.

Radiotherapy

Neuroblastoma is a radiosensitive tumor, and doses ranging from 15 to 36 Gy have been used for local irradiation depending on tumor site, volume, and age of the patient. The definition of the role and modalities of irradiation, either as external beam radiotherapy or more recent treatment modalities, is under continuous refinement. Local irradiation is also successfully used within palliative care on painful metastatic sites.

Targeted radio-pharmaceutical treatment is based on ^{131}I carried by mIBG (^{131}I-MIBG), which is incorporated into neuroblastoma cells by the norepinephrine transporter. The importance of dosimetry and radioprotection issues limits the use of this therapeutic approach to selected treatment centers.

Some groups have used radio-pharmaceutical treatments within first line strategies. More promising approaches have included general radio-metabolic therapy using ^{131}I-MIBG in a conditioning phase prior to ASCT, and current use as salvage treatment in relapsed disease is also under investigation.

Treatment of Patients With Low-Risk and Intermediate-Risk Neuroblastoma

Low-risk neuroblastoma refers to patients with neuroblastoma of stages INRG L1, L2 $<$18 months, and Ms $<$12 or 18 months, without MCN amplification, according to the definitions in **Table 3**, and account for nearly half of all newly diagnosed neuroblastoma patients. For these patients, therapeutic decisions aim to provide minimum treatment in order to minimize any medium- or long-term treatment-related sequelae, while maintaining excellent survival. Infants ($<$1 year) with a localized adrenal mass may probably be observed safely, even without histological documentation. For patients with localized disease that appears to be resectable due to the absence of IDRFs (INRG L1 stage) or on the basis of surgical evaluation, the tumor may be removed surgically. In the absence of MYCN amplification, a residual tumor is not considered a risk factor for relapse, with a 5-year event-free survival (EFS) of $>$90% and an overall survival (OS) of 99%–100% for these patients. For patients with low-risk neuroblastoma, INRG L2, or Ms, the overall therapeutic strategy depends on clinical symptoms and more precise prognostic factors, including the genomic profile. For

these patients, in the absence of clinical symptoms and in the case of a favorable genomic profile, careful observation can be proposed in the Ms stage, and the feasibility of such an approach is being studied in the context of international studies within the SIOPEN and COG cooperative groups (SIOPEN LINES Study—NCT01728155; COG ANBL1232—NCT02176967). In the presence of clinical symptoms, or as a result of unfavorable prognostic factors, in particular of an unfavorable genomic profile, chemotherapy treatment is indicated, minimizing the number of cycles. There is no indication for radiotherapy in these patients, and the indication of surgical resection of the primary tumor in the case of persistent IDRFs should be evaluated carefully.

Intermediate risk neuroblastoma is defined as INRG L2 stage >18 months or M <12 or 18 months, without MYCN amplification. For these patients, two to eight cycles of chemotherapy are proposed, with combination chemotherapy combining etoposide and carboplatin, and vincristine, cyclophosphamide, and doxorubicin. Surgical resection of the primary tumor is performed whenever possible. Chemotherapy alone for children with INRG L2 unresectable neuroblastoma >18 months with unfavorable histology without *MYCN* amplification might not be sufficient; their lower survival indicates that a more intensive treatment regimen including radiotherapy and perhaps high-dose chemotherapy is warranted. Based on these treatment approaches, the estimated overall 5-year survival of intermediate risk neuroblastoma is $>90\%$ for infants with stage M disease but only 70% of children >18 months with L2 disease. In the future, it is expected that treatment intensity of patients with intermediate-risk neuroblastoma will be adjusted according to treatment response and other tumor biology parameters.

Treatment of Patients With High-Risk Neuroblastoma

A high-risk neuroblastoma is defined by INRG stage M disease in children $>12/18$ months, or patients whose tumor harbor MYCN amplification. Although the 5-year survival rate has risen from $<15\%$ prior to the 1980s to the present 40%, further therapeutic advances are imperative. The current approach incorporates induction chemotherapy to reduce the primary tumor and metastases, local treatment by surgery and radiotherapy, consolidation with high-dose chemotherapy, and re-injection of ASCT, as well as maintenance therapy in residual disease with anti-GD2 monoclonal antibody and therapy of differentiation by isotretinoin (retinoic acid).

Induction chemotherapy aims to achieve complete metastatic remission. Patients achieving a complete or near-complete remission at the end of induction have a significantly greater event-free survival compared to partial or less than partial response, and this has led to increasing dose intensity in induction, with current regiments incorporating multiple pairs or triplets of active drugs in a sequential fashion. Current regimens include combinations of etoposide, carboplatin, cisplatin, cyclophosphamide, and vincristine, with or without doxorubicin, allowing a complete and partial response rate of 70%–80% at the end of induction in the protocols used in different cooperative groups (SIOPEN, GPOH, COG, and JNBST). A current study compares two induction regimens, one of which proposes cures every 10 days (rapid COJEC) and the other one every 3 weeks (modified N7 arm) (Study SIOPEN HR NBL-01, NCT01704716). For the 10%–15% of high-risk patients who are refractory to standard induction therapy, depending on the prior treatment, a combination of irinotecan and temozolomide, or topotecan, vincristine, and doxorubicin, has been proposed with an aim to achieve a secondary metastatic response. Another approach might be the use of therapeutic ^{131}I-MIBG, with an estimated 30% response rate in refractory disease.

Surgical resection of the primary tumor is proposed once metastatic remission has been achieved but is often difficult in high-risk neuroblastoma, even after chemotherapy, due to frequent encasement of renal or abdominal blood vessels, or invasion of neural foramina. Radiation therapy is frequently administered to the tumor bed at doses of 21–36 Gy to reduce the risk of local recurrence.

Myeloablative high dose chemotherapy followed by ASCT has resulted in improved EFS for patients with high-risk neuroblastoma. The collection of hematopoietic stem cells is carried out by peripheral blood cytapheresis, frequently once metastatic bone marrow remission has been achieved. Initially myeloablative regimens included total body irradiation (TBI) to treat metastatic foci, but this has now been abandoned due to the significant late effects of TBI such as infertility, growth delay, and secondary malignancies. Myeloablative treatment is now based on high-dose chemotherapy. The current SIOPEN trial compared a regimen of busulfan and melphalan (BuMel) to carboplatin, etoposide, and melphalan (CEM), with an advantage in terms of EFS in favor of the BuMel arm. This treatment also shows a more favorable short-term toxicity profile. A recent COG randomized trial consisting of tandem transplant using thiotepa/cyclophosphamide followed by CEM compared to CEM alone showed a significant improvement in EFS for the tandem regimen, providing further evidence for the importance of myeloablative therapy. Pilot studies are also testing targeted radiopharmaceutical treatment with ^{131}I-mIBG to try to eliminate residual metastatic disease prior to transplant.

A possible complication of high-dose chemotherapy is veino-occlusive disease, an obstructive micro-angiopathy most often occurring in the liver, but sometimes also in the lungs. Treatment with defibrotide may be proposed for VOD, in addition to symptomatic treatment.

Despite the improvement in EFS with myelosuppressive chemotherapy followed by ASCT, 50% of children will have a relapse. The addition of a molecule causing the differentiation of neuroblastic cells, isotretinoin or retinoic acid, given orally, has been shown to improve EFS. The development of anti-GD2 monoclonal antibodies, targeting the disialoganglioside GD2 expressed on tumor cells, has also led to an improvement in EFS. A large randomized trial using the chimeric anti-GD2 antibody ch14.18 showed a significant improvement in EFS following high dose chemotherapy and ASCT for the combination of ch14.18, interleukin 2, GM-CSF, and isotretinoin, compared to isotretinoin alone. Studies are currently ongoing to improve the treatment modalities (long-term infusion to reduce side effects such as pain) and its combination (with the immunomodulator IL2) for maintenance therapy. Overall, the administration of anti-GD2 treatment is now widely recognized as a standard component of maintenance treatment in high-risk neuroblastoma. Importantly, recent studies have compellingly suggested that anti-GD2-based treatment

might demonstrate increased efficacy when combined with chemotherapy, justifying its further evaluation not only in relapse but also in upfront induction chemotherapy schedules.

Management of Specific Complications

Spinal cord compression occurs in approximately 5%–10% of all patients with neuroblastoma, and is an absolute medical emergency. Immediate treatment aims to increase chances of neurological recovery. In addition to symptomatic treatment using high-dose corticosteroids, treatment options include chemotherapy or neurosurgical intervention, either by laminectomy or by laminotomy. Although many investigators prefer a chemotherapy approach based on the possibility of rapid tumor volume decrease and spinal cord decompression, detailed multidisciplinary discussions are necessary to allow decisions to reduce paraplegia or other sequelae.

OMS is associated with medium- and long-term neurological (in particular neurocognitive) sequelae, despite a favorable oncological prognosis. Due to the underlying autoimmune mechanism, high-dose corticosteroid therapy (bolus of dexamethasone) is proposed. In cases of insufficient response, this treatment may be combined with other immunosuppressive treatment approaches such as further chemotherapy, rituximab, or (if necessary) polyvalent immunoglobulins, immunomodulatory agents, or (in severe cases) plasmapheresis.

Relapse Management

In cases of relapse after treatment of a low-risk or intermediate risk neuroblastoma, high-risk-based treatment strategies can often be proposed. In cases of high-risk relapse, OS unfortunately remains very poor. Nevertheless, in these cases, new combinations of chemotherapy have succeeded in permitting partial or complete remission in some cases. Current treatments are based on chemotherapy combinations (combining topotecan with cyclophosphamide, irinotecan with temozolomide, or topotecan with temozolomide). Other salvage treatments use 131I-mIBG therapy. Overall relapse treatment strategies should now be incorporated into global "basket" treatment approaches.

Early clinical trials proposed within the cooperative groups COG in collaboration with NANT, or SIOPEN in close collaboration with ITCC are focusing more on biomarker-based biology-driven treatment strategies. An important aspect for this is the molecular characterization to search for targetable genetic alterations, often proposed within general precision medicine programs. The inclusion of robust predictive biomarkers in phase I/II clinical trials will enhance the success in drug development and reduce exposures and toxicity for populations unlikely to benefit from a particular therapy. These approaches will also enable to determine the indication of inclusion of novel therapeutic strategies in first-line treatment in particular of high-risk neuroblastoma patients.

Among these targeted therapies, ALK inhibitors can be offered to 10%–15% of patients with detected ALK mutation at relapse. Combinations strategies of ALK inhibitors, including newer inhibitors with higher efficacy on the activating ALK mutations, together with chemotherapy, are currently being evaluated. Further strategies seek to target MYCN. Aurora Kinase inhibitors have shown promising response rates in phase I studies and have also been combined with chemotherapy for neuroblastoma, as they have been shown to destabilize MYCN as well as inhibiting cell cycle with G2/M arrest. Bromodomain inhibitors, based on their inhibition of MYCN transcription by binding to the promotor site, and CDK1, CDK2, and CHEK1, which present synthetic lethality with MYCN, are currently under early phase development. Aberrant PI3K/mTOR activity in neuroblastoma correlates with poor outcome, drives oncogenic stabilization of MYCN, and can be targeted using clinical PI3K/mTOR inhibitors. Other promising targets are HDAC inhibitors, and targeted disruption of the TP53–MDM2 interaction.

Treatments with preclinical promise also include immunotherapy currently in phase I or II studies with checkpoint inhibitors, anti-GD2 antibody with NK cells, or anti-GD2 vaccines and T cells engineered to express chimeric antigen receptors targeting GD2. Finally, a simple strategy with oral etoposide is often proposed in the palliative care setting.

Long-Term Survival

Studies of high-risk survivors treated with modern multimodel therapy, which is based on intensive chemotherapy, surgery, radiation, and immunotherapy, suggest a high prevalence of significant late effects, including endocrinological disorders, renal dysfunction, hearing loss, and, importantly, second malignancy. Hypothyroidism in neuroblastoma survivors may be due to the repeated exposure to ^{131}I-MIBG, as well as external beam radiation. Female survivors of high-risk neuroblastoma demonstrate a high rate of pubertal dysfunction and primary ovarian failure, and males might have azoospermia or oligospermia based on exposure to alkylating agents. Avoidance of late toxicity can be enhanced by a refined definition of patient subgroups. The strategy of using clinical and biological features at diagnosis to determine which patients might need less therapy will result in an overall decrease in late toxicity burden.

Outlook

The evolution of therapeutic strategies for patients with neuroblastoma will depend on an improved understanding of oncogenesis, by precise characterization of genetically and epigenetically controlled mechanisms, taking into account the tumor

microenvironment as well as the host itself. By integrating new biological insights, the discovery of new therapeutic targets and the understanding of resistance mechanisms, we shall improve the therapeutic management by increasing the survival rate of patients with high-risk neuroblastoma and by reducing the risk of sequelae.

From a biological point of view, the development of better animal models including GEMM (genetically engineered mouse models), zebrafish models as well as patient-derived xenografts (PDX) will contribute to elucidate further the pathogenesis of this disease. These approaches will enable us to study how single or multiple gene alterations, noncoding genetic elements, and epigenetic changes might contribute to the oncogenesis of neuroblastoma. The recent development of CRISPR/Cas9 technologies and other advances in genetic engineering will greatly accelerate research on the developmental origin and oncogenesis of this disease.

More intense collaborations and worldwide efforts in the molecular characterization of neuroblastoma will contribute further to the identification of molecular pathways which play a role in neuroblastoma progression and treatment resistance. The complex intratumoral, spatial, and temporal genetic heterogeneity of neuroblastoma has to be unraveled further in order to take into account its role in the process of clonal evolution of neuroblastoma. Furthermore, the recent discovery of different cellular identities with the possibility of switch from one cellular identity to another adds an additional level of complexity to the understanding of neuroblastoma oncogenesis.

The importance of data integration enabling large transversal data analyses is now widely acknowledged, and significant efforts have been placed in the construction of large-scale clinical and clinicobiological and biological databases (iINRG: http://inrgdb.org/neuroblastoma-information/staging-system; R2: http://r2.amc.nl). A crucial step in the understanding of neuroblastoma treatment resistance will be the integration of pharmacogenetics and pharmacogenomics data. This will enable contributions both to the understanding of early and late toxicity as well as to response rates based on differential drug exposure.

Importantly, the development of new tools for diagnosis and disease follow-up will contribute significantly to a more precise staging and response assessment. Such new tools will take into account molecular and cell-based techniques. One important question is that of minimal residual disease, which might be assessed by high-resolution cytological techniques, as well as molecular techniques based on the detection of neuroblastoma-specific mRNAs or circulating tumor DNA. Circulating tumor DNA will also enable study of the evolution of genomic biomarkers over time and during treatment. Indeed, ctDNA markers have the potential to evolve toward both biomarkers for response and biomarkers for detection of decision-making genetic alterations in the field of precision medicine.

From an immunological point of view, further studies will allow better comprehension of the role of the immune system of the host in the defense against neuroblastoma cells. Newer immunotherapeutic approaches to be developed include CAR-T cell approaches, approaches based on expanded and activated NK cells, allogeneic transplant strategies including KIR mismatch, and new humanized antibody conjugates and vaccines.

Importantly, the integration of molecular constitutional and somatic genetic knowledge, and immunological information, into clinical study and trial designs will require new adaptive trial designs. The widespread scientific collaborations fostered through associations such as ANR (Advances in Neuroblastoma Research Association; https://www.anrmeeting.org/index.php) will prove crucial to these collaborative efforts in the future.

See also: Neuroblastoma.

Further Reading

Barone, G., Anderson, J., Pearson, A.D., et al., 2013. New strategies in neuroblastoma: Therapeutic targeting of MYCN and ALK. Clinical Cancer Research 19, 5814–5821.

Brisse, H.J., McCarville, M.B., Granata, C., et al., 2011. International neuroblastoma risk group project. Radiology 261, 243–257.

Brodeur GM, Pritchard J, Berthold F, et al., Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment (1993). Journal of Clinical Oncology 11, 1466-77

Cohn, S.L., Pearson, A.D., London, W.B., Monclair, T., Task Force, I.N.R.G., et al., 2009. The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. Journal of Clinical Oncology 27, 289–297.

Cohn, S.L., Pearson, A.D., London, W.B., et al., 2004. The International Neuroblastoma Risk Group (INRG) Classification System: AN INRG Task Force. Journal of Clinical Oncology 27, 289–297.

DuBois, S.G., Matthay, K.K., 2008. Radiolabeled metaiodobenzylguanidine for the treatment of neuroblastoma. Nuclear Medicine and Biology 35 (S1), S35–S48.

Eleveld, T.F., Oldridge, D.A., Bernard, V., et al., 2015. Relapsed neuroblastomas show frequent RAS-MAPK pathway mutations. Nature Genetics 47, 864–871.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: The next generation. Cell 144, 646–674.

Ladenstein, R., Pötschger, U., Pearson, A., et al., 2017. Busulfan and melphalan versus carboplatin, etoposide, and melphalan as high-dose chemotherapy for high-risk neuroblastoma (HR-NBL1/SIOPEN): An international, randomized, multi-arm, open-label, phase 3 trial. Lancet Oncology 18, 500–514.

Matthay, K.K., Maris, J.M., Schleiermacher, G., et al., 2016. Neuroblastoma. Nature Reviews. Disease Primers 2, 16078.

Matthay, K., Reynolds, P., Seiger, R.C., et al., 2009. Long-term results for children with high-risk neuroblastoma treated on a randomized trial of Myeloablative therapy followed by 13-cis-retinoic acid: A children's oncology group study. Journal of Clinical Oncology 7, 1007–1013.

Mody, R., Naranjo, A., Van Ryn, C., et al., 2017. Irinotecan-temozolomide with temsirolimus or dinutuximab in children with refractory or relapsed neuroblastoma (COG ANBL1221): An open-label, randomized, phase 2 trial. Lancet Oncology 18, 946–957.

Monclair, T., Brodeur, G.M., Ambros, P.F., et al., 2009. The international neuroblastoma risk group staging system: An INRG task force report. Journal of Clinical Oncology 27, 298–303.

Peifer, M., Hertwig, F., Roels, F., et al., 2015. Telomerase activation by genomic rearrangements in high-risk neuroblastoma. Nature 526, 700–704.

Pinto, N.R., Applebaum, M.A., Volchenboum, S.L., et al., 2015. Advances in risk classification and treatment strategies for neuroblastoma. Journal of Clinical Oncology 33, 3008–3017.

Schleiermacher, G., Janoueix-Lerosey, I., Delattre, O., 2014. Recent insights into the biology of neuroblastoma. International Journal of Cancer 135, 2249–2261.

Schramm, A., Köster, J., Assenov, Y., et al., 2015. Mutational dynamics between primary and relapse neuroblastomas. Nature Genetics 47, 872–877.

Shimada, H., Ambros, I.M., Dehner, L.P., et al., 1999. Terminology and morphologic criteria of neuroblastic tumors: Recommendations by the international neuroblastoma pathology committee. Cancer 86, 349–363.

Yu, A.L., Gilman, A.L., Ozkaynak, M.F., 2010. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. The New England Journal of Medicine 363, 1324–1334.

Neuroblastoma: Pathology and Genetics

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Abbreviations

ADCC Antibody dependent cell mediated cytotoxicity
AGM Aorta-gonad-mesonephros
ALK Anaplastic lymphoma kinase gene
ATRX Alpha thalassemia/mental retardation syndrome X-linked gene
BARD1 *BRCA1-associated RING domain 1 gene*
BMP Bone morphogenetic proteins
BsAb Bispecific antibody
CAR Chimeric antigen receptor
CNS Centrak nervous system
CTL Cytotoxic T cell
DCV Dense core vesicle
GD2 Disialoganglioside 2
GM-CSF Granulocyte-macrophage colony stimulating factor
GPC2 Glypican 2
GWAS Genome Wide Association Studies
HIF Hipoxia inducible factors 1α and 2α
HLA Human leukocyte antigen
HVA Homovanilic acid
Mab Monoclonal antibody
MAPK Mitogen activated protein kinase
MIBG Meta-iodobenzylguanidine
MKI Mitotis-Karyorrhexis Index
MYCN N-*myc proto-oncogene*
NB Neuroblastoma
NGF Nerve growth factor
NK Natural killer cell
OMAS Opsoclonus-myoclonus-ataxia syndrome
PHOX2B Paired-like homeobox 2b gene
SNP Single nucleotide polymorphism
SNS Sympathetic nervous system
TAM Tumor associated macrophage
TERT Telomerase reverse transcriptase gene
TRK Tropomyosin receptor kinase family of neurotrophin receptors: Trk-A, Trk-B
VMA Vanillylmandelic acid
WGS Whole genome sequencing

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in children, accounting for 8%–10% of all pediatric malignancies, and 12% of childhood cancer mortality (Whittle et al., 2017; Ahmed et al., 2017; Tolbert et al., 2017). There are approximately 20–50 cases per million, 650 new cases per year in the US (Ahmed et al., 2017; Matthay et al., 2016). The median age at diagnosis is 19 months, with 90% of patients being younger than 5 years (Whittle et al., 2017; Ahmed et al., 2017). Individuals of African ancestry show more aggressive forms compared to those of European descent (Whittle et al., 2017; Ahmed et al., 2017; Matthay et al., 2016). It is more common in boys than in girls although the underlying reasons for this gender preference remain unclear (Matthay et al., 2016).

NB is an embryonal tumor of neural crest origin that derives from sympathetic nervous system (SNS) precursors during development (Matthay et al., 2016; van Noesel, 2012; Cheung and Dyer, 2013; Ratner et al., 2016). It is a highly heterogeneous tumor in

regards to its presentation: age at diagnosis, stage, histological, molecular and genetic features lead to very different clinical behavior and outcome (Cheung and Dyer, 2013). Increasing knowledge on each of those areas has allowed for a more precise risk stratification of these patients that dictates management and prognosis, ranging from observation alone, with near 100% cure rates, to aggressive multimodal therapy with poor outcomes (Whittle et al., 2017; Ahmed et al., 2017; Cheung and Dyer, 2013; Kushner, 2017).

A better understanding of the relationship between normal development and tumor biology has also led to major therapeutic advances: expression of tissue related oncofetal differentiation proteins like GD2 or B7-H3 (used as antibody based immunotherapy targets); tissue differentiation inducers (like retinoids); and metabolic pathways (catecholamine metabolism) have led to diagnostic and therapeutic strategies that are improving outcomes of high risk patients (Cheung and Dyer, 2013; van Groningen et al., 2017; Ahmed et al., 2015). Whole genome sequencing (WGS) and genome wide association studies (GWAS) data are shedding some light into other potential genetic lesions relevant in NB (Tolbert et al., 2017; Matthay et al., 2016; Cheung and Dyer, 2013; Cao et al., 2017; Esposito et al., 2017). The low mutanome found in these tumors has hampered targeted therapies and immune based therapies (Esposito et al., 2017; Lee et al., 2017). Lack of mutations found in the majority of tumors at diagnosis, and recurrent patterns of whole chromosome or large segmental DNA copy number alterations, suggest that NB may be driven by gene copy number changes (Matthay et al., 2016). The goal of this article is to give an overview of the body of knowledge on NB biology that has influenced diagnosis, prognosis and therapeutic approaches to date.

Clinical Presentation Spectrum and Paraneoplastic Syndromes

NB is a tumor of neural crest origin that derives from SNS precursors, which give rise to the adrenal medulla and the sympathetic paravertebral ganglion chain (**Fig. 1**) (Cheung and Dyer, 2013). Primary tumor location follows this distribution pattern with up to 65% arising from the adrenal medulla and 35% from paravertebral ganglia (Cheung and Dyer, 2013).

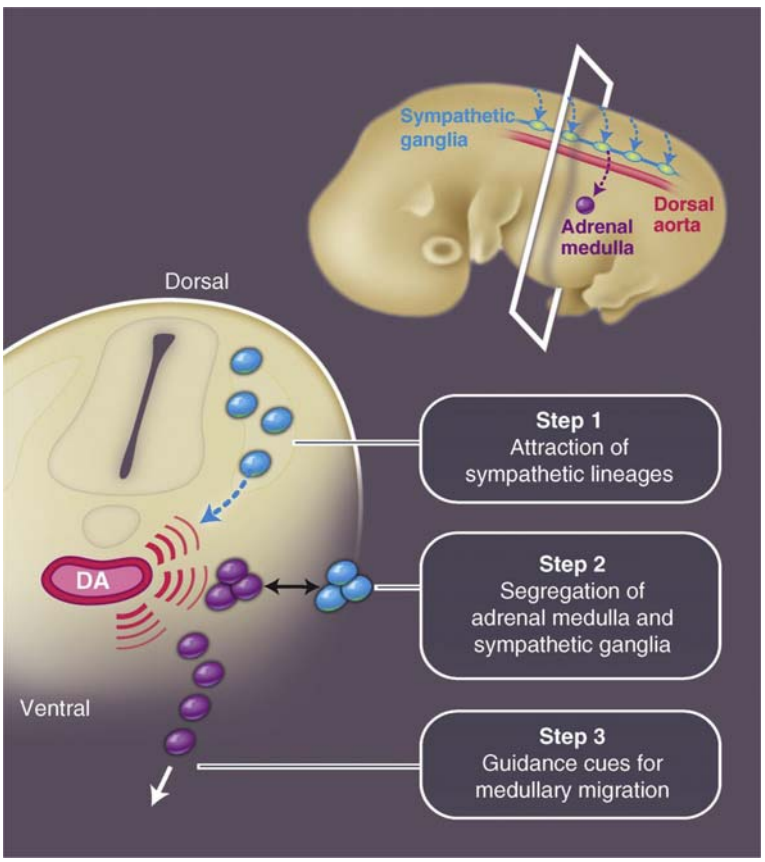

Fig. 1 *Early development of the sympathetic nervous system.* The dorsal aorta acts as a morphogenetic center for sympathetic lineages. Cells from the neural crest migrate ventrally and at the region where common precursors of sympatho-adrenal lineages migrate, receive signals from the dorsal aorta. Aortic signals determine the segregation of adrenal medullary cells from sympathetic ganglia. Finally, the dorsal aorta supports guidance molecule(s) for medullary cells to migrate ventrally. Reproduced with permission from AAAS, from Takahashi, Y., Sipp, D. and Enomoto, H. (2013). Tissue interactions in neural crest cell development and disease. *Science* **341**, 860–863, https://doi.org/10.1126/science.1230717.

Location influences clinical presentation: retroperitoneal masses may be asymptomatic or cause pain, abdominal distension and hypertension secondary to renal vasculature compression (Ahmed et al., 2017; Graeme Eisenhofer and Cheung, 2017). Paraspinal tumors may affect nerve structures causing Horner's syndrome (ptosis, miosis, enophthalmos, hemifacial anhidrosis) if located in the cervical/thoracic region or paresias or paraplegias from spinal cord compression, present in up to 15% of cases at presentation (Ahmed et al., 2017; Matthay et al., 2016). Half of the patients present with localized or regional disease, 35% having regional lymph node involvement at diagnosis (Ahmed et al., 2017). Bilateral disease is rare and should point at familial NB syndromes (Ahmed et al., 2017; Cheung and Dyer, 2013). The other 50% present with distant metastasis with bone marrow, bone and liver being the most common sites affected (Matthay et al., 2016; Graeme Eisenhofer and Cheung, 2017). Patients with distant metastasis limited to lymph nodes (4N), associated with younger age and favorable clinical features, have better outcome than those with generalized metastasis (Ahmed et al., 2017). NB has a predilection for metaphyseal, skull and orbital sites, the latter leading to periorbital ecchymosis at presentation ("racoon eyes") (Ahmed et al., 2017). In metastatic cases patients can develop constitutional syndrome with fever, weight loss and malaise (Ahmed et al., 2017; Matthay et al., 2016). Metastasis to other sites like lung or in the central nervous system (CNS) at initial diagnosis are rare (Matthay et al., 2016). However, relapse in CNS is increasingly common (Kramer et al., 2001; Cheung et al., 2012).

A particular pattern of dissemination seen in infants, classified as stage 4S (S for special), presents with skin, diffuse liver involvement, below 10% bone marrow involvement and small primary tumors (van Noesel, 2012). This particular distribution resembles that of early neural crest stem cells, which migrate to the skin and temporarily to the aorta-gonad-mesonephros (AGM), an embryologic structure from which fetal liver hematopoiesis and a small part of definitive bone marrow hematopoiesis derives (van Noesel, 2012). These neural crest stem cells that, from the AGM, migrate along fetal hematopoietic precursors, may be origin of 4S disease (van Noesel, 2012). These infants have a course of initial tumor growth followed by gradual simultaneous spontaneous regression over months, requiring therapy only if hepatomegaly causes life-threatening complications.

Paraneoplastic syndromes are rare and usually associated with more differentiated tumors. Secretory diarrhea resulting from tumor cell secretion of vasoactive intestinal peptide (VIP) resolves upon tumor removal (Graeme Eisenhofer and Cheung, 2017). Alternatively, a neuro-autoimmune process that cross-reacts with neurons, primarily of the cerebellum, called opsoclonus-myoclonus-ataxia syndrome (OMAS), is seen in up to 2%–3% of cases, and often leads to long term impairments despite tumor eradication (Whittle et al., 2017; Matthay et al., 2016; Ratner et al., 2016; Graeme Eisenhofer and Cheung, 2017). It is estimated that between 50% and 80% of patients with OMAS have underlying NB (Matthay et al., 2016). Interestingly, primary tumors from these patients have a dense inflammatory cell infiltrate (CD20+ B cells, CD4+ and CD8+ T cells), which is far less common in advanced stage NB (Ratner et al., 2016).

Development of the Sympathetic Nervous System

The neural crest is an embryonal structure that gives rise to different cell types, including peripheral neurons and glia of the sympathetic, parasympathetic and enteric nervous systems, the adrenal medulla, melanocytes, Schwann cells, craniofacial cartilage and bone cells (Graeme Eisenhofer and Cheung, 2017). The adrenal medulla hosts postganglionic neurons that respond to preganglionic neurons in the spinal cord releasing catecholamines (Graeme Eisenhofer and Cheung, 2017). The remainder sympathetic ganglions are distributed along the paravertebral chain from the neck down to sacral regions (Graeme Eisenhofer and Cheung, 2017) (**Fig. 1**). Migration and differentiation is controlled by the expression of specific markers that respond to different environmental signals (Graeme Eisenhofer and Cheung, 2017; Takahashi et al., 2013). Multipotency is maintained until destination is reached (Graeme Eisenhofer and Cheung, 2017). In the adrenal medulla and also at extraadrenal sites, these cells give rise to different populations: chromaffin cells (catecholamine producers), small intensely fluorescent (SIF) cells, neurons and glial cells (Graeme Eisenhofer and Cheung, 2017). For this specification process, bone morphogenic proteins (BMPs), synthesized in the dorsal aorta, are crucial in determining a chromaffin cell vs. sympathoneuronal fate, although the precise pathways involved are not completely clear (Graeme Eisenhofer and Cheung, 2017; Takahashi et al., 2013). NBs do not display the full chromaffin morphology, but instead resemble progenitors destined towards a neuronal path of differentiation (neuroblast) (Graeme Eisenhofer and Cheung, 2017). *N-myc proto-oncogene* (*MYCN*) blocks the transition of neural crest cells to a chromaffin cell fate, being a key gene in the development of these tumors (Graeme Eisenhofer and Cheung, 2017). Hypoxia pathways are also very relevant in the development of the sympathoadrenal system (Takahashi et al., 2013). Hypoxia is a stimulus for catecholamine secretion, and intact signaling pathways (HIF1α and HIF2α) are needed to ensure appropriate development of cathecholamine systems, which in turn facilitates fetal survival under hypoxic conditions (Graeme Eisenhofer and Cheung, 2017). HIF2α seems crucial for the survival of sympathetic neurons (Graeme Eisenhofer and Cheung, 2017). Expression of HIF2α in NB cells maintains them in an undifferentiated state, rendering more aggressive features (Graeme Eisenhofer and Cheung, 2017).

The observation that neuronal differentiation was driven by retinoids in vitro led to the use of isotretinoin (13-*cis*-retinoic acid) as a differentiating agent to treat high risk patients (Cheung and Dyer, 2013; Ratner et al., 2016). Despite being standard of care for a long time in the treatment of high risk patients, a recent meta-analysis showed no benefit when used after autologous stem cell transplantation (Peinemann et al., 2015). Resistance to isotretinoin therapy has been ascribed to RAF-MEK pathway activation; hence adding MEK inhibition may improve the activity of isotretinoin therapy (Ratner et al., 2016; Holzel et al., 2010).

Another relevant feature of neuroblastic cells is the expression of specific markers such as GD2, B7-H3, GPC2 also present in tumor cells, which are the target of antibody based therapies, rapidly becoming standard of care in high risk patients (Cheung and Dyer, 2013; Ahmed et al., 2015).

Histological Features and Classification

Peripheral neuroblastic tumors show different grades of morphological differentiation: NB is one of the small round blue cell tumors and its differential diagnoses requires consideration of clinical, radiological and histological features. The clinical pathology of NB typically shows nests of neuroblasts in various stages of differentiation, separated by fine fibrovascular septa and variable amount of Schwannian stroma (Ahmed et al., 2017; Wang et al., 2013). X-inactivation studies showed that neuroblastic and Schwannian-like stromal cells developed from the same progenitor, that led to the different cell fates (Mlakar et al., 2017). In these studies, chromosomal imbalances were seen only in the neuroblastic lineage, pointing at the fact that these chromosomal changes happen after cells have commited to a neuroblastic fate (Mlakar et al., 2017). Rosettes can be seen in up to one third of NB (Ahmed et al., 2017). The presence of neuropil and immunohistochemical findings (synaptophysin, chromogranin, tyrosine hydroxylase, nuclear Phox2B) can facilitate diagnosis (Graeme Eisenhofer and Cheung, 2017). In some instances, ganglion cells suggestive of neuroblast differentiation can be seen, leading to the diagnosis of ganglioneuroblastoma (where there are coexisting neuroblasts with terminally differentiated tumor derived ganglion cells) (Matthay et al., 2016; Wang et al., 2013). Ganglioneuroma (where tumor cells are terminally differentiated to ganglion cells) with no neuroblastic elements, possibly derived from a more differentiated lineage of the sympathetic system, requires only conservative management (Ahmed et al., 2017; Wang et al., 2013). Schwannian NB cells may derive from the malignant clone, whereas Schwann cells in mature ganglioneuromas may be normal infiltrating Schwann cells (Ratner et al., 2016) (**Fig. 2**).

Schwannian stroma poor tumors are the most undifferentiated appearing and aggressive (Ahmed et al., 2017). The Shimada histology-grading system is based on age plus histological features, which include the presence or absence of differentiation, Schwannian stromal content, and mitotis-karyorrhexis index (MKI) (Ahmed et al., 2017; Matthay et al., 2016; Cheung and Dyer, 2013). A more recent classification proposed by the International Neuroblastoma Pathology Committee (INPC), distinguishes favorable histology as any differentiation in NB, with low/intermediate MKI in patients younger than 18 months, and differentiating NB with low MKI in patients between 18 months and 5 years of age (Ahmed et al., 2017). Unfavorable histology includes undifferentiated or high MKI in any age, poorly differentiated and/or intermediate MKI in patients between 18 months and 5 years and any grade, any MKI in patients 5 years or older (Ahmed et al., 2017).

Catecholamine Synthesis and Metabolism: Diagnostic and Therapeutic Relevance

NBs are primitive catecholamine producing tumors (Graeme Eisenhofer and Cheung, 2017). In NB cells, catecholamines are usually synthesized and metabolized though not commonly secreted (Graeme Eisenhofer and Cheung, 2017). They are stored in dense core

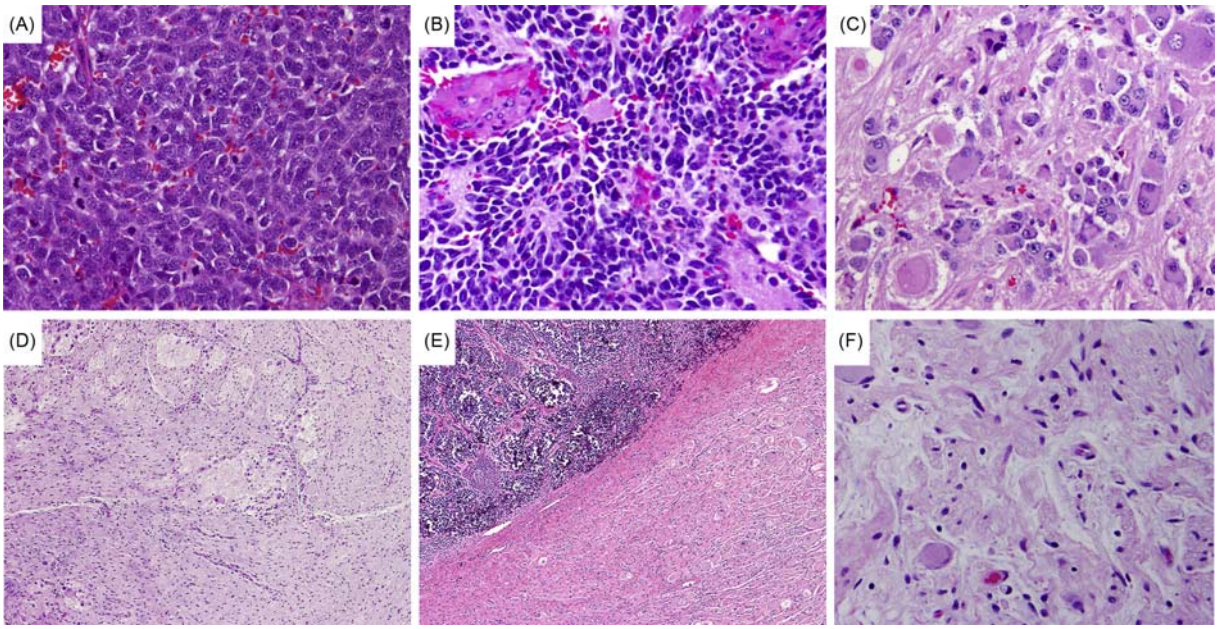

Fig. 2 *Hematoxylin and eosin staining of neuroblastic tumors.* Neuroblastoma: undifferentiated (A); poorly differentiated (B); differentiating NB (C). Low power GNB: intermixed (D); nodular (E). Ganglioneuroma (F). Pictures courtesy of Dr. Satish K. Tickoo, Department of Pathology, MSKCC.

vesicles (DCVs) which can be seen in NB cells (Cheung and Dyer, 2013). Most catecholamine metabolism results from leakage from the DCVs (Cheung and Dyer, 2013). Meta-iodobenzylguanidine (MIBG), an analogue of norepinephrine, is taken up by NB cells and a fraction stored in DCVs (Cheung and Dyer, 2013). Approximately 90% of NBs are MIBG avid (Ahmed et al., 2017; Matthay et al., 2016). Radioiodinated MIBG is used for diagnostic (^{123}I–MIBG) and therapeutic (^{131}I-MIBG) purposes (Cheung and Dyer, 2013) (**Fig. 3**).

In humans, homovanilic acid (HVA) and vanillylmandelic acid (VMA) are the main metabolic end products of dopamine metabolism, eliminated mainly through the urine (Graeme Eisenhofer and Cheung, 2017). Elevated urinary HVA and VMA is detected in most NB cases at diagnosis, and if coupled with bone marrow tumor infiltration determined through bone marrow aspirate or biopsy, will meet the diagnostic criteria of NB in the absence of a primary tumor sample (Ahmed et al., 2017). HVA and VMA can be determined in a random urine sample normalized to urine creatinine, as there is low diurnal variation and good correlation with 24 h urine collection levels (Graeme Eisenhofer and Cheung, 2017). Measurement in serum samples has less sensitivity, although in cases where urine sampling is not possible, determination of serum normetadrenalin, metadrenalin and methoxytyramine can be of use (Matthay et al., 2016; Graeme Eisenhofer and Cheung, 2017). A high ratio of HVA to VMA, of dopamine to VMA, or of dopamine to norepinephrine, indicates a relative deficiency in dopamine-B-hydroxylase, resulting in a decreased tumor cell conversion of dopamine to norepinephrine (Graeme Eisenhofer and Cheung, 2017). This immature metabolic pattern has been associated with more aggressive tumors and *MYCN* amplification (Ahmed et al., 2017; Graeme Eisenhofer and Cheung, 2017).

Molecular Genetics and Biology

NB can be classified into three groups based on its biological behavior. Type 1 includes those that differentiate into ganglioneuroblastomas (Graeme Eisenhofer and Cheung, 2017). Type 2 includes those regressing spontaneously overtime, which happens mostly in infancy, and is exemplified by stage 4S and some locoregional tumors despite residual microscopic disease (Graeme Eisenhofer and Cheung, 2017). The incidence of type 2 NB may be underestimated as it is thought that up to half may go undiagnosed (Matthay et al., 2016). Type 3 (>50%) includes those that progress and metastasize to distant organs (Graeme Eisenhofer and Cheung, 2017). Recent discoveries have helped understand the genetic basis of NB and Omics data should one day be integrated into the diagnosis and prognostication for accurate risk stratification and therapy selection for these patients. It is now estimated that approximately 10% of NB patients carry germline mutations in genes that might confer increased susceptibility to NB development (Tolbert et al., 2017). Many of the more highly penetrant mutations in NB predisposition, inherited or de novo, may affect the risk for other malignancies in survivors (Matthay et al., 2016). Here we will summarize the more relevant findings, and how they may relate to the biology and therapy of NB.

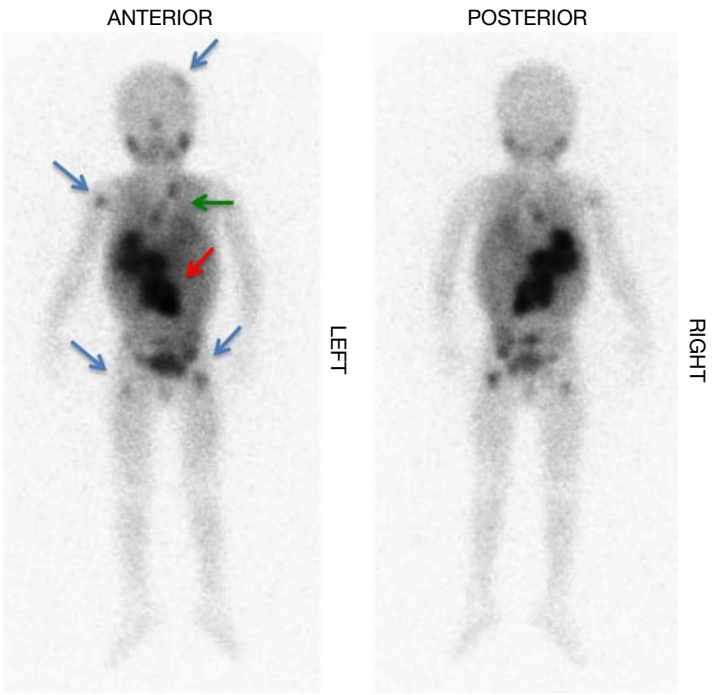

Fig. 3 *I123MIBG SCAN 24h.* Retroperitoneal mass and lymphadenopathies (*red arrow*), lymph node involvement (*green arrow*), multiple sites of bone involvement: skull, scapula, humerus, bilateral iliac and femurs (*blue arrows*).

Familial NB

Familial NB cases are rare, accounting for < 2% of all diagnosis (Tolbert et al., 2017; Cheung and Dyer, 2013). These patients do not seem to present at younger age, but may have multifocal primary disease (Ahmed et al., 2017; Tolbert et al., 2017; Matthay et al., 2016; Cheung and Dyer, 2013; Kamihara et al., 2017). Two genes have been identified within familial neuroblastoma cases: the *paired-like homeobox 2b* (*PHOX2B*) and the *anaplastic lymphoma kinase* (*ALK*) gene (Tolbert et al., 2017; Matthay et al., 2016; Cheung and Dyer, 2013; Cao et al., 2017). The disease is inherited in an autosomal dominant fashion, though pedigrees show considerable heterogeneity in the biology of tumors that arise in these families: benign ganglioneuromas and malignant NBs have been reported in the same family, and some pedigrees show incomplete penetrance (Tolbert et al., 2017; Kamihara et al., 2017).

A small proportion of patients have a clinically recognizable genetic syndrome: (a) most cases are related to abnormalities in neural crest development, like in congenital central hypoventilation syndrome (CCHS), aganglionosis of the colon (Hirschsprung disease), ROHHAD syndrome (rapid-onset obesity, hypothalamic dysfunction, hypoventilation, and autonomic dysfunction); (b) in the context of RASopathies such as Costello syndrome, Noonan syndrome, neurofibromatosis type 1; (c) epigenetic syndromes such as Beckwith–Wiedemann syndrome (BWS, including hemihypertrophy, or 11p overgrowth) (Kamihara et al., 2017). Germline mutations in *TP53* (in particular R337H), *SDHB*, *PTPN11* and *APC* have been reported to occur rarely in NB patients (Ahmed et al., 2017; Kamihara et al., 2017; Varan et al., 2016).

Inactivating mutations in *PHOX2B* were the first found in the study of syndromic NB cases, along with Hirschprung disease and/or CCHS (Matthay et al., 2016; Cheung and Dyer, 2013). Mutations in this gene account for a minority of familial NB cases (Tolbert et al., 2017; Matthay et al., 2016; Cheung and Dyer, 2013; Cao et al., 2017). It is a transcription factor that promotes cell cycle exit and neuronal differentiation of neural crest derived autonomic precursors (Cheung and Dyer, 2013). *PHOX2B* has two polyalanine repeat sequences (PARMS), and mutations in these are associated with a 1%–2% risk of developing tumors (Cheung and Dyer, 2013; Kamihara et al., 2017). On the other hand, nonpolyalanine repeat expansion mutations relate to the more severe phenotype including the NB-Hirschprung disease-CCHS association (Cheung and Dyer, 2013), and an increased risk (45%) of developing a neural crest tumor.

Activating mutations in *ALK* are accountable for up to 80% of familial NB cases (Tolbert et al., 2017; Matthay et al., 2016; Cheung and Dyer, 2013). It encodes for a receptor tyrosine kinase member of the insulin receptor superfamily (Ahmed et al., 2017). It is expressed in developing sympathoadrenal lineage of neural crest cells regulating multiple pathways, including the mitogen activated protein kinase (MAPK) ans Ras related protein 1 (Rap1) pathways (Cheung and Dyer, 2013). PHOX2B can directly regulate *ALK* expression, connecting both pathways found to be affected in familial NB cases (Cheung and Dyer, 2013). The overall penetrance of germline *ALK* mutations is estimated around 50% (Kamihara et al., 2017). There is a correlation between mutation type and penetrance: the R1275Q, the most common mutation seen in up to 45% of familial NB (Cao et al., 2017), leads to near complete penetrance, whereas G1128A renders a weaker activation and is correlated with a 25% risk of developing NB (Tolbert et al., 2017). Mutations in *ALK* can also be found in up to 6%–10% of sporadic NB tumors, while another 3%–4% carry amplifications, and overexpression is found in up to 33% of cases (Ahmed et al., 2017; Cheung and Dyer, 2013). The two most highly activated somatic hot-spot mutations (F1174 and F1245) were each observed in the germline once, associated with severe neurocognitive defects and brain stem abnormalities together with NB (Tolbert et al., 2017). ALK is a pharmacologic target: crizotinib, an ALK small molecule inhibitor, and newer agents for crizotinib resistant cases are being developed (Esposito et al., 2017). A specific *ALK* mutation (F1174L) has been used to develop a NB murine model (Cheung and Dyer, 2013; Graeme Eisenhofer and Cheung, 2017). *ALK* gain of function mutations in sympathetic precursor cells (using *dopamine-β-hydroxylase (Dβh)* promoter) require overexpression of *MYCN* (using *tyrosine-hydroxylase* (*Th*) promoter) to develop NB both in zebrafish and in a murine *Th* driven model (Matthay et al., 2016). The reason why *ALK* alone is enough to develop NB in F1174L transgenic animal model and not in others is unclear.

The search for additional familial NB genes continues: *GALNT14* was detected in three individuals from two families. Aberrant function of galactosaminyltransferases has been associated with tumor aggressiveness in various cancers, suggesting that *GALNT14* may be involved in NB predisposition or pathogenesis, yet further studies are required (Kamihara et al., 2017).

Genetic testing for mutations in genes known to be associated with NB predisposition should be considered in any individual diagnosed with NB who has a family history of NB, features suggesting predisposition such as bilateral or multifocal primary tumors, or clinical features associated with these other predisposition genes. Recently published guidelines recommend to start screening for tumor development once the mutation is identified, especially during the first decade of life (Kamihara et al., 2017).

Sporadic NB

In sporadic NB, *MYCN* amplification, defined as 10 or more copies per genome or fourfold larger signal relative to chromosome 2, is found in up to 20%–30% of cases, conferring poor prognosis (Ahmed et al., 2017; Cheung and Dyer, 2013) (**Fig. 4**). In normal development, several signaling pathways regulate its expression, which leads to proliferation, growth, differentiation and survival of neurons in developing central and peripheral nervous system (Cheung and Dyer, 2013). Intratumor heterogeneity of *MYCN* amplification has been described with coexisting amplified and nonamplified cells within tumors (Mlakar et al., 2017). Concomittant *ALK* mutation/amplification in addition to *MYCN* amplification is associated with highly lethal disease. The *MYCN* locus also encodes an antisense transcript: *MYCNOS* (encoding N-CYM), which is always coamplified and coexpressed with *MYCN*, and its

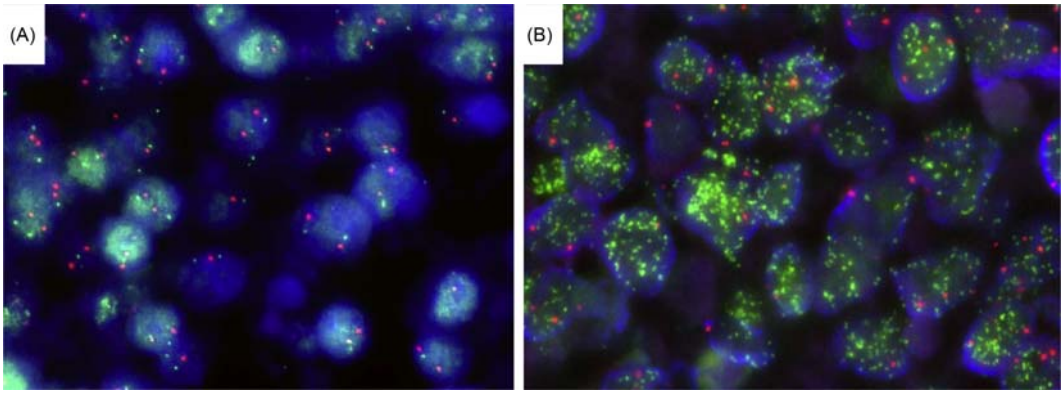

Fig. 4 Fluorescent in situ hybridization (FISH) image to detect *MYCN* amplification. The *green dots* represent MYCN and the *red signals* are for the centromere region of chromosome 2 and *blue* is DAPI counterstain labeling cell nuclei. (A) Shows a non *MYCN* amplified NB specimen with two copies for MYCN (*green dots*) and two centromeric regions in chromosome 2 (*red dots*), one per allele. (B) Shows a *MYCN* amplified NB specimen with over 10 copies of *MYCN* (*green dots*) per chromosome 2 centromeric region (*red dots*) per cell nuclei. Pictures courtesy of Dr. Yanming Zhang, Director of Cytogenetics Laboratory, Department of Pathology, MSKCC.

expression is associated with poor clinical outcome (Matthay et al., 2016). N-CYM stabilizes N-MYC by inhibiting its degradation, and mice transgenic for both *MYCN* and *MYCNOS* show frequent metastasis (Matthay et al., 2016). However, transgenic mice for *MYCNOS* alone do not develop NB (Matthay et al., 2016). MYC is a master transcription factor of cell proliferation and is considered a compelling target, though the knowledge of mechanisms affecting its expression, function and downstream targets has opened the possibility of an indirect therapeutic approach (Cheung and Dyer, 2013; Esposito et al., 2017) (**Table 1**).

The *alpha thalassemia/mental retardation syndrome X-linked* (*ATRX*) gene loss-of-function mutations are the most commonly found in older children and young adults, and its presence is mutually exclusive with *MYCN* amplification (Matthay et al., 2016; Cheung and Dyer, 2013). No *ATRX* mutations have been identified in patients <18 months, in contrast to 17% of children between 18 months and 12 years and 44% over 12 years with stage 4 disease at diagnosis, who have a poor prognosis (Cheung and Dyer, 2013). *ATRX* encodes for a SWI/SNF chromatin remodeling ATP dependant helicase (Cheung and Dyer, 2013). The role of *ATRX* in sympathoadrenal development or NB differentiation remains unknown (Cheung and Dyer, 2013). Mutations have also been associated with X-linked mental retardation and alpha-thalassemia, though in these patients no increased incidence of NB is seen (Cheung and Dyer, 2013). Most of *ATRX* mutated NBs have evidence of alternative lengthening of telomeres possibly related to a defect in histone H3.3 deposition at telomeres (Cheung and Dyer, 2013; Graeme Eisenhofer and Cheung, 2017). This highlits the importance of telomere content and length in NB, where in up to 30% of cases high telomerase activity is found, associated with adverse outcome (Cheung and Dyer, 2013). *ATRX* mutations have not been modeled although indirect targeted therapies are being evaluated.

Telomerase reverse transcriptase (*TERT*) rearrangements, have recently been detected in around 20%–30% of NB (Ahmed et al., 2017; Matthay et al., 2016; Graeme Eisenhofer and Cheung, 2017). These rearrangements are mutually exclusive from *ATRX* mutations and *MYCN* amplifications (Matthay et al., 2016). As *TERT* is also an N-MYC target, this may mean that all NB require a way to activate TERT or lengthening of telomeres, through *MYCN* amplification (*TERT* expression is elevated in *MYCN* amplified tumors), enhancer hijacking, or through *ATRX* mutations (Matthay et al., 2016).

By WGS, recurrent lesions have also been reported for genes involved in the Rac/Rho pathway and chromatin remodeling genes (*AT-rich interactive domain 1A* and *1B*: *ARID1A* and *ARID1B*) (Matthay et al., 2016; Cheung and Dyer, 2013). The role of these genes in NB initiation, progression and prognosis is yet to be determined (Cheung and Dyer, 2013).

The PI3K/AKT/mTOR pathway is involved in the development and progression of NB, though the activating molecular mechanisms of this pathway remain to be elucidated (Esposito et al., 2017). RAS pathway mutations are rare at diagnosis: *NF1* and *PTPN11* have been found mutated in 6 and 3% of patients (Holzel et al., 2010). Increased incidence of activating mutations in the MAPK pathway is seen in relapsed specimens; mutations in *ALK* as well as *NRAS*, *KRAS*, *HRAS*, *PTPN11* and *FGFR* have been described (Matthay et al., 2016).

In NB, P53 pathway (p53/MDM2/p16) is rarely mutated at diagnosis, but is frequently aberrant at relapse contributing to chemoresistance (Esposito et al., 2017). Targeting P53-MDM2 interactions, to prevent MDM2 mediated P53 inhibition, could be a therapeutic approach (Esposito et al., 2017). In NB this has shown to depend on *MYCN* status, as overexpression of *MYCN* sensitizes NB cells to MDM2 inhibition (Esposito et al., 2017).

Genetic Susceptibility for Sporadic NB

GWAS have helped pinpoint additional mutations/single nucleotide polimorphisms (SNPs) in genes that, when combined in the germline, may influence the probability of NB occurrence (Cheung and Dyer, 2013). DNA alleles that have been significantly

Table 1 Indirect MYCN—NMYC targeting strategies

Interaction	*Pathway*	*Function*	*Preclinical evidence*	*Trials*
MYCN expression inhibition	Retinoic acid PI3K/AKT/mTOR	*MYCN* promoter binding		Variable efficiency
N-MYC function/ degradation	Histone DeACelytales (HDAC)	Interaction with Sirt proteins (Marshall et al., 2011)	Favor N-MYC degradation	Vorinostat
	Aurora kinases (AURK)	Highly conserved serine/threonine kinases important in mitosis and its overexpression is associated with genetic instability and aneuploidy. Overexpressed in different human tumors. Its inhibition leads to cell cycle exit and apoptosis (Bavetsias and Linardopoulos, 2015)	AURKA inhibition leads to N-MYC degradation, as AURKA has been found to sequester N-MYC away from ubiquitin mediated proteolytic degradation. AURKB is a direct transcriptional target of N-MYC, and its increased expression is associated with poor outcomes (Esposito et al., 2017). In NB cell lines and murine models with *MYCN* amplification/ overexpression, the use of a pan-Aurora inhibitor decreased N-MYC expression and tumor growth (Bavetsias and Linardopoulos, 2015)	In clinical trials, AURKA inhibition in NB showed low efficacy, particularly in *MYCN* amplified patients. Newer drugs targeting protein-protein interaction are being developed (Esposito et al., 2017)
	N-MYC/MAX interaction	N-MYC requires the formation of an heterodimer with the MAX protein for the activation of transcription (Esposito et al., 2017)	Blockers of N-MYC/MAX interaction have been tested in vitro leading to apoptosis and neuronal differentiation. Yet to test in vivo efficacy (Esposito et al., 2017)	
	Bromodomain and extraterminal (BET)	BET proteins recognize and bind acetylated lysine residues on histone tails and are therefore considered "readers" (Varan et al., 2016)	One of the BET family members, BRD4, acts as a transcriptional coactivator of many genes including *MYC*. BRD4 inhibitors have been shown to reduce *MYC* expression by releasing BET proteins from *MYC* locus, indicating that BET proteins directly regulate *MYC* gene expression. In preclinical studies in NB cell lines, BET inhibition induced cell cycle arrest and apoptosis as well as downregulation of *MYC* expression. In murine NB models they decreased growth and significantly increased survival regardless of *MYC* amplification status. In addition to antitumor activity, neuronal differentiation was seen both in vitro and in vivo in *MYCN* amplified tumors (Varan et al., 2016)	Current open clinical trials exclude children younger than 16 years of age. Concerning potential side effects include defective spermatogenesis, viral reactivation and altered bone formation, which could impact growth (Varan et al., 2016)

Ornitine decarboxylase 1 (ODC1)	Rate limiting enzyme in polyamine synthesis, and its level is increased in highly metabolic active cells	Known target of N-MYC. Difluoromethylornithine (DFMO) is an anti-protozoal drug that causes irreversible inhibition of ODC1, and is being investigated as maintenance therapy in NB patients.	In a phase II study on high risk patients in remission after standard chemotherapy, the addition of DFMO maintenance was well tolerated (Esposito et al., 2017)
Tropomyosin Receptor Kinase (TRK) family of neurotrophin receptors	TrkA and its ligand, nerve growth factor (NGF), are required for neuronal differentiation	TrkA (coded by *NTRK1*) expression is increased in NB with favorable biology and spontaneous regression. NTRK1 polymorphisms have been have been associated with reduced survival. In high risk patients, TrkA expression is low or absent. TrkB (coded by *NTRK2*) and its ligand, Brain-derived neurotrophic factor (BDNF), are highly expressed in biologically unfavorable NB and associated with *MYCN* amplification, drug resistance and increased proangiogenic factors. No mutations or rearrangements have been found in *NTRK2*. Entrectinib, a novel ALK inhibitor, has shown to be effective for TrkB dependent NB in preclinical models. Preferential TrkB inhibitors are currently being developed with good in vitro activities (Esposito et al., 2017)	
ALK	ALK activation of the phosphoinositide-3-kinase (PI3K) signaling, stabilizes N-MYC. ALK also signals through RAS, with downstream activation of the MAPK pathway.	*ALK* and *MYCN* can be co-amplified, given its close localization in chromosome 2, associated with advanced stage at presentation and poor prognosis (Ahmed et al., 2017; Matthay et al., 2016). The synergy may be related to downstream activation of the MAPK pathway, which is frequently activated in relapsed NB	Targeted therapeutic interventions using ALK and MAPK pathway inhibition in NB, currently underway

associated with the development of high and low risk NB predisposition include *CASC15*, *BARD1*, *LMO1*, *LIN28B*, *HACE1*, *DUSP12*, *DDX4*, *IL31RA*, *HSD17B12*, *NEFL*, *TP53* and *NBPF23* (Cheung and Dyer, 2013).

A few germline mutations affecting DNA repair genes including *CHEK2*, *PINK1*, *BARD1* and *PALB2* have been found in NB patients (Cao et al., 2017). A GWAS study of high risk NB identified up to six SNPs in *BRCA1-associated RING domain 1* (*BARD1*), all affecting three different N-terminal introns (Tolbert et al., 2017). In depth studies into BARD1 have found an isoform, *BARD1b*, which lacked the RING domain for BRCA1 binding, as preferentially expressed in NB cell lines homozygous for the risk alleles (Tolbert et al., 2017). Knockdown of this isoform inhibits cell growth, whereas overexpression increases proliferation. Additionally, BARD1b was found to stabilize the Aurora family of kinases in NB cell lines, suggesting a possible mechanism of action and therapeutic strategy (Tolbert et al., 2017). Four SNPs on *LMO1* gene, were also shown to be associated with high risk disease and decreased survival (Tolbert et al., 2017). This gene encodes for a cysteine-rich transcriptional cofactor preferentially expressed in the nervous system. Both *BARD1* and *LMO1* risk alleles seem to be more prevalent in the African–American population, which may underlie the more aggressive disease observed in these patients (Cheung and Dyer, 2013).

LIN28B encodes an RNA binding protein developmentally regulated that blocks the expression of *let-7* family of micro-RNAs (Matthay et al., 2016). *LIN28B* and *let-7* are involved in stem cell differentiation with opposing effects, inhibiting and promoting differentiation, respectively. *LIN28B* was expressed at significantly higher levels in NB lines homozygous for the risk allele, which correlated with low levels of *let-7*, and its inhibition decreased cell growth (Matthay et al., 2016). In a murine model, forced expression of *LIN28B* under the *Dbh* promoter drives development of NB with high levels of N-MYC (Matthay et al., 2016). Mechanistic studies have shown that besides depleting *let-7* family of micro-RNAs, LIN28B modulates the activity of the GTP-binding nuclear protein RAN and the stability of Aurora Kinase A (AURKA), pointing at potential therapeutic benefits on using AURKA inhibitors in these patients (Matthay et al., 2016).

Chromosomal Instability

Ploidy is one of the strongest prognostic determinants in NB (Cheung and Dyer, 2013). In general, low/intermediate and 4S NB have whole chromosomal gains (hyperdiploidy) and high risk have intrachromosomal segmental rearrangements (Cheung and Dyer, 2013). It is thought that together with oncogene amplifications, large scale genomic alterations may deregulate messenger RNAs, micro RNAs and other non coding RNAs, that interfere with apoptosis, differentiation and immune surveillance (Cheung and Dyer, 2013). The incidence of segmental chromosomal aberrations increases with age at diagnosis (from infants to children) and is a predictor of outcome (Cheung and Dyer, 2013). Markers of poor prognosis include losses of 1p, 11q and gain of 17q, present in up to 35%, 40% and 50% respectively (Ahmed et al., 2017).

Loss of 11q is associated with older age (30%–60% in adolescents and young adults), no *MYCN* amplification and more chromosomal breaks (commonly associated with 17q gain and 3p loss) (Mlakar et al., 2017). It is reported in 20%–45% of all high risk NB, depending on detection method used (Matthay et al., 2016). Loss of heterozygosity has been detected in 34%–44% of cases, also associated with high risk features (Mlakar et al., 2017). 11q23 is the region most commonly deleted (Mlakar et al., 2017). Chromosomal instability is one of the main features of 11q deleted tumors (Mlakar et al., 2017). Mapping studies are trying to determine the candidate coding genes (*CCND1*,*CHK1*), and miRNAs within that region; none alone has to date mimicked 11q loss (Mlakar et al., 2017).

Chromothripsis, that occurs in 2%–3% of human tumors, has been described in up to 18% of stage 4 tumors though there is variability within different series (Cheung and Dyer, 2013).

Some recurrent segmental alterations also occur at relapse, including 1p and 6q deletions (Matthay et al., 2016). Presence of candidate tumor suppressor genes in deleted regions of 1p have been identified: *CHD5*, *CAMTA1*, *KIF1B*, *CASZ1* and *mir-34A* (*Matthay et al., 2016*).

NB Methylome

The NB genome is epigenetically distinct, being largely hypomethylated (Decock et al., 2016). Differences at an epigenomic level cluster in groups that correlate with clinicobiological features (Decock et al., 2016). NB methylation changes affect all chromosomes, with hypomethylated regions being enriched in chromosome 6, and hypermethylated regions being more abundant in chromosomes 1 and 5 (Decock et al., 2016).

DNA methylation of cytosines in the context of CpG dinucleotides is a major regulatory mechanism implicated in cellular processes critical for mammalian development (Gomez et al., 2015). At a CpG level, high risk NB tumors show higher levels of hypermethylation compared to low risk tumors, targeting mainly enhancers and polycomb repressed regions, affecting genes involved in differentiation, neuron development and biosynthetic processes. Low risk tumors show limited number of de novo CpG hypermethilated sites, mostly in promoter and enhancer related regions of genes, including *LIN28B*. Hypomethylation in low risk tumors is seen in genes related to development (*FOXP1*, *SOX13*, *RARRES3*) and in high risk tumors, in genes related to regulation of gene expression and RNA processing.

The methylome of human embryonic stem cells as well as adult human and mouse brain cortex is enriched for methylation in non-CpG sites (Gomez et al., 2015). Similar to CpG methylation, non-CpG methylation goes through a process of reconfiguration during development (Gomez et al., 2015). Significant non-CpG methylation changes have been found to correlate with NB clinicobiologic features: higher non-CpG methylation was found to be associated with favorable biology, wheras in

unfavorable tumors, non-CpG methylation was very low or absent (Gomez et al., 2015). *ALK* is one of the genes found to be regulated by non-CpG methylation in clinically favorable tumors rendering low *ALK* expression levels, in contrast to unfavorable tumors (Gomez et al., 2015). In pre- and post chemotherapy coupled samples where treatment related histological signs of differentiation were seen, higher levels of non-CpG site methylation were also found, showing that non-CpG methylation is modulated during treatment induced NB differentiation, and may be associated with changes in *ALK* gene expression(Gomez et al., 2015).

Tumor Regression

Several mechanisms have been proposed to explain the phenomenon of spontaneous regression of NB, including neurotrophin deprivation, humoral or cellular immune response, loss of telomerase activity or alterations in epigenetic regulation (Matthay et al., 2016; Ratner et al., 2016).

High TrkA, a neurotrophin receptor, is associated with favorable clinical and biological features. These tumors in vitro undergo neuronal differentiation when exposed to nerve growth factor (NGF, TrkA ligand), and these same cells die within a week when deprived from NGF. These culture conditions recapitulate the behavior of high TrkA tumors which either undergo differentiation or regression depending on the presence of NGF in the microenvironment. Migrating neural crest precursors (and favorable NB with high TrkA) can survive despite a lack of NGF in the microenvironment upon the expression of TrkAIII, a constitutively active alternatively spliced isoform. Thus, the conversion from TrkA, NGF independent to an NGF dependent tumor, could be a consequence of a developmentally programmed switch from TrkAIII to TrkA. NGF depletion in developing sympathetic neurons induces pro-apoptotic gene expression: *TP53*, *TP63*, *E2F1*, *UNC5D*, *KIF1B* and *PRUNE2*, which are also expressed at high levels in favorable NB tumors, including 4S (Matthay et al., 2016).

High risk disease evades the attack from the immune system by downregulating the expression of HLA class I molecules (Cheung and Dyer, 2013). On the contrary, patients with 4S NB express normal levels of class I HLA, which may augment immune surveillance and response (Ratner et al., 2016).

Telomeres are important for chromosome replication and stability, and its length is partly controlled by telomerases (Ratner et al., 2016). Telomerase activity is low in 4S NB, consistent with absence of *TERT* expression, suggesting that telomere crisis may also have a role in tumor regression (Ratner et al., 2016).

Finally, epigenetic developmental changes affecting DNA methylation, histone modifications and chromatin remodeling, may impact the survival of these cells and lead to spontaneous regression (Ratner et al., 2016).

Risk Stratification

Age and stage at diagnosis together with the presence of *MYCN* amplification, are the three strongest determinants of outcome (Cheung and Dyer, 2013). Those features, combined with loss of 11q, histological properties and ploidy, are the foundations of the current risk stratified classification of NB. There are two different staging systems currently in use, with the main difference being presurgical (International Neuroblastoma Risk Group, INRG) vs post-surgical (Intenational Neuroblastoma Staging System, INSS) tumor extent evaluation (**Table 2**). The benefit of the INRG classification is that it makes pretreatment patient cohorts comparable among different groups, with no surgical bias. Extent of disease is stablished upon the absence (L1) or presence (L2) of image defined risk factors (IDRFs) for locoregional tumors (Cohn et al., 2009). IDRFs refer to involvement of anatomical structures that increase surgical risk (Monclair et al., 2009). Stage M refers to widespread metastatic disease. Stage MS (S for special) refers to distant disease in children 0–18 months, limited to skin, liver and $<10\%$ bone marrow with no cortical bone involvement (Cohn et al., 2009). Besides staging evaluation, the INRG classification expands the infant group up to 18 months of age (Cohn et al., 2009), a major shift in risk classification that spares the 12–18 month age group of high dose chemotherapy and radiation. The INRG is a more granular definition of pretreatment risk group classification among the very low, low, intermediate and high risk categories (Cohn et al., 2009).

Table 2 International neuroblastoma staging system

Stage 1	Localized NB not crossing midline. Complete gross surgical excision. No metastasis to ipsilateral lymph nodes that were not attached to the tumor
Stage 2A	Localized NB not crossing midline but incomplete surgical resection
Stage 2B	Localized NB not crossing midline but presence of ipsilateral lymph node involvement
Stage 3	NB crossing midline and unresectable, with or without regional lymph node involvement Localized NB not crossing midline with bilateral lymph node involvement Localized NB with epidural extension
Stage 4	Any or no primary, with metastasis to distant sites (except 4S distribution)
Stage 4S	Localized primary (stage 1-2B), <1 yo, metastases limited to skin, liver or bone marrow ($<10\%$ involvement)

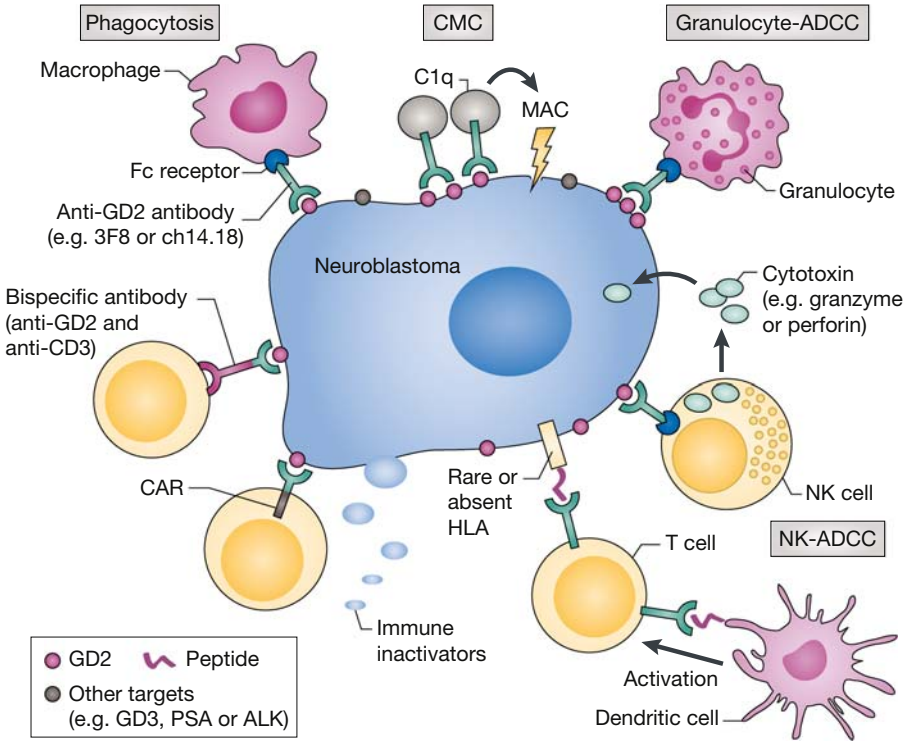

Fig. 5 *Immunotherapy against NB*: NB evades T-cells by downregulating or losing HLA expression, thereby interfering with the afferent arm (priming through dendritic cells), homing of T-cells to NB, and the CTL effector phase of adaptive immunity. Soluble inhibitors of immune response (e.g., FasL, gangliosides) are constantly released into the tumor stroma to impair cellular immunity. In addition, NB recruits pro-tumor macrophages and silences NK cells. Myeloid suppressor cells and T-reg can also suppress immunity. The paucity of mutations in NB compared to adult cancers like melanoma, the immaturity of the immune system in young patients, their massive disease and the intensive chemotherapy all combine to make NB poorly immunogenic for T-cells. Carbohydrate differentiation antigens (e.g., GD2, GD3 and polysialic acid (PSA)), all of which being classically T-independent antigens, offered alternative targets for antibody-based therapies. In the presence of monoclonal antibodies (e.g., 3F8 or ch14.18) specific for GD2, NB loses their defense and becomes highly susceptible to (1) NK (natural killer) cell mediated antibody-dependent cell mediated cytotoxicity (ADCC), (2) granulocyte mediated ADCC (3), complement mediated cytotoxicity by binding to C1q thereby activating the complement cascade, delivering membrane attack complex (MAC) to tumor cell membrane, and (4) monocyte-macrophage mediated cytotoxicity. Even polyclonal T-cells can be retargeted to kill NB through MAbs in the form of chimeric antigen receptors (CAR) or bispecific antibodies (anti-GD2 x anti-CD3). CARs are anti-tumor single chain Fv fragments (scFv) genetically fused through a transmembrane domain to T-cell activating motifs (CD3 ξ and CD28/41BB) and transfected into killer lymphocytes. Reprinted from Cheung, N. K, Dyer, M. A. (2013). Neuroblastoma: Developmental biology, cancer genomics and immunotherapy *Nature Reviews Cancer* **13**, 397–411, https://doi.org/10.1038/nrc3526.

Immune Microenvironment and Immunotherapy

Beyond the paucity of somatic mutations that confers NB low immunogenicity, these tumors have developed an immunosuppressive microenvironment to protect themselves from the development and function of effective immune response (Cheung and Dyer, 2013) (**Fig. 5**).

Immune Evasion

NB escapes T cells and natural killer (NK) cell surveillance by downregulation of HLA and adhesion molecules (Cheung and Dyer, 2013). NB cells can express or release proteins that inhibit or eliminate T cells and NK cells, and recruit tissue macrophages to help disable these lymphocytes (Cheung and Dyer, 2013). Patients older than 18 months with *MYCN* nonamplified tumors, have shown to have higher expression of inflammatory genes in tumor associated macrophages (TAMs), such as *CD33*, *FCGR3* (also known as *CD16*), *IL6R*, *IL10* and *CD14*, associated with adverse prognosis. Increased levels of prostaglandin E_2 have been seen in 11q deleted tumors (Matthay et al., 2016). How macrophages or other myelolid cells affect tumor growth is yet to be discerned: IL6R, expressed in NB cells, binds IL6, produced by TAMs, and activates signal transducer and activator of transcription 3 (STAT3) transcriptional program, which affects proliferation, drug resistance and immune evasion(Matthay et al., 2016).

NB cells also express on their surface ganglioside and sialic acid containing sugars and proteins important for migration, adhesion and metastasis (Cheung and Dyer, 2013). These epitopes are poorly immunogenic and in preclinical models immunosuppressive (Cheung and Dyer, 2013). Development of natural host antibodies against NB is rare; hence, NB survives in circulation despite the lack of complement decay accelerating factor (CD55). It can also express protectin (CD59) to resist complement mediated lysis

(Cheung and Dyer, 2013). While NB downregulates HLA to evade T cells, it can also re-express HLA to resist NK cell antibody dependent cell mediated cytotoxicity (NK-ADCC) when treated with monoclonal antibodies (MAbs) (Cheung and Dyer, 2013). NB may also escape to or hide in immune sanctuaries like the CNS (Cheung and Dyer, 2013). In fact, the increasing frequency of CNS, soft tissues and bone relapses, is creating a new challenge in the era of MAb therapy (Cheung and Dyer, 2013; Kramer et al., 2010).

Monoclonal Antibody (MAb) Therapies

GD2 is an oncofetal differentiation antigen expressed during fetal development in maturing neurons, pain fibers and skin cells (Cheung and Dyer, 2013). It belongs to a unique class of T cell independent carbohydrate antigens with high density, membrane proximity, homogeneity within and across NBs and rare occurrence of antigen loss (Cheung and Dyer, 2013). Research over the past 20 years has demonstrated that high risk patients can be maintained in remission with anti-GD2-specific MAb therapy, and therefore it is currently standard of care for these patients (Cheung and Dyer, 2013; Kushner, 2017; Cheung et al., 2012) (**Fig. 6**). Granulocyte and NK cell mediated ADCC are important effector mechanisms (Cheung and Dyer, 2013). By activating effector cells, anti-GD2 MAb therapy can be more effective, and for that purpose, regimens include concomitant administration of IL-2 and/or granulocyte-macrophage colony stimulating factor (GM-CSF) (Cheung and Dyer, 2013; Yu et al., 2010). IL-2 activates NK cells and has a modest effect against NB as a single agent, though has significant toxicity with the development of capillary leak syndrome (Cheung and Dyer, 2013). IL-15 has been shown to activate NK, NK-T and CD8 T cells without causing capillary leak syndrome, activation-induced cell death or increased T_{reg} activation in primates (Cheung and Dyer, 2013). It could be an alternative to IL-2.

Five different intravenous antibodies have been tested clinically: 14G.2a, chimeric 14.18 (ch14.18, dinutuximab, Unituxin™), hu14.18-K322A, murine m3F8 and humanized hu3F8. Intravenous ch14.18 administered with (GM-CSF), IL-2 and oral isotretinoin was shown to improve OS, but not EFS in a randomized trial. 3F8 with subcutaneous GM-CSF m3F8 + GM-CSF cleared BM of chemoresistant histologically-evident NB in 87% of patients with primary refractory disease (Cheung et al., 2014) and improving 10-year long-term survival of these patients (45%), as well as those treated in ≥second remission (44%) (Kushner et al., 2015) and in first remission (67%) (Cheung et al., 2012; Kushner et al., 2015), among high risk stage 4 patients. Dose is limited by pain, believed to be secondary to complement activation (Cheung and Dyer, 2013). Mutations in the Fc region and complement blocking antibodies have been tested to decrease complement activation, ameliorating though not eradicating pain (Cheung and Dyer, 2013). The presence of minimal residual disease (MRD) after two cycles of immunotherapy is predictive of treatment failure (Cheung and Dyer, 2013).

One critical observation in patients receiving anti-GD2 MAb therapy is the induction of a host anti-tumor or antiidiotype response, which may be important for long term tumor control (Cheung and Dyer, 2013). Human antimouse Ab (HAMA), an indirect measure of the host antiidiotype network, correlated with long term survival in patients that received murine 3F8 (Cheung and Dyer, 2013). This observation led to the development of antiidiotypic vaccines, though their efficacy may be limited by their narrow target coverage (Cheung and Dyer, 2013). The oligosaccharide GD2 antigen has also been conjugated to keyhole limpet hemocyanin (KLH) to overcome poor immunogenicity producing long term remission (Cheung and Dyer, 2013; Kushner et al., 2014).

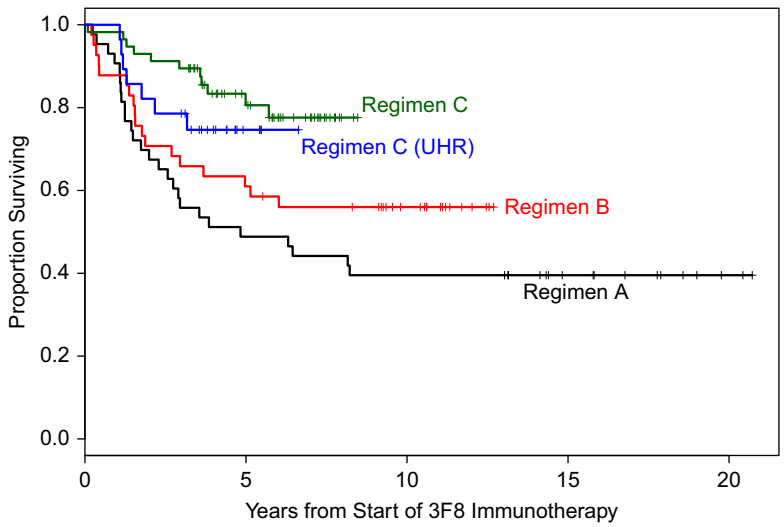

Fig. 6 *Kaplan Meier Survival curves of High Risk NB patients from initiation of anti-GD2 immunotherapy.* Overall survival (OS) among 169 patients with stage 4 neuroblastoma in first remission after consecutive immunotherapy regimens: 3F8 alone (regimen A–high risk [HR]; $n = 43$), 3F8 + intravenous granulocyte-macrophage colony-stimulating factor (GM-CSF) + 13-*cis*-retinoic acid (CRA; regimen B-HR; $n = 41$), and 3F8 + subcutaneous GM-CSF + CRA (regimen C-HR; $n = 57$ and regimen C–ultra HR [UHR]; $n = 28$); $P = 0.003$ (derived from log-rank test to compare OS among these four groups). Reprinted with permission. © (2017) American Society of Clinical Oncology. All rights reserved. Cheung, N. K. et al. (2012). Murine anti-GD2 monoclonal antibody 3F8 combined with granulocyte-macrophage colony-stimulating factor and 13-cis-retinoic acid in high-risk patients with stage 4 neuroblastoma in first remission. *Journal of Clinical Oncology*, **30**, 3264–3270.

GD2 has also been used as a target using antibodies for selective delivery of radioisotopes, nanoparticles and liposomes (Cheung and Dyer, 2013).

In the study of other potential surface targets, candidates such as GD3, GPC2, ALK, polisyalic acid, L1CAM and B7-H3 are being evaluated (Cheung and Dyer, 2013; Ahmed et al., 2015; Bosse et al., 2017). B7-H3 specific MAb 8H9 has been used successfully in compartimental radioimmunotherapy of CNS NB metastasis, a complication previously incurable (Kramer et al., 2010). More recently, a drug conjugated antibody against GPC2 has shown promising in vitro data (Bosse et al., 2017).

T Cell Mediated Immunity

Recovery of T cell function after high dose chemotherapy is required for effectiveness of T cell mediated strategies. NB antigens that can be recognized by cytotoxic T cells (CTLs) exist, such as cancer testes antigens, N-MYC and survivin; however, the absence or down regulation of HLA expression can severely limit CTL efficiacy (Cheung and Dyer, 2013). Despite preclinical efficacies in animal models, there has been limited success in clinical trials with the use of vaccines for classic T cell mediated adaptive immunity (Cheung and Dyer, 2013). Dendritic cell vaccines are also being developed without much clinical success (Cheung and Dyer, 2013).

The use of immune checkpoint inhibitors is being studied in many pediatric tumors including NB (Wagner and Adams, 2017). The Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4) was the first target for immune checkpoint inhibitiors (ICI), which binds to B7.1 and B7.2. CTLA4 expression occurs upon T cell activation, and once present curtails T cell activity (O'Donnell et al., 2017). Programmed Death 1 (PD-1) is another important checkpoint, when binding to its ligand (PD-L1), halts T cell activation and induces T cell exhaustion (O'Donnell et al., 2017). PD-L1 is expressed in a variety of different tumors, dendritic cells, macrophages and T cells (Wagner and Adams, 2017; O'Donnell et al., 2017). There is increasing evidence of increased PD-L1 expression with mutational burden or microsatellite instability in some pediatric sarcomas, NB and high-grade gliomas. Despite the expression of PD-L1 in up to 70%–100% NB tumors studied and the association of lower survival with higher PD-L1 expression, clinical results with ICIs have been disappointing (Wagner and Adams, 2017). The presence of inflammation (through interferon gamma) and hypoxia within a tumor have been shown to upregulate PD-L1 expression (Wagner and Adams, 2017). Interestingly, anti-GD2 therapy can induce cytokines within tumors, including interferon gamma, *ergo* enhancing PD-L1 tumor expression, suggesting a potential benefit of combined anti-GD2 and anti PD-L1 therapy to invigorate NK-ADCC (Wagner and Adams, 2017).

Given that autologous tumor reactive T-cells rarely develop in these patients, genetically redirected T cells, using chimeric antigen receptors (CARs) (Cheung and Dyer, 2013) and bispecific antibodies (BsAb) (Wu and Cheung, 2017) hold promise. Anti-GD2 and anti-L1CAM CAR-T cells have been tested, though the persistence of these cells was short-lived (Cheung and Dyer, 2013). To enhance survival, Epstein Barr virus (EBV) specific CTLs, which are life long from continuous antigenic challenge, have also been used(Cheung and Dyer, 2013). These dual specific T cells, anti-EBV through its T-cell receptor and anti-GD2 through the CAR, seem to persist and control the tumor for years (Cheung and Dyer, 2013). Similar strategies for NK and NKT cells are being explored. More recent studies highlight the exhaustion of CAR-T cells when they come into contact with tumor or antigen. BsAb (CD3xGD2) are showing great promise whereby polyclonal T cells can be activated without exhaustion to infiltrate neuroblastomas turning them from "cold" into "inflamed," overcoming PD-L1 resistance, leading to tumor ablation (O'Donnell et al., 2017). Here ICIs have shown benefit in preclinical models and clinical results should soon become available.

Conclusions and Prospective Vision

Neuroblastoma is a very heterogeneous disease which exemplifies how both tumor and host developmental biology intertwine at presentation, marked by a molecular and immunologic signature that determine tumor behavior and prognosis. While some tumors regress spontaneously with >95% cure rates, others are lethal despite multimodal therapy. Aside from *ALK* and *ATRX*, the low mutanome has limited the general application of gene-specific targeted therapies, though increased use of WGS and epigenomics may identify subgroups of high risk patients which might benefit from pathway-specific targeted therapies. On the other hand, NB has been one of the most successful stories for immunotherapy with the use of MAb, where prolonged survival is sustained over decades. Therapeutic strategies to engage T cells, vaccinate host immune system while decreasing treatment side effects, should further improve the prognosis for a metastatic solid tumor whose cure was unthinkable just two decades ago.

See also: Neuroblastoma: Diagnosis and Treatment.

References

Ahmed, M., et al., 2015. Humanized affinity-matured monoclonal antibody 8H9 has potent antitumor activity and binds to FG loop of tumor antigen B7-H3. Journal of Biological Chemistry 290, 30018–30029. https://doi.org/10.1074/jbc.M115.679852.

Ahmed, A.A., Zhang, L., Reddivalla, N., Hetherington, M., 2017. Neuroblastoma in children: Update on clinicopathologic and genetic prognostic factors. Pediatric Hematology and Oncology 1–21. https://doi.org/10.1080/08880018.2017.1330375.

Bavetsias, V., Linardopoulos, S., 2015. Aurora kinase inhibitors: Current status and outlook. Frontiers in Oncology 5, 278. https://doi.org/10.3389/fonc.2015.00278.
Bosse, K.R., et al., 2017. Identification of GPC2 as an oncoprotein and candidate immunotherapeutic target in high-risk neuroblastoma. Cancer Cell 32, 295–309e12. https://doi.org/10.1016/j.ccell.2017.08.003.
Cao, Y., et al., 2017. Research progress of neuroblastoma related gene variations. Oncotarget 8, 18444–18455. https://doi.org/10.18632/oncotarget.14408.
Cheung, N.K., Dyer, M.A., 2013. Neuroblastoma: Developmental biology, cancer genomics and immunotherapy. Nature Reviews. Cancer 13, 397–411. https://doi.org/10.1038/nrc3526.
Cheung, N.K., et al., 2012. Murine anti-GD2 monoclonal antibody 3F8 combined with granulocyte-macrophage Colony-stimulating factor and 13-Cis-retinoic acid in high-risk patients with stage 4 Neuroblastoma in first remission. Journal of Clinical Oncology 30, 3264–3270. https://doi.org/10.1200/JCO.2011.41.3807.
Cheung, N.K., et al., 2014. Key role for myeloid cells: Phase II results of anti-GD2 antibody 3F8 plus granulocyte-macrophage colony-stimulating factor for chemoresistant osteomedullary neuroblastoma. International Journal of Cancer 135, 2199–2205. https://doi.org/10.1002/ijc.28851.
Cohn, S.L., et al., 2009. The international Neuroblastoma risk group (INRG) classification system: An INRG task force report. Journal of Clinical Oncology 27, 289–297.
Decock, A., et al., 2016. Stage 4S neuroblastoma tumors show a characteristic DNA methylation portrait. Epigenetics. https://doi.org/10.1080/15592294.2016.1226739.
Esposito, M.R., Aveic, S., Seydel, A., Tonini, G.P., 2017. Neuroblastoma treatment in the post-genomic era. Journal of Biomedical Science 24, 14. https://doi.org/10.1186/s12929-017-0319-y.
Gomez, S., et al., 2015. DNA methylation fingerprint of neuroblastoma reveals new biological and clinical insights. Epigenomics 7, 1137–1153. https://doi.org/10.2217/epi.15.49.
Graeme Eisenhofer, S.G., Cheung, N.-K.V. (Eds.), 2017. Tietz textbook of clinical chemistry and molecular diagnostics. Elsevier, Saunders, St Louis, MO. Chapter 63.
van Groningen, T., et al., 2017. Neuroblastoma is composed of two super-enhancer-associated differentiation states. Nature Genetics. https://doi.org/10.1038/ng.3899.
Holzel, M., et al., 2010. NF1 is a tumor suppressor in neuroblastoma that determines retinoic acid response and disease outcome. Cell 142, 218–229. https://doi.org/10.1016/j.cell.2010.06.004.
Kamihara, J., et al., 2017. Retinoblastoma and Neuroblastoma predisposition and surveillance. Clinical Cancer Research 23, e98–e106. https://doi.org/10.1158/1078-0432.CCR-17-0652.
Kramer, K., Kushner, B., Heller, G., Cheung, N.K., 2001. Neuroblastoma metastatic to the central nervous system. The Memorial Sloan-kettering cancer center experience and a literature review. Cancer 91, 1510–1519.
Kramer, K., et al., 2010. Compartmental intrathecal radioimmunotherapy: Results for treatment for metastatic CNS neuroblastoma. Journal of Neuro-Oncology 97, 409–418. https://doi.org/10.1007/s11060-009-0038-7.
Kushner, B.H., 2017. Chemoimmunotherapy for high-risk neuroblastoma. The Lancet Oncology 18, 845–846. https://doi.org/10.1016/s1470-2045(17)30337-6.
Kushner, B.H., et al., 2014. Phase I trial of a bivalent gangliosides vaccine in combination with beta-glucan for high-risk neuroblastoma in second or later remission. Clinical Cancer Research. https://doi.org/10.1158/1078-0432.CCR-13-1012.
Kushner, B.H., et al., 2015. Prolonged progression-free survival after consolidating second or later remissions of neuroblastoma with Anti-GD2 immunotherapy and isotretinoin: A prospective Phase II study. Oncolmmunology 4, e1016704. https://doi.org/10.1080/2162402X.2015.1016704.
Lee, J.W., et al., 2017. ALK protein expression is related to neuroblastoma aggressiveness, but is not independent prognostic factor. Cancer Research and Treatment. https://doi.org/10.4143/crt.2016.577.
Marshall, G.M., et al., 2011. SIRT1 promotes N-Myc oncogenesis through a positive feedback loop involving the effects of MKP3 and ERK on N-Myc protein stability. PLoS Genetics 7, e1002135. https://doi.org/10.1371/journal.pgen.1002135.
Matthay, K.K., et al., 2016. Neuroblastoma. Nature reviews. Disease primers 2, 16078. https://doi.org/10.1038/nrdp.2016.78.
Mlakar, V., et al., 2017. 11q deletion in neuroblastoma: A review of biological and clinical implications. Molecular Cancer 16, 114. https://doi.org/10.1186/s12943-017-0686-8.
Monclair, T., et al., 2009. The international Neuroblastoma risk group (INRG) staging system: An INRG task force report. Journal of Clinical Oncology 27, 298–303.
van Noesel, M.M., 2012. Neuroblastoma stage 4S: A multifocal stem-cell disease of the developing neural crest. The Lancet Oncology 13, 229–230. https://doi.org/10.1016/s1470-2045(12)70012-8.
O'Donnell, J.S., Long, G.V., Scolyer, R.A., Teng, M.W., Smyth, M.J., 2017. Resistance to PD1/PDL1 checkpoint inhibition. Cancer Treatment Reviews 52, 71–81. https://doi.org/10.1016/j.ctrv.2016.11.007.
Peinemann, F., van Dalen, E.C., Tushabe, D.A., Berthold, F., 2015. Retinoic acid post consolidation therapy for high-risk neuroblastoma patients treated with autologous hematopoietic stem cell transplantation. Cochrane Database of Systematic Reviews 1, CD010685. https://doi.org/10.1002/14651858.CD010685.pub2.
Ratner, N., Brodeur, G.M., Dale, R.C., Schor, N.F., 2016. The "neuro" of neuroblastoma: Neuroblastoma as a neurodevelopmental disorder. Annals of Neurology 80, 13–23. https://doi.org/10.1002/ana.24659.
Takahashi, Y., Sipp, D., Enomoto, H., 2013. Tissue interactions in neural crest cell development and disease. Science 341, 860–863. https://doi.org/10.1126/science.1230717.
Tolbert, V.P., Coggins, G.E., Maris, J.M., 2017. Genetic susceptibility to neuroblastoma. Current Opinion in Genetics & Development 42, 81–90. https://doi.org/10.1016/j.gde.2017.03.008.
Varan, A., et al., 2016. Neurofibromatosis type 1 and malignancy in childhood. Clinical Genetics 89, 341–345. https://doi.org/10.1111/cge.12625.
Wagner, L.M., Adams, V.R., 2017. Targeting the PD-1 pathway in pediatric solid tumors and brain tumors. OncoTargets and Therapy 10, 2097–2106. https://doi.org/10.2147/OTT.S124008.
Wang, M., Zhou, C., Cai, R., Li, Y., Gong, L., 2013. Copy number gain of MYCN gene is a recurrent genetic aberration and favorable prognostic factor in Chinese pediatric neuroblastoma patients. Diagnostic Pathology 8, 5. https://doi.org/10.1186/1746-1596-8-5.
Whittle, S.B., et al., 2017. Overview and recent advances in the treatment of neuroblastoma. Expert Review of Anticancer Therapy 17, 369–386. https://doi.org/10.1080/14737140.2017.1285230.
Wu, Z., Cheung, N.V., 2017. T cell engaging bispecific antibody (T-BsAb): From technology to therapeutics. Pharmacology & Therapeutics. https://doi.org/10.1016/j.pharmthera.2017.08.005.
Yu, A.L., et al., 2010. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. The New England Journal of Medicine 363, 1324–1334. https://doi.org/10.1056/NEJMoa0911123.

Further Reading

Brodeur, G.M., Hogarty, M.D., Bagatell, R., Mossé, Y.P., Maris, J.M., 2015. Pizzo and Poplack: Principles and practice of pediatric oncology. Neuroblastoma, 7th Edition, pp. 772–792. Chapter 30.

New Rationales and Designs for Clinical Trials in the Era of Precision Medicine

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Glossary

Basket study A study that treats various cancers based on genomic characteristics and pools patients independent of histologic diagnosis.

Master protocol A study that combines the features of basket, umbrella, and platform studies testing multiple histologies with multiple platforms.

Matched therapy Providing a therapeutic regimen which incorporates at least one targeted agent which is specific to an identified tumor genetic alteration.

Molecular profiling The identification and documentation of the structure of a specific DNA, RNA, or protein molecules, in particular the alterations for standard present in tumors.

Octopus study A study that tests multiple drugs.

Platform study A study based on a technologic biomarker platform.

Precision medicine The customization of medical decisions, practices, or products which are tailored to the individual patient.

Umbrella study These studies enroll patients who share a tumor type, test molecular markers for a wide variety of potential molecular targets, and then assign patients to an arm of the study.

Introduction

The vast and rapidly expanding body of knowledge about cancer genomics has enabled the development of therapies targeted to specific molecular alterations or other biomarkers. While standard late-stage oncology clinical trials are histology specific, this model is suboptimal for assessing the efficacy of genomically targeted agents. It may also be suboptimal for evaluation of immunotherapy, as recent studies indicate that response to immunotherapy (checkpoint blockade with anti-PD-1/PD-L1 agents) can be predicted based on genomic markers such as microsatellite instability high (MSI-H) or high tumor mutational burden (TMB). The goal of precision medicine-based trials is to generate insight into relationships between biomarkers in tumors and responses. The most successful biomarkers to date have been genomic, but other biomarkers based on protein assays, transcriptomics, and more are emerging. One of the most important observations emerging from next generation sequencing (NGS) of solid tumors is that molecular alterations do not segregate by tumor organ of origin. Further, metastatic solid tumors harbor complex and unique genomic landscapes—no two are alike. The term "malignant snowflakes" has been coined to reflect this heterogeneity and distinctness.

These observations present a conundrum for the practice of precision medicine. In order to target tumors "precisely," it appears that therapy must be "personalized." Hence, the reality unveiled by genomic science, that is, the need for N-of-one treatments, does not fit well with canonical trial design that attempts to find commonalities between patients and treat them alike. Novel clinical trial designs are necessary. Most likely, these innovative designs will eventually focus on a unified strategy for assigning patients to treatment, but will permit different drugs for each patient based on the aberrations in their cancer. Herein, we review the evolution/revolution that is taking place in clinical research in the era of precision medicine.

Basket, Umbrella, Platform, Octopus, and Master Protocols

The cornerstone of traditional clinical trials in oncology has been finding commonalities between patients so that they can be fit onto a specific drug regimen (drug-centered trial). Historically, the commonality was organ of origin of the tumor (e.g., breast cancer trial or lung cancer trial). Newer designs base eligibility on biomarker commonalities. For instance, basket trials will treat a single gene alteration across multiple histologies (e.g., *BRAF*-altered basket trial). Other new types of trials include the following: umbrella trials, which examine multiple genomic alterations within a single histology; platform trials, which include multiple histologies and genomic or other biomarkers, but are based on a single technological platform (e.g., NGS) to interrogate the tumor; octopus trials (previously coined complete phase I trials when performed in the phase I setting) that have multiple arms testing different drug combinations; and master protocols, which can be comprised of multiple histologic arms (previously known as "broad phase II trials") or multiple basket or umbrella subtrials, trials, and/or multiple platforms. The ultimate evolution of the master protocol is to the patient-centered, N-of-one trial that permits individualized treatment based on the best available biomarkers.

Basket Trials

Basket trials treat various cancers based on genomic characteristics and pool patients independent of histologic diagnosis. These trials assume that response to targeted therapy can be evaluated independently from tissue of origin and independent of other molecular aberrations in the tumor; thus enrollment can be increased by combining patients with different tumors in the same studies. Some trials can be modified over time as new targets and treatments are identified. This design can result in certain arms opening and closing quickly. This methodology allows the testing of many potentially active drugs simultaneously.

Umbrella Trials

Umbrella studies enroll patients who share a tumor type, test molecular markers for a wide variety of potential molecular targets, and then assign patients to an arm of the study. Each arm represents a potentially effective treatment for the molecular marker. As with basket trials, some trials can be changed over time as new targets and therapies are discovered. This design can result in certain arms opening and closing rapidly, permitting the testing of many agents simultaneously.

Octopus Study

An octopus study design denotes multiple arms testing different drugs or drug combinations in a single trial. These were considered "complete phase I trials" (a terminology coined by Dan Von Hoff) when performed in the phase I setting.

Platform Study

Traditional

Traditional platform trials include multiple histologies and genomic or other biomarkers and are based on a single technological platform (e.g., next generation sequencing (NGS)) to interrogate the tumor. The standard platform trial design consists of a fixed number of treatments. There are several treatments and a control with equal randomization and a fixed number of patients in each arm. No additional treatment can be added beyond those included at the start of the trial. Each treatment is compared to the control. A modification of this strategy uses interim monitoring for success and futility at equally spaced intervals. If a treatment is dropped due to futility, the accrual on the remaining active arms is greater. The open platform employs an adaptive design where treatments can be dropped or added during the course of the trial. New treatments can be added to replace ineffective treatments during the trial. These trials have the potential to find effective treatment faster and with fewer resources than investigating one treatment per trial. They also do not require a new trial infrastructure for each treatment studied.

Innovative platform trials

Newer platform trials include multiple histologies and genomic or other biomarkers and are based on a single technological platform (e.g., NGS) to probe the tumor, but are not randomized.

Master Protocol

A master protocol uses either a single multiplex diagnostic assay to assign subjects to different candidate drugs or arms of the trial within the same trial or network of trials or it may use multiple discrete biomarker assays alone or in combination. Master protocols offer more options for patients and can also make screening and recruitment more efficient. A master protocol is an overarching protocol designed to answer multiple questions. Master protocols can include one or more interventions in multiple diseases or a single disease, with multiple interventions. Master protocols may include umbrella, basket, or platform trials.

Precision Medicine Clinical Trials and Studies With Results

Multiple studies have assessed the benefit of matching therapies to specific alterations. **Table 1** presents 19 studies with results. Of these studies, 4 were for a specific disease while 15 were for advanced cancers; 14 studies were prospective, 1 was retrospective, and 4 were registry-style studies. For these studies, the median number of patients screened was 698 with an interquartile range (IQR 25%–75%) 250–1360. The median percentage of patients matched was 17% with an IQR 12%–26%. Of the 11 studies that compared matched therapies to unmatched options, 10 of these studies (91%) showed a statistically significant benefit with the matched therapy. While these studies utilized different designs and had different objectives, collectively they show a net benefit in matching therapies to specific molecular alterations and provide motivation for evaluating new therapies with novel precision medicine-based trial designs.

Table 1 Precision medicine clinical trials with reported results (includes only studies where biomarkers were used to match patients)

Year	*First author/chairs*	*Trial name*	*Institute*	*Type of trial*	*Type of cancer*	*N (total screened)*	*% of patients matched*	*Type of biomarker*	*Outcome*	*Comment*	*References*
2010	Von Hoff	Bisgrove	US (9 sites)	Prospective navigation	Metastatic refractory cancer	106	62	IHC, FISH, microarray	27% of matched patients had a PFS ratio of $\geq$1.3 ($P = .007$)		Von Hoff et al. (2010)
2012	Tsimberdou Kurzrock	MD Anderson Personalized Cancer Therapy Initiative	MD Anderson	Registry style Navigational	Advanced cancer	1283	16	PCR-based	Response rate = 27% in matched vs. 5% unmatched ($P < .05$); Longer TTF (<0.001), OS (<0.001), and PFS ($P = .017$) in matched group		Tsimberidou et al. (2012)
2014	Johnson	N/A	Vanderbilt	Retrospective	Advanced cancers	103	17	NGS	N/A	No outcomes provided	Johnson et al. (2014)
2014	Kris	Lung cancer mutation consortium	14 sites	Prospective	Lung adeno-carcinoma	1537	18	Multiplex genotyping	Improved survival with matched therapy ($P = .006$)		Kris et al. (2014)
2014	Jameson	N/A	US sites	Prospective	Breast cancer	28	89	RRPA, cDNA MA, IHC, FISH	Time to progression with multiomic based treatment improved in 44%		Jameson et al. (2014)
2014	Tsimberdou Kurzrock	MD Anderson Initiative validation	MD Anderson	Registry style Navigational	Advanced cancers	1542	9	PCR-based	Longer response rate ($P < .0001$), PFS ($P = .001$), OS ($P = .04$) in matched group		Tsimberidou et al. (2014)
2015	Andre	SAFIR01/UNICANCER	Goustave Roussey (18 French sites)	Prospective	Metastatic breast cancer	423	13	aCGH/Sanger sequencing	9% response, 21% SD $>$16 weeks (matched group)		Andre et al. (2014)
2015	Le Tourneau	Shiva	Institut Curie (8 French sites)	Prospective Randomized	Refractory cancer	741	13	NGS	PFS not improved with matched therapy ($P = .41$)	$\sim$80% of patients received single agent hormone modulators or everolimus	Le Tourneau et al. (2015)
2016	Schwaederle Kurzrock	PREDICT	UC San Diego	Registry-type	Advanced cancers	347	25	NGS	Higher rates of SD $\geq$ 6 months/PR/CR, PFS, OS in matched group ($P = .039$)		Schwaederle et al. (2016)

2016 Wheler Kurzrock	MD Anderson personalized cancer therapy initiative	MD Anderson	Prospective Navigational	Advanced cancers	500	24	CGP/NGS	Higher rates of SD $\geq$ 6 months/PR/CR ($P = .024$), TTF ($P = .0003$), OS ($P = .05$) with better matching score		Wheler et al. (2016)
2016 Stockley	IMPACT/COMPACT	Princess Margaret (Canadian centers)	Prospective	Advanced cancers	1893	4.7	NGS	Higher overall response rate with matched therapy ($P < .026$)		Stockley et al. (2016)
2016 Aisner	Lung cancer mutation consortium II	16 sites	Prospective	Lung adeno-carcinoma	980	13	NGS	Improved survival with matched therapy ($P = .039$)		Aisner et al. (2016)
2016 Sohal	Cleveland clinic study	Cleveland clinic	Prospective	Advanced cancers	250	10	NGS	N/A	Outcomes not provided	Sohal et al. (2015)
2017 Sicklick Kurzrock	I-PREDICT	UC San Diego Avera	Prospective navigational	Advanced cancers	47	36	CGP	Longer PFS with matched therapy ($P = .0019$)	Preliminary results	Sicklick et al. (2017)
2017 Massard	MOSCATO	Goustave Roussey	Prospective	Advanced cancers	1110	18	aCGH, NGS, RNAseq	33% on matched therapy had improved outcome vs. prior baseline ($P = .004$)		Massard et al. (2017)
2017 Tsimberidou Kurzrock	IMPACT	MD Anderson	Registry-style Navigational	Advanced cancers	1436	27	PCR-based	Matched therapy with higher response ($P = .0099$), longer failure-free survival ($P = .001$), longer overall survival ($P < .001$)		Tsimberidou et al. (2017)
2017 Tredan	PROFILER	Centre Leon Berard (3 French sites)	Prospective	Advanced cancers	2676	5	NGS/aCGH	5 year OS 34.8% with matched vs. 28.1% for unmatched	Study is ongoing	Tredan et al. (2017)
2017 Hainsworth Kurzrock	MyPathway	Genentech (US sites)	Prospective	Advanced cancers	251	92	Genomic testing	23% response rate;38% HER2 group, 43% BRAF group	Study is ongoing; no comparison between matched therapy and prior therapy provided	Hainsworth et al. (2017)
2017 Kim	NEXT	Samsung Medical Center, Korea	Prospective	Advanced cancers	654	9	IHC/DNA sequencing	Improved clinical trial enrollment	Outcomes of matched therapy not provided	Kim et al. (2017)

Abbreviations: aCGH = Array comparative genomic hybridization, ASCO = American Society of Clinical Oncology, cDNA MA = cDNA microarray, CGP = comprehensive genomic profiling, FISH = fluorescence in situ hybridization, IHC = immunohistochemistry, N/A = not available, NGS = next generation sequencing, OS = overall survival, PCR = polymerase chain reaction, PFS = progression free survival, RRPA = reverse phase protein array, TTF = time to treatment failure.

Bisgrove Study

Von Hoff et al. (2010) performed a prospective navigational pilot study at nine United States sites attempting to match treatment regimens based on molecular profiling for patients with refractory metastatic cancer. A total of 106 patients were consented. Molecular profiling (IHC, FISH, and microarray analysis) was attempted in 81% ($N = 86$ patients) with molecular targets detected in 98% ($N = 84$): 68 of the screened patients (64%) were treated; 66 of the screened patients (62%) were treated according to molecular profiling and two patients (2%) were not. The use of matched therapy resulted in significantly longer progression-free survival as compared to each patient's own most recent prior progression-free survival from prior therapy (9.7 vs. 5 months, $P = .026$). Overall, 27% of matched patients had a progression-free survival ratio of greater than 1.3 as compared to their prior therapy ($P = .007$). The study concluded that molecular matching of therapy has a positive influence on the clinical course for an individual.

MD Anderson Personalized Cancer Therapy Initiatives

Four studies that described outcomes have been published. Matching rates ranged from 9%–27% of consented patients. All showed improvement in outcome parameters associated with matching. An exploratory Matching Score (number of matches divided by number of genomic alterations) for patients receiving combination therapies demonstrated significant correlation between higher Matching Scores and better outcomes (Wheler et al., 2016).

MD Anderson personalized initiative #1

Tsimberidou et al. (2012) reported an analysis of patients referred to the MD Anderson phase 1 clinical trials center with advanced cancer treated in the Clinical Center for Targeted Therapy aimed to determine if matching targeted therapies to detected molecular alterations would result in improved outcomes. Overall, 1283 patients were screened; 1144 patients (89%) had sufficient tissue for molecular analysis (polymerase chain reaction (PCR)-based genomic testing), and 36% ($N = 460$) were found to have at least one alteration. In total, 352 patients with molecular alterations were treated (27%). Matched therapy was administered to 211 (16%) patients and 144 (11%) did not receive matched therapy. The patients on matched therapy had improved response rates (27% vs. 5%, $P < .001$) and longer median progression-free (13.4 vs. 9.0 months, $P = .017$) and overall survival (5.2 vs. 3.1 months, $P < .001$).

MD Anderson personalized initiative validation

A follow-up validation registration-style navigational study with additional patients referred to the phase 1 clinical trial program at MD Anderson Cancer Center also explored differences between patients with advanced cancers who received targeted therapies and those who did not have a targeted option available (Tsimberidou et al., 2014). A total of 1542 patients were screened. Overall, 1276 patients (83%) had adequate tumor tissue for genomic analysis, 48% ($N = 739$) had at least one genomic alteration (PCR-based genomic testing). In total, 379 patients (25%) were treated: 143 patients (9%) received therapy matched to at least one alteration as compared to 236 (15%) who did not receive a matched therapy. The patients with matched therapy had greater median overall survival (11.4 vs. 8.6 months, $P = .04$), objective responses (12% vs. 5%, $P < .001$), and progression-free survival (3.9 vs. 2.2 months, $P = .001$). This was consistent with prior observations.

MD Anderson IMPACT

The Initiative for Molecular Profiling and Advanced Cancer Therapy (IMPACT) was a registry-style navigational study conducted at MD Anderson (Tsimberidou et al., 2017). Patients from the phase 1 clinical trials program with advanced cancer who underwent molecular analysis were assigned to clinical trials with a matched targeted agent when available. Of 1436 patients with molecular profiling by PCR-based genomic testing, 914 (77.5%) had a targetable mutation, 637 (44%) were treated: 390 with matched therapy (27%) and 2247 with nonmatched therapy (17%). Patients with matched targeted therapies had higher rates of response (11% vs. 5%; $P = .0099$), longer failure-free survival (3.4 vs. 2.9 months; $P = .0015$), and longer overall survival (8.4 vs. 7.3 months, $P = .041$).

MD Anderson/Foundation Medicine comprehensive genomic profiling prospective study

A prospective navigational single-center study at MD Anderson to evaluate the use of comprehensive genomic profile directed therapy enrolled 500 patients with advanced malignancies (Wheler et al., 2016). Molecular testing was performed on 339 patients (68%) using comprehensive genomic profiling/next generation sequencing performed by Foundation Medicine. Overall, 188 patients were treated (37%), 122 received matched therapy (24%), and 66 (13%) received unmatched therapy to at least one of the molecular alterations found on molecular profiling. Patients with high degrees of drug matches (high Matching Score) to molecular alternations were found to have significantly longer survival (hazard ratio [confidence interval] 0.65 [0.43–1.0], $P = .05$) and frequency of stable disease more than 6 months, partial response, or complete response (22% vs. 9%, $P = .024$).

UC San Diego

Two studies from UC San Diego have been published. Between 25% and 36% of consented patients received matched therapy. Both studies showed improvement in outcomes with matching.

Predict

The University of California San Diego PREDICT (Profile Related Evidence Determining Individualized Cancer Therapy) is a registry-type master protocol that aims to learn more about personalized cancer therapy including response to treatment and side effects. The study reviewed 347 consecutive patients with advanced solid tumors who had next generation sequencing performed (Schwaederle et al., 2016). In total, 180 patients (52%) were treated: 87 patients (25%) were treated with matched therapy as compared to 93 patients (27%) treated with unmatched therapy. Patients who had therapy matched to molecular alterations had better outcomes with longer overall survival (15.7 vs. 10.6 months, $P = .04$) and response (stable disease for more than 6 months, partial response, and complete response: 34.5% vs. 16.1%, $P = .02$).

UC San Diego I-PREDICT

The Investigation of Molecular Profile-Related Evidence to Determine Individualized Therapy for Advanced or Poor Prognosis Cancers (I-PREDICT) is a two-institution (UCSD and more recently Avera), prospective navigational study of personalized cancer therapy aimed to use genomic testing of tumor tissue to provide matched therapy recommendations to physicians at the University of California San Diego (Sicklick et al., 2017). Preliminary results demonstrated that of the 47 patients evaluated, 40 (85%) had complete genomic profiling (next generation sequencing) available, 22 (47%) were treated, 17 (36%) received matched therapies, and 11% received unmatched therapies. Overall, 53% of the matched patients achieved stable disease >6 months/PR/CR. Median progression-free survival for matched patients was 4.7 as compared to 1.0 months without matched therapy ($P = .0019$). The study is ongoing.

Shiva

The SHIVA trial (Le Tourneau et al., 2015) was an open-label prospective randomized phase 2 study of refractory cancer completed in France to assess the efficacy of several molecularly targeted agents. Molecular profiling of each patient's tumor was determined using next generation sequencing and patients were included with alterations in hormone receptors, the PI3 kinase/AKT, mTOR pathway, or the RAF/MEK pathways and were matched to 10 prespecified regimens. These regimens included 11 targeted agents: dasatinib, erlotinib, everolimus, imatinib, lapatinib with trastuzumab, letrozole, sorafenib, tamoxifen, and vemurafenib. Patients were randomly assigned to receive matched therapy with a molecularly targeted agent or the physician's choice. Overall, 741 patients were screened for the study and 293 were enrolled with 191 patients treated (26%): 99 patients (13%) were assigned to receive molecularly targeted therapy and 92 (12%) were in the control group. Median progression free survival was 2.3 vs. 2.0 months (hazard ratio [95% confidence interval]: 0.88 [0.65–1.19], $P = .41$). The study suggested that molecular matching of therapies outside of approved uses was not beneficial. Other reviews of the study have suggested that it was limited by a small number of molecular alterations and the therapeutic options included, since approximately 80% of patients in SHIVA were matched to single-agent hormone modulators or mTOR inhibitors.

MOSCATO

The MOSCATO 01 trial (Massard et al., 2017) was a prospective trial characterizing genomic alterations in advanced cancers performed at the Goustave Roussey Institute in France. Patients had molecular profiling with array comparative genomic hybridization, NGS, or RNA sequencing. A total of 1110 patients were screened, 843 (76%) had molecular profiling, 411 (37%) had an actionable mutation, and 199 (18%) received matched therapy. Of the patients treated on matched therapies, 33% of patients had an improved outcome compared to their prior baseline reference progression-free survival ($P = .004$); 11% had objective responses.

SAFIR-01/UNICANCER

The SAFIR-01/UNICANCER (High Throughput Technologies to Drive Breast Cancer Patients to Specific Phase I/II Trials of Targeted Agents; Andre et al., 2014) study was a prospective evaluation of women with metastatic breast cancer at 18 French study sites aimed to identify genomic abnormalities and provide matched targeted therapies. Molecular testing was performed using comparative genomic hybridization and Sanger sequencing. The study enrolled 423 patients, 195 (46%) had targetable mutations, and 55 (13%) received a matched therapy. Of the 43 evaluable patients receiving a matched, targeted therapy 4 had a partial response and 9 had stable disease for more than 16 weeks. No comparison was made between matched and unmatched outcomes in the publication.

Impact/Compact

The Princess Margaret Cancer Center led the Integrated Molecular Profiling in Advanced Cancers Trial (IMPACT) and Community Molecular Profiling in Advanced Cancers Trial (COMPACT) which were prospective studies at Canadian sites led by the Princess Margaret Cancer Centre with the goal of determining outcomes with matched therapies for advanced solid tumors (Stockley et al., 2016). Molecular profiling was completed with next generation sequencing. A total of 1893 patients were enrolled, 89 patients (4.7%) were given matched therapies. The response rate was 19% with matched therapies compared to 9% with unmatched therapies ($P < .026$).

PROFILER

The Program to Establish the Genetic and Immunologic Profile of Patient's Tumor for All Types of Advanced Cancer (PROFILER) is a prospective, nonrandomized, multicenter, cohort study which aimed to implement a personalized medicine approach by determining the genetic and immunologic profile of tumors for patients with advanced cancers (Tredan et al., 2017). The study was led by Centre Leon Berard and conducted at three French sites. Molecular testing was performed with next generation sequencing and array comparative genomic hybridization. Of the 2676 patients consented, 940 (35%) had at least one actionable mutation, 143 patients (5%) of patients were treated with matched therapies, and 502 patient did not received matched therapies (19%). The overall 5 year survival rate was higher with matched therapy (34.8% vs. 28.1%).

Lung Cancer Mutation Consortium

The Lung Cancer Mutation Consortium was a prospective multisite study which enrolled 1537 patients with metastatic adenocarcinoma of the lung and evaluated patients for mutations (Kris et al., 2014). Of these, 733 patients (48%) were genotyped using multiplex genotyping, 617 (40%) were treated, 275 (18%) received a targeted therapy, and 342 patients (22%) did not receive a matched therapy. Overall survival improved from 2.4 years to 3.5 years ($P = .006$) with the use of a matched therapy.

The Lung Cancer Mutation Consortium II study (Aisner et al., 2016) was a prospective study which enrolled 980 patients and obtained molecular profiles using next generation sequencing. Of these, 191 patients (19%) were treated, 127 (13%) received targeted therapy, and 74 (8%) did not received targeted therapy. Overall survival was again improved from 1.5 to 2.8 years with the use of targeted therapy ($P = .039$).

MyPathway

The MyPathway trial (Hainsworth et al., 2017) is a multicenter, nonrandomized, prospective study sponsored by Genentech of patients with advanced solid tumors who progressed after standard therapy or for which there is no approved treatment which is ongoing at United States sites. Patients had at least one potentially actionable genetic alteration as determined by a CLIA certified laboratory. Overall, 251 patients have been enrolled and 230 (92%) were matched into four groups: erlotinib (*EGFR* mutation), vemurafenib (*BRAF V600E* mutation), vismodegib (Sonic hedgehog pathway mutation), or pertuzumab/trastuzumab (*HER2* amplification). A total of 52 patients (21%) had objective responses. The *HER2* group had a 38% (14 out of 37 patients) response rate while the *BRAF V600* mutated group had a 43% (6 out of 14 patients) response rate. The publication did not compare matched to unmatched therapies or the current response on matched therapy to prior response on unmatched therapy. The study is currently ongoing.

Other Studies

Vanderbilt Ingram Cancer Center conducted a retrospective study to determine actionable mutations in advanced cancers (Johnson et al., 2014). Molecular profiling was completed using next generation sequencing. Of 103 patients receiving genotyping, 86 (83%) had potentially actionable mutations, however only 18 patients (17%) were treated on matched therapies. Outcomes of matched compared to nonmatched therapies were not provided in the publication.

A prospective pilot study of the use of multiomic molecular profiling looked at 28 women with metastatic breast cancer (Jameson et al., 2014). Molecular testing was preformed using cDNA microarray, immunohistochemistry, fluorescence in situ hybridization, and reverse phase protein microarray analysis. Of these, 25 (89%) successfully completed multiomic molecular profiling (MMP) and were treated with matched therapy. A growth modulation index greater than 1.3 (progression-free survival on MMP-selected therapy/time to progression on last prior treatment) was seen in 44% of these patients.

The Cleveland Clinic performed a prospective study with next generation sequencing of 250 patients to determine accessibility of genomic driven therapy (Sohal et al., 2015). Of the 250 patients sequenced, 109 (44%) had a potential matched therapy, but only 24 (10%) received matched therapy. Lack of trial access and clinical deterioration were the most common reasons for not receiving a matched therapy. Overall outcomes for matched vs. unmatched therapies were not provided in the publication.

The NEXT screening program was a study at the Samsung Medical Center in Korea and was a prospective molecular-screening program with the goal of facilitating enrollment on 13 ongoing biomarker-guided clinical trials (Kim et al., 2017). Genomic alterations were determined by immunohistochemistry and DNA sequencing. The study screened and consented 654 patients of which 418 (64%) were eligible for cancer treatment or biomarker-driven clinical trials and 60 (9%) patients were treated on biomarker selected clinical trials. The study concluded that molecular based screening can facilitate improved enrollment on biomarker-guided clinical trials, but overall outcomes for matched vs. unmatched therapies were not provided.

Correlative-Only Precision Medicine Clinical Trials

Several precision medicine studies did not attempt to use genotyping results to match therapies and instead only performed correlations between response to therapies and specific molecular markers. Table 2 represents four studies with results. These studies were all disease specific, prospective studies. The median number of patients screened was 334 and ranged 237–344.

BATTLE

The BATTLE trial (biomarker-integrated approaches of targeted therapy for lung cancer elimination trial) was a prospective study with adaptive randomization of chemotherapy refractory nonsmall cell lung cancer patients at MD Anderson Cancer Center (Kim et al., 2011). Molecular alterations were determined using PCR-based genomic testing, immunohistochemistry, and fluorescent in situ hybridization. A total of 341 patients were enrolled. Patients were randomized to four therapies (erlotinib for EGFR, erlotinib with bexarotene for retinoid-EGFR signaling, sorafenib for KRAS/BRAF, or vandetanib for VEGFR) after molecular tumor biomarker assessments. A total of 59 patients received erlotinib, 54 received vandetanib, 37 received erlotinib with bexarotene, and 105 received sorafenib. The 8 week disease control rate was 46% and median progression free survival was 1.9 months. The benefit rate was 79% with sorafenib as compared to 14% with erlotinib in the mutant KRAS/BRAF marker group and patients with EGFR mutations had better responses to erlotinib, vandetanib, and erlotonib with bexarotene than sorfaenib. The authors concluded that treatment efficacy was related to the mechanism of action of the therapy in the presence of individual biomarkers.

BATTLE-2 was a follow-up of BATTLE performed at MD Anderson Cancer Center and Yale (Papadimitrakopoulou et al., 2016). An adaptive trial design was used to assign patients with nonsmall cell lung cancer to erlotinib, sorafenib, erlotinib with Akt inhibitor (MK-2206), or MEK inhibitor (AZD6244) with MK-2206. Molecular profiling was performed with fluorescence in situ hybridization. Overall, 334 patients provided consent, 274 underwent molecular profiling, and 200 patients were randomly assigned at received treatment on the four treatment arms. Patients with *KRAS* mutations had significantly longer progression free survival if they were not treated with erlotinib (HR 1.95, 95% CI: 1.00–3.77, $P = .04$), but overall survival did not differ (HR 0.26, 95% CI: 0.65–2.46; $P = .50$). Those with *KRAS* wild-type tumors had better overall survival with erlotinib treatment (HR 0.66, 95% 0.45–0.97, $P = .03$). The study concluded that biopsy-mandated, biomarker driven adaptive study design was feasible for pretreated nonsmall cell lung cancer.

I-SPY Studies

I-SPY 1 (investigation of serial studies to predict your therapeutic response with imaging and molecular analysis, NCT00043017; Esserman et al., 2012) was a prospective study of women with locally advanced breast cancer receiving neoadjuvant chemotherapy. The study enrolled 237 patients. Patients were tested for hormone receptors (estrogen, progesterone) and HER2 with immunohistochemistry. The trial evaluated molecular biomarkers of treatment, MRI results, pathologic residual disease at time of surgery, and 3 year disease free survival. Hormone-receptor positive/HER2 negative cancers had only a 9% pathologic complete response (pCR) as compared to the 45% seen with hormone receptor negative/HER2 positive tumors. MRI volume was the best predictor for residual disease after initial therapy.

I-SPY2 compared standard chemotherapy with and without the addition of novel drugs. Patients were tested for hormone receptors (estrogen, progesterone), HER2, and MammaPrint (a 70 gene predictive test for distant recurrence prior to therapy). Patients were either MammaPrint high-risk, ER-negative, or HER-2 positive as determined by immunohistochemistry. Patients received a paclitaxel with or without novel therapy or control followed by doxorubicin and cyclophosphamide. HER-2 positive patients received either trastuzumab (control), neratinib, MK-2206 with trastuzumab, T-DM1 with pertuzumab, AMG386 with trastuzumab, or pertuzumab with trastuzumab. HER2-negative patients received paclitaxel alone (control) or in combination with ABT-888, MK-2206, AMG479 with metformin, or AMG386. The study utilized an adaptive trial design to allow agents that do well within a molecular signature subgroup to move through the trial more quickly. Preliminary results showed the additional of velaparib to standard chemotherapy ($n = 72$ patients) giving a 51% pCR rate with chemotherapy as compared to 25% with chemotherapy alone ($n = 44$ patients) (Rugo et al., 2016). Neratinib addition to standard chemotherapy improved pCR rates to 56% from 33% (Park et al., 2016).

Ongoing Precision Medicine Studies Without Results

There are multiple studies ongoing to evaluate the benefit of matched therapies. Table 3 represents 18 studies in progress. Seven studies were for a specific disease and 11 studies were for advanced cancers. Of these studies, 15 were prospective studies and 3 were registry-style studies. Studies with preliminary results are described in the precision medicine clinical trials and studies with results section and included in both Tables 1 and 3.

Table 2 Precision medicine clinical trials with reported results (biomarkers as correlative studies only)

Year	*First author/ chairs*	*Trial name*	*Institute*	*Type of trial*	*Type of cancer*	*N (total screened)*	*% of patients matched*	*Type of biomarker*	*Outcome*	*Comment*	*References*
2011	Kim Ki Hong	BATTLE	MD Anderson	Prospective Adaptive randomized	Lung cancer	341	N/A	PCR-based genomic, IHC, FISH	46% 8-week disease control rate	Randomized assignments to therapy (adaptive and nonadaptive)	Kim et al. (2011)
2012	Esserman	I-SPY 1	US sites (multiple)	Prospective Neoadjuvant	Neoadjuvant treatment for breast cancer	237	N/A	IHC	pCR differs by receptor subset		Esserman et al. (2012)
2016	Papadimitrako-poulou	BATTLE-2	MD Anderson Yale	Prospective	Nonsmall cell lung cancer	334	N/A	FISH	KRAS positive with longer PFS without erlotinib ($P = .04$); KRAS wild-type tumors with better OS on erlotinib ($P = .03$)		Papadimitrakopoulou et al. (2016)
2016	Park Rugo	I-SPY 2	Quantum-leap healthcare (US sites)	Prospective randomized	Neoadjuvant breast cancer	N/A	N/A	IHC, Mammaprint	Improved pCR rates in two study arms with drug addition	Results for 2 arms of study available	Park et al. (2016) and Rugo et al. (2016)

Abbreviations: FISH = Fluorescence in situ hybridization, IHC = immunohistochemistry, OS = overall survival, pCR = pathologic complete response, PCR = polymerase chain reaction.

Table 3 Ongoing precision medicine studies without results or with preliminary results

Year initiated	*Chairs*	*Trial*	*Institute (sites)*	*Type of trial*	*Type of cancer*	*Biomarker*	*NCT number*	*Comment*
2010	Park Rugo	I-SPY 2	Quantum-leap healthcare (US sites)	Prospective randomized	Neoadjuvant breast cancer	IHC, Mammaprint	NCT01042379	Ongoing study with preliminary results (see Table 1)
2010	Papadimitrako-poulou	BATTLE-FL	MD Anderson	Prospective navigational	Nonsmall cell lung cancer	FISH	NCT01263782	
2012	Schrauwen	SPECTAColor	European hospitals	Registry-type	Advanced colorectal cancer	NGS/IHC	NCT01723969	
2013	Chen	MPACT	NCI (US sites)	Prospective	Advanced cancer	NGS	NCT01827384	
2013	Soria Kurzrock	WINTHER	WIN consortium for personalized cancer therapy International	Prospective	Advanced cancers	NGS/transcriptomics	NCT01856296	Study has completed enrollment
2013	Blay Tredan	PROFILER	Centre Leon Berard (3 French sites)	Prospective	Advanced cancers	NGS/aCGH	NCT01774409	Ongoing study with preliminary results (see Table 1)
2014	Oxnard	ALCHEMIST	NCI (US sites)	Prospective	Early stage nonsmall cell lung cancer	Direct sequencing, FISH, CLIA certified genotyping	NCT02194738	
2014	Papadimitrako-poulou	Lung-MAP	NCI (US sites)	Prospective	Advanced squamous cell lung cancer	NGS	NCT02154490 NCT02785913 NCT02965378 NCT02785939 NCT02785952 NCT02926638 NCT02766335	
2014	Piccart	AURORA	Institut Jules Bordet, Brussels, Belgium (European hospitals)	Registry-type	Metastatic breast cancer	NGS/RNAseq	NCT02102165	
2014	Novartis	Signature	Novartis (multiple sites)	Prospective	Advanced cancers	Variable	NCT02187783 NCT02186821	
2014	Hainsworth Kurzrock	MyPathway	Genentech (US sites)	Prospective	Advanced cancers	Genomic testing	NCT02091141	
2014	Tsimberidou	IMPACT2	MD Anderson	Prospective	Metastatic cancer	Genomic testing	NCT02152254	
2014	Besse	SPECTALung	European hospitals	Registry-type	Thoracic tumors	NGS/IHC	NCT02214134	
2015	Flaherty	NCI-MATCH	NCI (US sites)	Prospective	Advanced cancers	NGS	NCT02465060	
2015	Sicklick Kurzrock	iPREDICT	UC San Diego Avera	Prospective navigational	Advanced cancers	CGP	NCT02534675	Ongoing study with preliminary results (see Table 1)
2016	Patel Chae Giles Kurzrock	DART	SWOG, Early Therapeutics and Rare Cancers Committees US sites (multiple)	Prospective	Rare cancers	NGS correlational testing: whole genomic, transcriptome, liquid biopsy (ctDNA) and immune signature	NCT02834013	
2016	Schilsky	TAPUR	ASCO (US sites)	Prospective	Advanced cancers	Genomic analysis or IHC	NCT02693535	
2017	Parsons	Pediatric MATCH	NCI-COG (US sites)	Prospective	Pediatric Advanced Cancers	CLIA-certified molecular testing	NCT03155620	

Abbreviations: aCGH = Array comparative genomic hybridization, ASCO = American Society of Clinical Oncology, CGP = comprehensive genomic profiling, CLIA = Clinical Laboratory Improvement Amendments, COG = Children's Oncology Group, FISH = fluorescence in situ hybridization, IHC = immunohistochemistry, N/A = not available, NCI=National Cancer Institute, NGS = next generation sequencing, RNA seq = RNA sequencing, WIN=Worldwide innovative networking in personalized cancer medicine.

NCI Studies

NCI-MATCH

The National Cancer Institute-Molecular Analysis for Therapy Choice (NCI-MATCH, NCT02465060) trial is a phase 2 basket trial design where patients with advanced tumors who either have progressed on standard treatment or have rare tumors with no standard treatments are assigned to receive treatment based on genomic sequencing regardless of tumor type. There are 19 treatment arms which enroll patients with specific molecular alterations to receive a prespecified study drug. The first reported results from screening of 800 patients found only 9% with an actionable mutation for a study-targeted drug.

NCI-IMPACT

The NCI based MPACT study (Molecular Profiling-Based Targeted Therapy in Treating Patients with Advanced Solid Tumors, NCT01827384) is a randomized prospective study to evaluate molecular profiling-based targeted therapy in treating advanced solid tumors. Patients will have sequencing of tumor tissues and only those with targetable mutations will remain in the study. The remaining patients will be randomized 2:1 to receive a matched therapy or a physician determined treatment that does not target the detected mutation. A total of 180 patients are planned to receive treatment. The therapy options in the study are veliparib with temozolomide, MK-1775 (WEE1 inhibitor), everolimus, and trametinib. Reponses and outcomes will be compared between with matched therapies and nonmatched therapy arms.

NCI pediatric MATCH

The NCI and Children's Oncology Group have partnered for the Pediatric MATCH (Molecular Analysis for Therapy Choice, NCT03155620) trial a study of children and adolescents with advanced solid tumors who have not responded to treatment or progressed on standard therapy. Molecular testing will be performed on tumor tissue for screening and patients who have a mutation targetable by one of the treatment arms will be enrolled in the appropriate arm. Germline testing will also be performed on peripheral blood at the time of enrollment.

SPECTA Studies

The SPECTAcolor (Screening platform for clinical trials in advanced colorectal cancer, NCT01723969) is a registry-style clinical trial for advanced colorectal cancer which aims to provide rapid access to novel drugs in clinical trials though molecular testing of tumors. The study is run by the EORTC (European Organization for Research and Treatment of Cancer) at European sites and is currently ongoing.

The SPECTAlung (Screening patients with thoracic tumors for efficient clinical trial access, NCT02214134) is a registry-style clinical trial for thoracic tumors including lung cancer, mesothelioma, and thymic malignancies which aims to screen patients for molecular markers with the goal of understanding molecular characteristics of the disease. The study is run by the EORTC (European Organization for Research and Treatment of Cancer) at European sites and is currently ongoing.

ASCO TAPUR

The American Society of Clinical Oncology is sponsoring the TAPUR study (Targeted Agent and Profiling Utilization Registry, NCT02693535). This is a prospective study to facilitate access to approved targeted therapies for patients with advanced cancer whose standard treatment options have been exhausted and tumors harbor a genomic variant known to be a drug target. The study will assess response to matched therapies and precision medicine. The study is ongoing and has recruited over 100 patients.

AURORA

The AURORA study (Aiming to Understand Molecular Aberrations in Metastatic Breast Cancer, NCT02102165) is a registry-style study in multiple European hospitals which aims to recruit 1300 breast cancer patients to better understand the genetic aberrations in metastatic breast cancer and understand mechanisms of response to therapy to help identify better understand how to personalize therapy. The study is currently ongoing.

ALCHEMIST

The ALCHEMIST (Adjuvant Lung Cancer Enrichment Marker Identification and Sequencing Trials, NCT02194738) clinical trials are NCI based studies of adjuvant matched therapies for early-stage nonsmall cell lung cancer. Patients will receive either erlotinib or crizotinib if they have EGFR or ALK mutations, respectively. Those lacking either mutation will be treated with nivolumab.

BATTLE-FL

BATTLE-FL (A Biomarker-Integrated Targeted Therapy Study in Previously Treated Patients with Advanced Nonsmall Cell Lung Cancer: Front Line, NCT01263782) an adaptive design for patients receiving front-line therapy to define how bevacizumab, cetuximab, or cixutumumab improve progression-free survival when combined with pemetrexed. The study is ongoing.

DART

The DART study (Dual anti-CTLA-4 and anti-PD-1 blockade in rare tumors, NCT02834013) is a Southwest Oncology group clinical trial of ipilimumab and nivolumab. Rare tumors (less than 6 in 100,000 incidence per year) who are registered in NCI-MATCH and do not have a treatment option available can enroll. Patients are treated with combination therapy with ipilimumab and nivolumab. The study plans to enroll 300 patients.

Signature

The Novartis "Signature" program is a novel signal-finding clinical trial protocol series which are tissue-agnostic, mutations specific protocols (NCT02187783, NCT02186821). Eight ongoing single agent clinical protocols were presented at ASCO 2015 which included buparlisib (PI3 kinase inhibitor), dovintinib (multikinase inhibitor), binimetinib (MEK inhibitor), encorafenib (RAF inhibitor), ribociclib (CDK 4/6 inhibitor), BGJ398 (FGFR inhibitor), ceritinib (ALK inhibitor), and sonidegib (SMO inhibitor). The study uses a Bayesian adaptive design with interim analyses for evaluating futility after the accrual of 10 patients and evaluating success after 15–30 patients. The study is currently ongoing.

Lung-MAP

Lung-MAP is a phase 2–3 master protocol of advanced squamous nonsmall cell lung cancer (NCT02154490, NCT02785913, NCT02965378, NCT02785939, NCT02785952, NCT02926638, NCT02766335). It incorporates a common biomarker screening platform. Patients were assigned to a subgroup based on biomarkers and those qualifying for more than one subgroup were randomly assigned to subgroups to allow lower prevalence biomarkers to receive more patients. Those without biomarkers were assigned a no-match group. Four targeted therapies and one therapy for the no-match group were used. The study has undergone several changes since opening. The no-match sub-study arm was designed to investigate MEDI4736 and transitioned to nivolumab compared to nivolumab with ipilimumab. The rilotumumab and MEDI4736 arms were discontinued and the remaining three biomarkers arms are taselisib, palbociclib and AZD4547.

MD Anderson IMPACT2

The Randomized Study Evaluating Molecular Profiling and Targeted Agents in Metastatic Cancer: Initiative for Molecular Profiling and Advanced Cancer Therapy (IMPACT 2, NCT02152254) study is a randomized prospective study at MD Anderson to further establish the benefit of personalized treatments. Patients will be randomized to a targeted therapy arm where they will receive therapies matched to molecular targets identified on their tumors through a clinical trial. If a clinical trial is not available, patients will receive a commercially available FDA approved targeted therapy. The comparator arm will be standard of care treatment not selected based on molecular alterations.

WINTHER

The Worldwide Innovative Networking Consortium for personalized cancer therapy (WINTHER, NCT01856296) was designed for patients with advanced cancer to match targeted therapies to each individual's molecular profile (Rodon et al., 2015). Either next generation sequencing or transcriptomics, which compares tumor to normal tissue and bioinformatics algorithms to prioritize drug selection, was used for the two arms of the study. The study has completed enrollment (about 300 patients consented) and is undergoing analysis.

Challenges in Precision Medicine Trials

Many precision medicine trials assume that response to targeted therapy can be evaluated independently from tissue of origin and independent of other molecular aberrations in the tumor, thus enrollment can be increased by combining patients with different tumors in the same studies. However it is unclear if this assumption is true for all molecular pathways. As an example, a phase 2 basket study of *BRAF V600* mutation positive nonmelanoma cancers explored the role of responses to vemurafenib by cancer type (Hyman et al., 2015). A total of 122 patients with *BRAF* mutations were treated. Response rates were highest for non-small-cell lung cancer (42%) and Erdheim-Chester disease (43%), whereas cholangiocarcinoma had a 12% response rate and anaplastic thyroid cancer 29% response rate. Colorectal cancer had a 0% response to vemurafenib alone and 4% response rate when combined with cetuximab. The majority of *BRAF* mutated tumors thus responded well across a diverse spectrum of hematologic and solid cancers.

Given that new mutations can arise in a tumor over the course of therapy, different results may arise in patients earlier in their treatment course even with in the same histology, which can alter outcomes. While some studies will limit treatment to patients with very advanced disease or who are medically unfit to withstand additional treatment for the initial testing of targeted therapies, this approach may ultimately make it difficult to assess the utility of novel targeted therapies. This approach can lead to high post-screening attrition rates as a result of deteriorating clinical status or death.

Protocol flexibility is needed for precision medicine trials to eliminate ineffective drugs or introduce new agents or cohorts as a trial evolves. Study designs must identify active arms as early as possible while acquiring enough data to understand why the drug failed in other arms. It is important to understand the biology behind the successes and failures. Histology-agnostic trials are generally nonrandomized and interim analyses are limited by small sample sizes, unbalanced data, and the heterogeneity of responses seen among patients with different malignancies. Current basket trial designs assume data can be aggregated across histologies, but it is also important to make sure that there is similar efficacy prior to combining the data. Thus novel designs for interim analyses are needed. Adaptive trials designs, as have been employed by some of the precision medicine trials described above, are incorporated to modify the course of structure of the trial. Early assessments of the clinical benefit or safety of the drug can be made to modify the response. A trial can be stopped early or extended depending on the emerging results and arms or doses can be dropped if there is no benefit. This allows for the identification of patients who are responding to investigational drugs and allows the randomization of proportions of the trial population or the rates at which data are accrued to be changed. It permits inclusion of multiple stages of drug development in a single trial.

Appropriate outcome measures must also be considered. While rate and duration of response has traditionally been used, responses may be short-lived and may not work well as surrogates for survival. An alternative end point may include the ratio of progression-free survival with the targeted therapy compared to progression free survival of prior line of therapy. A value of greater than 1.3 was felt to be clinically meaningful. However if the progression-free survival differs significantly between tumors of different origins, this end point can be of limited value.

Patient screening and recruitment can also present challenges. There need to be viable means to identify low-incidence patient subpopulations and to direct patients to clinical trials. These can be linked to umbrella and basket studies and global studies that can accept subjects from diverse screening routes. Screening programs are dependent on networks, collaborators, and reliable partners. The molecular testing platforms should enable broad, robust, and cost-effective tumor and patient profiling for the studies.

Evaluating a large number of therapies in a diverse patient population in a trial that can involve multiple countries can present difficulties in starting the trial. Considerable delays can occur in study approval and activation in the sites. While international sites may have an expedited process, rules about testing novel therapies and off-label uses of approved drugs can present a challenges. For combination therapies, the coordination and collaboration of multiple pharmaceutical companies can also present a challenge. Delays in molecular sequencing and drug acquisition can also delay treatment in precision medicine trials.

Conclusions

Significant advances have been made in genomic profiling which has enabled the administration of therapies matched to molecular alterations. Clinical trials and studies have evaluated the benefit of matching therapies to genomic information and have almost exclusively established a benefit for a tissue-agnostic approach. While significant challenges still remain in the implementation of precision medicine clinical trials and therapies, multiple trials are ongoing providing new hope for patients with advanced and metastatic cancer.

Prospective Vision

Genomic profiling has unveiled a new reality in oncology. Metastatic tumors are complicated; thus traditional study designs are not a good fit. The heterogeneity of metastatic solid tumors necessitates patient-centered studies and a personalized, N-of-one, approach. End-stage heavily pretreated patients do not fare as well with targeted therapies. While imatinib improved survival in CML from 4 to 5 years to 20 or more, the response rate in blast phase is only 10%. Thus, genomically targeted therapy may be more effective earlier in the course of the disease. Immunotherapy has provided another major breakthrough in oncology treatments and can work synergistically with targeted therapy to activate the immune system. Tumors with high microsatellite instability (MSI-high) and high tumor mutational burden (TMB-high) are more likely to respond to therapy. Immunotherapy may work better with complex genomic alterations and in combination with targeted therapies. The United States Food and Drug administration has provided a landmark drug approval to pembrolizumab for MSI-high or mismatch repair deficient tumors, thus representing the first tissue-agnostic approval. FDA-approvals are being granted based on correlative data which may provide an opportunity to perform retrospective data mining for future drug approvals.

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References

Aisner, D., Sholl, L.M., Berry, L.D., et al., 2016. Effect of expanded genomic testing in lung adenocarcinoma (LUCA) on survival benefit: The lung Cancer mutation consortium II (LCMC II) experience. Journal of Clinical Oncology 34, 11510.

Andre, F., Bachelot, T., Commo, F., et al., 2014. Comparative genomic hybridisation array and DNA sequencing to direct treatment of metastatic breast cancer: A multicentre, prospective trial (SAFIR01/UNICANCER). The Lancet Oncology 15, 267–274.

Esserman, L.J., Berry, D.A., DeMichele, A., et al., 2012. Pathologic complete response predicts recurrence-free survival more effectively by cancer subset: Results from the I-SPY 1 TRIAL–CALGB 150007/150012, ACRIN 6657. Journal of Clinical Oncology 30, 3242–3249.

Hainsworth, J., Meric-Bernstam, F., Swanton, C., et al., 2017. Targeted therapy for advanced solid tumors based on molecular profiles: Results from MyPathway, an open-label, phase IIa multiple basket study. Joural of Clinical Oncology in press.

Hyman, D.M., Puzanov, I., Subbiah, V., Faris, J.E., Chau, I., Blay, J.Y., Wolf, J., Raje, N.S., Diamond, E.L., Hollebecque, A., Gervais, R., Elez-Fernandez, M.E., Italiano, A., Hofheinz, R.D., Hidalgo, M., Chan, E., Schuler, M., Lasserre, S.F., Makrutzki, M., Sirzen, F., Veronese, M.L., Tabernero, J., Baselga, J., 2015. Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. New England Journal of Medicine 373 (8), 726–736.

Jameson, G.S., Petricoin, E.F., Sachdev, J., et al., 2014. A pilot study utilizing multi-omic molecular profiling to find potential targets and select individualized treatments for patients with previously treated metastatic breast cancer. Breast Cancer Research and Treatment 147, 579–588.

Johnson, D.B., Dahlman, K.H., Knol, J., et al., 2014. Enabling a genetically informed approach to cancer medicine: A retrospective evaluation of the impact of comprehensive tumor profiling using a targeted next-generation sequencing panel. The Oncologist 19, 616–622.

Kim, E.S., Herbst, R.S., Wistuba, I.I., et al., 2011. The BATTLE trial: Personalizing therapy for lung cancer. Cancer Discovery 1, 44–53.

Kim, S.T., Kim, K.M., Kim, N.K.D., et al., 2017. Clinical application of targeted deep sequencing in solid-Cancer patients and utility for biomarker-selected clinical trials. The Oncologist 22, 1169–1177.

Kris, M.G., Johnson, B.E., Berry, L.D., et al., 2014. Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. Journal of the American Medical Association 311, 1998–2006.

Le Tourneau, C., Delord, J.P., Goncalves, A., et al., 2015. Molecularly targeted therapy based on tumour molecular profiling versus conventional therapy for advanced cancer (SHIVA): A multicentre, open-label, proof-of-concept, randomised, controlled phase 2 trial. The Lancet Oncology 16, 1324–1334.

Massard, C., Michiels, S., Ferte, C., et al., 2017. High-throughput genomics and clinical outcome in hard-to-treat advanced cancers: Results of the MOSCATO 01 trial. Cancer Discovery 7, 586–595.

Papadimitrakopoulou, V., Lee, J.J., Wistuba, I.I., et al., 2016. The BATTLE-2 study: A biomarker-integrated targeted therapy study in previously treated patients with advanced non-small-cell lung Cancer. Journal of Clinical Oncology.

Park, J.W., Liu, M.C., Yee, D., et al., 2016. Adaptive randomization of Neratinib in early breast Cancer. The New England Journal of Medicine 375, 11–22.

Rodon, J., Soria, J.C., Berger, R., Batist, G., Tsimberidou, A., Bresson, C., Lee, J.J., Rubin, E., Onn, A., Schilsky, R.L., Miller, W.H., Eggermont, A.M., Mendelsohn, J., Lazar, V., Kurzrock, R., 2015. Challenges in initiating and conducting personalized cancer therapy trials: Perspectives from WINTHER, a Worldwide Innovative Network (WIN) Consortium trial. Annals of Oncology 26 (8), 1791–1798.

Rugo, H.S., Olopade, O.I., DeMichele, A., et al., 2016. Adaptive randomization of Veliparib-carboplatin treatment in breast Cancer. The New England Journal of Medicine 375, 23–34.

Schwaederle, M., Parker, B.A., Schwab, R.B., et al., 2016. Precision oncology: The UC San Diego Moores Cancer center PREDICT experience. Molecular Cancer Therapeutics 15, 743–752.

Sicklick, J.K., Leyland-Jones, B., Kato, S., et al., 2017. Personalized, molecularly matched combination therapies for treatment-naive patients. Journal of Clinical Oncology 35, 2512.

Sohal, D.P., Rini, B.I., Khorana, A.A., et al., 2015. Prospective clinical study of precision oncology in solid tumors. Journal of the National Cancer Institute 108.

Stockley, T.L., Oza, A.M., Berman, H.K., et al., 2016. Molecular profiling of advanced solid tumors and patient outcomes with genotype-matched clinical trials: The Princess Margaret IMPACT/COMPACT trial. Genome Medicine 8, 109.

Tredan, O., Corset, V., Wang, Q., et al., 2017. Routine molecular screening of advanced refractory cancer patients: An analysis of the first 2490 patients of the ProfilER Study. Journal of Clinical Oncology 35.

Tsimberidou, A.M., Iskander, N.G., Hong, D.S., et al., 2012. Personalized medicine in a phase I clinical trials program: The MD Anderson Cancer Center initiative. Clinical Cancer Research 18, 6373–6383.

Tsimberidou, A.M., Wen, S., Hong, D.S., et al., 2014. Personalized medicine for patients with advanced cancer in the phase I program at MD Anderson: Validation and landmark analyses. Clinical Cancer Research 20, 4827–4836.

Tsimberidou, A.M., Hong, D.S., Ye, Y., et al., 2017. Initiative for molecular profiling and advanced Cancer therapy (IMPACT): An MD Anderson precision medicine study. JCO Precision Oncology 2017.

Von Hoff, D.D., Stephenson Jr, J.J., Rosen, P., et al., 2010. Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. Journal of Clinical Oncology 28, 4877–4883.

Wheler, J.J., Janku, F., Naing, A., et al., 2016. Cancer therapy directed by comprehensive genomic profiling: A single center study. Cancer Research 76, 3690–3701.

Non-Hodgkin Lymphoma: Diagnosis and Treatment

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Abbreviations

ABC-DLBCL Activated B-cell diffuse large B-cell lymphoma
BTK Bruton's tyrosine kinase
CBC Complete blood count
CHOP Cyclophosphamide/doxorubicin/vincristine/prednisone
CNS Central nervous system
CVP Cyclophosphamide/vincristine/prednisone
CT Computed tomography
DLBCL Diffuse large B-cell lymphoma
ESMO The European Society for Medical Oncology
FDA US Food and Drug Administration
FDG F-18 2-deoxyglucose
FNA Fine-needle aspiration
FL Follicular lymphoma
FLIPI Follicular Lymphoma International Prognostication Index
GELF Groupe d'Etude des Lymphomes Folliculaires
HBV Hepatitis B virus
HSCT Hematopoietic stem cell transplantation
ICD-O International Classification of Diseases for Oncology
IPI International Prognostic Index
ISRT Involved site radiotherapy
NCCN US National Comprehensive Cancer Network
NHL Non-Hodgkin lymphoma
LDH Lactate dehydrogenase
PET Positron emission tomography
R [as first letter in therapeutic regimen acronyms] Rituximab
RT Radiotherapy
WHO World Health Organization

Definition

Non-Hodgkin lymphomas (NHLs) are one of the two groups of lymphomas (i.e., solid tumors of lymphoid tissues). NHLs are a large and very heterogeneous group of tumors, with more than 70 definitive or provisional entities listed in the current classification of the World Health Organization (WHO). They may originate from mature B cells (B-NHLs), T cells or NK cells (T/NK-NHLs), with the first of these categories (B-NHLs) accounting for up to 90% of all NHL cases. The most common NHL type is diffuse large B-cell lymphoma (DLBCL) followed by follicular lymphoma (FL). These two NHL subtypes taken together account for 50%–60% of all NHL cases. This entry, in addition to discussing clinical features and management of all NHLs in general, covers in detail these two NHL types.

Presentation and Diagnosis

Clinically, B-NHLs are categorized into low-grade and high-grade diseases. Low-grade NHLs are indolent, and tend to grow and spread slowly. They may transform into high-grade NHLs with time. High-grade NHLs are aggressive rapidly growing tumors, sometimes with a dramatic clinical course. Of the two NHL types discussed in this entry, FL is the most common among the low-grade NHLs (even though, a rare aggressive variant of FL exists) and DLBCL—among high-grade tumors.

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The clinical manifestations of NHL vary depending on the location of the lymphomatous process, the rate of tumor growth, and the function of the organ being compromised or displaced by the malignant process.

Low-grade NHLs give few signs and symptoms. Painless and slowly progressing peripheral adenopathy is the most common clinical presentation. As it can spontaneously regress (temporarily), it can potentially be confused with a manifestation of an infectious condition. Primary extranodal involvement and B symptoms (temperature over 38°C, night sweats, and over 10% weight loss within 6 months) are not common at presentation but they are frequent in patients with advanced, malignant transformation or end-stage disease. Bone marrow is often involved and this may be associated with cytopenia. Fatigue and weakness are more common in patients with advanced disease. FL most commonly involves bone marrow (60%–70% of cases), the liver (50%), and the spleen (40%). B symptoms occur in about 20% of patients. Symptoms resulting from the bone marrow dysfunction, such as anemia, leukopenia, or thrombocytopenia, are rare at presentation but can appear in advanced disease. Lymphocytosis, reflecting blood involvement, may also be present. A majority of patients present with stage III or IV disease.

Most patients with high-grade NHL present with adenopathy. Extranodal involvement is present in over one third of patients, the most common sites being the gastrointestinal tract, genitourinary tract, skin, bone marrow, sinuses, thyroid, and central nervous system. B symptoms are present in 30%–40% of patients. DLBCL patients usually present with a rapidly growing cervical lymph node bulk or abdominal mass, giving varied symptoms related to tissue infiltration and organ obstruction caused by the growing mass. Pain in the enlarged lymph node or organ may be noted. B symptoms occur in about 40% (stage I-II) to 60% (stage III-IV) of patients at initial presentation. Generalized pruritus (itchy skin) may also be present. Extranodular and extramedullary involvement occurs in over 40% of patients. Other symptoms can include anorexia, lower limb edema caused by extensive pelvic lymphadenopathy, fatigue, and chest discomfort or shortness of breath due to mediastinal lymphadenopathy.

The initial workup in case of a suspected NHL includes a thorough physical examination which should evaluate for hepatosplenomegaly, the presence of effusions, evidence of neuropathy, and signs of obstruction (e.g., extremity edema, superior vena cava syndrome, or spinal cord compression). Lymph node chains must be carefully examined, including the cervical, supraclavicular, axillary, epitrochlear, inguinal, femoral, and popliteal nodes. The lymph nodes are examined for size, multiplicity, consistency, and tenderness (lymphomatous involvement typically results in a rubbery consistency). The tonsils and oropharynx are also thoroughly examined. Waldeyer ring involvement mandates complete evaluation of the nasopharynx, oropharynx, and hypopharynx by endoscopy.

Laboratory testing includes complete blood count (CBC) with a differential, a comprehensive metabolic panel including liver and kidney function tests, serum lactate dehydrogenase (LDH), and serum β2 microglobulin. An abnormal blood count warrants a peripheral blood smear. Elevated LDH correlates with increased tumor burden and indicates poor prognosis. The LDH value is one of the parameters used to calculate the International Prognostic Index (IPI), a clinical tool to predict NHL patients' prognosis. Abnormal liver function tests may indicate hepatic involvement, hypermetabolic tumor growth, and/or chronic inflammation. Suspected DLBCL patients with elevated LDH levels should be assessed for spontaneous tumor lysis syndrome (TLS), which includes measurements of uric acid, potassium, phosphorus, calcium, and renal function. Hyperuricemia is a common manifestation of high-turnover-rate (aggressive) NHL. As some of the NHL treatments may lead to reactivation of hepatitis B virus (HBV), HBV testing is also recommended.

A chest radiograph is informative in approximately one fourth of NHL patients. It may identify hilar or mediastinal adenopathy, pleural or pericardial effusions, and parenchymal involvement. A computed tomography (CT) scan (with intravenous contrast) of the neck, chest, abdomen, and pelvis is routinely used to detect adenopathy, hepatosplenomegaly, or filling defects in the liver and spleen. Currently, it is the most widely used test for initial staging, and also for assessing treatment response and for follow-up. A bone scan is indicated in patients with bone pain and/or elevated alkaline phosphatase levels. Bone lesions are particularly frequent in DLBCL. Upper gastrointestinal series are performed if gastrointestinal involvement is suspected.

Functional imaging of the whole body using positron emission tomography (PET) with F-18 2-deoxyglucose (FDG) is usually recommended to complement staging of all patients with aggressive lymphoma, including DLBCL.

As a number of medical conditions may be associated with enlarged lymph nodes, a pathological evaluation of an incisional or excisional lymph node biopsy (or of biopsies of other sites in case of extranodal disease) is essential to establish an accurate diagnosis of any of the NHL types. The utility of fine-needle aspiration (FNA) biopsy remains controversial. In most clinical guidelines, FNA alone is considered insufficient to establish diagnosis but it may have an added value when used in combination with other techniques, like immunohistochemistry and flow cytometry. Moreover, according to the expert panel of the US National Comprehensive Cancer Network (NCCN), FNA alone may be sufficient to establish relapse.

Bone marrow biopsy to evaluate bone marrow involvement is commonly recommended as part of the diagnostic workup, in particular for high-grade lymphomas. However, some data suggest that FDG-PET is more accurate in detecting bone marrow involvement than bone marrow biopsy. Lumbar puncture and the analysis of the cerebrospinal fluid may be necessary in some patients with high-risk disease. Differentiating between disease subtypes is made based on immunophenotyping by flow cytometry and/or immunohistochemistry. Complementary cytogenetic or molecular analyses to detect characteristic chromosomal translocations or genetic alterations, or to establish clonality, may also be necessary in some cases. For detailed information on cell surface and genetic markers as well as on staging and risk stratification, refer to the entry in the "Pathology and Genetics" section.

Management and Therapy

The management of NHL patients varies depending on the tumor type and localization. In particular, the management strategies are very different between those for indolent (low-grade) and those for aggressive (high-grade) disease. This section describes specifically the management of FL (the most common of indolent tumors) and DLBCL (the prototype of the aggressive ones).

Follicular Lymphoma

Most FL patients present with advanced and disseminated disease. However, FL usually progresses slowly, frequently giving no or mild symptoms, and the prognosis is good in most cases. Therefore, immediate therapeutic intervention is not always necessary.

The "wait and watch" ("watchful waiting") approach (observation) may often be a reasonable option in early-stage FL, in particular in localized asymptomatic disease but also in advanced-stage disease in patients with a life expectancy shorter than 15 years. Observation is also common practice for patients with advanced but low tumor burden disease. The Follicular Lymphoma International Prognostication Index (FLIPI) and the criteria of the Group for Follicular Lymphoma Studies (Fr. Groupe d'Etude des Lymphomes Folliculaires; GELF) are used to determine whether the patient needs immediate treatment or whether observation is a viable option (for more information on these, refer to the "Pathology and Genetics" entry).

Otherwise, involved site radiotherapy (ISRT) remains the standard of care for localized nonbulky stage I-II FL. Combinations of RT with chemotherapy and with chemoimmunotherapy, as well as biological therapy are also used and none of the approaches has been shown to be superior to others. Surgical resection may additionally be considered in symptomatic localized disease. In selected cases, a monotherapy with rituximab (Rituxan®, Biogen Idec and Genentec, USA), an FDA-approved monoclonal anti-CD20 antibody, may be considered to avoid the side effects of radiation. Patients with bulky stage I-II disease are managed with systemic therapy like stage III-IV patients.

Stage III and IV FL is considered to be an incurable disease. The therapeutic most frequently used as first-line treatment of later-stage FL, either as monotherapy or as part of a multiagent therapy, is rituximab. Adding rituximab to chemotherapy regimens has been shown to improve both progression-free and overall survival in FL patients. The European Society for Medical Oncology (ESMO) recommends rituximab monotherapy for stage III-IV patients with mild symptoms, and combined rituximab/chemotherapy for those with high tumor burden. These combined regimens are the following: rituximab with cyclophosphamide/doxorubicin/vincristine/prednisone (R-CHOP) and rituximab with bendamustine. Rituximab with cyclophosphamide/vincristine/prednisone (R-CVP) may also be considered but some reports suggest that it may be less effective than the other two regimens. Full courses of purine analogue-based schemes: fludarabine/cyclophosphamide (FC) or fludarabine/mitoxantrone (FM), are not recommended due to higher hematological toxicities, but a brief course of chemoimmunotherapy with full rituximab course is an alternative in elderly patients, with good efficacy and low toxicity. The R-CHOP regimen is recommended if there is any evidence of an aggressive FL type and/or transformation. A novel strategy, combining lenalidomide, an immunomodulatory agent, with rituximab for the initial management of FL is currently being evaluated in clinical trials.

Since the introduction of rituximab, single-agent systemic therapy with chlorambucil or cyclophosphamide, or with the purine analogs fludarabine and cladribine, is used increasingly less frequently but can still be effective in some patients. Antibody monotherapy with rituximab or using radio-immunoconjugates, like ibritumomab tiuxetan (Zevalin®), or treatment with chlorambucil plus rituximab, remain alternatives for lower risk patients or when conventional chemotherapy is contraindicated. Rituximab or radioimmunotherapy are recommended for consideration as maintenance therapy following the combined rituximab/chemotherapy regimen in patients with high-burden tumors. Combining rituximab with bortezomib (a proteasome inhibitor) and bendamustine may also provide some benefit as the first-line treatment of FL patients.

Myeloablative consolidation followed by autologous hematopoietic stem cell transplant (HSCT) prolongs progression-free survival after chemotherapy, but its benefit after a rituximab-containing induction is minor. Therefore, this approach is not recommended as the first-line therapy of responding patients.

Multiple options exist for the treatment of patients who relapse or progress after the first-line therapy. However, the relapse should be histologically evaluated to exclude transformation into an aggressive FL. Bendamustine is approved in the US for use in patients with rituximab-refractory indolent B-cell lymphoma, including FL. Many practitioners administer bendamustine in combination with rituximab. Fludarabine-based regimens are another option for patients who relapse after an alkylator-based therapy. However, they may not be suitable for heavily pretreated elderly patients in whom rituximab (in case of rituximab-sensitive disease) or radioimmunotherapy may be better options. For patients who are refractory to rituximab, the combination of bendamustine and obinutuzumab (type II anti-CD20 monoclonal antibody) was shown to be superior to bendamustine alone with respect to progression-free survival. Obinutuzumab is also under investigation in the frontline setting.

Allogenic or autologous HSCT may be considered in younger patients with relapsed or recurrent disease. Allogeneic HSCT can induce long-term remissions but is associated with high mortality. Autologous HSCT has a low transplant-associated mortality and prolongs progression-free survival. Still, most patients eventually relapse and there is an increased rate of secondary malignancies. Therefore, this option is only recommended for young patients with adverse presentations, including early relapse after first-line treatment. HSCT is usually performed following a high-dose chemotherapy. Subsequent rituximab maintenance therapy may improve the outcome. However, the utility of HSCT in the rituximab era is to be redefined.

A relatively novel option for the treatment of relapsed or refractory FL is idelalisib, an inhibitor of the phosphatidylinositol 3-kinase δ (PI3Kδ). Idelalisib is approved by FDA for treatment of relapsed FL which has not responded to at least two prior systemic therapies (double-refractory FL). Moreover, a variety of other novel therapeutic agents for FL treatment, such as inhibitors of Bruton's tyrosine kinase (BTK), antibody-drug conjugates, novel anti-CD20 monoclonal antibodies, chimeric antigen receptor T-cell therapy, and immune checkpoint inhibitors are being developed.

Diffuse Large B-Cell Lymphoma

DLBCL is curable in 50%–80% of cases. Localized nonbulky DLBCL (stage I and II disease) can be successfully managed by an abbreviated course of a rituximab-doxorubicin-containing regimen (R-CHOP) followed by ISRT. Another approach is full-course chemotherapy with or without subsequent RT. Bulky stage I–II disease as well as disseminated (i.e., stage III–IV) disease is treated with full-course CHOP chemotherapy, with the addition of rituximab if the tumor is CD20-positive. In patients with concurrent CNS disease, methotrexate is incorporated as part of the treatment plan. CNS prophylaxis should be applied in patients with a high risk of the CNS involvement.

Other chemotherapy regimens, like R-ACVBP (rituximab, doxorubicin, vindesine, cyclophosphamide, bleomycin and prednisolone) or DA-EPOCH (dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, adriamycin) are also used but there is no convincing evidence that they provide a benefit over R-CHOP treatment and they are not recommended by the existing clinical guidelines.

Complete restaging to assess response to treatment is mandatory, with FDG-PET/CT being the recommended standard for post-treatment assessment in DLBCL. Overall, over 30% of DLBCLs will ultimately relapse.

Few patients with limited refractory disease may possibly be salvaged with ISRT if the involved area is not extensive but this has never been evaluated in clinical trials. In most cases, salvage chemotherapy, followed by autologous HSCT in responsive patients, is the preferred approach for relapsed/refractory disease. Second-line chemotherapy regimens often employ high-dose cytosine arabinoside or gemcitabine, corticosteroids, and platinum-containing chemotherapeutic agents with or without etoposide. The most commonly used regimens are the following: DHAP (dexamethasone, high-dose cytarabine, cisplatin), ESHAP (etoposide, methylprednisolone, high-dose cytarabine, cisplatin), MIME (methyl-GAG, ifosfamide, methotrexate, etoposide), ICE (ifosfamide, carboplatin, etoposide), and GDP (gemcitabine, dexamethasone, cisplatin). Any of these regimens could be combined with rituximab if the patient is deemed to be responsive to this agent. However, the actual clinical impact of this practice is not clear. Patients who achieve at least partial remission following the salvage chemotherapy are considered for autologous HSCT. Allogeneic HSCT may be most reasonable in young patients who have a suitable donor match and who do not fall into the favorable categories for benefit from autologous transplantation. Patients who are not candidates for HSCT receive nonplatinum-based chemotherapy. However, enrolment in a clinical trial is the best option for these patients.

Progressing knowledge of pathological and molecular heterogeneity of DLBCL has stimulated a search for personalized molecular therapy approaches. For example, the activated B-cell (ABC) DLBCL subtype has been shown to have a worse prognosis when compared with germinal centre B-cell in patients treated by R-CHOP. This subtype is characterized by a constitutive activation of the NF-κB pathway which could be targeted by different agents, such as bortezomib and lenalidomide. Preliminary studies of treatment regimens involving these two agents have given promising results and phase II clinical trials are currently ongoing. Furthermore, ibrutinib, a novel oral BTK inhibitor, has shown selective activity in ABC-DLBC. Several clinical trials evaluating this agent for treatment of DLBCL and other lymphomas are under way. Finally, a number of recurrent genetic alterations found in DLBCL and other NHL lymphomas which have been identified by recent genomic analyses give hope that at least some of them may become either prognostic biomarkers or useful targets for next-generation NHL treatment.

See also: Non-Hodgkin Lymphomas: Pathology and Genetics.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer.

Dreyling, M., et al., 2016. Newly diagnosed and relapsed follicular lymphoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Annals of Oncology 27 (suppl. 5), v83–v90.

Kahl, B.S., Yang, D.T., 2016. Follicular lymphoma: Evolving therapeutic strategies. Blood 127 (17), 2055–2063.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. B-Cell Lymphomas. Version 2.2018 – February 26, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site

Tilly, H., et al., 2015. Diffuse large B-cell lymphoma (DLBCL): ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Annals of Oncology 26 (suppl. 5), v116–v125.

Relevant Websites

https://clinicaltrials.gov/—Clinical trial information at the US National Library of Medicine, National Health Institutes.

Non-Hodgkin Lymphomas: Pathology and Genetics

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Glossary

Follicular helper T cells (T_{FH} cells) Are resident T cells of the germinal center (GC) which are necessary for the formation and maintenance of the GC reaction, a hallmark of adaptive immunity T_{FH} cells play a major role in supporting and regulating the selection and survival of B cells in the GC. T_{FH} cells are CD3+ CD4+ cells that express BCL6, critical for maintaining the T_{FH} transcriptional program have surface expression of CXCR5, PD-1, and ICOS and secrete different cytokines and chemokines, in particular CXCL13 and IL-21.

Somatic hypermutation (SHM) An AID-dependent process that introduces point mutations and occasional small deletions or duplications at a very high rate into DNA sequences of IGVH and VL gene regions (variable regions of the heavy and light-chains IG genes) of germinal center (GC) B cells, is mechanistically linked to lymphomagenesis. Somatic hypermutation in normal GC B cells is not restricted to IGV regions but also targets the 5′ sequences of BCL6, a transcription repressor essential to the GC reaction expressed at high levels in GC B cells, as well as in the FAS gene. Some of these mutations may be selected during lymphomagenesis for their activity in deregulating BCL6 gene expression. In a large proportion of diffuse large B-cell lymphomas, an aberrant hypermutation activity may target multiple loci including the 5′ untranslated or coding sequences of several other protooncogenes, including MYC. Interestingly, the hypermutable genes are susceptible to chromosomal translocations in the same region, consistent with a role for hypermutation in generating translocations by DNA double-strand breaks. Thus, by mutating multiple genes, and by favoring chromosomal translocations, aberrant hypermutation may represent a major contributor to lymphomagenesis.

Ongoing SHM Analysis of the pattern of somatic hypermutations may indicate whether these occurred as a result of antigen selection. The presence of intraclonal variation within lymphoid neoplasms is taken as indicative of ongoing mutations, and therefore a relationship to the germinal center.

Definition and Principles of Classification

Lymphoid neoplasms represent a large group of clonal proliferations derived from immature or mature lymphoid cells. They encompass a variety of distinct diseases with different clinical presentations, pathological features and biological behavior. In general, leukemia is the term used for disseminated lymphoid tumors mainly involving the blood and the bone marrow, and lymphoma refers to lymphoid neoplasms presenting as tumors in lymph nodes or other hematopoietic or nonhematopoietic organs, but not infrequently overlapping presentations are encountered. In the World Health Organization (WHO) classification —the universal reference for the definition and diagnosis of hematological malignancies—the definition of disease entities incorporates multiple parameters: morphology, immunophenotype, genetic and molecular features, clinical characteristics, and, whenever possible, the postulated normal cell counterpart from which the tumor is derived (Fig. 1) (2017 #8370). Both lymphomas and lymphoid leukemias are included in this classification, since both solid and circulating phases are present in many lymphoid neoplasms, and distinction between them is artificial.

Lymphoid neoplasms derive from B cells, T cells or NK cells at various stages of differentiation and retain many features (i.e., normal genetic events, gene expression, immunophenotype, morphology, homing patterns) of their normal counterparts, which in large part dictate the clinical behavior of the diseases. Therefore, the knowledge of normal lymphoid differentiation provides a useful framework for understanding the biology of lymphomas (Fig. 2). There are two major categories of lymphoid neoplasms, based on stage of differentiation: precursor (lymphoblastic) neoplasms, derived from immature lymphoid cells, and peripheral or mature neoplasms, derived from mature lymphoid cells. The usual presentation for neoplasms derived from immature B or T lymphoid cells, which are primarily diseases of children, is an acute leukemia. Most mature lymphoid neoplasms present as lymphomas, or, less commonly, as chronic leukemias. Neoplasms derived from plasma cells (terminally differentiated B cells) constitute another group of usually primarily bone marrow-based lymphoid malignancies, with disease manifestations related to bone destruction, excess of monoclonal immunoglobulin production, inhibition of normal host resistance to infection, and/or tissue immunoglobulin deposition. Among lymphomas, Hodgkin lymphomas (HL) are segregated from all other forms, which constitute a much larger group referred to as non-Hodgkin lymphoma (NHL). There are two major groups of NHL, based on cell derivation: those derived from B cells (B-NHL), by far most frequent, and those derived from T cells or NK cells (T/NK-NHL), which account for $< 10\%$ of all NHLs in western countries and many parts of the world (Table 1). Overall there are more than 70 definitive or provisional NHL entities listed in the current WHO classification.

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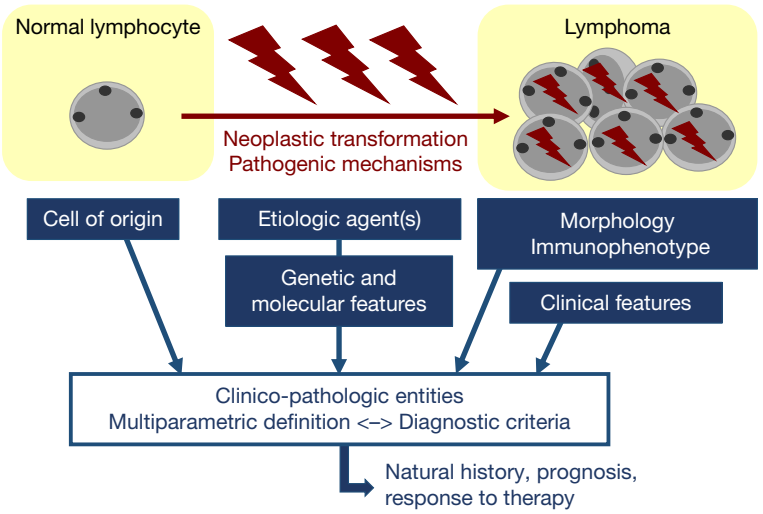

Fig. 1 Principles of lymphoma classification in the WHO classification.

Epidemiology

Worldwide, lymphomas are more common in developed countries with the highest incidence in the United States and in Europe. They are much less frequent in China, and have an intermediate incidence rate in South America, Africa and Japan. According to epidemiological data from the United States and United Kingdom, NHL represents the 6th most frequent cancer in those countries (4.3% of all cancers in the United States and 4% in the United Kingdom). For 2017 about 72,000 new NHL cases diagnoses and about 20,000 NHL-related deaths in the United States were estimated. Incidence is higher among men than women, and in the United States it is also higher in nonhispanic white people than in black people, while no significant variation by ethnicity is observed in the United Kingdom. For US patients, the median age at diagnosis is 67 years, the median age at death is 76 years and a overall 5 years survival of 71%. There has been a marked increase in NHL incidence over the past decades, with a near doubling of the incidence rate between 1975 and 1995. Since then, there has been little variation or even a trend toward a minimal decrease in incidence (in a range $<1\%$) in the United States, while in the United Kingdom, the NHL incidence has continued to slightly increase in the recent years, essentially in the older age groups. It is not clear whether this is a true increase in the incidence of the disease, or a reflection of improved medical surveillance of elderly people or improved diagnostic testing.

Etiology and Risk Factors

The etiology of NHL remains poorly understood. The best established factors affecting an individual's risk of developing NHL are immune disorders and infections. Other factors include genetic and racial features, familial predisposition, lifestyle factors, environmental or occupational exposures.

Immune Disorders

Increased incidence rates of NHL are observed in immunosuppressed populations (those receiving immunosuppressive treatment following solid organ transplantation, and individuals with the acquired immunodeficiency syndrome (HIV/AIDS)). The majority of immunodeficiency-related lymphoid malignancies are B-cell derived and frequently associated to EBV. Individuals with inherited defects of immune function, like ataxia-telangiectasia, Wiskott–Aldrich syndrome, X-linked lymphoproliferative disorder, severe combined immunodeficiency syndrome and others, are at increased risk of NHL as well. On the other hand, autoimmune disorders also represent a risk factor for the development of NHL. Evidence of an association is confirmed among individuals with Sjogren's syndrome, rheumatoid arthritis, systemic lupus erythematosis, autoimmune thyroiditis and celiac disease.

Infectious Agents

A variety of infectious agents has been identified as either linked to or truly causative of lymphomas (Table 2). Among viruses, the Epstein-Barr virus (EBV) is associated with the largest variety of lymphoproliferations. EBV is present in nearly 100% of endemic Burkitt lymphoma and in 40% of sporadic and HIV-associated cases, is detected in virtually all cases of extranodal nasal type T/NK-cell lymphoma, and is clearly implicated in the pathogenesis of lymphoproliferations occurring in the setting of immunodeficiency. The human herpesvirus-8 (HHV8) is found in association with different lymphoproliferative diseases linked to HIV

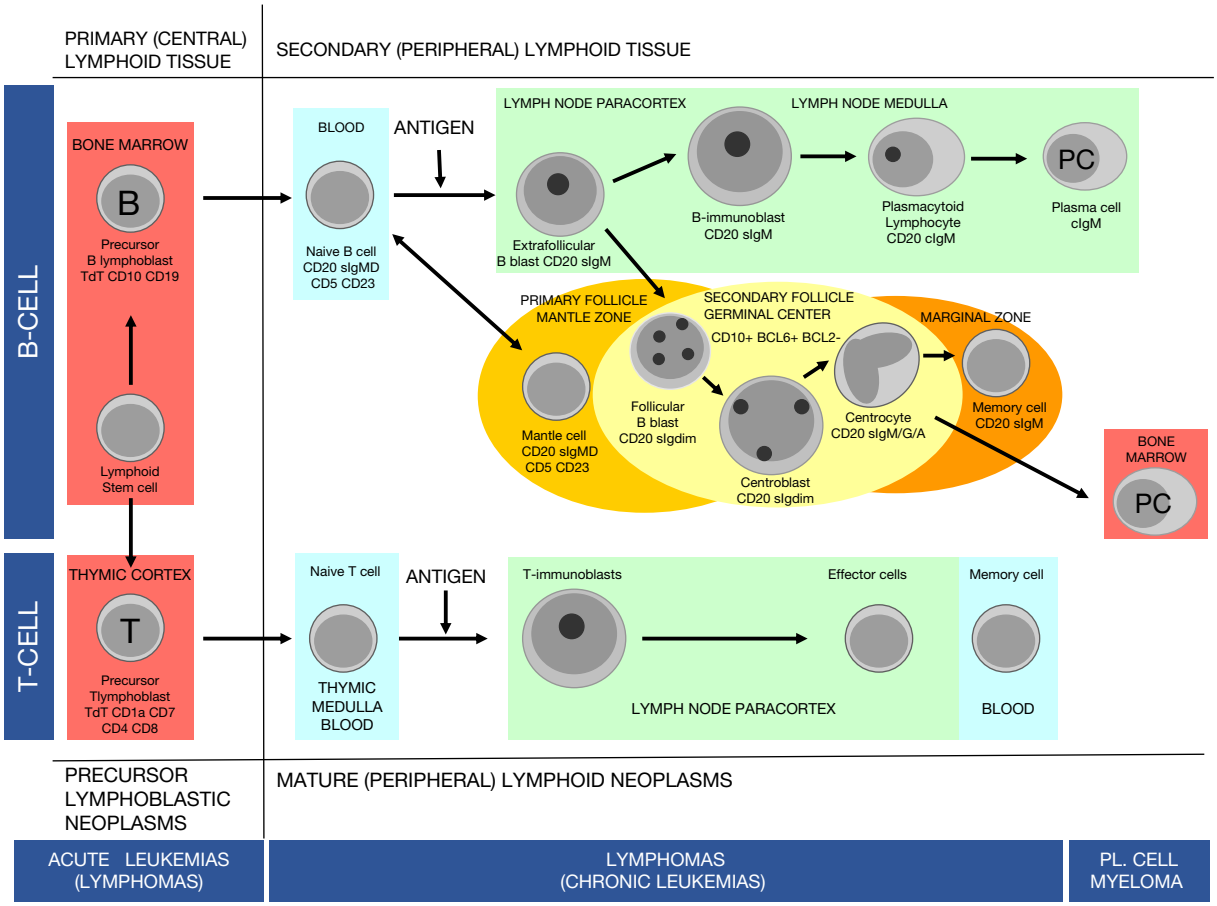

Fig. 2 Normal lymphoid differentiation and corresponding lymphoid neoplasms. Each stage occurs at a specific anatomic site and architectural nichewithin lymphoid organs, and is characterized by changes in surface antigen expression. The antigen-independent phase of lymphoid differentiation occurs in the primary lymphoid organs (bone marrow and thymus). A postulated common lymphoid stem cell gives rise to both B and T-cell lineages. The early stages are stem cells and lymphoblasts (precursor T and B cells), which are self-renewing. Antigen-independent differentiation results in mature, naïve T and B cells that are capable of responding to antigen that binds to their surface antigen receptors (T-cell receptor, TCR and B-cell receptor, BCR or surface immunoglobulin, sIg). Naïve B cells are found in the blood and in primary follicles and mantle zones of secondary follicles. Naïve T cells are found in the blood, in the thymic medulla and also in the paracortex of lymph nodes. On exposure to antigen in the secondary lymphoid organs (lymph nodes, spleen, mucosal-associated lymphoid tissue (MALT)), the naive lymphocyte undergoes "blast transformation," and becomes a large, proliferating cell, which gives rise to progeny that are antigen-specific effector or memory cells. Antigen-dependent B-cell differentiation may occur along two pathways: T-cell independent reactions (immunoblastic paracortical reaction leading to the generation of short-lived IgM secreting plasma cells) or the germinal center (GC) reaction, hallmark of T-cell dependent responses. B-blasts activated in the paracortex upon antigen encounter migrate to the center of a primary follicle, proliferate and differentiate into centroblasts which further mature to centrocytes. In the GC, B cells undergo somatic mutations of the variable region of the immunoglobulin (IGV) genes regions and class switching of the Ig heavy chain (IGH) gene allowing for expression of IgG, IgA or IgE. Only cells with favorable IGV gene mutations—that are those resulting in a higher affinity of the BCR for the antigen—are positively selected and survive. These centrocytes may then differentiate to effector cells (usually IgG or IgA secreting plasma cells) homing to the bone marrow, or to memory cells, which may collect into a marginal zone. Antigen-dependent T-cell differentiation occurs in the paracortex of the lymph node and results in expanded clones of effector CD4+ and CD8+ T cells (helper and cytotoxic) and memory cells that may pass into the blood. TdT: terminal deoxynucleotidyl transferase; CD: cluster of differentiation; sIg: surface immunoglobulin expression; cIg: cytoplasmic Ig expression.

infection (primary effusion lymphoma, HHV8-associated multicentric Castleman's disease, HHV8+ diffuse large B-cell lymphoma) as well as in the indolent and rare germinotropic lymphoproliferative disorder in immunocompetent individuals. HHV8 is capable of establishing a latent infection in B cells and its oncogenic mechanisms involve the promotion of cell survival and proliferation, through the release of cytokines and antiapoptotic factors. In multicentric Castleman's disease, viral protein IL6 produced by infected cells is as a major mediator of the systemic symptoms and of hypergammaglobulinemia associated to the disease.

The human T-cell leukemia virus type 1 (HTLV1) is causally linked to adult T-cell leukemia/lymphoma, a disease endemic in Japan and in the Caribbean area. The lifetime risk for development of ATLL among seropositive individuals is overall inferior to 5%, indicating the necessity for additional events for neoplastic transformation. Clonally integrated HTLV1 is found in all cases. The antisense gene product HBZ is thought to represent major driver of T-cell transformation by inducing chronic proliferation,

Table 1 2017 WHO classification of non-Hodgkin lymphomas

Precursor B-cell neoplasms
- B-lymphoblastic leukemia/lymphoma, NOS and multiple variants based on genetic abnormalities

Mature B-cell neoplasms
- *Predominantly disseminated*
  - Chronic lymphocytic leukemia/B-cell small lymphocytic lymphoma
  - Monoclonal B-cell lymphocytosis
  - B-cell prolymphocytic leukemia
  - Splenic marginal zone B-cell lymphoma
  - Splenic B-cell lymphoma/leukemia, unclassifiable[a]
  - Splenic diffuse red pulp small B-cell lymphoma[a]
  - Hairy cell leukemia
  - Hairy cell leukemia variant[a]
  - Lymphoplasmacytic lymphoma
  - IgM monoclonal gammopathy of undetermined significance
  - Heavy chain diseases
  - Plasma cell neoplasms
- *Primary extranodal*
  - Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT)
- *Predominantly nodal*
  - Follicular lymphoma (FL)
  - In situ follicular neoplasia
  - Duodenal-type FL
  - Testicular FL
  - Pediatric-type FL
  - Large B-cell lymphoma with *IRF4* rearrangement[a]
  - Primary cutaneous follicle center lymphoma
  - Mantle cell lymphoma
  - In situ mantle cell neoplasia
  - Nodal marginal zone B-cell lymphoma
  - Pediatric nodal marginal zone lymphoma[a]
  - Diffuse large B-cell lymphoma (DLBCL) not otherwise specified (NOS), germinal center B-cell type (GCB-DLBCL-NOS)
  - DLBCL-NOS activated B-cell type (ABC-DLBCL-NOS)
  - T-cell/histiocyte-rich large B-cell lymphoma
  - Primary DLBCL of the central nervous system
  - Primary cutaneous DLBCL, leg type
  - EBV-positive DLBCL-NOS
  - EBV-positive mucocutaneous ulcer
  - DLBCL associated with chronic inflammation
  - Fibrin-associated DLBCL
  - Lymphomatoid granulomatosis
  - Primary mediastinal (thymic) large B-cell lymphoma
  - Intravascular large B-cell lymphoma
  - ALK-positive DLBCL
  - Primary effusion lymphoma
  - Multicentric Castleman disease
  - HHV8-positive DLBCL-NOS
  - HHV8-positive germinotropic lymphoproliferative disorder
  - Burkitt lymphoma
  - Burkitt-like lymphoma with 11q aberration[a]
  - High-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements
  - High-grade B-cell lymphoma NOS
  - B-cell lymphoma unclassifiable with features intermediate between DLBCL and classical Hodgkin lymphoma

Immunodeficiency-associated lymphoproliferative disorders
- Posttransplantation lymphoproliferative disorders (PTLD)
  - Nondestructive PTLD (plasmacytic hyperplasia, infectious mononucleosis, florid follicular hyperplasia)
  - Polymorphic PTLD
  - Monomorphic PTLD
  - Other iatrogenic immunodeficiency-associated lymphoproliferative disorders

Precursor T-cell neoplasms
- T-lymphoblastic leukemia/lymphoma
- Early T-cell precursor lymphoblastic leukemia
- NK-lymphoblastic leukemia/lymphoma

Mature T-cell neoplasms
- *Disseminated/leukemic*
  - T-cell prolymphocytic leukemia
  - T-cell large granular lymphocytic leukemia
  - Chronic lymphoproliferative disorder of NK cells[a]
  - Aggressive NK-cell leukemia
  - Systemic EBV-positive T-cell lymphoma of childhood[a]
  - Chronic active EBV infection of T- and NK-cell type, systemic form
  - Adult T-cell leukemia/lymphoma
- *Nodal*
  - Angioimmunoblastic T-cell lymphoma
  - Follicular T-cell lymphoma
  - Nodal peripheral T-cell lymphoma with T follicular helper phenotype
  - Anaplastic large-cell lymphoma, ALK-positive
  - Anaplastic large-cell lymphoma, ALK-negative
  - Peripheral T-cell lymphoma, not otherwise specified
- *Extranodal*
  - Extranodal NK/T-cell lymphoma, nasal type
  - Enteropathy-associated T-cell lymphoma
  - Monomorphic epitheliotropic intestinal T-cell lymphoma
  - Indolent T-cell lymphoproliferative disorder of the gastrointestinal tract[a]
  - Hepatosplenic T-cell lymphoma
  - Subcutaneous panniculitis-like T-cell lymphoma
  - Breast implant-associated anaplastic large-cell lymphoma[a]
- *Cutaneous*
  - Mycosis fungoides
  - Sezary syndrome
  - Primary cutaneous CD30 + lymphoproliferative disorders
    - Lymphomatoid papulosis
    - Primary cutaneous anaplastic large-cell lymphoma
  - Primary cutaneous $\gamma\delta$ T-cell lymphoma
  - Provisional CD4+ or CD8+ entities[a]
  - Hydroa vacciniforme-like lymphoproliferative disorder
  - Severe mosquito bite allergy

[a]Provisional entities.

Table 2 Infectious agents associated with the development of NHL

Infectious agent	*Lymphoid neoplasm*
Epstein-Barr virus (EBV)	Burkitt lymphoma
	Posttransplantation lymphoproliferative disorders
	EBV-positive diffuse large B-cell lymphoma, NOS
	Plasmablastic lymphoma
	Lymphomatoid granulomatosis
	Angioimmunoblastic T-cell lymphoma[a]
	Extranodal NK/T-cell lymphoma, nasal type
	Pediatric EBV-positive T-cell neoplasms of childhood
Herpesvirus-8 (HHV8)	Primary effusion lymphoma
	HHV8-positive diffuse large B-cell lymphoma, NOS
	Multicentric Castleman disease
Human T-leukemia lymphoma virus-1 (HTLV1)	Adult T-cell leukemia/lymphoma
Hepatitis C virus (HCV)	Lymphoplasmacytic lymphoma
	Marginal zone lymphomas (splenic and nodal types)
Helicobacter pylori	Gastric MALT lymphoma
Borrelia burgdorferi	Cutaneous MALT lymphoma
Chlamydia psittaci, pneumonia and trachomatis	Ocular adnexal MALT lymphoma
Campylobacter jejuni	Intestinal MALT lymphoma associated to alpha chain disease

[a]EBV present in a population of B cells in the tumor but not in the neoplastic T cells; MALT: mucosa-associated lymphoid tissues; NOS: not otherwise specified.

apoptotic resistance, multiple organ invasion, and drug resistance. TAX oncoprotein activates multiple signaling pathways in infected T cells, but is not expressed in ATLL neoplastic cells in many cases Further genetic and epigenetic changes lead to development of malignancy.

Different types of B-NHL are associated with hepatitis C virus (HCV) infection (relative risk of about 1.5), including splenic and nonsplenic marginal zone lymphomas, lymphoplasmacytic lymphoma and diffuse large B-cell lymphomas in extranodal localizations. The pathophysiology of HCV-associated lymphomas involves two nonexclusive mechanisms of transformation: chronic antigenic stimulation and to a lesser extent direct transformation via insertional mutagenesis or expression of oncogenic proteins. Sustained virologic response obtained with antiviral treatments might induce the remission of indolent lymphomas.

Bacteria, or at least immune responses to bacteria, have also been implicated in the pathogenesis of extranodal B-cell marginal zone lymphoma of the MALT type. The most convincing and best documented example is that of gastric MALT lymphoma arising from acquired MALT induced by *Helicobacter Pylori* infection. In early stage, MALT lymphoma cells preserve B-cell properties and their growth may be driven by antigenic stimulation. In this condition, as lymphoma growth in vitro is dependent on the presence of T cells activated with *H. pylori* antigens, antibiotic treatment causes regression of the lymphoma in many patients. In later stages of gastric MALT lymphoma, acquisition of genetic abnormalities, such as t(11;18)(q21,q21) or t(1;14)(p22;q32) is associated with tumor escape from its growth dependency. Similarly, *Borrelia burgdorferi* has been implicated in the pathogenesis of cutaneous MALT lymphoma and *Chlamydia psittaci* in that of ocular adnexa MALT lymphoma. Intestinal MALT lymphoma and immunoproliferative small intestinal disease (IPSID)/alpha chain disease has been associated with mixed bacterial infections and more specifically with *Campylobacter jejuni* infection.

The development of pyothorax-related lymphoma, strongly associated with EBV infection, is closely related to antecedent *Mycobacterium tuberculosis* infection.

Inherited Factors

An increased risk of developing lymphoid malignancies has been reported for relatives of patients. In particular, chronic lymphocytic leukemia seems to comprise a relatively more substantial familial component than other entities. Genome-wide association studies have identified genetic variants at different loci that are associated to an increased risk of developing different types of B-cell NHL, and less frequent genetic determinants associated to specific diseases.

Lifestyle and Environmental Factors

Lifestyle factors like obesity and tobacco smoking have been suggested as risk factors for B-cell NHL. In particular, a high body-mass index has been consistently associated with an increased risk of diffuse large B-cell lymphoma. Several studies have suggested a possible protective effect of sun exposure and ultraviolet radiation. Exposure to herbicides and pesticides have been incriminated in the development of B-cell NHL, but despite numerous studies, no specific association has been found that is thought to be causal.

Genetics of NHL

In contrast to many carcinomas, which display random genomic instability, lymphomas tend to have relatively stable genomes and generally lack defects in DNA mismatch repair genes responsible of microsatellite instability. Conventional cytogenetics has been instrumental in identifying recurrent translocations and in cloning the major oncogenes involved in B-cell lymphomagenesis. Over the last years, technological and analytical advances in genetic, epigenetic and molecular profiling methods and next-generation sequencing technologies have markedly accelerated the characterization of lymphoma-associated molecular signatures and genetic profiles, and the discovery of genetic lesions, which collectively have greatly enhanced our understanding of lymphoma pathogenesis. These studies have revealed genetic events shared between different disease types, as well as extensive genetic heterogeneity within a single disease entity. A nonexhaustive list of common genetic alterations in lymphomas is presented in **Table 3**.

Chromosomal Translocations and Gene Fusions

Reciprocal chromosomal translocations represent a major mechanism of protooncogene activation in NHL, and are a hallmark of many types of B-cell lymphomas (**Table 3** and **Fig. 3**). Many translocations associated with NHL involve a protooncogene in the proximity of the chromosomal recombination site, whose expression is altered as a consequence of the juxtaposition to heterologous regulatory sequences derived from the partner chromosome, a mechanism referred to as transcriptional deregulation. The most common translocations involved in lymphoid neoplasms place a gene that is normally silent in resting cells under the influence of a promoter associated with either an immunoglobulin (IG) or T-cell receptor (TCR) gene, resulting in deregulated expression of the gene and giving the cell either a growth or a survival advantage. Examples include the t(14;18)(q32;q32) of follicular lymphoma, or the t(11;14)(q13;q32) of mantle cell lymphoma, which place the *BCL2* or *BCL1* gene under the IG promoter and deregulate their expression.

Translocations in B-NHL result from chromosomal breaks that are aberrant by-products of the enzymes that rearrange the IG gene segments to produce the B-cell receptor (BCR) in normal B cells, and are usually caused by either activation-induced deaminase (AID) or the recombination activating gene (RAG) complex. Physiologically, AID is the master regulator of antibody diversification involved in the initiation of somatic hypermutation (see Glossary) and IGH locus class switch recombination in germinal center B cells, while the RAG complex is involved in VDJ recombination of IG and TCR genes in immature lymphoid cells.

Translocations in B-NHL tend to associate preferentially but not exclusively with a specific type of lymphoma. Translocations involving the same protooncogene may involve different breakpoints and arise by different mechanisms in different lymphoma types. Some translocations are seen in all cases of a specific lymphoma type. Paradigms include the involvement of *MYC* in virtually all cases of Burkitt lymphoma and of *BCL1* in virtually all cases of mantle cell lymphoma; these translocations may therefore be useful as a diagnostic marker. Moreover, within specific histologic subtypes, specific gene rearrangements may identify prognostic groups, and hence their detection is useful for the characterization of these lymphomas.

An alternative mechanism of oncogene activation by chromosomal translocation consists of the juxtaposition of coding sequences derived from the two involved genes, resulting in gene fusion coding for a novel chimeric protein. This type of translocation which is common in myeloid and lymphoblastic neoplasms, is less frequent in mature NHL. For example, the t(2;5)(p23;q35) in anaplastic large-cell lymphoma, produces a hybrid nucleophosmin–ALK (anaplastic lymphoma kinase) protein, and there are alternative translocation partners to ALK. Another example is the t(11;18)(q21;q21) in MALT lymphoma, which produces a BIRC3(API2)-MALT1 fusion protein.

Translocations can be detected by cytogenetics showing derivative chromosomes, or by fluorescence in situ hybridization (FISH), using probes to specific chromosomes or segments, or by molecular techniques (PCR or RT-PCR). Since an important consequence of many translocations is the overexpression of a protein not ordinarily found in that cell type, immunohistochemistry can be used as a surrogate to detect the translocation-induced deregulated protein, for example, ALK or BCL2. Over the last years, with high-resolution deep sequencing technologies, paired-end sequencing, RNA sequencing and integration of mate-pair and RNA sequencing, have led to the discovery of several new fusion genes that are recurrent in NHL and which mechanistically may correspond to translocations, intrachromosomal deletions or inversions.

Genetic Imbalances

Genomic imbalances are common in NHL, in particular in subsets of diffuse large B-cell lymphoma, and comprise genomic gains and losses that correspond to deregulated expression of putative oncogenes (*BCL2*, *FOXP1*, *SPIB*, *REL*, *MDM2*) and tumor suppressor genes (*CDKN2A*, *REL*, *PTEN*, *MDM2*). Primary mediastinal large B-cell lymphoma is characterized by both rearrangements and/or copy-number variations of several genes mapping to 9p24 (*PDCD1LG1*, *PDCD1LG2*, and *JAK2*). Several lymphoma types show frequent 6q deletion (6q21-6q23 and 6q25-6q27) which encompasses several candidate tumor suppressor genes (*FOXO3A*, *PRDM1*, *HACE1*). Deletions of the long arm of chromosome 13 (13q14) found in a subset of chronic lymphocytic leukemias involve the microRNA cluster miR-15a/miR-16-1, which functions as a tumor suppressor by targeting *BCL2*.

Table 3 Common translocations and gene fusions in NHL

	Translocation-fusion	*Genes*	*Biologic function*	*Lymphoma*
			Anaplastic lymphoma kinase = tyrosine kinase	
				Anaplastic large cell lymphoma (75%)
	t(2;5)(p23;q35)	*NPM/ALK*	Nucleophosmin–ALK fusion	Diffuse large B-cell lymphoma (rare)
	t(1;2)(q21;p23)	*TPM3-ALK*	Tropomyosin 3–ALK fusion	Anaplastic large cell lymphoma (15%)
				Anaplastic large cell lymphoma (2%)
	t(2;17)(p23;q23)	*CLTC/ALK*	Clathrin heavy chain–ALK fusion	Diffuse large B-cell lymphoma, ALK+
	t(2;3)(p23;q21)	*TFG-ALK*	TRK-fused gene–ALK fusion	Anaplastic large cell lymphoma (2%)
ALK	inv (2) (p23;q35)	*ATIC-ALK*	ATIC enzyme–ALK fusion	Anaplastic large cell lymphoma (2%)
				Mantle cell lymphoma (virtually all)
				Hairy cell leukemia (some)
BCL1	t(11;14)(q13;q32)	*BCL1/IGH*	Cell cycle regulator	Multiple myeloma (15%)
	t(14;18)(q32;q21)	*BCL2/IGH*	Negative regulator of apoptosis	Follicular lymphoma (80%)
	t(2;18)(q11;q21)	*BCL2/IGK*		Diffuse large B-cell lymphoma (20%)
BCL2	t(18;22)(q21;q11)	*BCL2/IGL*		
BCL3	t(14;19)(q32;q13)	*BCL3/IGH*	NF-kappaB subunit	B-CLL/SLL (rare)
	t(3;14)(q27;q32)	*BCL6/IGH*		Diffuse large B-cell lymphoma (30%)
BCL6	der (3)(q27)	*BCL6/var*	Transcriptional repressor necessary for GC formation	
BCL10	t(1;14)(p22;q32)	*BCL10/IGH*	Activator of the NF-kappaB pathway	MALT lymphoma (<5%)
		ICOS-CD28	CD28 : TCR costimulatory receptor	
CD28	Fusions	*CTLA4-CD28*	TCR signaling	Peripheral T-cell lymphomas (overall 5%)
		CIITA-PD-L1	CIITA: MHC class II transactivator reduced MHC II	Mediastinal large B-cell lymphoma (38%)
CIITA	16p13.13 break apart	*CIITA-PD-L2*	expression in rearranged cases	Testicular large B-cell lymphomas (10%)
				MALT lymphoma (18%) (other than gastrointestinal & pulmonary)
	t(14;18)(q32;q21)	*MALT1/IGH*	Paracaspase, binds to BCL10	
MALT1	t(11;18)(q21;q21)	*BIRC3/MALT1*	Fusion protein increases NF-kB activity	MALT lymphoma (50%)
	t(8;14)(q24;q32)	*MYC/IGH*	Transcription factor regulating	Burkitt lymphoma (100%)
	t(2;8)(p11;q24)	*MYC/IGK*	Cell proliferation	Diffuse large B-cell lymphoma (10%)
MYC	t(8;22)(q24;q11)	*MYC/IGL*		
				Multiple myeloma (rare)
MUM1(IRF4)	t(6;14)(p25;q32)	*MUM1/IGH*	Transcription factor involved in plasma cell differentiation	Large B-cell lymphoma with IRF4 rearr.
			Transcription factor regulating B cell proliferation and	Lymphoplasmacytic lymphoma (rare)
PAX-5	t(9;14)(p13;q32)	*PAX5/IGH*	differentiation	Multiple myeloma (rare)
PDL1 (CD274)	9p24.1 break apart		PDL1/2: immune checkpoint inhibitors	Mediastinal large B-cell lymphoma (20%)
				Testicular large B-cell lymphoma
PDCD1LG			Overexpression of PDL1/PDL2	Central nervous system large B-cell lymphoma
			ITK: IL2-inducible T-cell kinase	Follicular peripheral T-cell lymphoma (20%)
			SYK: spleen tyrosine kinase	Angioimmunoblastic T-cell lymphoma (rare)
SYK	t(5;9)(q33;q22)	*SYK-ITK*	ITK-SYK mimics TCR signaling	

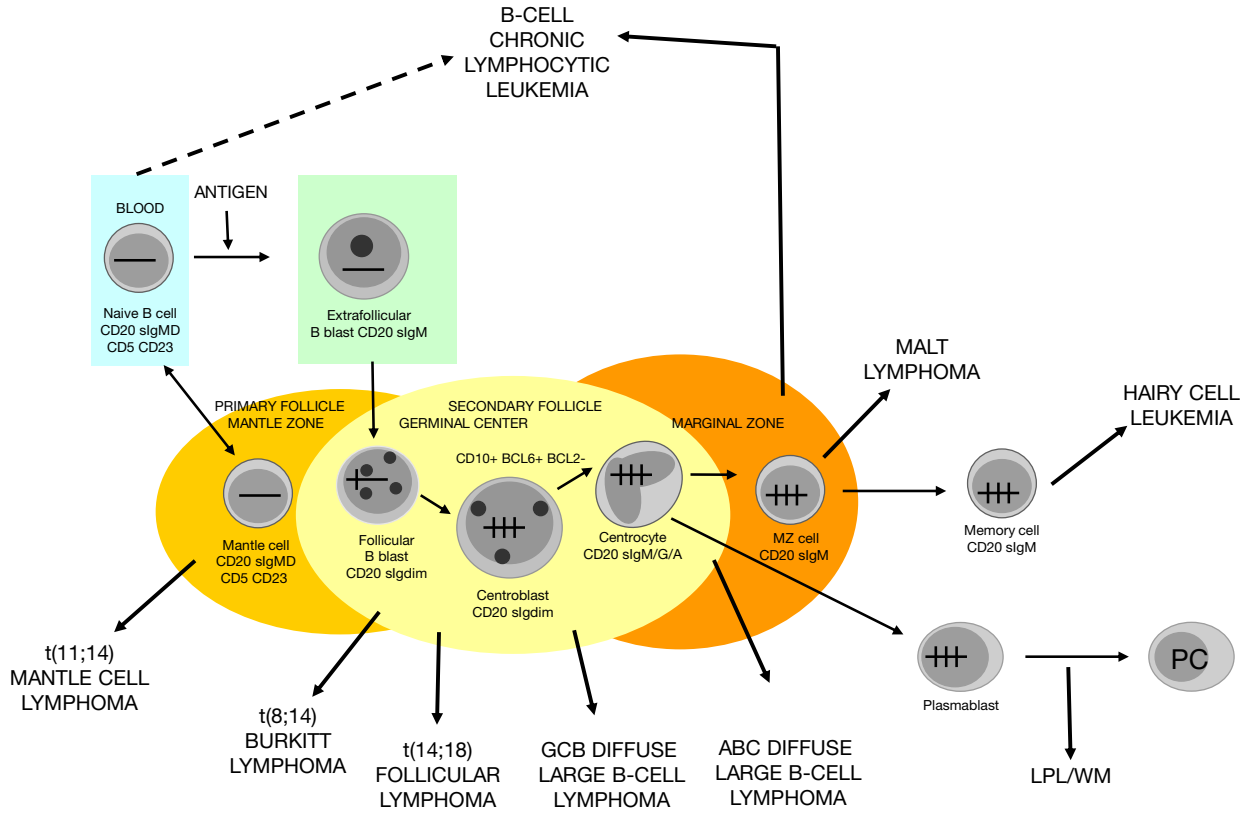

Fig. 3 Histogenetic and pathogenetic model of mature B-cell neoplasms. The follicular (T-cell dependent) and extrafollicular pathways of normal B-cell differentiation are represented. Rearrangement of the Ig heavy chain gene is symbolized with an horizontal bar in the nucleus, and somatic mutations in the IgV region are symbolized with vertical lines. The most common types of mature B-cell neoplasms are linked to their putative normal counterpart. The genetic lesion most frequently associated with each lymphoma category is also indicated.

Tumor Suppressor Genes

The tumor suppressor genes involved in the pathogenesis of NHL included *TP53*, *P16*, *RB1* and *ATM* (ataxia-telangiectasia mutated). Inactivation of tumor suppressor genes often appears as a secondary event associated with lymphoma evolution or transformation, rather than a primary genetic abnormality. The mechanisms of inactivation include point mutations, gross deletions and promotor hypermethylation; inactivation usually occurs through deletion of one allele and nonsense/missense mutation or hypermethylation of the other.

Mutational Landscapes

The mutational landscape of most common NHL have been extensively characterized over the past years. Even if an exhaustive descritpion of these is beyond the scope of this review and the most importnat mutaitons are mentioend in histotype-specific sections, it is worth mentioning some general points. The genomic landscapes of the most common NHL entities show wide heterogeneity within and across subtypes, but also similar altered pathways and mechanisms of oncogenesis overlapping across different entities. Various genes belonging to multiple functional pathways are mutated, and the pattern of mutations for each gene is variable: some genes are characterized by one or a few hotspot alterations, or the mutations may be more heterogeneous. Sometimes distinct mutations in the same gene may be either activating or loss-of-function, and mutations with apparently discordant functional impact may be encountered in different cases of the same disease. Some disease entities are associated to a very characteristic mutational profile, involving one or a few genes, while other diseases are genetically more heterogeneous.

Mature B-Cell Neoplasms

Mature B-cell neoplasms comprise over 80% of NHL. The most common types are follicular lymphoma and diffuse large B-cell lymphoma, which together account for more than half of all NHL. B-NHL entities comprise a range of morphologically, phenotypically, genetically and clinically distinct malignancies. The majority of B-NHL harbor somatically mutated IG genes and therefore are derived from germinal center (GC) or post-GC B cells. The correspondence to normal B-cell subpopulations initially assessed by

a combination of morphology and immunophenotype, has been refined by the comparison of gene expression signatures of lymphomas and normal B-cell subpopulations across the full range of normal B-cell differentiation. Fig. 4 illustrates the model of histogenesis and pathogenesis of B-cell neoplasms, and Table 4 summarizes the phenotypic, genetic and molecular features of most common mature B-cell neoplasms.

Clinically, B-NHL are categorized into those indolent or low-grade diseases, which tend to grow and spread slowly, and have few symptoms usually not requiring immediate chemotherapeutic intervention; and aggressive or high-grade lymphomas associated with a rapid growth and progressive clinical course. This clinical segregation corresponds to two broad categories of diseases and pathological entities. Indolent lymphoma essentially equates to small B-cell lymphoma entities, the most frequent and prototype being follicular lymphoma, followed by small lymphocytic lymphoma/chronic lymphocytic leukemia, lymphoplasmacytic lymphoma, marginal zone lymphoma (of extranodal, nodal and splenic types) and hairy cell leukemia. Mantle cell lymphoma, although composed usually of small B cells, is typically characterized by an aggressive behavior. Despite slow disease evolution and treatment responsiveness, indolent lymphomas essentially represent incurable chronic diseases, and encompass a cumulative risk of transformation to a high-grade lymphoma. Diffuse large B-cell lymphomas represent the prototype of aggressive B-cell

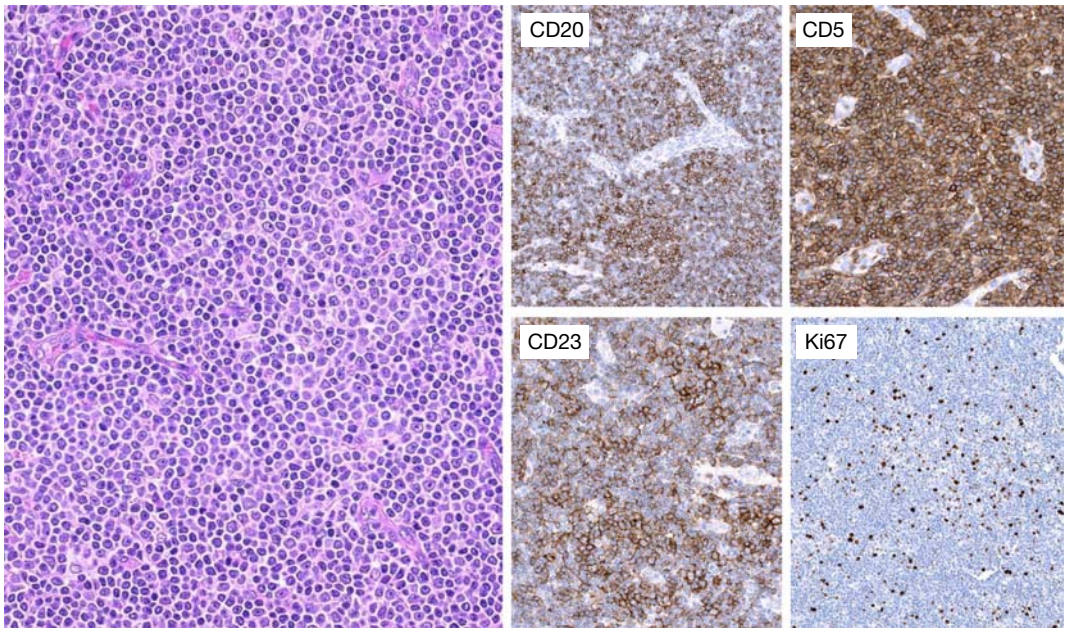

Fig. 4 Small lymphocytic lymphoma/chronic lymphoid leukemia. On a standard hematoxylin & eosin stained section, SLL shows a diffuse proliferation of small lymphocytes, admixed with a small number of larger cells, forming a proliferation center. The lymphoma cells are faintly positive for CD20, coexpress CD5 and CD23, and show a low proliferation fraction (<5%) by Ki67.

Table 4 Pathologic and genetic features of most common B-cell neoplasms

Neoplasm	*CD5*	*CD10*	*BCL6*	*BCL2*	*CD23*	*CD43*	*Cyclin D1*	*Genetic abnormalities*
B-SLL/CLL	+	−	−	+	+	+	−	Trisomy 12; del 13q
LPL	−	−	−	+	−	+/−	−	*MYD88* mutation
HCL	−	−	−	+	−	+	+/−	*BRAF* V600E
SMZL	−	−	−	+	−	−	−	del 7q
MALT	−	−	−	+	−/+	−/+	−	Trisomy 3 t(11;18)*BIRC3-MALT1*
FL	−	+	+	+	−/+	−	−	t(14;18) BCL2-IGH
MCL	+	−	−	+	−	+	+	t(11;14) BCL1-IGH
DLBCL	−	−/+	+/−	+/−	−	−/+	−	t(3q;X) BCL6 translocations t(14;18) *BCL2-IGH* t(8;14) *MYC-IGH*
PMBL	−	−/+	+/−	+/−	+/−	−	−	*CIITA* rearrangements 9p24 2p16 amplifications
BL	−	+	+	−	−	−	−	t(8;14) *MYC-IGH*

+: positive in the vast majority of cases; +/−: positive in >50% of the cases, −/+: positive in <50% of the cases; −: usually negative.

lymphomas, while Burkitt lymphoma and high-grade B-cell lymphomas, not otherwise specified or "double hit" lymphomas, represent less common aggressive B-cell lymphoma entities.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (B-CLL/SLL)

CLL is a neoplasm of small mature CD5+ CD23+ B cells with a blood count of at least 5 × 10e9 monoclonal B cells/L. It represents the most common leukemia of adults in western countries. SLL designates the same disease with tissue involvement and a lesser circulating count of monoclonal cells, and accounts for <5% of NHL. On blood smears, CLL cells appear like small lymphocytes with clumped chromatin and scant cytoplasm admixed with a small proportion of slightly larger prolymphocytes. In tissues (Fig. 4) SLL, is characterized by a diffuse accumulation of small lymphocytes, and the presence of proliferation centers comprising a continuum of smaller to larger cells (prolymphocytes and paraimmunoblasts). Proliferation centers represent a peculiar microanatomical site where CLL/SLL cells interact with accessory cells and the key site of CLL proliferation. CLL cells are positive for B-cell associated antigens, usually with weak expression of CD20 and surface IG expression, coexpress CD43 and are negative for cyclin D1.

CLL development is preceded by an asymptomatic phase named monoclonal B-cell lymphocytosis (MBL), defined by the presence of circulating CLL-like monoclonal B cells amounting to <5 × 10e9/L. MBL progresses to CLL at a rate of approximately 1%/year.

CLL/SLL comprises two disease subtypes based on the mutational status of the variable region of IG genes: the IGHV-mutated subtype (60% of the cases) associated to a good prognosis, and the IGHV-unmutated subtype (40% of the cases) that tend to have more advanced clinical stage at presentation and shorter median survival (8–10 vs. 15–25 years). Both mutated and unmutated subtypes display a gene expression signature that is related to the profile of CD27+ memory B cells, suggesting an origin from post-GC memory cells in mutated cases and from antigen-experienced B cells having developed outside the GC in the unmutated cases.

In CLL/SLL, chromosomal translocations are very uncommon but the t(14;18)(q32;q21) resulting in *IGH-BCL2* can be found in some cases. Deletion of 13q14 region is the most frequent genetic lesion, in 50%–60% of the patients, associated to the mutated subtype; trisomy 12 occurs in 15% of the cases independently of the mutational status; deletion of the 11q22-23 region (which encompasses the *ATM* gene) is found in 20% of the patients, preferentially with unmutated IGHV. Deletion of 17p (comprising *TP53*) is found in <10% of the patients, most commonly in unmutated CLLs, and is associated to a *TP53* mutation on the other allele in most instances, which imparts a bad prognosis. Besides *TP53*, other recurrently mutated genes include *NOTCH1* (NOTCH signaling pathway) and *SF3B1* (RNA processing machinery) in roughly 10% of patients each, and *BIRC3*, *POT1* and *MYD88* in <10% of the cases.

Clinical progression has been associated with histologically aggressive forms, characterized by expanded proliferation centers or increased proliferation fraction (Ki67 > 40%) or an increased proportion of prolymphocytes (>10%) in the peripheral blood. The sudden transformation of CLL into an aggressive disease—Richter's syndrome (RS)—occurs in 5%–10% of the cases. Histologically, most cases are represented by DLBCLs. Most RS represent clonal evolution of the preexisting SLL/CLL, but some are clonally unrelated to the CLL and represent secondary malignancies. A higher proportion of *TP53* alterations found in clonally related cases, and clonally unrelated RS are associated to a significantly longer survival compared to clonally related RS who do very poorly.

B-Cell Prolymphocytic Leukemia (B-PLL)

This extremely rare disease (<1% of B-cell leukemias) is a malignancy of B-prolymphocytes (medium-sized round lymphoid cells with a single, prominent nucleolus) affecting blood (and accounting for >55% of peripheral blood lymphoid cells), bone marrow and spleen. Patients typically present with very high white blood counts ($>100 \times 10^9$) and their median survival is 30–50 months. Cases of CLL with increased prolymphocytes or prolymphocytoid transformation, as well as lymphoproliferations associated to a t(11;14) translocation involving *CCND1* are by definition excluded. Numerical or structural abnormalities of the *MYC* gene are recurrently found in B-PLL.

Lymphoplasmacytic Lymphoma (and Waldenström's Macroglobulinemia) (LPL)

Lymphoplasmacytic lymphoma (LPL), a rare lymphoma occurring in older adults is a neoplasm of small lymphocytes, plasmacytoid, lymphocytes and plasma cells, usually involving the bone marrow and sometimes lymph nodes and spleen. A subset of cases occur in association with chronic HCV infection. The majority of LPL patients have Waldenström's macroglobulinemia, (WM), which is defined as bone marrow involvement by LPL and presence of an IgM serum paraprotein of any concentration, with or without hyperviscosity syndrome. More than 90% of LPL have *MYD88* L265P mutation, but this abnormality, which leads to NF-kappaB signaling, is not specific as it is also encountered in other small B-cell lymphomas and in a subset of DLBCLs. About 30% of the patients harbors somatic mutations in *CXCR4* (most commonly the nonsense truncating S338X, or frameshift mutations in the C-terminal domain) in addition to the activating *MYD88* L265P mutation, and this genotype tends to correlate with higher disease activity.

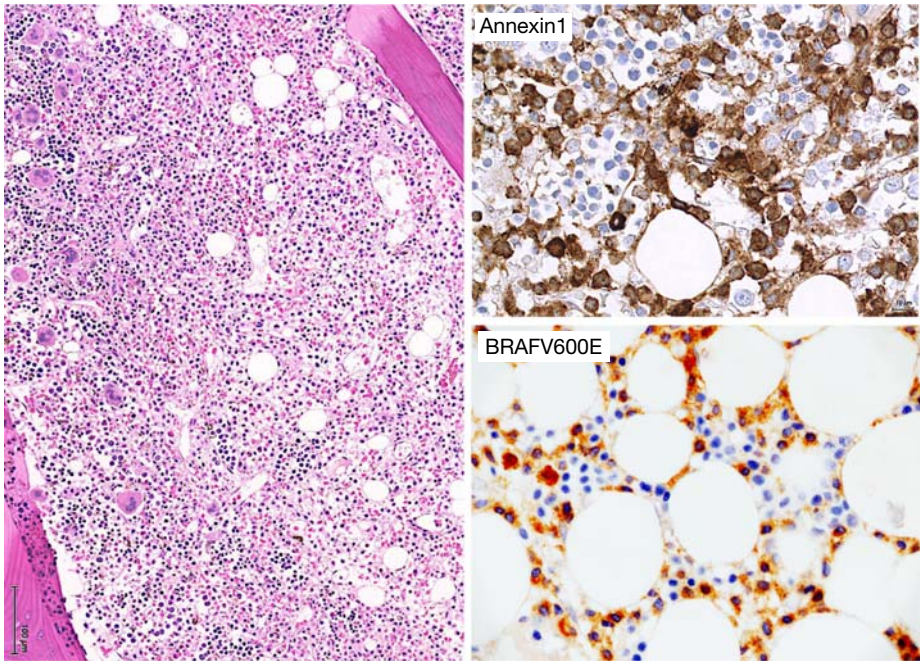

Fig. 5 Hairy cell leukemia. This bone marrow section shows a diffuse infiltrate by small lymphoid cells, which by immunochemistry are positive for Annexin1, and show expression of the *BRAF* V600E variant, as demonstrated by this mutation-specific antibody.

Hairy Cell Leukemia (HCL)

HCL (Fig. 5) is a rare lymphoid leukemia in older adults that has a distinct cytology (small lymphoid cells with an oval nucleus and abundant cytoplasm with "hairy" projections) and immunophenotype (expression of CD103, CD25, CD11c and annexin A1). Leukemic cells are present in low numbers in the peripheral blood but show a diffuse bone marrow and splenic red pulp infiltration, responsible of patients' pancytopenia and susceptibility to infections. Almost all cases carry a *BRAF* V600E kinase-activating mutation, which aberrantly activates the RAF-MEK-extracellular signal-regulated kinase (ERK) signaling pathway, leading to uncontrolled proliferation. Accompanying mutations of the *KLF2* transcription factor or the *CDKN1B* (p27) cell cycle inhibitor are recurrent in 16% of patients with HCL and likely cooperate with BRAF-V600E in HCL pathogenesis.

Marginal Zone Lymphomas (MZL)

Marginal zone lymphomas (MZL) of splenic, nodal and MALT type correspond to postgerminal center memory cells of marginal zone type that derive from and proliferate in splenic, nodal and extranodal tissues (Fig. 6).

Splenic marginal zone lymphoma (SMZL)

Splenic marginal zone lymphoma (SMZL) is a rare, indolent B-cell neoplasm involving the spleen by replacing the normal B-cell follicles and showing a marginal zone differentiation, which may have circulating neoplastic cells with short "villous" projections. Patients typically present with splenomegaly, bone marrow involvement and lymphocytosis, usually without peripheral lymphadenopathy. SMZL cells express IgM, IgD, B-cell antigens (CD19, CD20, CD22), BCL2 are usually CD5-negative, and typically lack CD10, CD43, CD23, BCL6, cyclin D1, CD11c and CD25.

Allelic loss of 7q21-32 allelic is detected in up to 40% of the cases. Other cytogenetic abnormalities include gains of 3q or 12q and 6q deletion. Recurrent mutations disrupting genes involved in the normal process of marginal zone differentiation, *NOTCH2* and *KLF2*, are found in 10%–25% and in up to 44% of the cases, respectively. These mutations, as well as infrequent *TP53* mutations, have been associated to adverse clinical outcome.

Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type (MALT lymphoma)

Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type (MALT lymphoma), the third most common type of B-cell lymphoma, occurs in organs that normally lack organized lymphoid tissue. About half involve the gastrointestinal tract, and they represent 40%–60% of lymphomas in the ocular adnexa, thyroid, lung, breast, and salivary gland; skin or soft tissue may also be the primary site. The majority of patients present with localized (stage I or II) extranodal disease. MALT

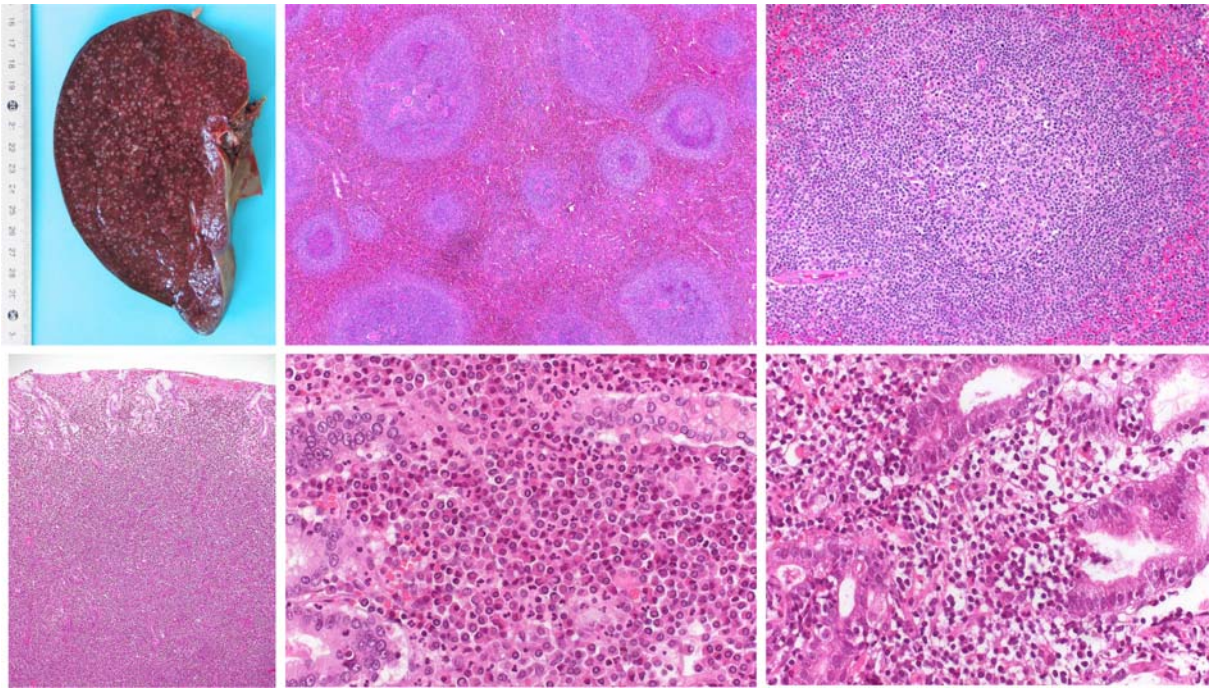

Fig. 6 Marginal zone lymphomas. Splenic marginal zone lymphoma (upper panels); gross picture of a splenectomy specimen showing increased size of the spleen, and micronodular pattern on the cut section; a low-power view of the splenic parenchyma shows a nodular expansion of the white pulp, with prominent marginal zones at their periphery. Gastric MALT lymphoma (lower panels): on a low-power view of a gastric section specimen, a diffuse lymphoproliferation obliterating the mucosa is seen, with a pale appearance. The middle panel shows a case with marked plasmacytic differentiation, and the right panel shows a mucosal infiltrate by lymphoma cells showing epitheliotropism with the formation of lymphoepithelial lesions.

lymphomas run an indolent natural course. Dissemination or recurrence may occur, often in other localized extranodal sites, with long disease-free intervals.

"Acquired MALT" secondary to autoimmune disease, in particular Sjögren syndrome or Hashimoto thyroiditis, or infection such as *H. pylori* gastritis, represents the substrate for lymphoma development. MALT lymphoma reproduces the morphologic features of normal MALT, with a polymorphous infiltrate of small lymphocytes, marginal zone (centrocyte-like) B cells, and plasma cells, admixed with scattered blast cells, occupying the marginal zone of reactive follicles and in some cases "colonizing" and disrupting these follicles. In epithelial tissues, neoplastic cells typically infiltrate the epithelium, forming so-called lymphoepithelial lesions. In MALT lymphomas, when clusters or sheets of blasts are present, a separate diagnosis of large B-cell lymphoma is made and these cases are associated with a worse prognosis. MALT lymphoma cells express sIg (M > G > A), lack IgD, and may show plasmacytoid differentiation (40% of the cases). They express B-cell antigens (CD19, CD20, CD22, CD79a), may rarely be positive for CD5, and do not express CD10 and BCL6 and cyclinD1.

The most common numerical chromosomal abnormality is trisomy 3 (about 60% of cases), but is not specific for this lymphoma type. Three main recurrent translocations are encountered (see **Table 2**). The t(11;18)(q21;q21) fusing *BIRC3(API2)* and *MALT1* is the most common, found in roughly 20% of the cases with the highest prevalence in lung (40%) and gastric (25%) MALT lymphomas. The t(11;18)(q21;q21) is a marker for cases of gastric MALT lymphomas that do not respond to *H. pylori* eradication, but these cases are less likely to undergo transformation to diffuse large B-cell lymphoma. The t(14;18)(q32;q21) leading to deregulated expression of the *MALT1* gene mainly occurs in a small proportion of MALT lymphomas of the ocular adnexa and lung and is rare in other localizations. The t(1;14)(p22;q32) causing overexpression of BCL10, is rare (< 5% of the cases, mostly gastric and pulmonary). These mutually exclusive translocations deregulate the activation of the canonical and/or noncanonical NF-kappaB pathways. Inactivation of *TNFAIP3* (*A20*) by 6q23 deletion and deleterious mutations, mainly seen in MALT lymphomas of the ocular adnexa, thyroid and salivary glands where translocations are infrequent, impairs the repression of NF-kappaB normally induced by TNFAIP3.

Nodal MZL

Nodal MZL is the least frequent form of MZL encompassing an adult type and a pediatric-type. It morphologically resembles nodal involvement by MZL of MALT type or splenic type, but without evidence of extranodal or splenic disease. The lymphoma cells frequently coexpress CD43. Some genetic features are shared with splenic and MALT lymphomas, including *NOTCH2* and *KLF2* mutations, but translocations associated to MALT lymphomas and 7q deletion are not seen.

Follicular Lymphoma (FL)

FL, the most common form of indolent lymphoma and second most common lymphoma in western countries, affects predominantly older adults. FL remains essentially an incurable disease; moreover, subsets of patients may progress toward transformation to a high-grade B-cell lymphoma and a subset of patients may progress or relapse early after receiving first-line therapy. Transformation of FL usually occurs by a nonlinear divergent evolution from a common progenitor cell, and is associated to a poor outcome.

FL is a neoplasm of follicular center B cells composed of a mixture of centrocytes and centroblasts, which usually has, at least partially, a follicular pattern (Fig. 7). Histological grading along a three-grade scale is based on the abundance of centroblasts by microscopic counting per high-power field (HPF). Grade 1 cases have 0–5 centroblasts per HPF, grade 2 cases have 6–16 centroblasts per HPF and grade 3 have >15. Grade 3 is further subdivided into 3a, when centrocytes are still present, and 3b, composed of centroblasts only, biologically closer to diffuse large B-cell lymphoma. The majority of cases are grade1–2. Several studies have shown a more aggressive course for grade3 FL, but the difference may be alleviated by the use of anthracycline-containing chemotherapy and/or rituximab. Most cases have a predominantly follicular pattern, and the presence of diffuse areas in grade 1–2 FL is thought to not be clinically significant, but diffuse areas of grade 3 qualify for a diagnosis of diffuse large B-cell lymphoma. Rare cases are predominantly diffuse, a variant frequently occurring as large inguinal tumors.

FL cells are usually sIg + (IgM > IgG > IgA). The tumor cells express pan-B-cell associated antigens (CD19, CD20, CD22, CD79a), are usually CD10+, BCL6+, BCL+, CD5− and CD43−. CD23 may be positive, in particular in the purely diffuse variant. Grade 3 FL may be negative for BCL2 and CD10. Meshworks of follicular dendritic cells (FDCs) are present in follicular areas and may be highlighted by CD21or CD23. IG heavy and light-chain genes are rearranged, with extensive and ongoing somatic mutations, similar to normal germinal center cells, and consistent with a germinal center derivation.

The pathogenesis of FL is driven by the t(14;18)(q32;q21) translocation which is the hallmark and most recurrent feature of FL, detected in 85%–90% of the cases and inducing ectopic BCL2 protein overexpression; and the genetic aberrations of epigenetic modifiers. The *BCL2* translocation is an early event occurring in the bone marrow in pre-B cells and is considered the initiating oncogenic hit, providing a survival advantage by activating an antiapoptotic program and favoring the acquisition of additional genetic lesions during repeated transits through germinal centers. As for alterations of epigenetic modifiers in FL, mono- or biallelic inactivation of the *KMT2D* (*MLL2*) histone H3K4 methyltransferase represents the most highly recurrent lesion (about 80% of the cases). Alterations in other chromatin modifiers include inactivating mutations of the histone acetyltransferases *CREBBP* and *EP300*, in about 60% and 10% of the cases, respectively, and activating mutations of *EZH2* histone H3K27 methyltransferase (about 25% of the cases). The deregulated epigenetic mechanisms have been implicated in predicting

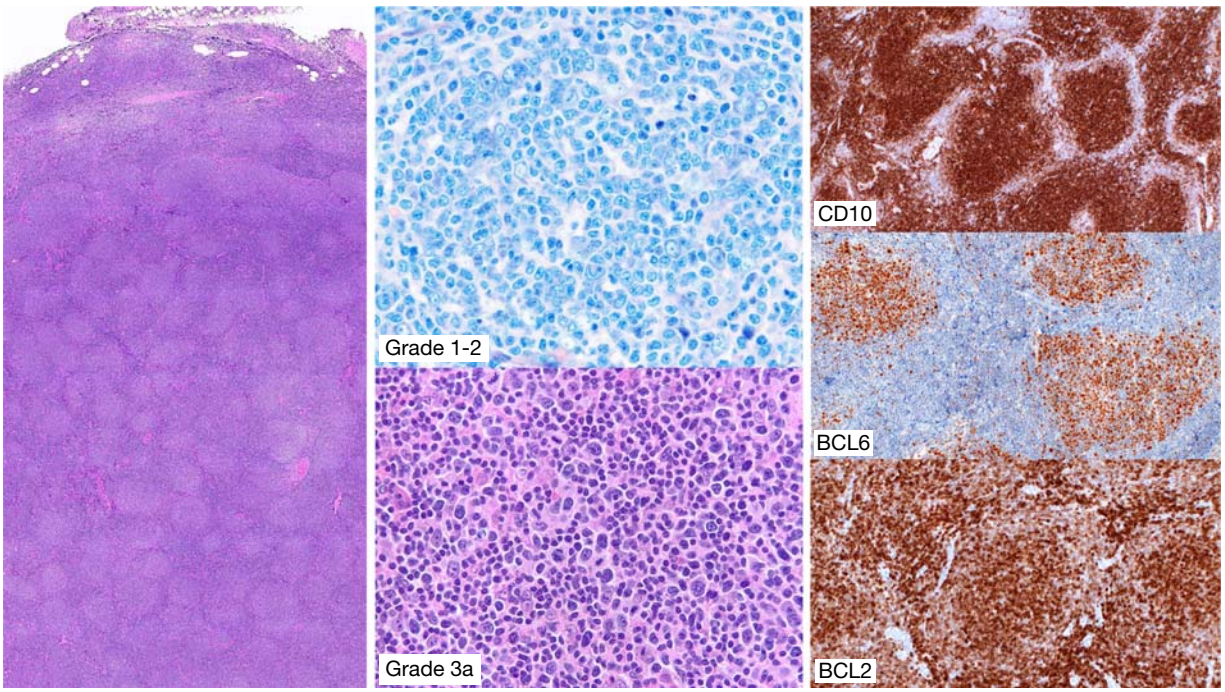

Fig. 7 Follicular lymphoma. At low-power view, lymph node parenchyma is replaced by multiple small nodular structures; this lymphoproliferation extends beyond the capsula into the adjacent fat. A Giemsa stain and hematoxylin & eosin stain illustrate to the high-power view of two cases, one grade 1–2 follicular lymphoma, comprising <10 centroblasts per high-power field, and a grade 3a case, comprising an admixture of centrocytes with numerous centroblasts (>15 large cells/hpf). The typical immunophenotype of follicular lymphoma is CD10+, BCL6+, BCL2+.

patient outcome, and a prognostic model, named m7-FLIPI, that links seven mutations including five with epigenetic functions, to patient prognosis has been proposed.

A small proportion of FL do not have *BCL2* gene rearrangement and do not express BCL2. In particular grade 3 FL tend to be negative for BCL2 more frequently than grade1–2 cases, and purely diffuse FL are typically negative for *BCL2* translocation, while showing recurrent 1p36 deletion (containing *TNFRSF14*), a feature common to all forms of FL. Various other chromosomal gains and losses affecting multiple chromosomes are frequently encountered as well. Abnormalities of 3q27 and/or *BCL6* rearrangement are found in about 5%–15% of the cases.

In situ follicular neoplasia (ISFN)

In situ follicular neoplasia (ISFN) designates partial or total colonization of germinal centers by light-chain restricted clonal B cells harboring the *BCL2* translocation characteristic of FL, and strongly expressing BCL2 and CD10 in otherwise reactive lymph nodes. It is thought to be the tissue equivalent of circulating FL-like B cells that are detected in the blood of otherwise healthy individuals. ISFN is detected in about 2% of randomly selected reactive lymph nodes; if found incidentally with no evidence of concurrent FL by staging, the risk of subsequent FL is $<5\%$.

Duodenal-type FL

Duodenal-type FL is a variant of FL with very indolent behavior: it involves the duodenum as small polyps and is typically diagnosed incidentally in women undergoing upper gastrointestinal endoscopy for other reasons; the lymphoma is usually purely follicular and grade 1–2, harboring a *BCL2* rearrangement, with an immunophenotype similar to that of nodal FL, frequent expression of IgA and of the intestinal homing receptor alpha4beta7 integrin. There is a very low risk of progression to nodal disease.

Testicular FL

Testicular FL occurs in children or less commonly in adults, and usually consists of grade3 lesions that are negative for BCL2 and lack *BCL2* rearrangement. It is usually diagnosed on surgical excision of a testicular mass and has a very good prognosis.

Pediatric-type FL

Pediatric-type FL is an uncommon form of nodal FL occurring in children or young adults, most often presenting as localized disease involving cervical lymph nodes. Cytologically, the lesions are composed of large blastic cells (grade3) and have a high proliferation index, which contrasts with the very indolent behavior. Pediatric FL lacks *BCL2*, *BCL6* or *IRF4* translocations, often features deletions of 1p36, and about half of the cases have deletions and mutations of *TNFRSF14*, and/or *MPA2K1* mutations.

Primary cutaneous follicle center lymphoma (PCFCL)

Primary cutaneous follicle center lymphoma (PCFCL) is a cutaneous lymphoma composed of centrocytes, centroblasts and large multilobated cells, showing a follicular, follicular and diffuse, or purely diffuse growth pattern. It usually presents as localized skin lesions on the head, forehead or scalp. PCFCL is positive for B-cell antigens and expresses BCL6, but CD10 is variably detected., BCL2 immunohistochemical expression is usually negative or faintly positive, and *BCL2* rearrangement is generally absent. Grading is not applied to FCFCL; the proliferation fraction may be high, especially in diffuse cases composed of large cells, and the prognosis is excellent.

Mantle Cell Lymphoma (MCL)

Mantle cell lymphoma (MCL) is an aggressive lymphoma of middle-aged to older adults (median survival 3–5 years) with a marked male predominance (75%). It accounts for about 7% B-NHL. Most patients have disseminated disease at diagnosis, with lymphadenopathy, hepatosplenomegaly and bone marrow and blood involvement. Extranodal involvement, especially of the gastrointestinal tract (lymphomatous polyposis), is common.

MCL is typically composed of a monotonous population of small to medium-sized lymphoid cells, with slightly irregular or "cleaved," nuclei which may have a diffuse, nodular, mantle zone, or mixed pattern in lymph nodes (**Fig. 8**). The blastoid variant, composed of cells resembling lymphoblasts with fine chromatin and a high mitotic rate, and the pleomorphic variant, which comprises many large irregular cells with prominent nucleoli, are considered more aggressive cytological variants. A marginal zone variant may morphologically mimic marginal zone lymphoma. MCL is not graded but the mitotic count or proliferation index have an important prognostic impact.

MCL cells strongly express sIgM and IgD, more often lambda than kappa light chains and are positive for B-cell antigens; most cases coexpress CD5, and CD43, and they usually lack CD23, CD10 and BCL6. Nuclear expression of cyclin D1 is present in $>95\%$ of the cases and represents the hallmark of the disease. Expression of SOX11 is detected in most cases including the rare subset of cyclinD1-negative MCL. MCL has unmutated or only minimally mutated IGHV regions, consistent with an origin from naïve B cells.

The t(11;14)(q13;q32) (involving *CCND1* gene) is present in $>95\%$ of the cases is the primary genetic event and results in overexpression the cyclin D1, a protein involved in the regulation of the cell cycle transition between the G1 to the S phase not normally expressed in lymphoid cells. Deregulated expression of cyclin D1 overcomes the cell cycle suppressive effect of RB and p27 but is not sufficient in itself to induce MCL. Abnormal SOX11 is thought to be another important pathogenic factor. MCL also carries numerous chromosomal aberrations—gains and losses—and some of these aberrations may correlate with an aggressive clinical

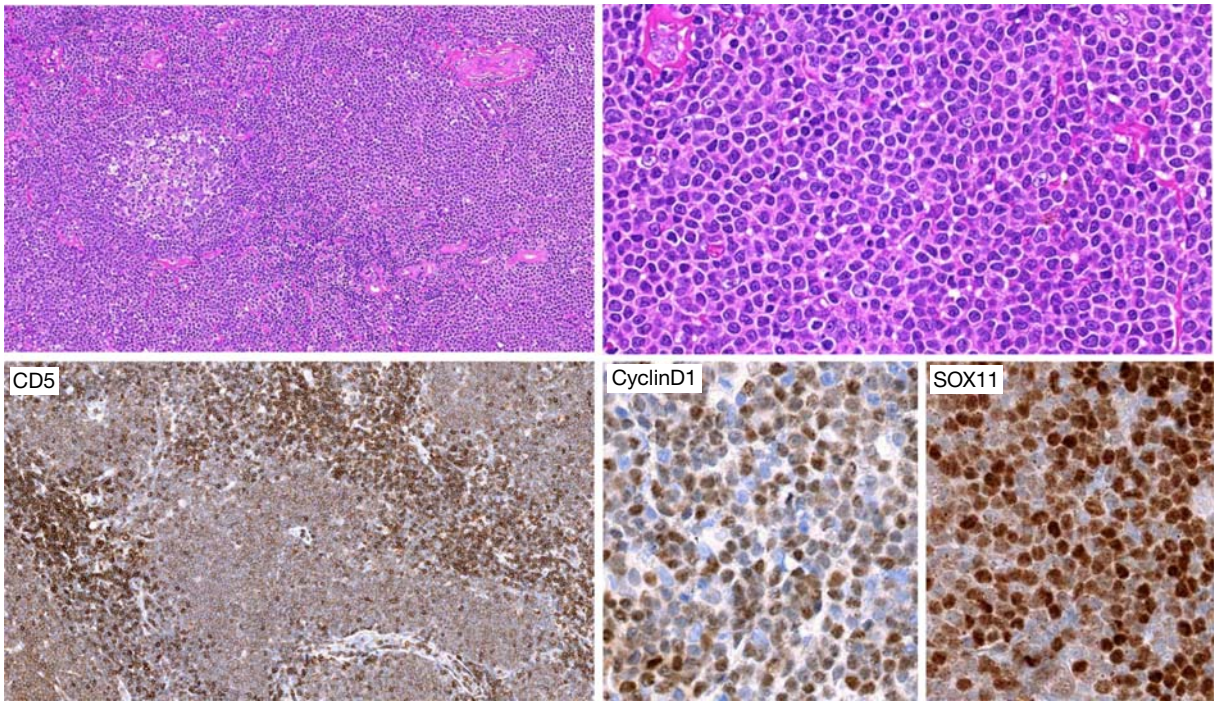

Fig. 8 Mantle cell lymphoma. A medium-power view of a lymph node involved by mantle cell lymphoma (upper left panel) shows a residual germinal center surrounded by a monotonous lymphoproliferation of small lymphoid cells; the upper right panel shows the cytological features of the lymphoid cells, of small size with irregular nuclei (classical variant of mantle cell lymphoma). By immunochemistry, the lymphoma cells are faintly positive for CD5, and most nuclei show expression of cyclin D1 and SOX11.

parameters. Mutations in genes involved in cell cycle and other pathways, including *ATM*, *KMT2D* (*MLL2*), *NOTCH1/2* are found in a variable proportion of the cases. Mutations of *TP53* and homozygous deletions of *CDKN2A* are commonly found in highly proliferative cases. A small subset of the cases are negative for cyclinD1 and lack *CCND1* translocation; about half of these cases carry an alternative *CCND2* or *CCND3* translocation.

In addition to the usual nodal MCL, two subsets of MCL with an indolent behavior, namely in situ mantle cell neoplasia and nonnodal, leukemic MCL are now recognized.

In situ mantle cell neoplasia

In situ mantle cell neoplasia designates the presence of cyclinD1-positive B cells harboring a *CCND1* translocation, in the mantle zones of otherwise reactive lymph nodes. This condition is very rare (much less frequent than in situ follicular neoplasia) and carries a very low risk of progression to overt MCL. Compared to classical MCL, in situ lesions tend to be CD5-negative and are variably positive or negative for SOX11.

Leukemic nonnodal MCL

Leukemic nonnodal MCL is defined by clinical presentation of MCL with blood and bone marrow involvement, sometimes splenomegaly, and no significant lymphadenopathy. Leukemic cells are usually small, positive for cyclin D1, carry the t(11;14) translocation, but tends to be negative for SOX11 and have hypermutated IGHV regions. Despite a usually indolent clinical course, which can remain stable for many years, these cases may transform, sometimes to a pleomorphic blastoid morphology. Routes of transformation include *TP53* mutations or other oncogenic events.

Diffuse Large B-Cell Lymphoma (DLBCL)

DLBCL accounts for about one-third of B-NHL and represents the most common type of lymphoma. It is defined as a neoplasm of medium to large B cells more than twice the size of small lymphocytes, with a diffuse growth pattern. DLBCL encompasses marked biological heterogeneity. It may occur de novo or as a high-grade transformation of a small B-cell lymphoma. Median age at presentation is in the 7th decade, but may occur at any age. Patients typically present with a rapidly enlarging mass, with B symptoms in one-third of the cases. About 50% have localized disease involving nodal or in up to 40% of the cases various extranodal sites. Overall, $>30\%$ of the patients will not respond to therapy or will relapse with resistant disease. The International Prognosis Index (IPI) stratifies patients into risk groups and is predictive of outcome.

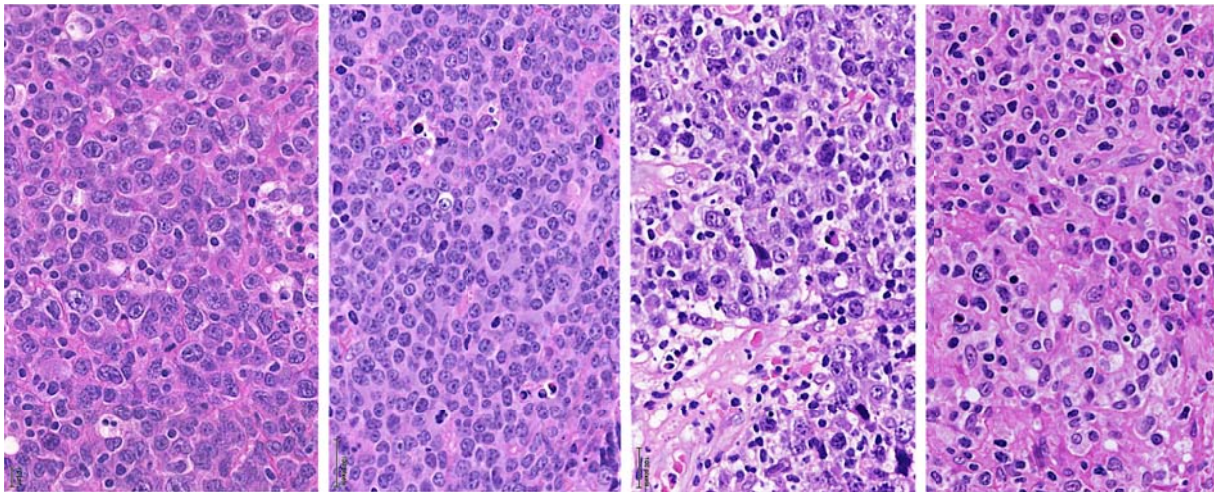

Fig. 9 Morphological variants of diffuse large B-cell lymphoma.

Diffuse large B-cell lymphoma, not otherwise specified (DLBCL-NOS)

In its usual form, DLBCL is a diffuse proliferation of large lymphoid cells that may be classified as centroblastic (most common variant, may be monomorphous composed entirely of centroblasts, or polymorphous comprising an admixture of centroblasts and immunoblasts), immunoblastic (comprising >90% immunoblasts, i.e., large lymphoid cells with a centrally located nucleolus), or anaplastic (composed of large pleomorphic cells that are often CD30+ and tend to form cohesive sheets with a sinusoidal pattern of growth)(Fig. 9). Immunoblast-rich tumors have been found to have a worse prognosis in several studies. Bone marrow involvement in DLBCL seen in about 15% of the cases may appear either as a large-cell infiltrate, or slightly more often as an infiltrate of small atypical B cells ("discordant" marrow involvement); the latter is not associated with a worse prognosis than cases without marrow involvement.

DLBCL express CD45, one or more B-cell-associated antigens (CD19, CD20, CD22, CD79a), PAX5, and often surface with or without cytoplasmic Ig (IgM > IgG > IgA). Therapy of B-cell lymphoma with chimeric antibodies against CD20 can result in the loss of CD20 antigen expression. About 5%–10% of de novo DLBCLs (i.e. excluding Richter's syndrome) express CD5. CD5+ DLBCLs are reported to occur in elderly women, with a predilection for extranodal involvement, especially bone marrow and spleen and to be associated with shorter survival in comparison with CD5+ tumors. CD30 expression is seen in 10%–20% of the cases, especially those with anaplastic morphology. Ki67 proliferation index is usually >40% and may be >90%. Expression of BCL2 is reported in 50%–80% of the cases, and about 30%–40% of the cases are MYC-positive. Approximately one-third of the cases express CD10, 70% express BCL6, and 50% are positive for MUM1/IRF4; unlike normal B cells, DLBCL frequently coexpresses BCL6 and MUM1.

Evaluation of DLBCL gene expression profiles by cDNA microarray techniques identified three molecularly distinct subtypes of DLBCL: the GCB subtype, characterized by the expression of genes normally expressed by GC B cells and showing ongoing somatic hypermutation; the ABC subtype, characterized by the features of BCR-activated B cells entering plasmablastic differentiation, showing activation of the NF-kappa B pathway, upregulation of genes involved in plasmacytic differentiation, and no ongoing hypermutations; and an unclassified group (15%–30% of the cases) (type 3). Patients with GC-like DLBCLs have better outcomes than those with activated B cell-like and type 3 DLBCLs. Different immunohistochemistry-based algorithms based the expression of markers associated to one or the other signature, have been developed as surrogates to microarray-based gene expression profiling in order to define the molecular subtypes; one of the most commonly used is the Hans algorithm, relying on the expression of three markers (CD10, BCL6 and MUM1/IRF4).

Compared to other lymphomas, DLBCL shows a higher degree of genomic complexity, harboring between 50 and >100 lesions per case. The most frequent recurrent cytogenetic aberration consists of rearrangement of *BCL6* at the 3q27 locus in about 30% of the cases, both of GCB and ABC subtypes. In addition, *BCL6* is the target of somatic mutations abrogating its promoter regulatory sequences in its 5′ untranslated region in 70% of the cases, and BCL6 expression is deregulated in many cases as the consequence of mutations in other genes that are normally involved in regulating its function and activity or its degradation. Altogether there is ample evidence that BCL6 deregulation is an essential pathogenic event common to both molecular subtypes. Inactivating mutations and deletions of the histone acetyltransferases *CREBBP* and *EP300*, and the histone methyltransferase *KMT2D* (*MLL2*) are also highly recurrent in both molecular subtypes and may favor tumor development by reprogramming the cancer epigenome. The immune surveillance pathway is frequently impaired through genetic loss of *B2M* or *HLA-I* genes or loss of the gene encoding CD58 ligand, allowing to escape from cell-mediated cytotoxicity. Mutations of *TP53* gene are detected in about 20% of DLBCL, in both molecular subtypes, and are associated with clinical drug resistance and poor outcome. Translocations of *BCL2* and

MYC occur in about 20%–25% and 10% of cases respectively, and almost exclusively in GCB-DLBCL. These also harbor gain-of-function mutations of *EZH2* in 20%–25% of the cases. Constitutive activation of the NF-kappaB pathway is the hallmark of ABC-DLBCL, the underlying mechanisms being heterogeneous and including gain-of-function mutations in signal transduction components of the BCR, and toll-like receptor pathways (*CD79B*, *CARD11*, *MYD88*), and loss-of-function mutations in *TNFAIP3* (*A20*). ABC-DLBCL is also characterized by defective plasma cell differentiation caused by lesions deregulating *BCL6* and inactivating *PRDM1/BLIMP1*.

T-cell/histiocyte-rich large B-cell lymphoma (T/HRLBCL)

This variant of DLBCL comprises $<10\%$ large neoplastic B cells (which may resemble the neoplastic cells of nodular lymphocyte predominance Hodgkin lymphoma or even Reed–Sternberg cells of classical Hodgkin lymphoma) scattered in a background of nonneoplastic T cells with or without histiocytes. T/HRLBCL usually presents with advanced-stage disease with nodal, splenic and bone marrow involvement. Immunophenotypic studies are essential to distinguish this variant from peripheral T-cell lymphoma and Hodgkin lymphoma.

Plasmablastic lymphoma

Plasmablastic lymphoma was initially described as a tumor occurring in the oral cavity in HIV-infected patients but since then similar lesions have been reported in other anatomical sites as well as in non-HIV+ individuals. The tumor cells are immunoblastic in morphology, with or without plasmacytoid differentiation, with a plasmacytic phenotype. They are negative for CD45 and CD20, negative or weakly positive for PAX5, may be positive for CD79a, and are positive for MUM1/IRF4 and CD138. EBV is detected overall in 60%–75% of the cases, including the majority of HIV-associated cases. *MYC* rearrangement, usually to an *IG* locus, is detected in about half of the cases and translates into strong MYC positivity by immunohistochemistry.

ALK-positive large B-cell lymphoma

This a very rare aggressive lymphoma is composed of large immunoblast-like B cells expressing ALK and featuring a plasma cell immunophenotype (weakly positive for CD45, lacking expression of CD20 and PAX5, positive for IRF4/MUM1 and CD138) with expression of EMA (Epithelial Membrane Antigen) and cytoplasmic IgA. CD30 is usually negative. ALK expression is usually granular and cytoplasmic, reflecting expression of the CTCL-ALK fusion protein.

Primary mediastinal (thymic) large B-cell lymphoma (PMBL)

PMBL comprises 7% of large B-cell lymphomas and 2.4% of all NHL. This lymphoma has clinical, morphological, molecular and genetic features distinct from usual DLBCL. PMBL tends to occur in young patients (median age of about 35 years), and affects women more commonly than men. Patients present with an anterior mediastinal mass originating in the thymus, with symptoms related to local invasion. Relapses tend to occur in distant extranodal sites such as central nervous system and the gastrointestinal tract. PMBL is associated to a better prognosis than GCB and ABC-DLBCL subtypes.

PMBL is cytologically heterogeneous, but clear cells, multilobated nuclei and compartmentalizing sclerosis are common features. The neoplastic cells express the B-cell antigens CD19, CD20, and CD79a but frequently lack surface immunoglobulin (Ig), despite expression of the appropriate transcription factors and have defective expression of HLA class I and/or class II molecules. A minority of cases are positive for CD10; BCL6 staining is found in more than half of the cases, and IRF4/MUM1 is expressed in the majority of cases. Most cases are BCL2+. Most cases express CD30 (at variable extent and intensity), CD23, the MAL antigen, and programmed cell death ligands (PD-L1 and PD-L2).

PMBL has a unique gene expression signature that differs from that of other DLBCL and partly overlaps with that of classic Hodgkin lymphoma, and reflects activation of the nuclear factor-κB and the Janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling pathways. This not only supports PMBL as a distinct clinicopathologic entity, but also the concept of "gray zone" lymphomas with features overlapping between those of PMBL and classical Hodgkin's lymphoma. Overexpression of the MAL gene is a characteristic feature of the PMBL signature that is not found in DLBCL arising in other sites. MLBCL has been postulated to derive from medullary thymic asteroid B cell. PMBL cells have isotype-switched IG genes with a high load of somatic mutations without evidence of ongoing mutations.

BCL2, *BCL6* or *MYC* rearrangements are extremely infrequent, but half of the cases harbor rearrangements or mutations of the *CIITA* transactivator resulting in downregulated HLA class II molecules, creating an immune-privileged phenotype. PMBL also exhibits gains at 9p24, including the *JAK2/PDCD1LG2/PDCD1LG1* locus in 70% of the cases, an aberration that explains the overexpression of PD-L1 and PD-L2 in PMBL. About half of the cases have gains at 2p16 including *REL* that encodes a transcription factor of the nuclear factor-kappaB family and *BCL11A*. Constitutive activation of the JAK/STAT signaling pathway results from *JAK2* amplifications, frequent inactivating mutations of *SOCS1* and *PTPN1*, and *STAT6B* mutations.

Primary central nervous system lymphoma (PCNSL)

PCNSL is a rare and aggressive lymphoma that is confined to the brain, eyes, spinal cord, or leptomeninges without systemic involvement. PCNSL can develop in immunosuppressed (HIV/AIDS, organ transplant, immunosuppressive agents) or immunocompetent patients. PCNSL in immunocompetent patients is rare and represents 4% of all intracranial neoplasms. The outcome is worse than for non-CNS DLNCL.

Pathology reveals highly proliferative large lymphoid cells disposed in an angiocentric growth pattern, diffusely infiltrating the CNS. By immunohistochemistry to identify DLBCL molecular subgroups, most PCNSLs are nongerminal center subtype. Accordingly, frequent recurrent mutations in *MYD88* and *CD79B* are identified in PCNSL. Many cases have defective HLA class I and/or class II antigen expression. Moreover, PCNSL exhibit frequent 9p24.1 copy-number alterations and infrequent translocations of 9p24.1 and associated increased expression of the programmed cell death protein 1 (PD-1) ligands, PD-L1 and PD-L2, suggesting that immune evasion might play a role in PCNSL.

Primary cutaneous DLBCL, leg type (PCLBCL)

PCLBCL represents $<5\%$ of primary cutaneous lymphomas, and arises in older patients, with a median age at presentation >70 years. Clinically, patients present with solitary or multiple rapidly growing bluish-red nodules or plaques, which may be ulcerated, usually on one or, rarely, both legs.

Histologically, the dermis is infiltrated by dense, diffuse sheets of centroblasts and immunoblasts, often extending into the subcutis. PCLBCL is typically positive for pan-B-cell markers, CD10−, BCL6+, MUM1+, BCL2+, IgM+, and shows an ABC signature by gene expression profiling. Genetic alterations within the NF-kappaB pathway, include mutations in MYD88 in 60% of the cases, or less commonly, mutations in CD79B or CARD11. Translocations of *BCL2* do not occur but amplifications of the locus at 18q21 likely explains the usually intense BCL2 expression. Loss of *CDK2NA/CDKN2B*, by deletion and/or promoter methylation occurs in $>50\%$ of the cases and associates with a poor outcome.

Large B-cell lymphoma with IRF4 *rearrangement*

Large B-cell lymphoma with *IRF4* rearrangement is characterized by strong expression of MUM1/IRF4, usually as a consequence of *IRF4* rearrangement. This is an overall rare lymphoma, affecting predominantly the pediatric age group, consisting of a follicular and/or diffuse proliferation of large B cells and manifesting as tumors in the tonsils or Waldeyer ring or head and neck lymph nodes. The outcome is favorable.

Intravascular large B-cell lymphoma

This entity consists of a disseminated intravascular proliferation of large B cells involving small blood vessels, without an obvious extravascular tumor mass or leukemia, maybe related to defective expression of homing receptors. The central nervous system, kidney, lung, and skin are commonly involved, and patients present with symptoms related to organ dysfunction secondary to vascular occlusion. Many reported cases are diagnosed at autopsy.

EBV-positive large B-cell lymphomas

Several EBV-positive DLBCL entities are recognized: EBV-positive DLBCL, not otherwise specified (**Fig. 10**); EBV-positive mucocutaneous ulcer; lymphomatoid granulomatosis; and DLBCL associated with chronic inflammation.

EBV-positive DLBCL-NOS

EBV-positive DLBCL-NOS occurs in older patients where age-related immunodeficiency is thought to represent the predisposing conditions, but also in younger patients without known immunodeficiency. Peculiar morphologic features that are frequently encountered include the presence of large very atypical lymphoid cells, resembling those seen in Hodgkin lymphoma, a variable inflammatory background sometimes mimicking T-cell/histiocyte-rich large B-cell lymphoma, and areas of geographic necrosis. The neoplastic cells have an activated immunophenotype, frequent CD30 expression, and express a type II or type III EBV latency program. The disease is aggressive in older individuals but has a good prognosis in younger patients.

EBV-positive mucocutaneous ulcer

EBV-positive mucocutaneous ulcer manifests as superficial ulcerated lesions in the skin, oral mucosa, gingiva or mucosal sites in patients with age-related or iatrogenic immunosuppression. The course is typically indolent and some lesions may spontaneously regress.

Lymphomatoid granulomatosis (LyG)

Lymphomatoid granulomatosis (LyG) is an angiocentric and angiodestructive EBV+ large B-cell lymphoma with a T-cell-rich background. Necrosis is common. Patients typically present with extranodal disease, most commonly involving lung, CNS, and/or kidneys. Evidence of past or present immunosuppression may be found. LyG is graded (1 to 3) according to the number of large B cells, which correlates with clinical aggressiveness.

DLBCL associated with chronic inflammation

DLBCL associated with chronic inflammation occurs in the setting of chronic longstanding inflammation, like pyothorax, osteomyelitis, metallic implant insertion, or chronic skin ulcers. LDBCL associated with chronic inflammation forms tumor masses and is usually localized. The tumor may show plasmacytic differentiation and not uncommonly coexpress T-cell antigens. A type III EBV latency is characteristic. *TP53* mutations and *MYC* amplifications are common.

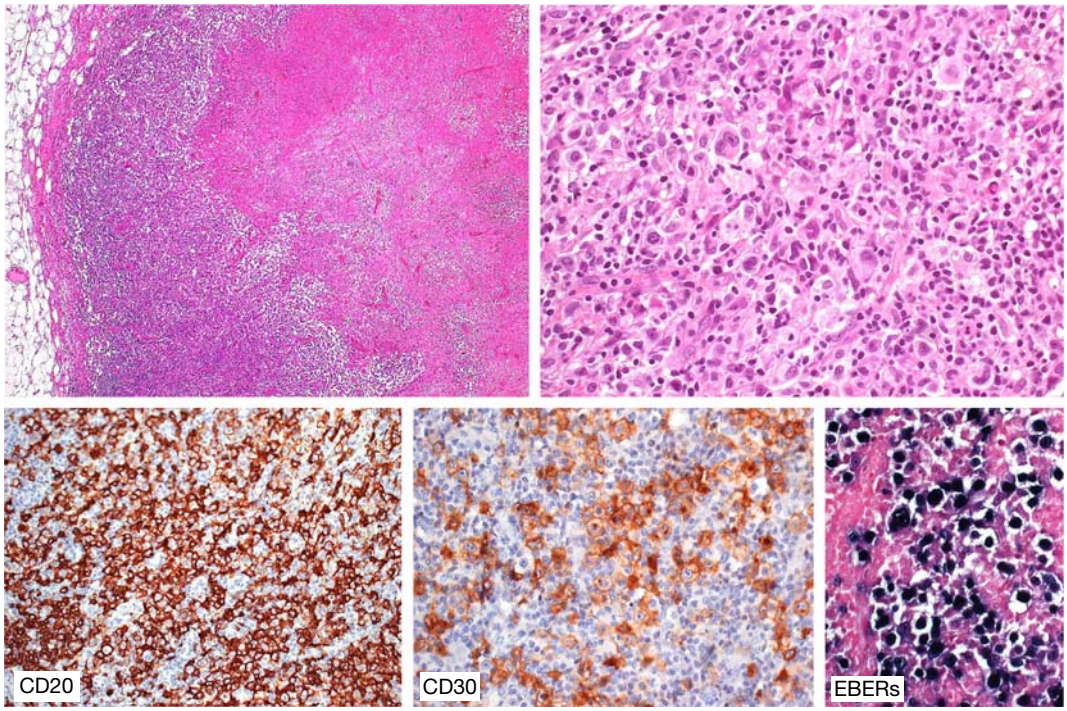

Fig. 10 EBV-positive diffuse large B-cell lymphoma, not otherwise specified. A low-power view of the lymph node (upper left panel) shows a diffuse lymphoproliferation associated to large areas of geographic necrosis. A closer view (upper right panel) shows a polymorphous infiltrate comprising many large lymphoid cells, including some resembling Reed–Sternberg cells, admixed with an inflammatory infiltrate. The atypical lymphoid cells are positive for CD20, coexpress CD30, and EBV infection is demonstrated by EBERs in situ hybridization.

Primary effusion lymphoma (PEL)

This rare and aggressive disease originally identified in AIDS patients, may also occur in other clinical settings. Patients present with effusions in serous cavities, generally with no formation of tumor masses. Rare HHV8+ DLBCLs may occur as solid tumor masses and are designated "extracavitary PELs." PEL is composed of large, often pleomorphic cells. They are usually CD45+ but lack expression of B-cell antigens and sIg and are often positive for MUM1 and CD138 as well as for CD30. The tumor cells are characteristically infected by the human herpesvirus-8 (HHV8) and most cases are coinfected with EBV. The gene expression profile of PEL shows features intermediate between those of immunoblasts and plasma cells, suggesting a plasmablastic derivation. The genome of PEL is complex.

Burkitt Lymphoma (BL)

Burkitt lymphoma (BL) is a well-defined aggressive B-cell lymphoma with characteristic morphological, immunophenotypical and molecular features with a simple karyotype and a homogeneous gene expression profile. Three distinct forms of BL are recognized: endemic BL (primarily found in Africa), sporadic BL (comprising 30% of pediatric lymphomas in western countries), and immunodeficiency-associated BL (most often affecting HIV+ patients). In all groups, the majority of patients are male. Patients present with rapidly growing often extranodal tumor masses. In endemic cases, the jaws and other facial bones are often involved. In sporadic cases, the majority of patients present with abdominal tumors. Immunodeficiency-related cases more often involve lymph nodes, and both these and sporadic cases may present as acute leukemia. BL is highly aggressive but potentially curable; intensive chemotherapy results in cure rates of up to 90%.

BL cells are monomorphic, medium-sized, with round nuclei, multiple nucleoli, and basophilic cytoplasm with small lipid vacuoles that are best seen on cytological smears (Fig. 11). There is a very high rate of proliferation and a "starry-sky" pattern is imparted by macrophages that have ingested apoptotic tumor cells. Some cases may show more pleomorphism or evidence of plasmacytic differentiation.

The immunophenotype, with expression of pan B-cell antigens, germinal center markers CD10 and BCL6 and lack of BCL2 protein, is quite constant and part of the definition. Strong MYC protein expression is detectable in the majority of neoplastic cells by immunohistochemistry. The proliferation rate, as assessed by MIB1 staining, should be close to 100% of tumor cells. More than 90% of BL show translocations of the *MYC* oncogene, usually with the immunoglobulin heavy chain locus, and, less frequently, with the light-chain loci. Immunoglobulin heavy and light-chain genes are rearranged, with somatic mutations in most cases and intraclonal heterogeneity, consistent with their putative derivation from early follicular blasts.

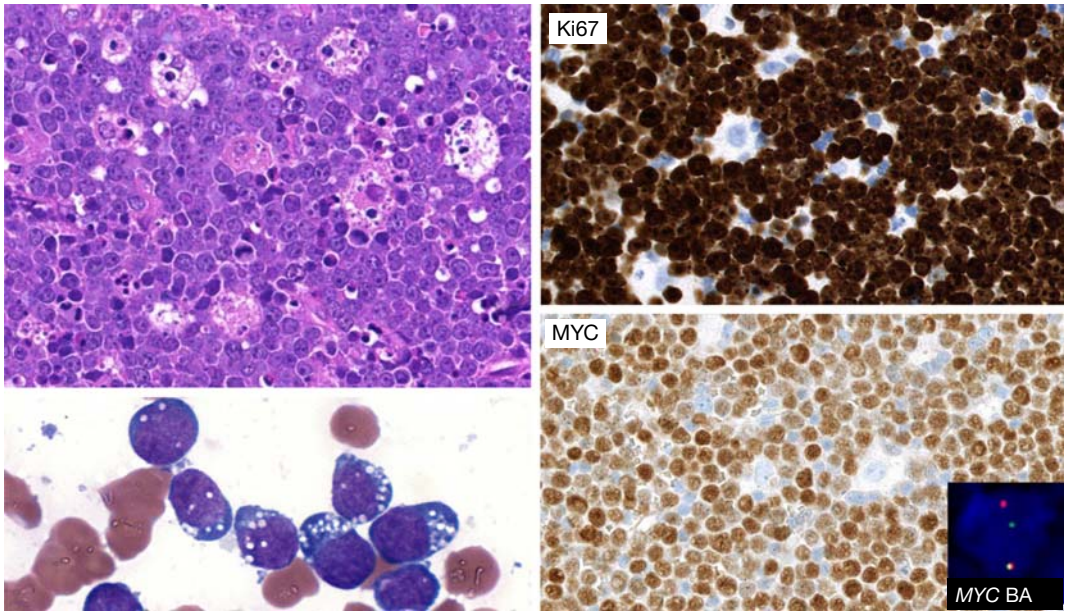

Fig. 11 Burkitt lymphoma. The histological and cytological features of a typical case are depicted in upper and lower left panels. Burkitt lymphoma is characterized by a very high proliferation fraction (almost 100% nuclei are Ki67 positive). MYC overexpression occurs as a consequence of a *MYC* rearrangement (inset, FISH using a break-apart probe for the *MYC* locus, showing a one red, one green and one yellow pattern, indicative of a *MYC* break).

Most cases have a translocation of MYC from chromosome 8q to either the IGH region on 14q—t(8;14)(q24;q32)—or IGK or IGL loci on 2q—t(2;8)—or 22q—t(8;22). Virtually all endemic cases contain EBV genomes, as do 25%–40% of sporadic and immunodeficiency-associated cases. In EBV-positive cases, the tumor cells contain clonal viral episomal genome, and display a type I latency program of gene expression (EBERs and EBNA1 expression). The exact role of EBV in the pathogenesis of BL is not understood. Next-generation sequencing analyses have revealed mutations of the transcription factor *TCF3* or its negative regulator *ID3* resulting in activation of the BCR signaling, in about 70% of the cases, and other mutations observed at lower frequency in various genes including *CCND3*, *TP53*, and *RHOA*. In general, cases that are EBV-positive tend to harbor a lesser number of mutations.

Recent studies have identified rare cases fulfilling the morphological and phenotypical criteria of BL and showing a similar gene expression profile, but lacking *MYC* translocations. In some of these cases, recurrent alterations of 11q23, namely high level gains including 11q23.2-q23.3 and telomeric losses of 11q24.1-qter were identified. Their clinico-pathological spectrum is slightly different from BL with more frequent nodal disease, higher morphological variability, lower levels of MYC expression and more complex karyotypes. The characteristic gain/loss pattern of 11q is associated with overexpression of *PAFAH1B2* and loss of *ETS1*.

High-Grade B-Cell Lymphoma

High-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements, and high-grade B-cell lymphoma, not otherwise specified, were introduced in the most recent WHO classification to encompass a group of highly aggressive lymphomas, that in the past were in part categorized as unclassifiable B-cell lymphoma with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma. Patients with high-grade B-cell lymphomas tend to have a very aggressive clinical disease with extensive tumor dissemination, often with bone marrow involvement, elevated LDH, rapid progression and poor response to standard therapy.

High-grade B-cell lymphomas may morphologically resemble DLBCL, have features intermediate between DLBCL and Burkitt lymphoma (high proliferation fraction and a starry sky appearance, with cells larger or more pleomorphic than the spectrum of classical Burkitt lymphoma or a morphology resembling Burkitt lymphoma with a discordant immunophenotype, typically strong BCL2 expression), or have a blastoid morphology. A sizeable fraction of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements, also simply termed "double hit" or "triple hit" lymphomas, represent transformed follicular lymphomas. *MYC* plus *BCL2* double hit lymphomas are usually GCB and those with *MYC* and *BCL6* rearrangement are usually ABC. High-grade B-cell lymphomas NOS by definition do not have double or triple hits, but a significant proportion carries a *MYC* rearrangement (alone).

Mature T/NK-Cell Neoplasms

Neoplasms of mature NK or T cells (PTCLs) show important variations in their geographic distribution: while comprising approximately 10% of all lymphomas in Western world, in Asia, where EBV-associated extranodal NK/T-cell lymphoma (and adult T-cell

leukemia/lymphoma) are more frequent, they represent up to 20% of lymphomas. Most entities are clinically aggressive with a dismal prognosis.

Many distinct T/NK-cell diseases have a broad range of cellular composition, from pleomorphic small cells to anaplastic large cells; immunophenotypic profiles may vary within a disease category, while similar antigen combinations may be found across different entities; and the genetics of many of these diseases is heterogeneous with few diagnostic abnormalities. Thus the delineation of mature NK/T-cell neoplasms into entities along the WHO scheme is not as sharp as for the B-cell tumors, in regard to the numerous T-cell subsets and their functional plasticity, and reflecting the fact that, despite the cell of origin being a major determinant of PTCL biology, the cellular derivation of many PTCL entities is heterogeneous or remains poorly characterized (Fig. 12). There are currently >25 different T-cell neoplasms (Table 1), which broadly segregate into leukemias (summarized in Table 5), lymphomas with predominant nodal involvement, extranodal, or cutaneous manifestations. Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), angioimmunoblastic T-cell lymphoma (AITL), and the anaplastic large-cell lymphomas (ALCL), are the most frequent T-cell lymphoma entities with somewhat varying incidence between the Americas, Asia, and Europe.

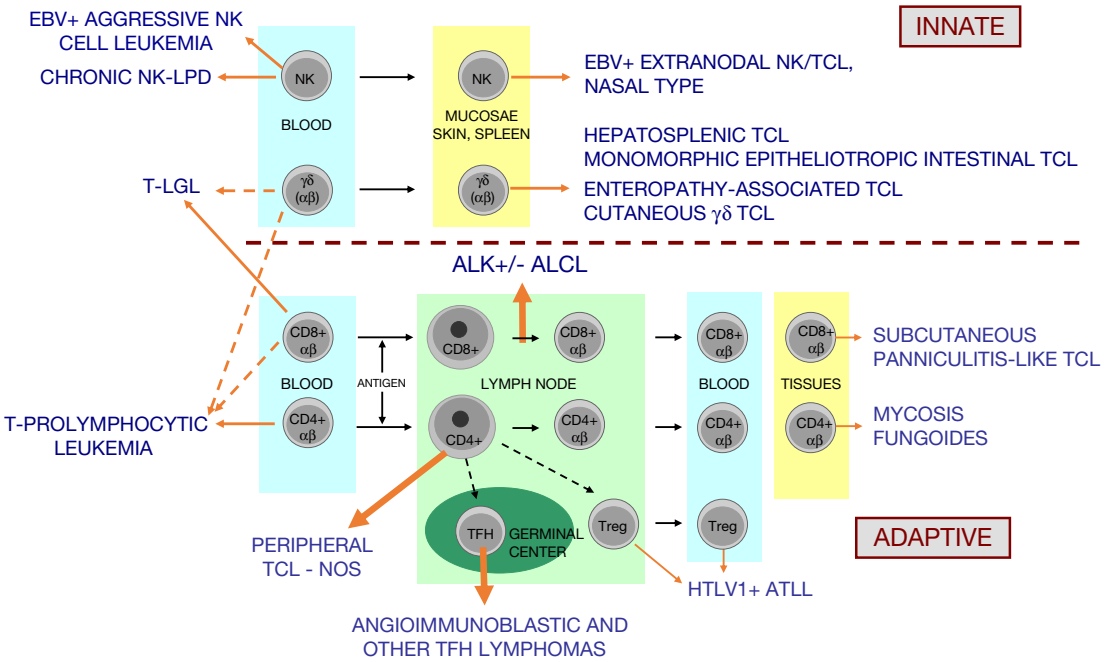

Fig. 12 Cellular derivation of PTCLs from normal T-cell and NK-cell subsets. Normal mature T cells either express the αβ or γδ isoforms of the T-cell receptor (TCR) and both express surface and cytoplasmic CD3. Alpha–beta T cells comprise CD4+ (mainly helper) and CD8+ (mainly cytotoxic) subsets. Gamma–delta T cells (CD4−CD8− or CD4−CD8+) comprise <5% of T cells and are preferentially distributed in the skin, mucosae, and to the splenic red pulp. NK cells are distinguished by the absence of TCR rearrangement and membrane TCR expression. NK cells share some markers with T cells such as CD2, CD7, CD45RO, and cytoplasmic (but not surface) CD3. NK cells are usually CD4−CD8− but may be CD8+, and they express one or several of the "NK-associated" antigens (CD11b, CD16, CD56, CD57, NK receptors), which are, however, not entirely specific. Both NK cells and activated cytotoxic T cells express cytotoxic proteins, T-cell intracellular antigen (TIA)-1 (a marker of cytotoxic cells in general), perforin and granzyme B (both expressed upon activation and not in the resting stage). Functionally, the majority of αβ T cells recognizing the antigen in a MHC-restricted fashion in the presence of an antigen-presenting cell, are part of the adaptive immune system, which features specificity and memory of the immunological response, whereas NK cells, a subset of the γδ T cells and a minor subset of αβ T cells are part of the innate immunity. Reflecting the paucity and distribution of normal γδ T cells, γδ PTCLs comprise rare, mostly extranodal cytotoxic malignancies, with aggressive behavior and poor outcome. The WHO classification comprises three lymphoma entities usually derived from γδ T cells, that are hepatosplenic T-cell lymphoma, monomorphic epitheliotropic intestinal T-cell lymphoma, and primary cutaneous γδ T-cell lymphoma. In addition minor subsets of other entities (T-LGL, NKTCL, EATL) as well as a small minority of PTCL, NOS, also have a γδ phenotype. Mature naïve T cells, either CD4+ or CD8+, are found in the thymic medulla, in the circulation and in the paracortex of lymph nodes; some may give rise to cases of T-cell prolymphocytic leukemia (T-PLL) and some peripheral T-cell lymphomas. Antigen-dependent reaction occurs in the paracortex of lymph nodes, and many peripheral T-cell lymphomas (peripheral T-cell lymphomas not otherwise specified, PTCL-NOS; angioimmunoblastic T-cell lymphoma, AITL; anaplastic large-cell lymphoma, ALCL) appear to correspond to proliferating peripheral T cells. From the immunoblastic reaction come antigen-specific T cells of either CD4 or CD8 type, as well as memory cells, that may recirculate and home to peripheral tissues. Some cases of peripheral T-cell lymphomas are thought to correspond to effector T cells. Mycosis fungoides (MF) corresponds to a mature, effector CD4+ T cell; T-cell large granular lymphocyte leukemia (T-LGL) to a mature effector CD8+ cell and many extranodal T-cell lymphomas to cytotoxic T cells. Gamma/delta T cells are usually CD8+; they constitute a minority of circulating T cells and have a predilection for homing to the spleen, skin and mucosae. These cells are thought to give rise to most cases of hepatosplenic T-cell lymphomas as well as some enteropathy-associated T-cell lymphomas (ETCL) and a subset of subcutaneous T-cell lymphomas (SPTCL). Natural killer (NK) cells appear to derive from a common progenitor with T cells. Immature NK cells are thought to be the precursor to blastoid NK-cell lymphoma/leukemia. Some types of LGL leukemia and extranodal T/NK lymphoma appear to correspond to mature NK cells.

Table 5 Features of leukemic/disseminated mature T/NK-cell neoplasms

Entity	*Morphology*	*Cell derivation*	*Functional features*	*Virus*	*Genetics*
T-cell PLL *Aggressive (1–2 years survival)*	Prolymphocyte (or small-cell or cerebriform variants)	αβ T cell (CD4 + CD8 − or less commonly CD4 + CD8 + or CD4 − CD8 +) sCD3 + CD5 − CD7 +	Noncytotoxic	No	Inv(14)(q11;q32), t(14;14) (*TCL1*), t(X;14) (*MTCP1*) *JAK1-JAK3* mutations (35%) *STAT3-STAT5B* mutations (35%)
T-cell LGL leukemia *Indolent*	Large granular lymphocyte	αβ T cell (CD8 ≫ CD4) more rarely γδ T cell) sCD3 + CD5 −/+ CD7 + CD16 + CD56 + CD57 +	Cytotoxic (A)	No	*STAT3* mutations (30%) *STAT5B* mutations (rare)
Chronic LPD of NK cells *Indolent*	Large granular lymphocyte	NK-cell (CD4 − CD8 −) sCD3 − CD5 − CD7 − CD16 + CD56 + CD57 +	Cytotoxic (A)	No	*STAT3* mutations (30%)
Aggressive NK-cell leukemia *Aggressive to fulminant*	Similar to but larger than large granular lymphocytes	NK-cell (CD4 − CD8) sCD3 − CD5 − CD7 − CD16 +/− CD56 + CD57 −	Cytotoxic (A)	EBV	6q21–23 deletion
Adult T-cell leukemia/lymphoma *Acute and lymphomatous: aggressive; chronic and smoldering: protracted course*	Flower cells (blood); pleomorphic cells (tissues)	αβ T cell (CD4 + CD8 −) sCD3 + CD5 + CD7 − CD25 + CD30 −/+	T regulatory (FOXP3 +)	HTLV1	14q31.1 fragile site deletion (*NRXN3* locus) (60%) *PLCG1* and *PRKCB* mutations (about 35% each) *CARD11, VAV1, FYN* mutations (25%, 20% and 5%) *CD28* amplifications, mutations and fusions *CCR4* truncating mutations (25%) Prominent CpG island DNA hypermethylation

T-PLL: T-cell prolymphocytic leukemia; T-LGL: T-cell large granular lymphocyte leukemia; NK-LGL: NK-cell large granular lymphocyte leukemia; ATLL: adult T-cell leukemia/lymphoma; Cytotoxic: denotes TIA-1, perforin, and/or granzyme expression; +/−: > 50% positive; −/+: < 50% positive.

Nodal Lymphomas Derived From Follicular Helper T Cells

Nodal lymphomas derived from follicular helper T cells (TFH cells) (see Glossary) is an umbrella term to embrace a large group of PTCL that have in common an immunophenotype overlapping with that of normal TFH cells, and share a similar genetic landscape. Angioimmunoblastic T-cell lymphoma (AITL), a disease entity encompassing systemic symptoms, biological abnormalities (such as anemia or hypergammaglobulinemia), usually generalized lymphadenopathy and a polymorphous infiltrate in lymph nodes with prominent proliferation of vessels and follicular dendritic cells, represents the prototypic and most common TFH-PTCL entity (Fig. 13). The two other nodal TFH-derived PTCL entities are: follicular T-cell lymphoma (FTCL), which is rare and defined by a predominantly follicular growth pattern and PTCL of TFH immunophenotype (TFH-PTCL), designating a subset of PTCL "unspecified" by morphology showing expression of a TFH phenotype. Worldwide, AITL is the second most common PTCL after PTCL-NOS, with the highest prevalence in North America and Europe. In France, AITL currently represents >35% of newly diagnosed noncutaneous PTCLs, largely outnumbering PTCL-NOS.

AITL usually diffusely involves lymph nodes and comprises a relatively small proportion of neoplastic cells (medium-sized atypical cells with clear cytoplasm), admixed to an abundant reactive microenvironment composed of small lymphocytes, histiocytes or epithelioid cells, B-cell immunoblasts, eosinophils, and plasma cells. The neoplastic cells are mature CD4+CD8− T cells, frequently show aberrant loss or reduced expression of one or several T-cell antigens and express several markers of T_{FH} cells (CD10, CXCL13, CXCR5, CD154, CD279/PD-1), CD278/ICOS (inducible T-cell costimulatory), SAP (SLAM-associated protein), BCL6, and/or c-MAF. The presence of large B-cell blasts, often infected by EBV is a feature common to AITL and other nodal T_{FH} lymphomas. Accordingly, the gene expression signature of AITL comprises a strong microenvironment imprint, with overexpression of B-cell- and FDC-related genes, chemokines/chemokine receptors, and genes related to extracellular matrix and vascular biology; conversely the neoplastic cell signature, quantitatively minor, is enriched in genes normally expressed by T_{FH} cells. The TFH phenotype and functionality of AITL neoplastic cells likely explains the peculiar pathological and biological traits of the disease, with the secretion of various soluble factors by TFH cells promoting recruitment, activation and differentiation of other cell types, for example CXCL13 promoting B-cell expansion and plasmacytic differentiation, causing hypergammaglobulinemia and Coombs-positive hemolytic anemia commonly found in AITL patients.

Broadly, the numerous recurrent genetic aberrations that have been recently identified in AITL and other TFH lymphomas interfere with two major processes: T-cell receptor (TCR) signaling pathway, and DNA methylation. Among the genes related to TCR

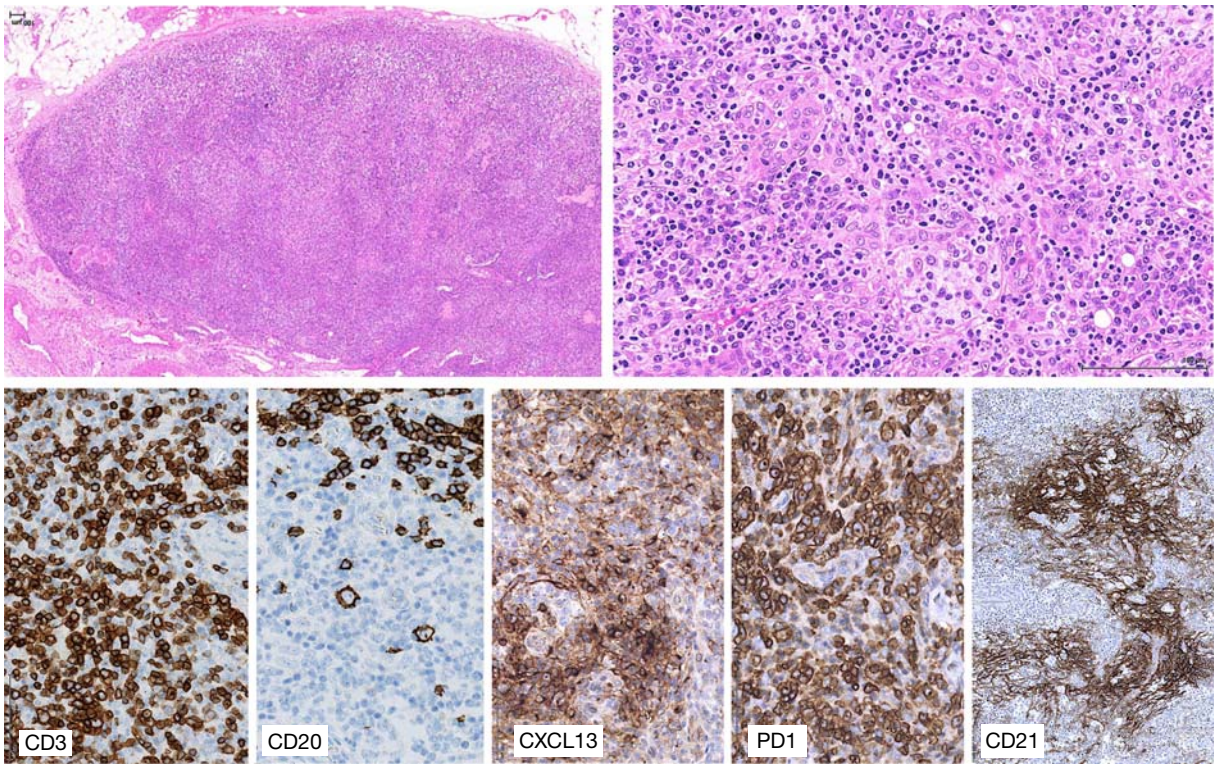

Fig. 13 Angioimmunoblastic T-cell lymphoma. A low-power view of the lymph node (upper left) shows a diffuse obliteration of the architecture, with a somewhat pale appearance. A higher magnification (upper right) shows abundant high endothelial venules, and a polymorphous cellular infiltrate comprising atypical medium-sized lymphoid cells with clear cytoplasm, that represent the neoplastic component. Most cells are CD3+ while CD20 highlights scattered large lymphoid cells. CXCL13 and PD-1 highlight a T_{FH} immunophenotype in the neoplastic component. CD21 shows an irregular proliferation of follicular dendritic cells which is characteristic of that entity.

signaling pathway, *RHOA*, which encodes a small GTPase is the most frequently mutated (60%–70% of the cases). Most mutations are hotspot generating p.Gly17Val RHOA dominant-negative variant which specifically binds to guanine nucleotide exchange factor VAV1, an interaction enhancing the adaptor function of VAV1, and ultimately leading to increased TCR signaling. In addition, activating mutations in genes encoding proximal TCR signaling elements (FYN), costimulatory receptors (CD28), or key intracellular effectors of signal transduction (PLCG1, PI3K, CTNNB1, GTF2I) are detected in up to half of TFH-PTCLs. Oncogenic TCR activation may also result from gene fusions, that is, the rare t(5;9)(q33;q22) translocation producing an ITK-SYK chimeric protein, and overall infrequent gene fusions involving *CD28* with *CTLA4 or ICOS*. The second group of genes frequently altered are involved in DNA methylation: *TET2*and *DNMT3A* harbor mono- or biallelic inactivating mutations distributed along their coding sequences in 50%–75% and 20%–30% of TFH-PTCLs, respectively. In addition, about 20%–30% of AITLs have gain-of-function missense *IDH2* mutations at the R172 residue which induces the production of an oncometabolite (hydroxyglutarate), which inhibits various deoxygenases including TET2 and histone demethylases. In AITL, *DNMT3A* and *IDH2* mutations almost always occur in association with *TET2* mutations, in contrast with myeloid neoplasms where they are usually mutually exclusive. While *RHOA* and *IDH2* mutations are present in tumor cells only, *TET2* and *DNMT3A* mutations have been found also in hematopoietic progenitors. In most cases, *RHOA* mutations are observed in *TET2* mutated tumors, with the allelic burden for *TET2* or *DNMT3A* mutations being higher than for *RHOA*, suggesting cooperation between impaired RHOA function preceding TET2 loss of function contributing to AITL pathogenesis.

Anaplastic Large-Cell Lymphomas

Anaplastic large-cell lymphoma (ALCL) encompasses four disease entities that differ by their clinical presentation and genetic features: anaplastic lymphoma kinase (ALK)-positive and ALK-negative (systemic) ALCLs, each accounting for 7%–8% of PTCL, primary cutaneous ALCL (pcALCL) and breast implant-associated ALCL (BI-ALCL) (**Fig. 14**). ALCLs share a common core of morphological and immunophenotypical features: they are composed of large anaplastic cells, including so-called "hallmark cells" characterized by an eccentric kidney- or horseshoe-shaped nucleus and a prominent Golgi region; they are homogeneously strongly positive for the activation marker CD30; they often present a cohesive pattern of growth and sinusoidal dissemination in the lymph nodes; they frequently exhibit so-called "null" immunophenotype with defective expression of the TCR/CD3 and of many T-cell antigens, despite a T-cell genotype; and usually express cytotoxic-associated antigens.

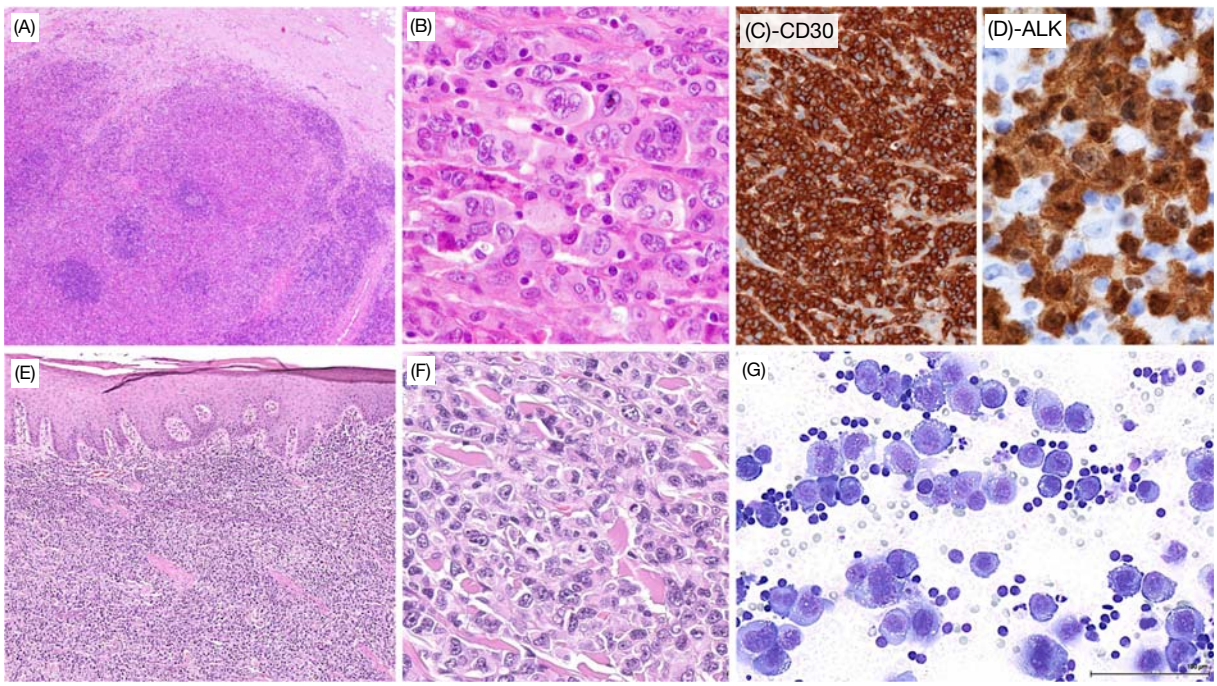

Fig. 14 Anaplastic large-cell lymphomas. ALK-positive anaplastic large-cell lymphoma (upper panels): low-power view of the lymph node shows a fibrous thickening of the capsule, and partial obliteration of the architecture (A); at higher magnification, the neoplastic cells are very large, sometimes gigantic, with horseshoe-shaped or multilobulated nuclei (B); the tumor cells are strongly positive for CD30 (C) and show nuclear and cytoplasmic positivity for ALK (D) which is indicative of t(2;5) (NPM–ALK) translocation. Primary cutaneous anaplastic large-cell lymphoma (lower left panels): the lesion is characterized by a diffuse lymphoproliferation occupying the dermis and sparing the epidermis (E); the neoplastic cells are large and grow in diffuse cohesive sheets; nuclei are irregular; mitotic figures are seen (F). Breast implant-associated anaplastic large-cell lymphoma (lower right panel): Giemsa stain of a cytological smear of seroma associated BI-ALCL, comprising large pleomorphic cells with abundant cytoplasm (G).

ALK+ ALCL

ALK+ ALCL preferentially affects children and young adults. Overall ALK+ ALCLs are aggressive neoplasms with good response to therapy and a significantly better survival as compared to ALK-negative ALCL (perhaps as a result of the younger age of ALK+ ALCL patients) and other PTCLs. Among the variety of *ALK* translocations, which induce the expression of chimeric fusion proteins having constitutive ALK tyrosine kinase activation, the most common fuses *ALK* gene @2p23 to the *nucleophosmin* gene (*NPM*) @5q35. The type of translocation determines the subcellular distribution of ALK but has no prognostic significance. ALK oncoproteins represent the major oncogenic driver in ALK+ ALCL: in vitro, constitutively activated ALK chimeras induce cellular transformation, enhance cell proliferation and survival, and account for the silencing of the T-cell phenotypic markers. These properties result from engagement of multiple signaling pathways, including the JAK/STAT and the PI3K/Akt pathways. No etiologic agent has been linked to ALCL, but there have been case reports of systemic ALK+ ALCL presenting with skin lesions occurring after an insect bite, suggesting the possible role of inflammatory mediators released upon the bite in eliciting lymphoma development. A recent study presented a model of peripheral ALCL pathogenesis where the malignancy is initiated in early thymocytes, before TCR β-rearrangement, which is bypassed in CD4/NPM–ALK transgenic mice following NOTCH1 expression. However, TCR is required for thymic egress and development of peripheral murine tumors, yet TCR must be downregulated for T-cell lymphomagenesis. These observations suggest that children affected by ALCL may harbor thymic lymphoma-initiating cells capable of seeding relapse after chemotherapy.

ALK-negative ALCL

ALK-negative ALCL tends to occur in older individuals than ALK+ ALCL, and to have more preserved T-cell immunophenotype, with less frequent expression of cytotoxic molecules. ALK− ALCL by definition contains no *ALK* translocation and is negative for ALK protein expression. While distinct signatures have been derived from the comparison of ALK+ and ALK− ALCL, other studies have also evidenced much commonality in RNA or protein expression in ALK+ and ALK-negative ALCL, and in ALK-negative ALCL and a subset of PTCL, NOS with strong CD30 expression. ALCL, irrespectively of ALK status, is molecularly distinct from other PTCLs and express a distinct set of transcripts, including CD30 (*TNFRSF8*), *BATF3*, and *TMOD*, and demonstrate low expression of genes associated with TCR signaling.

Two types of recurrent translocations were discovered in ALK− ALCL by massive parallel sequencing: (1) the most frequent rearrangements involving the 6p25.3 locus which are rather specific to ALK− ALCLs (both systemic and primary cutaneous), and virtually absent in other PTCL entities, and involve either *IRF4* or *DUSP22* (which encodes a dual-specificity phosphatase that inhibits TCR signaling) with various partners; (2) the less frequent *TP63* rearrangements encoding fusion proteins homologous to ΔNp63, a dominant-negative p63 isoform that inhibits the p53 pathway, which have been found also in PTCL-NOS. The most common rearrangement resulting from inv(3)(q26q28), fuses *TP63* to *TBL1XR1*. Rearrangements of *DUSP22* and *TP63* in ALK- ALCL are mutually exclusive and detected in 19%–30% and 7%–8% of the cases, respectively. *DUSP22*-rearranged ALCLs have a prognosis similar to ALK+ ALCL, while, conversely, *TP63* rearrangements are associated with a poor outcome (17% 5-year OS). Cases lacking all three genetic markers have an intermediate prognosis. In addition there are diverse mechanisms convergent to induce constitutive oncogenic activation of the JAK/STAT pathway in a substantial proportion of ALK− ALCL including occasionally co-occurring activating mutations of *STAT3* and *JAK1*, and recurrent gene fusions involving a transcription factor (*NFKB2* or *NCOR2*) with tyrosine kinase genes (*ROS1* or *TYK2*). Thus, STAT3 activation represents a shared oncogenic mechanism in both ALK+ and ALK− ALCLs.

Primary cutaneous (pc)ALCL

Primary cutaneous (pc)ALCL is the second most common cutaneous T-cell lymphoma (after mycosis fungoides). pcALCL presents as solitary or less commonly multiple skin nodule(s) or tumor(s) that may regress and recur, and usually carries a good prognosis. Its pathological and genetic features overlap with those of systemic ALK− ALCL, including rearrangements of *DUSP22* in up to 30% of the cases, rearrangements of *TYK2* in 13% of the cases, occasional *STAT3* mutations and rearrangements of *TP63*.

Breast implant-associated ALCL (BI-ALCL)

Breast implant-associated ALCL (BI-ALCL) is a very rare form of ALK-negative ALCL that arises in association with various kinds of breast implants with an estimated risk of one case per 500,000 to 3,000,000 women with breast implants. Most cases are confined to the periprosthetic effusion and capsule have excellent outcomes, while a minority of patients present with a breast tumor mass, which is an adverse prognostic factor. Information on the genetics of BI-ALCL is essentially limited. Next-generation sequencing findings in a few primary cases consistently showed either no alteration or a small number of somatic alterations, including activating mutations of *STAT3* or *JAK1*. *DUSP22* and *TP63* rearrangements were not found in a reported series.

Peripheral T-Cell Lymphoma, Not Otherwise Specified (PTCL-NOS)

PTCL-NOS comprises a heterogeneous group of mature T-cell neoplasms, which do not qualify for any other specific entity. PTCL-NOS make up to 40% of mature T-cell malignancies in historical series, but their relative frequency in more recent registries is lower now that cases with of a TFH immunophenotype are considered in the group of AITL-related PTCLs.

Lymph nodes usually are diffusely involved and cytology is typically pleomorphic, with predominantly smaller or larger cells. Reactive eosinophilia and/or histiocytes may be prominent. Many cases are CD4+CD8−, a subset are CD4−CD8+, and more rarely tumors are either double-negative or -positive for CD4 and CD8. Most cases derive from α/β T cells, a minority are of γδ

derivation, or TCR-silent. CD30 is often detected in a variable proportion of tumor cells. Up to 50% of PTCL-NOS are EBV-positive usually in a small subset of cells, likely bystander B cells, and this feature has been associated with a poor survival. A subset of PTCL-NOS, most commonly CD8+, feature a cytotoxic immunophenotype which in general correlates with a poorer prognosis.

Gene expression profiling has identified two subgroups of PTCL-NOS characterized by high expression of either *GATA3* or *TBX21* transcription factors and downstream target genes, associated with different prognosis. These findings can be translated to routine immunohistochemistry: PTCL-NOS with high expression of GATA3 or TBX21/T-bet appear to be essentially nonoverlapping and the high GATA3 group was shown to portend a significantly worse prognosis in two independent series.

Conventional cytogenetics and comparative genomic hybridization have shown recurrent genetic aberrations and imbalances, usually complex, but have not allowed to capture specific driver alterations. Very rare recurrent translocations involve breaks in the TCR genes. The t(6;14)(p25;q11.2) translocation involving the *IRF4* locus, has been reported in clinically aggressive cytotoxic PTCL. Expression and constitutive activation of PDGFRα and SYK tyrosine kinases appear to represent features common to many PTCLs, and although their role in lymphomagenesis remains poorly understood, they represent novel potential therapeutic targets. The mutational landscape of PTCL-NOS is not fully characterized; targeted sequencing analyses have highlighted a heterogeneous pattern of alterations including recurrent mutations in epigenetic mediators, regulators of signaling pathways, and tumor suppressor genes.

Extranodal NK/T-Cell Lymphoma (ENKTCL)

ENKTCL, nasal type (ENKTCL) represent the prototype of EBV-positive NK- or T-cell neoplasm (**Fig. 15**, upper panels). ENKTCL is not exceptional in western countries, but predominantly affects middle-aged men in Asia, Mexico and South America. It presents as tumors or destructive lesions in the nasal cavity, maxillary sinuses or palate, and despite a localized presentation in most patients, tends to relapse locally or at other extranodal sites, such as the skin, with an overall 40%–50% 5-year survival rate. "Extranasal NK/T-cell lymphomas" otherwise similar to the nasal NKTCL, may present in other localizations, especially in the skin, gastrointestinal tract, or testis, with a more adverse clinical outcome. Aggressive NK-cell leukemia, also EBV-associated and derived from NK cells, is regarded as the systemic form of NKTCL.

ENKTCL cells usually express cytoplasmic CD3 (CD3ε+), are CD2+,CD5− CD7+ CD16+/− CD56+, with an activated cytotoxic profile. Consistent PD-L1 expression by neoplastic cells was reported in the majority of the cases. Most cases are derived from NK cells and have a germline *TCR* configuration; however, in some series up to more than half of the cases derived from clonal T cells, with a $\gamma\delta$ or more rarely $\alpha\beta$ TCR configuration.

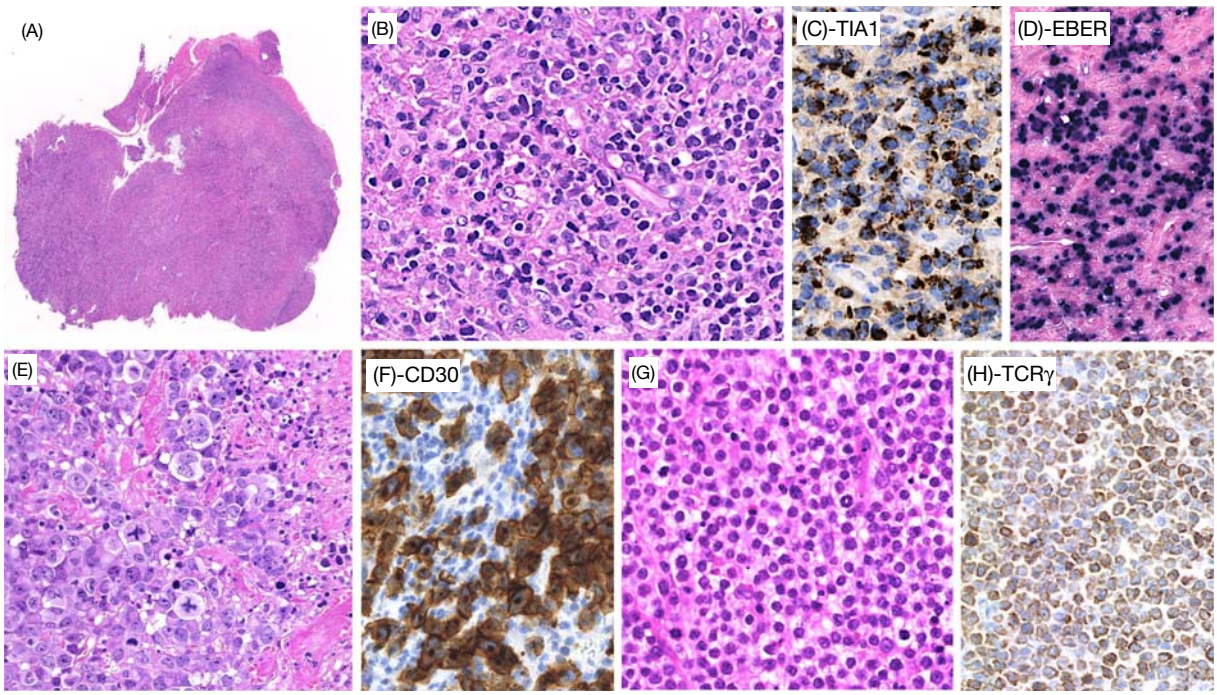

Fig. 15 Extranodal NK/T-cell lymphomas. Extranodal NK/T-cell lymphoma, nasal type (upper panels): this low-power biopsy shows a piece of sinonasal mucosa, with surface ulceration, diffuse necrosis and focal areas of viable tumor (A); the latter comprises an admixture of atypical lymphoid cells, including large cells (B); the tumor cells are positive for TIA1 (C) and EBV (D) as demonstrated by in situ hybridization with EBER probes. Intestinal T-cell lymphomas (lower panels): enteropathy-associated T-cell lymphoma composed of large cells with anaplastic morphology, associated to necrosis (E) and showing strong expression of CD30 (F). Monomorphic epitheliotropic intestinal T-cell lymphoma comprising a monotonous proliferation of small to medium in size lymphoid cells with pale cytoplasm (G) and showing TCR-gamma membrane expression (H).

By definition, all cases are associated with EBV, with a type II latency program (expression of EBERs, LMP1 and EBNA2). EBV is clonally present in an episomal form and exerts oncogenic effects through the production of cytokines such as IL-9 and IL-10, and the upregulation of IP10/MIP2 chemokines that may contribute to vascular damage and secondary necrosis. TNFα production may explain the common hemophagocytic syndrome. Partial deletion of chromosome 6 (6q21-25) is a recurrent aberration in ENKTCL. Several candidate tumor suppressor genes, such as *PRDM1*, *ATG5*, *AIM1* and *HACE1*, are mapping to that region and their inactivation by deletion and/or methylation might be involved in lymphomagenesis. Compared to normal NK cells, ENKTCL is characterized by activation of PDGFRA and of the AKT, JAK/STAT, MYC and nuclear factor-kappaB pathways. While ENKTL consistently shows nuclear localization of p-STAT3, very different results have been published regarding the actual frequency of activating *JAK3* mutations in this disease. Based on whole exome or large gene panel sequencing, the most frequent mutations have been found in *BCOR* (a corepressor acting selectively with the POZ domain of BCL6 (21%, loss-of-function mutations), *DDX3X* (a RNA helicase, mutated in 16% of the cases, and recurrently methylated as well), *TP53* (15%) and *STAT3* (13%).

Intestinal T-Cell Lymphomas

Primary intestinal T-cell lymphomas derived from intraepithelial T lymphocytes overall account for 5%–8% of PTCLs (**Fig. 15**, lower panels). These diseases tend to occur in adults, usually manifest with single or multiple lesions/tumors in the small intestine, and have a poor prognosis. They include two entities: enteropathy-associated T-cell lymphoma (EATL), and monomorphic epitheliotropic intestinal T cell lymphoma (MEITL).

EATL is most common overall, especially in Northern Europe where celiac disease is more prevalent. EATL occurs as a complication of gluten-sensitive enteropathy, but in up to half of the patients, it is the first manifestation of enteropathy. EATL is composed of pleomorphic, medium to large, occasionally anaplastic lymphoid cells, often comprises necrosis and an inflammatory infiltrate rich in histiocytes and eosinophils. The neoplastic cells are usually CD3+, CD5−, CD4−, CD8−, CD30+ CD56−, have an activated cytotoxic immunophenotype and usually express TCRαβ+ and the intraepithelial homing integrin CD103.

MEITL lacks the association to celiac disease in the majority of cases and is overall very rare, but represents the more prevalent intestinal T-cell lymphoma type in Asian-Pacific studies. It comprises a monomorphic proliferation of medium-sized cells, without necrosis and inflammation spreading to the adjacent mucosa with marked epitheliotropism. The neoplastic cells are usually CD3+, CD5−, CD7+, CD4−/CD8+, CD30−, CD56+, with an activated cytotoxic immunophenotype. Aberrant expression of CD20 or other B-cell markers is seen in some cases. MEITL appears heterogeneous in terms of TCR expression with the majority of cases being of γδ origin.

EATL and MEITL share common recurrent chromosomal imbalances (chromosome 9q gains with almost mutually exclusive losses of 16q12.1, gain of chromosome 7 and losses of 8p22-23.2, 16q21.1, 11q14.1- q14.2 and 9p21.2-p21.3), and also distinctive genetic alterations. MEITL is characterized by significantly more frequent gains of the *MYC* oncogene locus and less frequent gains of chromosomes 1q and 5q as compared with EATL. Additionally the loss of 3p21.31 has been reported as recurrent in MEITL but not in EATL.

A few studies have examined the mutational landscape of these lymphomas. Whole exome sequencing analysis of MEITL led to the discovery of highly recurrent alterations of the tumor suppressor *SETD2* gene encoding a nonredundant H3K36-specific trimethyltransferase, in the majority of the cases (14/15) (93%). *SETD2* alterations were often biallelic, mainly by loss-of-function mutations and/or loss of the corresponding locus (3p21.31). *SETD2* alterations are found in both γδ and αβ-expressing tumors and consistently correlated with defective SETD2 expression and H3K36 trimethylation. In a subsequent study, *SETD2* alterations were identified in a small percentage of EATL as well. In a T cell-specific *SETD2* knockout mouse model, an expansion of γδ T cells was observed. Interestingly, *SEDT2* is also most frequently mutated gene (about one-third of the cases) in Hepatosplenic T-cell lymphoma, an entity having in common with MEITL to be highly aggressive and often derived from γδ T cells. In another study from Asia, *GNAI2* was mutated gene in 24% of MEITL cases. Both EATL and MEITL harbor recurrent mutations in both the JAK/STAT and in MAPK (*TP53*, *BRAF* and *KRAS)* signaling pathways with *STAT5B* and *JAK3* most frequently mutated. In MEITL mutation-induced alterations in the JAK-STAT pathway, mainly affecting *STAT5B* (60%–65%), *JAK3* (35%–46%) and *SH2B3* (20%), are almost constant.

Hepatosplenic T-Cell Lymphoma (HSTL)

HSTL mostly derives from γδ T cells of the splenic pool with vδ1 gene usage, but there are rare cases with an αβ phenotype and similar clinicopathologic and molecular features. HSTL predominantly affects young male adults and may arise in the setting of chronic immune suppression or prolonged antigenic stimulation, particularly in solid organ transplant recipients or in children treated by azathioprine and infliximab for Crohn's disease. The disease typically presents with hepatosplenomegaly, thrombocytopenia and systemic symptoms, without lymphadenopathy or peripheral blood involvement.

HSTL comprises a monotonous infiltrate of atypical medium-sized lymphoid cells, with a prominent sinusoidal pattern in the splenic red pulp, liver and bone marrow. The usual immunophenotype is CD3+ CD5− CD56+ CD4−/CD8− TCRγδ+ with a nonactivated cytotoxic profile. Irrespective of TCR cell lineage, HSTL has a unique gene signature, with elevated levels of transcripts of *SYK*, NK-cell-associated molecules and oncogenes (*FOS* and *VAV3*), and low expression of the tumor suppressor AIM1 due to promoter hypermethylation.

The majority of the cases carry an isochromosome 7q, and trisomy 8 and loss of a sex chromosome seem to be associated with progression of the disease. Deep sequencing analyses have highlighted frequent mutations in epigenetic modifiers in 62% of cases, most commonly *SETD2* but also *INO80*, and *ARID1B*. Other frequent abnormalities include activating mutations of *STAT5B* (31%) and *STAT3* (9%), and of *PIK3CD* (9%).

Conclusion

Lymphoid neoplasms are a diverse group of tumors arising from cells of the immune system. The normal counterpart of many but not all of these tumors can be recognized. With available techniques, a large number of clinically and biologically distinct tumors can be defined; in daily practice fewer than 10 of these make up the majority of the cases seen by clinicians and pathologists. New genetic data are enhancing our ability to define clinically relevant subsets of heterogeneous groups such as diffuse large B-cell lymphomas. Recognizing distinct diseases is important for future progress in understanding the pathogenesis of diseases and in defining optimal therapies.

See also: Non-Hodgkin Lymphoma: Diagnosis and Treatment.

Further Reading

Basso, K., Dalla-Favera, R., 2015. Germinal centres and B cell lymphomagenesis. Nature Reviews. Immunology 15 (3), 172–184.
Elenitoba-Johnson, K.S.J., Lim, M.S., 2018. New insights into lymphoma pathogenesis. Annual Review of Pathology 13, 193–217.
Fabbri, G., Dalla-Favera, R., 2016. The molecular pathogenesis of chronic lymphocytic leukaemia. Nature Reviews. Cancer 16 (3), 145–162.
Ott, G., 2017. Aggressive B-cell lymphomas in the update of the 4th edition of the World Health Organization classification of hematopoietic and lymphatic tissues: Refinements of the classification, new entities and genetic findings. British Journal of Haematology 178 (6), 871–887.
Robbiani, D.F., Nussenzweig, M.C., 2013. Chromosome translocation, B cell lymphoma, and activation-induced cytidine deaminase. Annual Review of Pathology 8, 79–103.
Rosenquist, R., Bea, S., Du, M.Q., Nadel, B., et al., 2017. Genetic landscape and deregulated pathways in B-cell lymphoid malignancies. Journal of Internal Medicine 282 (5), 371–394.
Schreuder, M.I., van den Brand, M., Hebeda, K.M., et al., 2017. Novel developments in the pathogenesis and diagnosis of extranodal marginal zone lymphoma. Journal of Hematopathology 10 (3–4), 91–107.
Shaffer 3rd, A.L., Young, R.M., Staudt, L.M., 2012. Pathogenesis of human B cell lymphomas. Annual Review of Immunology 30, 565–610.
Smedby, K.E., Ponzoni, M., 2017. The aetiology of B-cell lymphoid malignancies with a focus on chronic inflammation and infections. Journal of Internal Medicine 282 (5), 360–370.
Taylor, J., Xiao, W., Abdel-Wahab, O., 2017. Diagnosis and classification of hematologic malignancies on the basis of genetics. Blood 130 (4), 410–423.
Vallois, D., Dobay, M.P., Morin, R.D., et al., 2016. Activating mutations in genes related to TCR signaling in angioimmunoblastic and other follicular helper T-cell-derived lymphomas. Blood 128 (11), 1490–1502.

Nonsmall-Cell Cancers of the Lung: Pathology and Genetics

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Glossary

APOBEC family Is a family of enzymes with cytidine deaminase function that catalyze the deamination of cytidine to uridine in the single stranded DNA substrate. Beside antiviral function they also have mutagenic effect later in oncogenesis.

Comparative genomic hybridization Is a molecular technique to screen chromosomes for discrete chromosomal regions, of which the copy number has increased or decreased; that is, gained or lossed compared to the standard of two alleles (one maternal and one paternal allele in each nucleus). For pathology diagnosis this technique is now replaced by *shallow sequencing*, providing similar information with less input material.

Mutational load of a neoplasm Is the cumulative result of all acquired mutational changes in a tumor, since the first division of the cell.

Next generation sequencing Is a term covering high-throughput (multiple parallel) sequencing technologies, enabling the identification of coding-sequence alterations down to single-base-pair resolution with a fairly high level of precision and accuracy in a short period of time (days). Coverage is a term used in next generation sequencing denoting the number of reads in a sample for a given DNA fragment.

Synthetic lethality An occurrence in which the disruption of two or more gene products leads to cell death, but the inhibition of either one alone does not.

Transcriptome alterations Analyses in tumor specimens have led to several important observations regarding the effects of DNA-sequence alterations on RNA transcripts, splice-site mutations, and gene fusions.

Whole exome sequencing Is a transcriptomics technique for sequencing all of the protein-coding genes in a genome (which is called the exome). The size of the exome in humans is approximately 1% of the genome and covers approximately 30 million base pairs.

Whole genome sequencing The process of determining the complete DNA sequence of an organism's genome at a single time. The size of the human genome is 3 billion base pairs.

Pathology of Nonsmall Cell Lung Carcinoma

Introduction

Until the beginning of the 21st century, the pathological classification of lung cancer for guiding of systemic treatment was quite simple: a division existed in small cell lung carcinoma and the other carcinomas (nonsmall cell lung cancer, NSCLC), each having their own (but different) chemotherapy treatment. NSCLC is a nondescriptive term that comprises the large majority of malignant lung tumors and several histologic subtypes, including squamous cell carcinoma, adenocarcinoma and large cell or undifferentiated carcinoma. Pulmonary carcinoid, mesothelioma, mucoepidermoid carcinoma, and other rare forms of lung tumors are considered separately from this general SCLC and NSCLC classification. The World Health Organization (WHO) classification of 2004 and earlier classifications for decades had been based on resection specimens but this has been influenced by two developments. One was the finding in several clinical studies that in some histological categories (adenocarcinomas (AdC) and large cell carcinomas) a chemotherapeutic agent (pemetrexed) worked better than other chemotherapic agents, but less optimal than in squamous cell carcinomas (SqCC). In parallel, it was discovered that genetic alterations which drive tumorigenesis (driver mutations, like *EGFR* activating mutations) were predominantly found in adenocarcinomas and their presence predicts the response to small molecule inhibitor treatment. The latter development opened a new dimension in pulmonary pathology, since after the diagnosis of lung cancer an additional (predictive) test was needed that is, a test that, when positive, directs toward alternative treatment. As a result the pathological diagnosis of lung cancer has become a multistep process, beginning with morphology on routine hematoxylin and eosin stain (H&E) and immunohistochemistry (IHC), to discriminate benign from malignant processes and metastases from primary lung cancer. In case of primary nonsquamous NSCLC, this will be followed by molecular characterization. We will first discuss the diagnostic process and subsequently the predictive procedures.

Small Sample Diagnosis

Squamous cell carcinoma is recognized by signs of squamous differentiation, that is, keratinization or intercellular bridges. In contrast, adenocarcinomas present different patterns: acinar, micropapillary, rarely papillary, solid component with intracellular

mucin, and/or lepidic growth (i.e., growth along alveolar walls). Large cell carcinoma is a diagnosis by exclusion: it is a carcinoma that has no characteristics fitting small cell carcinoma, adenocarcinoma, or squamous cell carcinoma. Thus, in morphological terms large cell carcinoma is an undifferentiated carcinoma. The diagnosis of large cell carcinoma is only made on resection specimens, because resection specimens provide larger fragments of the tumor for examination. If histological criteria characteristic for adenocarcinoma or squamous cell carcinoma are focally present (arbitrarily in $>10\%$), the tumor is diagnosed accordingly. Biopsies invariably are of small size, and as a consequence the proportion of cases without distinct differentiation by H&E stain is as high as 30%–40%. This morphological pattern in biopsies is called "nonsmall cell carcinoma, not otherwise specified" (NSCLC-NOS, a term only used on biopsies). In daily practice approximately 70%–80% of all lung cancer diagnoses are made on small samples, the proportion of NSCLC-NOS is relatively high and this diagnosis does not provide any direction for decisions on systemic treatment. To provide arguments for therapy decisions, additional markers have to be added in the diagnostic process, as elaborated in the 2015 WHO classification of lung cancer. A set of three markers is used, pointing either to squamous cell carcinoma (immunohistochemistry strongly positive for p40 (or p63) in all undifferentiated tumor cells) or adenocarcinoma (immunohistochemistry for TTF-1 positive in most tumor cells in 60%–70% of adenocarcinomas, and histochemical mucin stain, which may be complementary to TTF-1), as shown in Fig. 1. The use of this marker panel reduces the NSCLC-NOS category to $<10\%$, and reliably can be used for guiding treatment.

Genetics of Nonsmall Cell Lung Carcinoma

Introduction

Since the discovery of DNA it became gradually clear that acquired (somatic) DNA changes in DNA are fundamental to tumor development. In lung cancer, smoking (local pollution) is the main cause of the acquired (somatic) changes. The somatic driver changes roughly corroborate with the histologic subtypes, but several additional (also called passenger) mutations are responsible for a tremendous diversity. Lung cancer is not one disease, but turns out to be a spectrum of different diseases. In this article the general mutational process in carcinogenesis is discussed and subsequently the somatic changes related to the different histological subtypes.

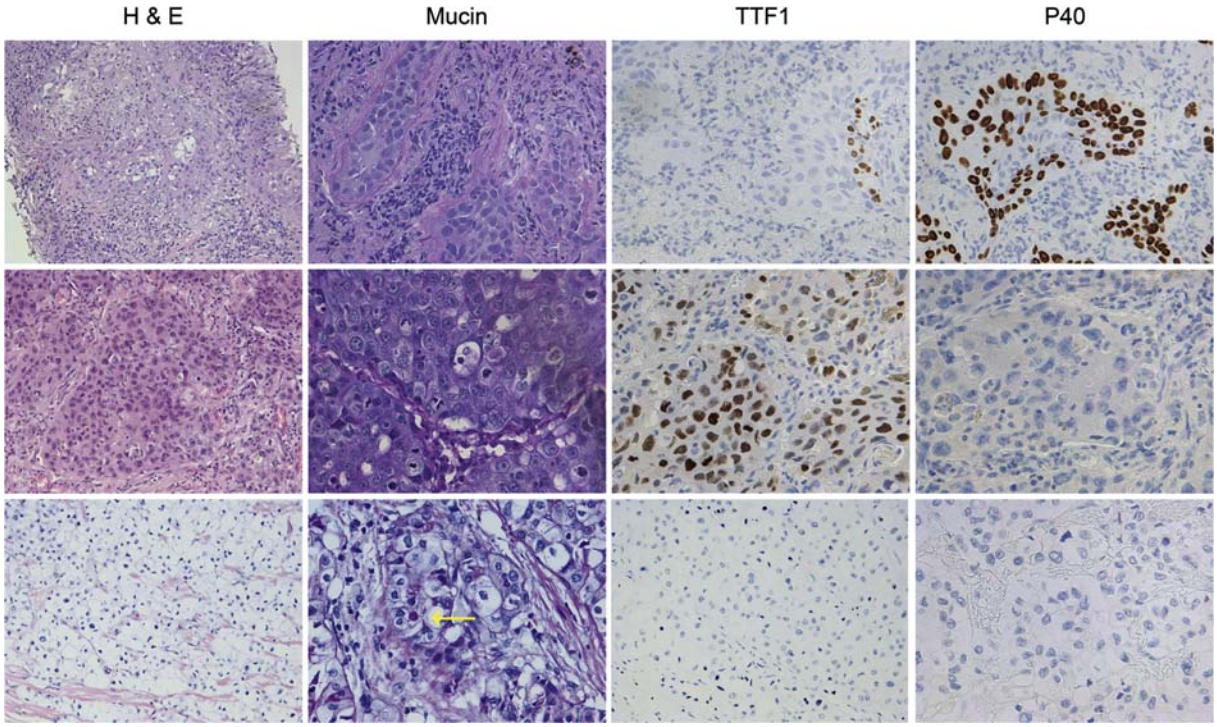

Fig. 1 For three different lung cancer cases (three rows) of nonsmall cell lung carcinoma not otherwise specified (NSCLC-NOS) the results of additional stains is shown. The top row shows positivity for p40 for a diagnosis of nonkeratising squamous cell carcinoma. The two rows below show adenocarcinoma based on positivity for TTF1 and mucin, respectively. H&E at $100\times$ and additional stains at $200\times$ magnification. In case of lack of morphological clues for the distinction between squamous cell carcinoma and adenocarcinoma on routine histological staining, or when the pathologist is in doubt, additional diagnostic stains are requested. The left panels show staining patterns and the diagnoses with which these are associated. The right panels list the predictive tests per diagnostic category. ESMO guideline indicates that molecular testing is only recommended in adenocarcinomas and NSCLC-NOS and not in patients with SqCC, except for never/former light smokers (<15 pack years).

General Somatic Changes

Smoking is the major cause of lung cancer (85% of cases). For the remaining 15%, environmental exposure, for example, diesel fumes, is likely to be responsible. With each inhalation, about 10^{14} oxidant molecules leave a cigarette, of which 10^{7} reach the lungs. These toxic components may enter airway cells and are then modified by cellular enzymes which metabolize xenobiotics, which facilitates excretion via liver and kidneys. There is some variability of the genes encoding the enzymes involved in the xenobiotic metabolism in the human population, leading to minor interindividual differences in enzyme activity. Variants of the involved genes are associated with the risk of the development of lung cancer. Some of the xenobiotics metabolites bind to DNA, creating so called DNA adducts which may result in DNA mutations. The original DNA sequence can be maintained through the removal of DNA adducts by DNA repair enzymes. DNA repair is a highly accurate process which is responsible for more than 3000 repairs per nucleus per day. Genes encoding for DNA repair enzymes also show some level of variability in the polulation, which is associated with variations in DNA repair enzyme activity. This provides a partial explanation to the fact that while many heavy smokers get lung cancer, some do not. The long latency period between smoking and the development of lung cancer (25–30 years) is one of the reasons why it has been difficult to convince smokers to quit smoking. The average life expectancy of heavy smokers is 10 years less than that of nonsmokers, due to cardiovascular and (chronic obstructive) pulmonary disease and to cancer of the lung and of other organs. The failure of epidemiological data to convince heavy smokers to quit has given rise to more personal approaches in the "quit smoking" campaigns, for example with the message that each smoked cigarette reduces life expectancy with approximately 15 min. Awareness that "smoking each cigarette is a mini-suicide attempt" may be more effective as an incentive to stop smoking.

Even though DNA repair is highly accurate, errors do occur. A single nucleotide repair error will change a single nucleotide, known as a point mutation. Point mutations may be in noncoding DNA or may not be associated with a change in the quality of the encoded protein. Such "passenger mutations" will not result in any noticeable damage. However, when a mutation in a protein coding gene changes the characteristics of the encoded protein this may profoundly impact cell behavior. Mutations in genes encoding proteins that stimulate cell proliferation, known as potential oncogenes, can convert such a gene in *a dominant oncogene* which drives uncontrolled cell proliferation (dominant because mutation of only one allele is responsible for the effect). Mutations may also be in genes inhibiting cell proliferation. Failure to repair double strand breaks may be responsible for rearrangement of some DNA strands. For tumor suppressor genes both alleles need to be mutated or functionally impaired to become oncogenic. Yet another possibility during rearrangement is the fusion between parts of two different genes, leading to a fusion protein, which may have an oncogenic driver function.

The mean somatic mutation rate in malignant tissue from the lungs of tobacco smokers is 8–10 mutations per megabase (1 million base pairs), regardless of the histologic subtype. In nonsmokers the somatic mutation rate is 10 times lower.

At nucleotide level three patterns of mutations are discerned. In smoking related lung cancer a frequent early nucleotide change is a cytosin to adenine transition (C > A), also called the "smoking signature." A similar pattern may also occur in nonsmokers, if exposure happens to similar toxins by, for example, occupational exposure. The second signature is an age related change: C > T transition at CpG sites. The third arises during cancer progression, during which additional mutations from cytosine to guanine or thymidine (C > G or C > T) develop. This pattern is associated with APOBEC enzyme-mediated mutagenesis creating the so called "APOBEC signature." Interestingly, despite maintained carcinogen exposure, tumors from smokers showed a relative decrease in smoking-related mutations over time compared to the APOBEC related mutations, which indicates that further expansion of the genetic derangement/chromosomal instability occurs. Interestingly, cessation of smoking for a longer period is associated with a reduction of the smoking signature. This implies that the tumor mutation spectra in former smokers reflects not only quantity of smoking exposure, but also time since smoking cessation.

The general assumption is that DNA mutations develop stochastically, but nonetheless the distribution of mutations is not random over the whole genome, as the folding of DNA in chromatin varies between cell types. In morphological terms, heterochromatin in a nucleus contains densely packed DNA strands, while euchromatin contains transcriptionally active DNA which is more loosely packed and more vulnerable to DNA adduct formation.

Regarding molecular oncogenesis of lung cancers, it is assumed that following early (tabacco related) mutations genome doubling occurs, along with chromosomal instability responsible for gain or loss of chromosomes chromosomal frangments, responsible for so called "copy number alterations." The pattern of copy number alterations, visualized by comparative genomic hybridization, shows some similarities between adenocarcinomas and squamous cell carcinomas, but also many differences, allowing to distinguish between these two categories in approximately 80% of the cases. In line with genomic instability as one of the hallmarks of cancer, in squamous dysplasia, the morphologically recognizable premalignant precursor lesion of respiratory epithelium, the presence of abundant copy number alterations mark progression to squamous cell carcinoma. Dysplasia without abundant copy number variation remain stable or even regress. Abundant copy number alterations in precursor lesions are not reversible.

Cell of Origin

The epithelium of the respiratory tract consists of basal, parabasal, neuroendocrine, ciliated, goblet cells, type I and type II pneumocytes. Depending on the cell type, some genes will be active, while others will be silent. Inhaled toxic components may affect any cell and cause a mutation, which raises the question whether the various cell types of the respiratory tract differ in sensitivity for mutagenesis. Ciliated cells and type II pneumocytes are terminally differentiated cells, which will undergo apoptosis at the end of their

life-span and will then be replaced freshly matured cells derived from local stem cells. In such terminally differentiated cells a mutation will not initiate cancer. In contrast, if a mutation develops in a cell with inherent proliferative capacity (e.g., basal, neuroendocrine, alveolar type II, and Clara cells), this may be the initial molecular event responsible for malignant transformation of a cell that might become the cell of origin of a tumor. Oncogenesis, however, requires the accumulation of multiple mutations in genes responsible for vital cellular functions (e.g., regulating cell proliferation and apoptosis).

The question of cell of origin of lung cancer has been studied by introducing specific gene mutations in specific cell types in mouse models, including exposure to toxins. Cell-of-origin appeared to be associated with the histopathological characteristics of the resulting tumor. The neuroendocrine cell appears to be the cell of origin of small cell lung cancer. For KRAS mutated adenocarcinomas Clara cells or alveolar type II cells serve as the initiating cell type. Of note, basal cells, Clara cells and alveolar type II pneumocytes may also serve as initiating cell types for squamous cell carcinoma, with SOX2 activation as the event responsible squamous differentiation.

Surprisingly, only 10% of the smoking population develops lung cancer and that even after a long latency period, in spite of the enormous daily toxic burden of a regular smoker. This is (at least in part) due to the efficacy of the DNA repair process as well as the fact that many mutations will occur in terminally differentiated cells which will not become cancer initiating cells.

Clonal Development

Once the cell has acquired a sufficient number of mutations conferring autonomic growth, autonomous clonal expansion will occur. During this process all cells of the clone will contain the same set of initiating mutations. To reach the volume of a detectable tumor (>0.5 cm) at least 30 doubling cycles have to be accomplished. With a tumor volume doubling time for malignant tumors varying between 50 and 400 days, the time it takes to develop a detectable tumor is counted in years. During this time frame additional mutations will have developed but not necessarily in all tumor cells. When samples from different regions of a large tumor are extensively studied with whole genome and/or exome sequencing, different parts of the tumor will show different mutational profiles, even though the set of initiating mutations is retained. Mapping these regions analogous to a phylogenetic tree structure, mutations present in all regions of the tumor are called "trunk mutations," heterogeneous mutations present in only some regions of the tumor are called "branch mutations," and mutations that are present only in one region of the tumor are called "private branch mutations." Trunk mutations are acquired early on during carcinogenesis while branch mutations have occurred later. Private branch mutations discovered with high sequencing depth might confer significant growth advantage, resulting in clonal expansion and this will turn them into branch mutations. This concept illustrates ongoing accumulation of somatic genome aberrations due to genome instability, continued background mutagenesis (age-related signature), and genetic drift. Intratumor heterogeneity is manifested in multiple subclones that coexist in different regions of one tumor.

Somatic Genome Changes in Adenocarcinoma

Comprehensive analysis of the genomic landscape of adenocarcinomas identified a very high number and a large variety of somatic gene aberrations, including mutations and/or copy number alterations (76%), as listed in **Table 1**. Some genes are frequently affected, but in most of the listed genes aberrations occur at a frequency $<10\%$. Affected genes are usually abnormally active in one or more cellular signaling pathways. We will discuss gene aberrations in adenocarcinomas, including those affected in AdC as well as in SqCC, according to functional categories. Some genes fit into more than one functional category, and for reasons of simplicity have been put together under the heading "deregulated proliferation."

Deregulated Apoptosis

TP53 is a tumor suppressor gene, implying that both alleles need to be aberrant for the carcinogenic effect. An important physiological function of p53 is detection of DNA damage which affects transcription of downstream targets, leading to growth arrest and apoptosis. When one allele of *TP53* is mutated and the other allele is lost, p53 is inactive which permits continuation of cell growth and cell survival in spite of DNA damage. *TP53* is the most frequently mutated gene in NSCLC, and in addition it is most frequently (92%) associated with mutations of other genes.

Deregulated Receptor Tyrosine Kinases

EGFR (ERBB1) is a transmembrane protein with receptor tyrosin kinase function. EGFR is activated by ligand binding and subsequent dimerization, which activates autophosphorylating capacity of the intracytoplasmic domain. This results in conformational changes of the protein, which activates downstream signaling via RAS (see *KRAS*). In AdC, EGFR mutations in the intracellular domain confer driver oncogene properties to the protein, as it is activated without ligand-binding. This results in oncogenic activities largely similar to those of *KRAS* mutations.

ERBB2, previously called her2/neu, is a member of the EGFR growth factor receptor family. Signaling promotes cell proliferation and opposes apoptosis and requires tight control. In AdC deregulation (mutation, amplification, and overexpression) the tight control is lost and oncogenic effect occurs.

Table 1 Gene aberrations in pulmonary *adenocarcinomas*, with aberration frequency

Gene	Also in SqCC	Aberration	
		M%	Lesion type
TP53	Yes[a]	54	
EGFR		10–50[b]	
KRAS		30–10[b]	
Keap 1		15	
STK11		14	
SMARCA4		9	L
RBM10		6	
RB1	Yes	5	L
ARID1	Yes	7	L
CDKN2A	Yes[a]	4	L
PIK3CA	Yes[a]	5	
NF1	Yes	10	
ALK		2–3	F
ROS1		1	F
BRAF		1–8	
ERBB2		2–4	
MET		1–3	A
ATM		8	
SETD2		5	
FTSJD1		4	
SMAD4		3	L
ARID2		5	L
NTRK1		0,1	F

M%: frequency of gene aberrations, including mutations, small insertions/deletions, and chromosomal rearrangements.
Lesion type other than point mutations: *F*, fusion; *G*, gain; *L*, loss in CNV analysis; *A*, amplification.
[a]Higher frequency in SqCC than in AdC.
[b]In western countries prevalence for *EGFR* 10%–15% and for *KRAS* 25%–30%, while in eastern countries prevalence for *EGFR* is 30%–60% and for *KRAS* 10%.

The *MET* gene (tyrosine-protein kinase Met or hepatocyte growth factor receptor) encodes for the c-MET protein. The c-MET protein plays a role in embryogenesis and is expressed on epithelial cells, but also some mesenchymal cells (endothelial cells, neurons, hematopoietic cells, melanocytes, and neonatal cardiomyocytes). In cancer two changes are relevant: amplification and deletion of exon 14. This exon contains an ubiquitin binding site. Deletion maintains an active c-MET protein with continuous activation of oncogenic pathways (RAS, PI3K, STAT3, b-catenin) and neoangiogenesis.

The *NTRK1-3* genes encode for proteins belonging to the tropomyosin kinase receptor family. The proteins have prosurvival and differentiation functions. Fusions occur at low frequency in several cancer types, and lead to constitutive oncogenic tropomysin kinase activity, also via MAPK pathway.

Deregulated Proliferation

KRAS is a dominant oncogene. When one allele is mutated it translates into a continuously active protein, resulting in persistent downstream signaling of the RAF/MEK/ERK pathway. This stimulates cell proliferation. It also activates the PI3K/AKT pathway, which stimulates cell survival (e.g., protein synthesis, cell motility).

The *RB1* gene codes for the retinoblastoma protein, a transcription repressor inhibiting cell growth. When pRB is phosphorylated it becomes inactive, allowing cell cycle progression. *Rb1* mutations result in an inactive protein, likewise allowing cell cycle progression. *RB1* is mutated at high rates in SCLC, and much lower in squamous cell carcinomas and adenocarcinomas.

CDKN2A gene (cyclin-dependent kinase inhibitor 2A) encodes for two proteins, p16 and p14arf, through an alternative reading frame. Both are cell cycle regulating tumor suppressor proteins; p16 inhibits CDK4 and CDK6 and thereby activates pRb, while p14arf activates the p53 tumor suppressor. Mutations will allow cells to continue to cycle.

PIK3CA encodes the phosphatidylinositol-3-kinase catalytic subunit alpha, that has a role in cell cycle control, differentiation, cytoskeleton reorganization, and intracellular trafficking. In lung cancer activation of the PI3K pathway allows uncontrolled cell proliferation.

The *NF1* gene encodes the protein neurofibromin, a negative regulator of the RAS signal transduction pathway. A germline *NF1* mutation is responsible for neurofibromatosis. The role of somatic *NF1* mutations in lung cancer is not clear; they may be driver mutations, but may also contribute to therapy resistance.

In NSCLC the *ALK* gene is involved in chromosomal translocation, resulting in a fusion gene. There are different fusion partners. The fusion gene is transcribed as fusion protein, containing the kinase domain of *ALK*. The fusion protein results in abnormal ALK signaling, which stimulates cell growth. Wild type ALK protein is normally not expressed in the lung. Screening for ALK aberrations in NSCLC can therefore be performed by immunohistochemical staining for the ALK protein.

The *ROS1* gene is also involved in chromosomal translocation in NSCLC. There are several different fusion partners (*CD74*, *EZR*, *FIG1*, *CCD6C*, *KDELR2*, *LRIG3*, *SDC4*, *SLC34A2*, *TPM3*, and *TPD52L1*). Transcription of the fusion gene usually contains the kinase domain of ROS1. This leads to constitutive activation of MAP kinase ERK, PI3K, mTOR, JAK, and STAT pathways. Like ALK, ROS1 can be detected using immunohistochemistry, but ROS1 staining may be positive in nonmalignant cells (e.g., type II pneumocytes).

BRAF is part of the RAS signal transduction pathway. Mutations result in activation of the MAPK pathway which stimulates cell growth.

SMAD4 (mothers against decapentaplegic homolog 4) also known as DPC4 (deleted in pancreatic cancer-4) is member of the SMAD protein family, that functions as signaling molecules downstream of the TGFβ receptor and so modulate gene transcription. SMAD4 activates the expression of genes that inhibit cell growth. In NSCLC *SMAD4* is inactivated by homozygous deletion or mutation.

Deregulated Oxidative Stress

KEAP1 encodes the Kelch-like ECH-associated protein 1 (KEAP1). KEAP1 interacts with NRF2 in the cytoplasm and has a repressive effect on NRF2. In lung cancer, mutations in *NRF2* and *KEAP1* are mutually exclusive.

STK11 encodes serine/threonine kinase 11, a cytoplasmic protein that functions as upstream kinase of adenosine monophosphate-activated protein kinase. It is involved in cell polarity and a necessary element in cell metabolism, required for maintenance of energy homeostasis, especially in a low energy state. Mutations in *STK11* lead to disorganization of cell polarity and facilitates tumor growth under energetically unfavorable conditions.

Deregulated DNA Repair/Chromatin Modeling

The SWI/SNF family is a multisubunit chromatin remodeling complex, including two mutually exclusive ATPase activities (SMARCA2 and SMARCA4). These reposition nucleosomes, which has an impact on gene expression. Mutations have been found most commonly in the SMARCA4 subunit but also in other subunits, and are thought to confer functional specificity (ARID1A, ARID1B, PBRM1, and ARID2). In NSCLC, loss of SMARCA4 can be demonstrated using immunohistochemistry and this is associated with a high mutation rate.

ATM is a gene encoding a serine/threonine protein kinase, that senses the presence of oxidative DNA damage, including double-strand DNA breaks, and facilitates subsequent repair. Insufficient DNA repair increases genome instability and contributes to cancer progression.

SETD2 protein is a histone methyltransferase, specific for lysine-36 of histone H3. Methylation of this residue prevents inappropriate transcriptional initiation, supports repair of damaged DNA, and regulates pre-mRNA splicing. Mutations of *SETD2*, or mutations at or near lysine-36 of histone H3, are associated with cancer development.

Deregulated Transcription

RBM10 encodes for an RNA binding protein involved in alternative splicing. Somatic alterations in *RBM10* lead to loss of function.

FTSJD1 (also known as *CMTR2*) was recently discovered and encodes an enzyme that methylates the ribose of the second transcribed nucleotide of messenger RNA and small nuclear RNA. In lung cancer frame shift mutations of *FTSJD1* have been found.

Somatic Genome Changes in Squamous Cell Carcinoma

Comprehensive analysis of the genomic landscape of SqCC identified a very high rate and a large variety of somatic gene alterations, both mutations (74%, with a mutation rate per megabase of 3–10 times more than in other cancers) and/or copy number alterations (76%). Consequently, most SqCCs (95%) have potentially targetable molecular alterations and almost two-thirds (>70%) have two or more alterations. This high rate of molecular changes reflects the mutagenic effects of tobacco in this strongly smoking-associated histological subtype. Some of the most significant somatic alterations of lung SqCCs identified in the Cancer Genome Atlas Research Network (TCGA) and in several studies are listed in **Table 2**. The genes carrying aberrations in squamous cell carcinomas only, excluding those that can be mutated in AdC as well as SqCC (which have been discussed earlier), are summarized

Table 2 Gene aberrations in *squamous cell carcinomas* of the lung with aberration frequency

Gene	Also in AdC	Aberration	
		M%	Lesion type
TP53	Yes[a]	51–81	92% co-mutations
MLL2		20	
PIK3CA	Yes[a]	3–16	G (34–43)
CDKN2A	Yes[a]	9–18	L (22–29) Me+(21)
NFE2L2		15	
KEAP1		12	
PTEN		8–10	L (17)
NOTCH1		8	
RB1	Yes	7	
DDR2		4	
FAT1		7	
RASA1		6	
CUL3		5	
PASK		4	
SOX2		21	G (21)
TP63		16	SNP (16)
FGFR1		16–21	G (16–22)
F (3)			
FGFR2		2–3	F (3)
FGFR3		2–3	F (3)
EGFR		7–14	G (7–14)
PDGFRA/KIT		8	G (8)
CCND1		14	G (14)
MET	Yes	3–21	G (3–21)
NF1	Yes	8	
ARID1A	Yes	5	
NOTCH1		5	

M% = frequency of gene aberrations, including point mutations, small insertions/deletions, chromosomal rearrangements.
Lesion type if not (only) point mutations (frequency in % of other lesion types) *F*, fusion; *G*, gain; *L*, loss in CNV analysis; *SNP*, single nucleotide polymorphism; *Me+*, gene silencing due to promoter hypermethylation.
[a]Higher frequency in SqCC than in AdC.

according to functional categories. Genes that fit into more than one functional category have been combined where appropriate under "deregulated proliferation."

Deregulated Receptor Tyrosine Kinases

FGFR 1-3 are proto-oncogenes and encode fibroblast growth factor receptor proteins, that belong to the fibroblast growth factor receptor family. FGFR proteins are involved in several signaling pathways including phospholipase C/PI3K/AKT, Ras subfamily/ERK, protein kinase C, and STAT. FGFR alterations as a rule are gain-of-function changes (amplification/fusion), but the exact mechanism is not always clear.

PDGFRa gene encodes platelet-derived growth factor receptor, a cell surface tyrosine kinase receptor. PDGFR proteins are involved in several signaling pathways including phospholipase C/PI3K/AKT, Ras subfamily/ERK, protein kinase C, and STAT3. Alterations of *PDGFR* in cancer include amplification, mutations, and rearrangements making the receptor constitutively active, and alternative *PDGFR* splicing.

NOTCH1 Notch homolog 1, translocation-associated (Drosophila), also known as NOTCH1, is a human gene encoding a single-pass transmembrane receptor, that belongs to the NOTCH family. In lung embryogenesis NOTCH1 is expressed in pluripotent progenitor cells in human bronchial epithelium. NOTCH1 suppresses multiple pathways, and when functionally disturbed it impacts on cancer-associated processes including proliferation, cell survival, epithelial–mesenchymal transition, metastasis, and angiogenesis.

PASK encodes the PAS domain-containing serine/threonine-protein kinase, which is involved in nutrient signaling. *PASK* mutations have recently been reported in lung cancer. The downstream oncogenic mechanism is unclear.

DDR2 discoidin domain-containing receptor 2 (also known as CD167b) encodes a receptor tyrosine kinase. Mutations are oncogenic, but the downstream mechanism is unclear.

Deregulated Proliferation

The *CCND1* gene encodes the cyclin D1 protein that belongs to the cyclin family, which show periodicity in protein abundance during the cell cycle. Cyclin D1 is required for progression through the G1 phase of the cell cycle. Constitutive overexpression of cyclin D1 leads to increased growth.

NFE2L2 (nuclear factor erythroid-derived 2-like 2, also known as Nrf2), encodes a basic leucine zipper (bZIP) protein. This transcription factor regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. Aberrant activation of the NRF2 signaling pathway has an oncogenic effect, because of crucial functions in cell survival and proliferation.

PTEN is a tumor suppressor gene which encodes phosphatase and tensin homolog protein, and functions as tumor suppressor through negative regulation of the Akt/PKB signaling pathway. Its inactivation in cancer leads to increased cell proliferation and reduced cell death.

RASA1 encodes p21 protein activator 1 or RasGAP (Ras GTPase activating protein), which is part of the GAP1 family of GTPase-activating proteins. RASA1 is located in the cytoplasm and stimulates GTPase activity of normal RAS p21, acting as suppressor of RAS function, thereby allowing control of cellular proliferation and differentiation.

FAT1 a tumor suppressor gene encoding a protein that is a member of the cadherin superfamily. FAT1 is expressed in fetal epithelium and has a supporting role in controlling cell proliferation.

Deregulated Oxidative Stress

CUL3 is a tumor suppressor gene encoding a core scaffolding protein in an E3 ubiquitin ligase complex that includes KEAP1 and RBX1, which plays a role in the oxidative stress response pathway. Loss of *PTEN* and *CUL3* synergize to activate NFE2L2 signaling. The oncogenic mechanism is unclear, possibly decreased production of superoxide might be protective for tumor cells.

Deregulated Migration

P63 encodes the transformation-related protein 63, that is part of the p53 gene family. P63 is expressed in the basal layer of most stratified epithelia and plays a role in epithelial development and differentiation. Even though p63 expression is used in the diagnostic process of NSCLC to make a diagnosis of SqCC, strictly speaking p63 is not a marker of squamous differentiation. *TP63* is frequently amplified and overexpressed of in a variety of epithelial cancers, which has led to the assumption that this protein behaves as an oncogene. Reduction of p63 expression levels has been associated with epithelial mesenchymal transition and metastasis.

SOX2 encodes a protein (sex determining region Y-box 2, SRY), that is a member of the Sox family of transcription factors. SOX2 is essential for maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells. SOX2 is frequently amplified in SqCC and its overexpression activates cellular migration and anchorage-independent growth.

Deregulated DNA Repair/Chromatin Modeling/Transcription

MLL2 is a tumor suppressor gene encoding histone H3 lysine 4 (H3K4) mono-methyltransferase, that colocalizes with lineage determining transcription factors on transcriptional enhancers and is essential for cell differentiation and embryonic development. Mutations occur in several cancer types. The oncogenic mechanism is not clear. One possibility is that in situations of transcriptional stress RNA polymerase II malfunctions (pausing, stalling, and backtracking), resulting in abnormalities in early replicating fragile sites within the chromosome. In NSCLC, most genes are affected in $< 10\%$ of the cases except for *TP53*, which is frequently mutated in NSCLC. Development of malignancy does not require aberrations in all of these genes. Genes such as *TP63*, *SOX2*, and *PIK3CA* reside in the same chromosomal region (chromosomal region 3q) and co-amplification of these three genes with *FGFR1* amplification (chromosomal region 8p12), may lead to cross-activation.

Predictive Biomarkers in Nonsmall Cell Lung Carcinoma

Once the diagnostic process has been completed, predictive testing is a second equally important step that needs to be performed in patients with advanced lung cancer. The outcome of predictive testing is essential for the choice of drug treatment. This may also include information on xenobiotic metabolism, which influences drug dosage. For practical purposes this article will only discuss NSCLC testing for the choice of drug. Predictive testing does not only assess acquired genetic changes (**Table 3**), but also includes expression of specific proteins. The prevalence of aberrations in many of the relevant genes is low and therefore many tests (10 to almost 100) need to be performed to have even a single positive result, directing the choice for a patient toward a specific drug. This makes predictive testing rather expensive.

Most genome alterations occur in nonsmokers. Rearrangements of *EGFR*, *ALK*, and *ROS1* are predominantly found in young patients. *ALK* and *ROS1* alterations occur in adenocarcinomas with a solid or cribriform growth pattern and/or the presence of signet ring cells. *KRAS* mutations are most frequent (they occur in nearly 30% of NSCLC), predominantly in smokers, but they

Table 3 Genes examined for predictive testing in NSCLC

Gene	*Histology*	*Alteration*	*Frequency (%)*	*Method(s)*
EGFR	Adenocarcinoma	Mutation	10–60	Sanger sequencing/NGS
ALK	Adenocarcinoma	Rearrangement	2–5	IHC, FISH, NGS
ROS1	Adenocarcinoma	Rearrangement	1	IHC, FISH, NGS
BRAF	Adenocarcinoma	Mutation	1–3	Sanger sequencing/NGS
ERBB2	Adenocarcinoma	Mutation	2–4	Sanger sequencing/NGS
MET	Adenocarcinoma	Amplification	1–3	ISH
MET	Adenocarcinoma	Mutation	1–3	Sanger sequencing/NGS
PD-L1	NSCLC	Expression	20–30	IHC

IHC, immunohistochemistry; *FISH*, in situ hybridization; *NGS*, next generation sequencing.

are not (yet) targetable. An important role of the pathologist is to characterize the tumor and choose the tumor sample for testing. This includes testing for responsiveness to immune checkpoint inhibitors. As in about 80% of NSCLC patients only small cell/tissue samples are available (small biopsies or cytology specimens), the pathologist needs to make most efficient use of the tumor specimens and prioritize the analyses. The pathologist will select tissue for DNA extraction to be used for next-generation-sequencing (NGS), will assess chromosomal rearrangements using fluorescence in situ hybridization (FISH) and will also assess PD-L1 expression, to select patients for treatment with PD1/PD-L1 inhibitors.

NSCLC tissues are heterogeneous and composed of a mix of nonneoplastic stromal cells (immune cells, fibroblasts, endothelial cells) and neoplastic cells. This implies that tumor DNA is "diluted" with nontumor DNA from the stromal compartment. Any molecular test needs to be sufficiently sensitive (analytical sensitivity) to detect a mutation: when a molecular test can detect a minimum of 10% of mutated DNA, assuming that the mutation is heterozygous this will require the presence of 20% of malignant cells in the sample. When the fraction of neoplastic cells is lower, macro- or microdissection can be used to arrive at a higher fraction of tumor DNA. Morphological assessment of the percentage tumorcells in a sample, on H&E stained sections on both sides of the series of sections used for DNA extraction, is mandatory for reliable results.

For most PCR-based NGS techniques, a minimum of 5–10 ng of total DNA (including DNA from neoplastic and nonneoplastic cells) is required, which is equal to approximately 1000 cells in formalin-fixed and paraffin-embedded tissue material. Hybrid capture-based NGS technologies, necessary for RNA sequencing, require 100–200 ng of RNA or DNA, which may not be attainable in up to one third of small biopsy samples.

EGFR

The first responses to EGFR tyrosin kinase inhibitors in NSCLC were observed in metastatic patients who no longer responded to conventional treatment. Sequencing of the *EGFR* gene in these tumors led to the discovery that the gene sequence coding for the intracytoplasmic domain of EGFR contained activating mutations in exons 18–21, notably 15–18 base pair deletions in exon 19 and point mutations in exon 21 (p. L858R). Progression free survival improved, compared to standard chemotherapy, which resulted in approval of gefitinib, erlotinib, and afatinib for first-line treatment of *EGFR*-mutated advanced NSCLC. However, after a few months to a few years progression occurred, indicating resistance to treatment. Second and third generation EGFR inhibitors have been developed (such as osimertinib, designed against a resistance mutation T790M).

ALK Rearrangement

Anaplastic lymphoma kinase (*ALK*) gene rearrangements in NSCLC result in expression of ALK fusion-proteins with strong oncogenic properties. Screening for ALK rearrangement can be done by immunohistochemistry, which requires limited equipment and is available in pathology laboratories worldwide. The method allows detection of the presence of the fusion protein even in a small number of tumor cells. Interpretation of ALK stained sections is quite straight forward, as ALK protein is not expressed in normal epithelial lung tissue. Other nonneoplastic cells will stain, such as light dot-like cytoplasmic staining in alveolar macrophages, nerve and ganglion cells, epithelial cells of bronchial glands, extracellular mucin, and necrotic tumor areas. In case of doubt, Fish can be used to confirm a rearrangement. ALK positive NSCLC is treated with ALK-inhibitors crizotinib and ceritinib and the results are superior to those of standard chemotherapy. Recently, alectinib has been shown to be superior to crizotinib.

ROS1 Rearrangement

Rearrangements of the *ROS1* gene have been discovered recently and can be found in 1%–2% of lung cancer patients. Treatment with Crizotinib has been approved for *ROS1* rearranged NSCLC patients. Data on the predictive value of immunohistochemical staining for ROS1 are still evolving. *ROS1* rearranged tumors invariably show diffuse cytoplasmic staining, varying significantly in intensity. At low staining intensity a *ROS1* rearrangement might not be found by FISH as more cases stain positive by immunohistochemistry than are amplified by FISH. An additional issue is expression of ROS1 in normal reactive pneumocytes and

macrophages. Current guidelines suggests screening for ROS1 by immunohistochemistry and subsequent confirmation of immunohistochemically positive cases by FISH or any molecular method.

PD-L1

PD-L1 expression on tumors cell is one of the mechanisms of immune evasion, since this inhibits functional activity of cytotoxic lymphocytes which will then not attack tumor cells. For NSCLC, PD-L1 expression by immunohistochemistry is the first predictive biomarker for checkpoint blocking immunotherapy. Interpretation of PD-L1 immunohistochemistry requires familiarity with expression patterns, since PD-L1 is expressed on dendritic cells, macrophages, mast cells, T and B lymphocytes, endothelial and tumor cells. Currently, there are no universally accepted test protocols. Results may be different depending on the used antibody, thresholds for calling a test positive have not been universally validated and to what extent immunostaining of immune cells rather than tumor cells needs to be taken into consideration has not been clarified. This is exemplified by the observation that of patients with negative PD-L1 staining by immunohistochemistry who received anti-PD-1/PD-L1 treatment, as many as 9% will still have a clinical response. This may be due to heterogeneity in PD-L1 expression, with some subclones completely negative while others strongly positive. A form of sampling bias may also be involved: PD-L1 immunostaining will as a rule be performed on the sample on which the initial diagnosis was made. Chemotherapy may have affected PD-L1 expression, which might be resolved by taking a biopsy of the recurrent tumor. This is, however, not always feasible.

Other Emerging Biomarkers

New targeted therapies gave encouraging results in early phase clinical trials for NSCLC patients with *BRAF*, *HER2*, or exon 14 *MET* mutations, and *RET* or *NTRK* rearrangements.

Development of Resistance

Although treatment targeting EGFR, ALK, and ROS1 has proven a major step forward in lung cancer management, in most patients at some point in time a relapse occurs. Biopsy of the recurrent lesion, especially after tyrosine kinase inhibitors targeting EGFR, has provided insight into resistance mechanisms. Apparently, under the selection pressure of targeted therapy, drug sensitive tumor cells undergo apoptosis or at least become quiescent, while other tumor cells survive and continue to proliferate. The mechanisms determining resistance are not yet fully understood but three mechanisms have been proposed. Firstly, the target gene of the inhibitor may acquire an additional mutation, which reduces the binding affinity of the drug to the target, or may be amplified. After treatment with EGFR tyrosine kinase inhibitors (gefitinib, erlotinib, and afatinib), the most frequent resistance mechanism is a second mutation in exon 20 (p. T790M, occurring in $\sim$50% of secondary resistant cases). This mutation changes protein conformation at the ATP binding site, which is also the EGFR TKI binding site, resulting in constitutive activation. Remarkably, the proportion of tumor DNA carrying T790M is usually lower than that of the initial driver mutation. When the fraction of tumor cells is close to the detection threshold and the initial driver mutation was barely detectable, in such a situation it may be difficult to exclude a T790M. The relevance of rebiopsy after a recurrence is underscored by the development of a drug (osimertinib) specifically against EGFR with the T790M mutation. Rebiopsy should be included as standard of care in a setting of targeted therapy.

Secondly, activation of alternative pathways such as mutation or amplification of *ERBB2* or *MET* may bypass the originally targeted pathway.

Thirdly, histological transformation, for example, of NSCLC to small cell lung cancer or epithelial mesenchymal transition may develop (**Fig. 2**).

As ALK targeting treatment is more recent, less is known about the mechanism(s) involved in the development of secondary ALK resistance. Treatment with the first generation ALK inhibitor crizotinib has resulted in secondary resistance and in about 30% of the cases a second acquired mutation or occasional amplification have been found (**Fig. 3**). Even against second generation ALK inhibitors resistance develops and in such cases in 45% additional ALK resistance mutations have been reported.

Further elucidation of mechanisms involved in development of resistance is important. To this end it is important to obtain tissue samples at the time of disease progression. Liquid biopsies and ever more sensitive sequencing methods will help to better document mechanisms of resistance to targeted therapies in lung adenocarcinoma.

Prospective Vision

Molecular analysis of a tumor tissue sample has traditionally been highly informative but to obtain a tissue sample usually requires an invasive procedure. A less invasive method is taking a blood sample (what is now customarily called a liquid biopsy) as in the circulation tumor cells but also free circulating DNA and RNA are present. Analysis of a blood sample is rapid, precise, easy to interpret and might provide information relevant for therapeutic management of the patient. The quantity of circulating free DNA is low, which currently limits extensive testing and the liquid biopsy momentarily is used to obtain information on specific molecular events such as EGFR T790M mutation, which when found has direct impact on therapy choice. Isolation of circulating tumor cells requires sophisticated equipment and low numbers of circulating cells render clinical application less informative. It remains to be seen which position the liquid biopsy will ultimately will take in the molecular analysis toolbox.

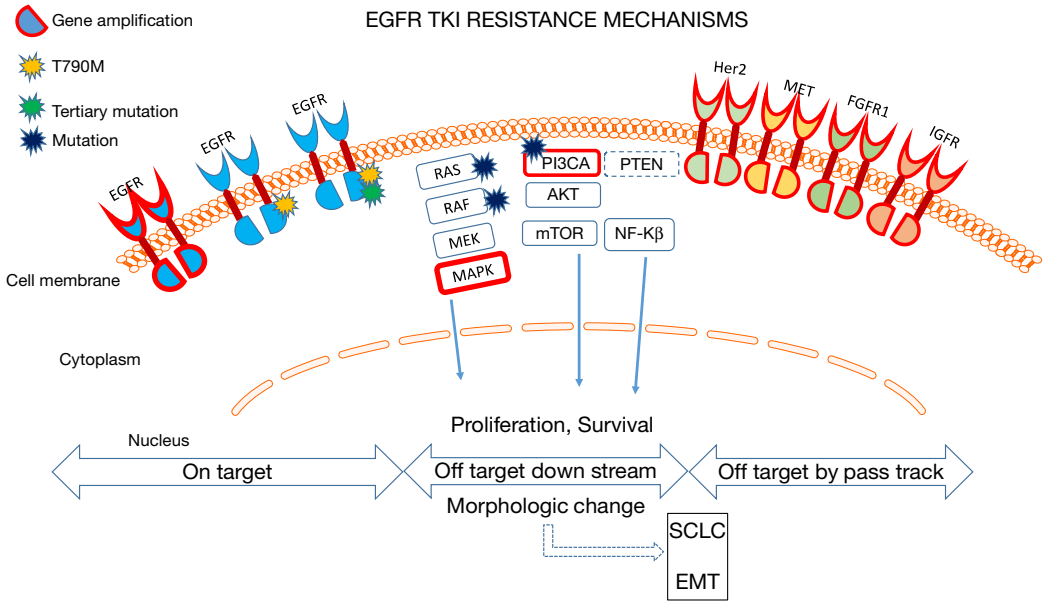

Fig. 2 EGFR resistance.

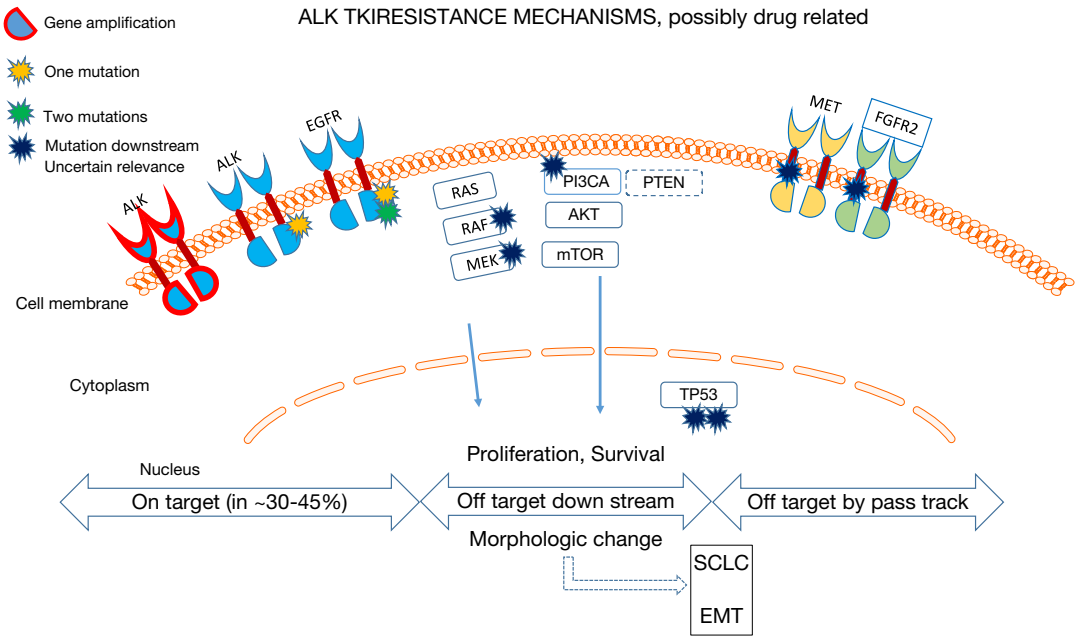

Fig. 3 ALK resistance.

In an immunotherapeutic perspective, testing for expression of PD-L1 is gradually entering into clinical practice. Although this immunohistochemical test currently provides the best information, it is likely that more discriminative testing modalities will be developed. As the mutation rate in NSCLC is high, many neo-antigens will be generated driving an antitumor immune response. A surrogate marker for response to PD-L1 blocking therapy may be the tumor mutational burden, which can also be determined on a liquid biopsy. It is likely that more markers will emerge to provide a more comprehensive and specific appreciation of the antitumor immune status of individual patients, to be used in decisions on modalities of immunotherapy.

In the future, pathologists will be required to provide detailed three dimensional assessment of tumor morphology and the host response to the tumor but also information on the mutation status of the tumor tissue, allowing clinicians to target molecular aberrations with high specificity as well as choosing immunotherapeutic modalities with the highest likelihood of response.

Further Reading

Bubendorf, L., Lantuejoul, S., de Langen, A.J., et al., 2017. Nonsmall cell lung carcinoma: Diagnostic difficulties in small biopsies and cytological specimens. European Respiratory Review 26, 17007.

Campbell, J.D., Alexandrov, A., Kim, J., et al., 2016. Distinct patterns of somatic genome alterations in lung adenocarcinomas and squamous cell carcinomas. Nature Genetics 48, 607–616.

Gainor, J.F., Dardaei, L., Yoda, S., et al., 2016. Molecular mechanisms of resistance to first- and second-generation ALK inhibitors in ALK-rearranged lung cancer. Cancer Discovery 6, 1118–1133.

Jamal-Hanjani, M., Wilson, G.A., et al., 2017. Tracking the evolution of non-small-cell lung cancer. The New England Journal of Medicine 376, 2109–2121.

Macintyre, G., Ylstra, B., Brenton, J.D., 2016. Sequencing structural variants in cancer for precision therapeutics. Trends in Genetics 32, 530–542.

Sacher, A.G., Komatsubara, K.M., Oxnard, G.R., 2017. Application of plasma genotyping technologies in non-small cell lung cancer: A practical review. Journal of Thoracic Oncology 12, 1344–1356.

Schallenberg, S., Merkelbach-Bruse, S., Buettner, R., 2017. Lung cancer as a paradigm for precision oncology in solid tumors. Virchows Archiv 471, 221–233.

Swanton, C., Govindan, R., 2016. Clinical implications of genomic discoveries in lung cancer. NEJM 374, 1864–1873.

Thunnissen, E., Unger, M., Flieder, D.B., 2013. Epidemiological and clinical aspects of lung cancer. In: Hasleton, P., Flieder, D.B. (Eds.), Spencer's pathology of the lung, 6th edn. Cambridge University Press, Cambridge, pp. 945–1003.

Thunnissen, E., Allen, T.C., Adam, J., 2018. Immunohistochemistry of pulmonary biomarkers: A perspective from members of the Pulmonary Pathology Society. Archives of Pathology & Laboratory Medicine 142, 408–419.

Obesity and Cancer: Epidemiological Evidence

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Glossary

Abdominal obesity Also known as central obesity, is the status that excessive body fat is accumulated around the stomach and abdomen. Waist circumference is commonly used as an indirect measurement of abdominal obesity.

Body mass index (BMI) Defined as body weight in kilograms divided by the square of height in meters (kg/m^2). For adult over 20 years old, normal or healthy weight is defined as BMI 18.5–24.9, overweight is BMI 25–29.9, and obesity is BMI $\geq$ 30. The obesity category is further classified as class I (BMI 30–34.9), class II (BMI 35–39.9), and class III (BMI $\geq$ 40) obesity.

Obesity Status of abnormal or excessive fat accumulation that may impair health.

Obesity Definition and Measurements

Body Mass Index

Obesity is defined as excessive fat accumulated in the body. A most commonly used tool to assess obesity is the body mass index (BMI), a simple index of weight-for-height. It is defined as a body weight in kilograms divided by the square of height in meters (kg/m^2). In 1832, Adolphe Quetelet (1796–1874), a Belgian astronomer, mathematician, and statistician, found that normal body weight in kilograms was proportional to the square of the height in meters in adults. In 1972, Ancel Keys found that the Quetelet index (kg/m^2) was strongly correlated with body adiposity measured by skinfold thickness and body density measurements in men in Europe, United States, South Africa, and Japan. Later studies also found that BMI was strongly correlated with more direct measures of body fat obtained from bioelectrical impedance, densitometry (underwater weighing), dual energy X-ray absorptiometry, computed tomography, or magnetic resonance imaging.

Since Keys renamed the Quetelet index to the BMI, BMI has been the most practical, inexpensive, and easy-to-measure method to screen for weight category. For adult over 20 years old, weight categories recommended by the World Health Organization (WHO) are underweight (BMI $<$ 18.5), normal or healthy weight (BMI 18.5–24.9), overweight (BMI 25–29.9), and obesity (BMI $\geq$ 30). The obesity category is further classified as class I (BMI 30–34.9), class II (BMI 35–39.9) and class III (BMI $\geq$ 40) obesity. Studies found that for the same BMI, percent of body fat differs by sex, age, and race/ethnicity. It has been debated whether the cut-off points for overweight and obesity are lower for Asian populations. For reporting purposes to facilitating international comparisons, it is recommended to use the additional cut-off points of 23, 27.5, 32.5, and 37.5 kg/m^2.

For children and adolescents, overweight and obese are based on age- and sex-specific growth standard. WHO definition for overweight and obesity in children under 5 years of age is weight-for-height greater than 2 and 3 standard deviations, respectively, above WHO Child Growth Standards median. For children aged between 5 and 19 years, overweight is BMI-for-age greater than 1 standard deviation above the WHO Growth Reference median, and obesity is greater than 2 standard deviations above the WHO Growth Reference median.

BMI has been widely used to examine the relationship between body fatness and diseases, including cancer, in numerous epidemiologic studies. Because people tend to over-report their height whereas underreport their body weight, BMI calculated from self-reported height and weight in observational studies may be lower than their actual BMI. However, the difference between self-reported BMI and measured BMI was negligible and did not introduce substantial bias in epidemiologic evaluation of body fatness and cancer association in large epidemiologic studies.

Waist Circumference

Waist circumference is also widely used as an indirect measurement of body fatness, particularly for central or abdominal obesity. Waist circumference is typically at the natural waist, just above hipbones. However, some studies measured at umbilicus or naval. People tended to underreport their waist circumference, in part, due to difficulties in measuring circumference around the waist. Waist circumference is positively correlated with visceral adipose tissue that is more metabolically active than subcutaneous fat. A consensus on the cut-off points for high risk for metabolic complications (e.g., diabetes, hypertension, cardiovascular diseases) is $>$102 cm in men and $>$88 cm in women. However, lower cut-off points have been proposed for Asians.

Imaging-Based Methods

There are several imaging methods that assess body fatness more accurately than BMI. Bioelectrical impedance analysis (BIA) measures body fatness as well as lean mass by assessing the impedance or resistance to a small electric current passed across body tissues. The greater the fatty tissue, the greater the resistance to the current. However, the bioelectrical impedance analysis

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depends on individual's hydration status. The dual-energy X-ray absorptiometry (DXA) measures bone mineral density as well as fat and fat-free mass. Magnetic resonance imaging (MRI) and computed tomography (CT) can assess total body fat as well as visceral and subcutaneous adipose tissue, separately. However, these imaging-based methods are expensive and not readily available in clinics and studies. Also, there is no standardized visceral adipose tissue assessment method used across studies.

Prevalence of Obesity

The global prevalence of obesity tripled between 1975 and 2016 and increased in all age groups and every country. Age-standardized prevalence of obesity (BMI $\geq$ 30) increased from 3.2% in 1975 to 10.8% in 2014 in adult men, and from 6.4% to 14.9% in adult women. Overweight and obesity has increased not only high-income countries but also in low- and middle-income countries, particularly in urban areas. In 2016, 671 million adults were obese, and 1.3 billion adults were overweight while 125 million and 213 million children and adolescents aged 5–19 years were obese and overweight, respectively. If this trend continues, child and adolescent obesity is expected to surpass underweight by 2022, and one out of five adults in the world will be obese by 2025.

Obesity and Cancer

In 2016, the International Agency for Research on Cancer (IARC)WHO convened a Working Group to review extensive evidence on the effect of obesity on cancer risk. The Working Group reviewed more than 1000 epidemiologic studies and also experimental studies and concluded that excess body fatness causes cancer of the esophagus (adenocarcinoma), gastric cardia, liver, gallbladder, pancreas, colon and rectum, kidney, thyroid, breast (postmenopausal), endometrium, and ovary as well as meningioma and multiple myeloma.

Another independent group of investigators also conducted an umbrella review of 95 meta-analyses that reported the association between body fatness and cancer in cohort studies. After a rigorous evaluation for strength and validity of reported associations in meta-analyses, the umbrella review also made similar conclusions that current evidence supports the positive association of obesity with esophageal adenocarcinoma, multiple myeloma and cancers of gastric cardia, biliary tract system, pancreas, colon, rectum, kidney, ovarian, endometrium (premenopausal women), and breast (postmenopausal) cancer.

Similarly, recent reports from the World Cancer Research Fund that evaluate and summarize a body of evidence on obesity in relation to cancer concluded that obesity increased the risk of esophageal adenocarcinoma, gastric cardia, colorectal, gallbladder, pancreas, liver, kidney, thyroid, endometrial, postmenopausal breast, and ovarian cancer and multiple myeloma.

Head and Neck Cancer

Head and neck cancer includes cancers in oral cavity, pharynx, and larynx. Earlier case-control studies suggested that obesity was inversely related to the risk of head and neck cancer, particularly among smokers and alcohol drinkers. However, case-control studies have significant methodological limitations such as selection bias and reverse causality, which may lead to an inverse association between obesity and risk of head and neck cancer. Healthier patients were more likely to participate in a case-control study, and obese patients may already have significant weight loss before cancer diagnosis, thus have lower BMI at the study entry and misclassified as having normal weight. Also, given that smoking is a strong risk factor for head and neck cancer, and smokers tended to have lower BMI than nonsmokers, the inverse association observed in smokers suggested residual confounding (i.e., incomplete adjustment) by smoking.

Later, a large pooled analysis of 20 prospective cohort studies found that obesity was associated with modestly increased risk of head and neck cancer in never smokers. Every five unit increase in BMI was associated with 15% increased risk of head and neck cancer in never smokers. There was no association between BMI and head and neck cancer in former or current smokers. In addition, abdominal obesity assessed by waist circumference was related to a higher risk of head and neck cancer in never and former smokers.

Esophageal Cancer

Esophageal cancer is the six leading cause of cancer death in the world. The most common types of esophageal cancer are squamous cell carcinoma that arises from the epithelial cells lining of the esophagus and adenocarcinoma that arises from glandular cells in the lower third of the esophagus. There is strong evidence that obesity increases the risk of esophageal adenocarcinoma, but not squamous cell carcinoma. Obese individuals had two to threefold increased risk of esophageal adenocarcinoma compared with normal weight individuals. It is hypothesized that obesity increases intragastric pressure, which subsequently relaxes the lower esophageal sphincter, exposing the lower esophagus to gastric acid and increasing the risk of gastro-esophageal reflux disease. Thus, the effect of obesity on esophageal cancer is mediated by gastro-esophageal reflux disease. However, a positive association between obesity and esophageal cancer was found in people with a history of gastro-esophageal reflux disease as well as those without the disease, suggesting an independent effect of obesity on esophageal cancer.

In addition, waist circumference and waist-to-hip ratio are related to a significantly increased risk of esophageal cancer. Every 10 cm increase in waist circumference is related to approximately 80% increased the risk of esophageal adenocarcinoma. Increase in waist-to-hip ratio (per 0.1 unit) was associated with a twofold increase in esophageal adenocarcinoma risk.

Stomach Cancer

Stomach (gastric) cancer is the third leading cause of cancer death in the world and more common in eastern and central Europe, Asia, and central and South America. The effect of obesity on gastric cancer differs by subtypes defined by the tumor location—cardia and noncardia. The cardia region is the top of the stomach, close to the esophagus, and the noncardia region is the main area of the stomach. Noncardia stomach cancer is associated with *Helicobacter pylori* infection. A body of evidence supports that obesity increases the risk of stomach cancer in cardia and is not related to the risk of stomach cancer in noncardia. Obesity is related to about 1.5- to 2-fold increase in the risk of cardia stomach cancer. The positive association between obesity and cardia gastric cancer was consistently found in studies conducted in Asia, Europe, and North America.

Interestingly, emerging evidence suggests that adolescent obesity is associated with an increased risk of noncardia stomach cancer in mid-life. Compared with people with normal weight at age 16–19 years, people who were obese at age 16–19 years had 1.8 times higher risk of noncardia stomach cancer in later adulthood in a cohort of over 1.7 million Israeli men and women who underwent health examinations at the age of 16–19 between 1967 and 2002.

Colorectal Cancer

An extensive body of literature showed that body fatness was associated with increased risk of colorectal cancer in both men and women. There is a clear dose–response relationship between BMI and colorectal cancer. Compared with normal weight individuals, overweight individuals had 10%–15% increased risk of colorectal cancer, and obese individuals had approximately 30% increased risk of colorectal cancer. The association between obesity and colorectal cancer is stronger in men than in women. The positive association with obesity was found in all subtypes by tumor locations—proximal and distal colon and rectal cancer.

Abdominal (central) obesity measured by waist circumference is also related to significantly increased risk of colorectal cancer. Every 10 cm increase in waist circumference is related to approximately 6%–15% increased risk of colon cancer and 2%–6% increased risk of rectal cancer. Weight gain during adulthood is also associated with higher risk of colorectal cancer. Five kilograms increased in weight gain during adulthood was related to a 6% increased risk of colon cancer. Accumulating evidence also indicates that being overweight or obese at adolescence and young adulthood are associated with an increased risk of colorectal cancer in later life.

Liver Cancer

Liver cancer is the second leading cause of cancer deaths and the fifth most common cancer in men and the ninth in women in the world. Hepatitis B or C virus infection and heavy alcohol use are well-known risk factors for liver cancer. Causes of liver cancer differ among populations and geographical locations. However, convincing evidence exists for the harmful effect of obesity on liver cancer in all populations and geographic regions, including Asia, Europe, and America. In general, obesity almost doubles the risk of liver cancer. Compared with normal weight individuals, obese individuals have 1.5- to 2-fold increased risk of liver cancer. The positive association between obesity and liver cancer is stronger in men than in women. A recent large pooled analysis of 14 US based prospective cohort studies found the positive association between obesity and liver cancer in people with sera-negative for hepatitis, but not in people with sera-positive for hepatitis, suggesting the role of obesity in liver cancer may differ by viral hepatitis infection status.

Waist circumference, marker of central obesity, was also related to a higher risk of liver cancer. A recent study examining BMI measured at age 17–19 years in Swedish men in 1969–96 found that the late adolescence BMI was related to the risk of liver cancer in later life. Compared with normal weight (BMI 15.5–22.5), overweight and obesity in late adolescence were associated with 1.5- and 3.5-fold, respectively, increased risk of liver cancer in later adulthood.

Gallbladder Cancer

Although gallbladder cancer is rare, it is the most common tumor among biliary tract cancers. Earlier studies, although small, suggested that body fatness was associated with increased risk of gallbladder cancer. Recently, a large pooled analysis of prospective cohort studies found a clear link between obesity and gallbladder cancer. Compared with normal weight, overweight and obesity were related to 27% and 64%, respectively, increased risk of gallbladder cancer.

Central obesity measured by waist circumference was also positively associated with risk of gallbladder cancer. Every 5 cm increase in waist circumference was related to 9% increased risk of gallbladder cancer. Obesity at adolescence or young adulthood (18–21 years) was also associated with higher gallbladder cancer risk in later life.

Pancreatic Cancer

The increased risk of pancreatic cancer in obese men and women is well established. Obese individuals are more likely to be diagnosed with pancreatic cancer at younger age. There is a clear dose–response relationship between BMI and the risk of pancreatic cancer. Compared with normal weight people, obese people had 1.5–2 times higher risk of pancreatic cancer. Also, abdominal (central) obesity is associated with an increased risk of pancreatic cancer. Every 10 cm increase in waist circumference was related to approximately 23% increased risk of pancreatic cancer.

The link between obesity and pancreatic cancer was also confirmed in a study using the Mendelian randomization approach that eliminated confounding by diabetes and other metabolic risk factors. When obesity-associated single-nucleotide polymorphisms (SNPs) found in the large genome-wide association studies were used as an unconfounded marker of obesity (i.e., genetic instrument for BMI), the risk of pancreatic cancer increased by 34% per 4.6-unit increase in BMI.

Studies also have found that obesity in late adolescence and young adulthood was associated with increased risk of pancreatic cancer in late life. Every five unit increase in young adulthood BMI was associated with 18% increased risk of pancreatic cancer in later life.

Lung Cancer

The role of obesity in lung cancer is inconsistent. Many studies reported that higher BMI was associated with lower risk of lung cancer. However, the inverse association between BMI and lung cancer was likely due to residual confounding (i.e., incomplete adjustment) by smoking in the analysis. Smokers who are at high risk for developing lung cancer tend to have lower BMI than nonsmokers of the same age and sex. Therefore, obesity may appear to be protective against lung cancer. When the obesity and lung cancer relation was examined in smokers and nonsmokers, separately, obesity was not associated with lung cancer in nonsmokers but was related to a lower risk of lung cancer in smokers. A Mendelian randomization study of obesity and lung cancer using a genetic score for BMI as instrumental variable to eliminate reverse causation and reduce confounding found that the genetic risk score for obesity was associated with increased risk of lung cancer.

In addition, waist circumference—a proxy for abdominal obesity—was positively associated with lung cancer. Each 10 cm increase in waist circumference was associated with a 10% increased risk of lung cancer in both smokers and nonsmokers. Indeed, a genetic risk score for obesity in the GAME-ON consortium was positively associated with lung cancer, suggesting that the observed inverse associations with BMI may be noncausal.

Melanoma and Nonmelanoma Skin Cancer

The association between obesity and melanoma is less clear. Some studies found that obesity was related to an increased risk of melanoma, particularly in men, but not in women, while others found no association between obesity and melanoma. Sun exposure and skin type are important risk factors for melanoma, but most studies did not adjust for these factors, raising concern about residual confounding (i.e., incomplete adjustment) in the analyses. It has been suggested that obese people tended to spend less time outdoors and avoid sunbathing compared with normal weight people. Studies that adjusted for sunlight exposure observed a positive association between obesity and risk of melanoma in women.

In contrast, although limited, studies suggested that obesity was related to a lower risk of nonmelanoma skin cancer, including squamous cell carcinoma and basal cell carcinoma, even after controlling for sun exposure or UV radiation susceptibility. More studies with measures of sun exposure or sun-seeking behaviors are needed to investigate the role of obesity in melanoma and nonmelanoma skin cancer.

Breast Cancer

Obesity is an established risk factor for postmenopausal breast cancer in women. Five unit increase in BMI increases risk of postmenopausal breast cancer by approximately 15%. The effect of obesity on breast cancer is stronger in postmenopausal women who never used the exogenous hormones (i.e., hormone replacement therapy) than those who used. The effect of obesity on breast cancer also differs by tumor hormone receptor status (i.e., estrogen and progesterone receptor). Obesity is associated with 1.5- to 2-fold increased risk of both estrogen and progesterone receptors positive breast cancer, but not with both estrogen and progesterone receptors negative breast cancer. Weight gain during adulthood or after menopause also increased risk of postmenopausal breast cancer, especially among women who never used hormone replacement therapy.

On the other hand, obesity is related to a lower risk of premenopausal breast cancer. The inverse association with premenopausal breast cancer seems to be restricted to estrogen receptor-positive breast cancer. Triple-negative breast cancer that lacks expression of estrogen and progesterone receptors and human epidermal growth factor receptor 2 (HER2) tended to be diagnosed at younger age and have aggressive disease course. Several studies have shown that higher BMI and greater waist circumference are associated with a two to three times increased risk of triple-negative breast cancer among premenopausal women.

Interestingly, accumulating evidence suggests that obesity is related to an increased risk of breast cancer in men as well. Studies found that obesity was associated with approximately 30% higher risk of breast cancer in men. Also, obesity during adolescence or early adulthood was related to an increased risk of breast cancer in men.

Cervical Cancer

Cervical cancer is the fourth most common cancer in women in the world and is caused by persistent infection of human Papilloma Viruses (HPV). However, the majority of women with HPV do not develop cervical cancer, and other environmental factors are required for cancer to develop. Cervical cancer screening is effective in detecting precancerous lesions that can be treated and cured before they develop into cancer. The association between obesity and cervical cancer is not clear. Some studies found a weak but significantly positive association between obesity and cervical cancer while others found no association. Most of these studies did not have cervical cancer screening information that may have confounded the association.

Recently, a large retrospective cohort study of over 900,000 women who underwent cervical cancer screening (i.e., cytology and human papillomavirus DNA testing) found that overweight and obese women had an increased risk of cervical cancer. The positive association between obesity and cervical cancer was consistently found in HPV positive women as well as in HPV negative women. Obesity was also related to an increased risk of both subtypes—glandular and squamous. However, the study found that the increased risk of cervical cancer in obese women was likely due to low efficacy of cervical cancer screening in obese women, resulting in underdiagnosis of precancerous lesions, thus increased risk for cervical cancer.

Endometrial Cancer

Obesity is a well-established risk factor for endometrial cancer. There is a clear dose-response relationship between BMI and endometrial cancer. Every five unit increase in BMI is associated with 50% increased risk of endometrial cancer. The positive association between obesity and endometrial cancer was found in premenopausal and postmenopausal women and in hormone replacement therapy users and nonusers. The detrimental effect of obesity on endometrial cancer tended to be stronger in women who never used hormone replacement therapy than those who used. Obesity was also related to an increased risk of type I (mostly endometrioid adenocarcinomas) and type II (nonendometrioid such as serous, clear cell, and carcinosarcoma) endometrial cancer. Mendelian randomization study also found that genetic risk scores for BMI was positively associated with the risk of endometrial cancer.

Abdominal (central) obesity assessed by waist circumference is also associated with increased risk of endometrial cancer. Every 5 cm increase in waist circumference was related to a 13% higher risk of endometrial cancer risk. Weight gain in adulthood also increases the risk of endometrial cancer, particularly in women who never used hormone replacement therapy. Obesity at young adulthood (age 18–25 years) is also associated with higher risk of endometrial cancer later in life.

Ovarian Cancer

Emerging evidence suggests that obesity is related to a modest increase in the risk of ovarian cancer, particularly in premenopausal women. Every five-unit increase in BMI is associated with approximately 10% increase in ovarian cancer risk. The positive association between obesity and ovarian cancer is stronger in premenopausal women than in postmenopausal women. Some studies suggest that obesity is related to certain subtypes of ovarian cancer such as low-grade serous and invasive endometrioid and mucinous tumors.

Abdominal obesity assessed by waist circumference is also related to a small increase in the risk of ovarian cancer. Every 10 cm increase in waist circumference was associated with a 6% increase in ovarian cancer. Weight gain in adulthood seems to increase risk of ovarian cancer, especially in postmenopausal women who never used hormone replacement therapy. Interestingly, studies also found that obesity at young adulthood (age 18–29) was associated with higher risk of ovarian cancer at later life.

Prostate Cancer

Findings from earlier studies that examined the association between obesity and the risk of prostate cancer were inconsistent. Studies conducted in North America found no or inverse association between obesity and prostate cancer, whereas European studies found a weak but significantly positive association. It was postulated that the differential rate of prostate-specific antigen (PSA) screening between these regions was attributable to the inconsistent findings. PSA screening that resulted in a diagnosis of early-stage less aggressive cancer was common in North America, but not in Europe. Therefore, European studies tended to have advanced and fatal prostate cancer.

When the obesity and prostate cancer relation was examined by prostate cancer aggressiveness—nonadvanced, advanced, and fatal prostate cancer in later prospective cohort studies, a clear pattern emerged. Obesity was associated with an increased risk of advanced or fatal prostate cancer. Every five unit increase in BMI was related to approximately 8% increased risk of advanced prostate cancer. The risk of advanced prostate cancer also increased by 12% per 10 cm increase in waist circumference. In contrast, obesity was associated with a lower risk of nonadvanced prostate cancer.

Recent studies suggest that the relation of obesity to prostate cancer differs by molecular subtypes of tumor as well. Two studies found that obesity was inversely associated with risk of TMPRSS2:ERG (T2E)-positive prostate cancer, but not in T2E-negative prostate cancer.

Bladder Cancer

The association of obesity with bladder cancer is less certain. A meta-analysis of prospective cohort studies found a weak but significantly positive association between obesity and bladder cancer. Given that smoking is an established risk factor for bladder cancer, and smokers tended to have lower BMI than nonsmokers, most studies of obesity in relation to bladder cancer may have suffered from residual confounding (i.e., incomplete adjustment) by smoking. A large prospective cohort study found a positive association between waist circumference and bladder cancer, but it was limited to men.

Kidney Cancer

There is convincing evidence that obesity increases risk of kidney cancer (mostly renal cell carcinoma). The effect of obesity on kidney cancer is independent of hypertension that is another established risk factor for kidney cancer. In general, obesity is associated with a 1.5- to 2-fold increased risk of kidney cancer in both men and women. Abdominal obesity assessed by waist circumference is also positively related to the risk of kidney cancer. Every 10 cm increase in waist circumference increased risk of kidney cancer by approximately 11%. Weight gain in adulthood is also associated with increased risk of kidney cancer.

On the other hand, several studies that examined the association between obesity and cancer survival in kidney cancer patients found a contradicting result. Patients who were obese at the time of kidney cancer diagnosis tended to have better survival compared with patients with normal weight at cancer diagnosis. It was suggested that obese patients were more likely to have indolent tumors, leading to better survival.

Brain Tumors

Meningioma is slow-growing brain tumor that arises in the membranes surrounding the brain and the spinal cord. Although a limited number of studies examined obesity in relation to meningioma, they consistently found that obesity was associated with increased risk of meningioma. Overall, compared with normal weight individuals, obese individuals had a 1.5-fold increased risk of meningioma. In contrast, obesity is not associated with risk of glioma, the most common intracranial tumor that starts in the glial cells of the brain or the spine.

Thyroid Cancer

Obesity has been consistently associated with modestly increased risk of thyroid cancer. Every five unit increase in BMI was related to a 6% increase in thyroid cancer risk. The positive association between obesity and thyroid cancer was found in both men and women, but it tended to be stronger in men than in women. Obesity was related to an increased risk of all major histological types of thyroid cancer except medullary thyroid carcinoma. Similarly, abdominal obesity measured by waist circumference was associated with a higher risk of thyroid cancer. Emerging evidence also suggested that young adulthood obesity was related to approximately 13% increased risk of thyroid cancer in later life.

Non-Hodgkin Lymphoma

Non-Hodgkin lymphoma is a cancer originated from the lymph system, a part of immune system. The most common non-Hodgkin lymphoma subtypes are diffuse large B-cell lymphoma, follicular lymphoma, and chronic lymphocytic lymphoma/small lymphocytic lymphoma. Earlier studies on obesity and non-Hodgkin lymphoma reported inconsistent results. Some studies found a weak to moderate positive association between obesity and non-Hodgkin lymphoma whereas others found no association. When each subtype of non-Hodgkin lymphoma was examined separately, it became clear that obesity was related to an increased risk of diffuse large B-cell lymphoma, but not other subtypes. Compared with normal weight people, obese people had approximately 25% increased risk of diffuse large B-cell lymphoma. Recent studies also suggest that obesity during adolescence or young adulthood is associated with a higher risk of diffuse large B-cell lymphoma in later life.

Multiple Myeloma

Multiple myeloma is a rare but fatal malignancy. Mounting evidence indicates that obesity is related to an increased risk of multiple myeloma in men and women. A large pooled analysis of 20 prospective cohort studies found that obesity was associated with approximately 20% increased risk of multiple myeloma, compared with normal weight. The positive association of obesity with multiple myeloma seems to be stronger for obesity at adolescence or young adulthood (age 18–21 years). Waist circumference was also related to a higher risk of multiple myeloma, suggesting the harmful effect of abdominal obesity.

Leukemia

The role of obesity in leukemia is less clear. Limited evidence suggests that obesity is associated with a modestly increased risk of leukemia. The positive association with obesity was found for all leukemia subtypes such as acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, and chronic lymphocytic leukemia.

Obesity at Adolescence or Young Adulthood and Cancer

Emerging evidence suggests that excess body weight in early life increases risk of cancer in later life, particularly those known to be related to obesity in late adulthood. Due to the methodological challenges in ascertaining height and body weight in childhood, adolescence, and young adulthood and following participants for several decades for cancer outcomes, a limited number of studies examined the association between obesity in early life and cancer risk in later life. Some studies linked historical mandatory health examination data (e.g., for military service) to a national cancer registry data, and others collected self-reported recalled height and weight at early life period.

Although limited, studies consistently found that obesity at adolescence or young adulthood was associated with increased risk of stomach, colorectal, liver, gallbladder, pancreas, and thyroid cancer and diffuse large B-cell lymphoma and multiple myeloma in late adulthood. In contrast, obesity at early life was related to a lower risk of both premenopausal and postmenopausal breast cancer.

Weight Gain During Adulthood and Cancer

On average, young adults gain approximately 0.5 kg per year, which is translated into approximately 10 kg weight gain or 3.1–3.5 BMI increase during 18 years. Most people move into a higher BMI category by middle age. People who experienced early and rapid weight gain at young adulthood are more likely to continue to gain weight throughout the adulthood. The weight gain during adulthood is mostly due to increase in body fat, particularly around the waist. It is postulated that weight gain during adulthood, which can be measured easily, is a good surrogate of body fatness. A meta-analysis of studies of adult weight gain and cancer found that adult weight gain was associated with postmenopausal breast, endometrial, and ovarian cancer and cancers in colon and kidney. The increased risk of female cancers tended to be higher among hormone replacement therapy nonusers than among users. In general, every 5 kg weight gain during adulthood was related to approximately 10% increased risk of cancers.

Intentional Weight Loss and Cancer

A body of literature provides convincing evidence that obesity increases risk of numerous cancers. However, whether or not intentional weight loss reduces cancer risk is unclear. It is, in part, due to the difficulties in intended weight loss and long-term maintenance of weight loss in large observational studies. Therefore, bariatric surgery patients who had significant and sustained weight loss represent a unique population to examine the effects of long-term intentional weight loss on cancer risk.

Studies found that intentional weight loss by bariatric surgery in morbidly obese individuals was related to a lower risk of cancer. Compared with people with severe obesity but no bariatric surgery, people who received bariatric surgery had approximately 30% lower risk of cancer overall. The risk reduction was more pronounced for obesity-related cancers. There was a 40% risk reduction for postmenopausal breast cancer, 60% reduction for endometrial cancer, 40% reduction for colon cancer, and 50% reduction for pancreatic cancer in people who underwent bariatric surgery compared with those who did not.

Obesity Paradox in Cancer Survival

In contrast to the harmful effect of obesity on most cancers, some studies found that obesity was related to a lower risk of cancer death among cancer survivors. This obesity paradox (i.e., obese people live longer) was suggested in some studies of survivors of colorectal cancer, renal cell carcinoma, diffuse large B-cell lymphoma, and melanoma. Several methodological limitations in studies were proposed to explain the observed inverse association between obesity and cancer survival.

First, residual confounding (i.e., incomplete adjustment) due to inadequate or unmeasured confounders such as smoking, comorbidities, socioeconomic status, and lifestyle factors may cause a spurious positive association between BMI and cancer survival. Second, given that weight loss often precedes cancer diagnosis and is associated with an increased risk of mortality in cancer survivors, reverse causation may have occurred in the analysis. The third is a collider stratification bias that occurs in data analysis stratified by a variable affected by exposure and outcome. For example, obese people may have developed cancer due to obesity or smoking whereas normal weight people developed cancer due to smoking in the absence of obesity. Therefore, obese cancer patients are less likely to be smokers, while nonobese cancer patients are more likely to be smokers. Because smoking is a strong risk factor for mortality, obese patients appear to have lower mortality risk than nonobese patients in the analysis of cancer survivors.

In addition, although BMI is the most commonly used measurement to define obesity, BMI may not well represent body fatness in cancer survivors who often experienced changes in body weight and body composition. Studies that measured body fat mass and muscle mass using computed tomography images found that overweight cancer patients tended to have a normal body composition whereas normal weight cancer patients had low muscle mass, similar to underweight. Thus, cancer patients with normal weight at diagnosis may have different body composition compared with normal weight noncancer individuals. The common BMI category may not applicable to cancer patients. Lastly, there are significant heterogeneities in study design and methods, including time of BMI ascertainment (e.g., few months to years before or after cancer diagnosis), adjustments of potential confounders, and

duration of follow-up. These limitations in current literature contribute to inconsistent findings. More studies are needed in this emerging field.

Biological Mechanism

There are several biological mechanisms that may explain links between obesity and increased risk of cancer.

Sex Hormones

Estrogen has been hypothesized to play a role in mostly female cancers such as breast, endometrial, and ovarian cancer. Estrogen is predominantly produced in the ovary, but after menopause, it is produced primarily in adipose tissue through a cellular pathway involving the enzyme aromatase that converts androgens to estrogen. Postmenopausal obese women have higher concentration of total and free circulating estrogen levels compared with normal weight women. Women with higher concentration of circulating various estrogen metabolites and lower concentration of sex hormone binding globulin had an increased risk of postmenopausal breast cancer.

Animal and experimental studied showed that estrogen promotes tumor growth; stimulates cellular proliferation; inhibits apoptosis via estrogen receptor-α agonism; and induces vascular endothelial growth factor and angiogenesis. Estrogen is also suggested to promote free-radical mediated DNA damage, genetic instability, and mutation. Selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene and aromatase inhibitors are effective breast cancer treatments by blocking the effects of estrogen.

In men, obesity was related to high estrogen, low testosterone, and low sex hormone binding globulin levels, which led to greater availability of estrogen. The majority of male breast cancer is estrogen receptor positive.

Insulin and Insulin-Like Growth Factor-1

High levels of insulin and insulin-like growth factor (IGF)-1 have been hypothesized to promote the development of cancer. Obesity is positively associated with circulating levels of insulin and IGF-1. Many obese people are insulin resistance and develop type 2 diabetes. Circulating insulin binds to the insulin receptor on the cell surface and promotes hyperactivity of phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signaling pathways that stimulate cell growth and cell proliferation. Metformin that is a type 2 diabetes medication increases insulin sensitivity and activates the AMP-activated protein kinase (AMPK) pathway, thereby counteracting the PI3K/AKT/mTOR signaling pathway. Metformin has been suggested as a chemopreventive agent for cancer prevention.

Chronic Inflammation

Obesity is a common cause of chronic inflammation, both systemically and at the tissue level. Obesity is associated with systemic chronic inflammation markers such as C-reactive protein, tumor necrosis factors (TNFs), interleukin (IL)-1β, IL-6, IL-8, and vascular endothelial growth factor. TNF-α, IL-1β, and IL-6 promote tumor growth in mouse models of obesity, and IL-6 and CRP have been associated with the development and progression of breast tumors. Also, chronic inflammation in the tissue microenvironment such as pancreatitis and gallstone are known risk factors for pancreatic and gallbladder cancer, respectively.

Adipokines

Adipose tissues, particularly white adipose tissue, also secrete various cytokines, known as adipokines that modulate the chronic inflammatory state. Leptin and adiponectin are the most studied adipokines in relation to cancer and play opposing roles in cancer. Leptin is a proinflammatory adipokine while adiponectin is an antiinflammatory adipokine. Leptin activates cell proliferation and multiple signaling pathways including the PI3K, MAPK, and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways. Leptin can also induce IL-6 production, suggesting its contribution to the systemic inflammation.

On the other hand, adiponectin inhibits cell growth, increases apoptosis, and activates AMPK that induces cell cycle arrest and inhibits mTOR activity, therefore plays a protective role in cancer. Obesity is associated with higher leptin level and lower adiponectin level. It is suggested that adipose tissue in tumor microenvironment is also a rich source of pro-inflammatory mediators, stimulating tumor growth, and is more relevant than total body fatness to local tumor development.

Microbiome

Emerging studies suggest that microbiome also play a role in obesity and vice versa. The microbiome is microbes such as bacteria, bacteriophage, fungi, protozoa and viruses that live inside and on the human body. Growing evidence indicates that microbiomes in guts predispose individuals to obesity, and obesity also changes the gut microbiota, creating environment prone to

carcinogenesis. Studies showed that microbiomes were related to proinflammatory and immunosuppressive responses. It has been hypothesized that the microbiome in gut affects multiple pathways by which obesity acts on cancer.

Burden of Obesity on Cancer

Assuming that obesity causes cancer, it is estimated that approximately 4% of all new cancers in the world in 2012 was due to high BMI ($\geq$25 kg/m^2). The cancer burden attributable to obesity varied by cancer site in men and women. The population attributable fraction that is a contribution of obesity to a cancer is approximately 30% for esophageal, 10% for colon, 8% for pancreatic, and 18% for kidney cancer in men and 30% for endometrial, 10% for postmenopausal breast, 8% for colon, 8% for pancreatic, and 25% for kidney cancer in women. Also, approximately 10%–13% of cancers in liver, gallbladder, and gastric cardia and 7%–9% of multiple myeloma were attributable to high BMI in both men and women. It is estimated that the global increase in obesity prevalence since 1980s contributed to 25%–32% of obesity-related cancers in 2012.

Obesity also attributed to 120.1 million disability-adjusted life-years in 2015, which made the obesity one of the largest contributors to global disability-adjusted life-years. The cancer burden, including the disability-adjusted life-years, due to obesity is likely to increase if the obesity prevalence continues to rise worldwide.

Future Directions

Although convincing evidence indicates that obesity increases risk of numerous cancers, several challenges remain to advance the understanding of body fatness and weight management in the cancer control continuum.

Accumulating evidence suggests that body fatness in adolescence or early adulthood is associated with increased risk of several cancers at later life. People who experienced excessive weight gain at early life are more likely to keep their excess weight or gain even more weight throughout adulthood. Therefore, body fatness in early life could be an indicator of life-long exposure to obesity. Even though we do not know the biologically relevant time period for obesity to cause cancer, considering a long latency period of cancer, obesity in early life may play a role in carcinogenesis during adulthood, leading to cancer diagnosis at later life. However, there are gaps in the understanding of the effect of obesity at different life course and duration of obesity on cancer risk. In addition, whether intended weight loss at mid-life lowers cancer risk in later life is less clear. More studies are needed to investigate the time course of weight management-either gain or loss- in a life course and its effect on cancer development as well as cancer survival after diagnosis.

The role of obesity in cancer survival is the emerging area of interest. The inconsistencies in the current literature on the effect of obesity on survival among cancer patients indicate the need for a comprehensive examination of body composition, including both body fat mass and muscle mass, in relation to cancer survival. However, there are no practical and standardized methods to measure body composition in large-scale observational studies. A practical and economical method to assess body composition is needed to close a significant gap in understanding of the role of body fatness in cancer survival. Future research on the effect of body fatness and body composition after cancer on patients' overall health and well-being is warranted.

With increasing recognition of tumor heterogeneities in cancer, more and more cancers are reclassified into subtypes according to their molecular characteristics. It is hypothesized that the etiology of one molecular subtype of cancer differs from other subtypes. Further research to understand the role of obesity, including its biological pathways, in the etiology of each molecular subtype of cancer may contribute to find more effective cancer prevention strategies, including development of chemopreventive agents and targets for cancer treatment.

Given the increasing trend in obesity prevalence worldwide, the burden of obesity on cancer is likely to increase continuously. As obesity is a preventable and modifiable risk factor for cancer, obesity prevention and management can contribute significantly to cancer prevention. More efforts are needed to develop multilevel but personalized and sustainable approaches for a healthy lifestyle that maintains healthy weight and prevents weight gain throughout individual's life-course.

See also: Breast Cancer: Pathology and Genetics. Cancer-Related Inflammation in Tumor Progression. Endometrial Cancer: Pathology and Genetics. Esophageal Cancer: Pathology and Genetics. Hormones and Cancer. Prevention and Control: Nutrition, Obesity, and Metabolism.

Further Reading

Arnold, M., Pandeya, N., Byrnes, G., et al., 2015. Global burden of cancer attributable to high body-mass index in 2012: A population-based study. Lancet Oncology 16 (1), 36–46.

Committee on Accelerating Progress in Obesity Prevention, 2012. Accelerating progress in obesity prevention: Solving the weight of the nation. National Academies Press (US), Washington, DC.

Iyengar, N.M., Gucalp, A., Dannenberg, A.J., Hudis, C.A., 2016. Obesity and cancer mechanisms: Tumor microenvironment and inflammation. Journal of Clinical Oncology 34 (35), 4270–4276.

Khandekar, M.J., Cohen, P., Spiegelman, B.M., 2011. Molecular mechanisms of cancer development in obesity. Nature Reviews Cancer 11 (12), 886–895.

Keum, N., Greenwood, D.C., Lee, D.H., et al., 2015. Adult weight gain and adiposity-related cancers: A dose–response meta-analysis of prospective observational studies. Journal of the National Cancer Institute 107 (2), djv088.
Kyrgiou, M., Kalliala, I., Markozannes, G., et al., 2017. Adiposity and cancer at major anatomical sites: Umbrella review of the literature. BMJ 356, j477.
Lauby-Secretan, B., Scoccianti, C., Loomis, D., et al., 2016. Body fatness and Cancer—Viewpoint of the IARC Working Group. New England Journal of Medicine 375 (8), 794–798.
NCD Risk Factor Collaboration, 2017. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: A pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. Lancet 390 (10113), 2627–2642.
Park, Y., Peterson, L.L., Colditz, G.A., 2018. The plausibility of obesity paradox in cancer. Cancer Research 78 (8), 1898–1903.
Renehan, A.G., Zwahlen, M., Egger, M., 2015. Adiposity and cancer risk: New mechanistic insights from epidemiology. Nature Reviews Cancer 15 (8), 484–498.
Schauer, D.P., Feigelson, H.S., Koebnick, C., Caan, B., Weinmann, S., Leonard, A.C., Powers, J.D., Yenumula, P.R., Arterburn, D.E., 2017. Bariatric surgery and the risk of cancer in a large multisite cohort. Annals of Surgery (Epub ahead of print).

Relevant Website

https://www.wcrf.org/int/research-we-fund/continuous-update-project-findings-reports—World Cancer Research Fund Continuous Update Project findings and reports.

Oncology Imaging

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Advances in Technology

Computed Tomography

Computed tomography (CT) continues to be the workhorse in oncology imaging, since it is fast, extremely reliable, and can be performed in patients who are severely ill, suffer from pain, require monitoring of vital functions, or even mechanical ventilation. Following the first publication of the principle in 1990 (Kalender et al., 1990), spiral CT was rapidly introduced, followed by the implementation of multirow detectors. In contemporary scanners, the beam geometry is cone-shaped to cover an array with 64 to >300 detector rows. The rotation time of the tube now lies below 1 s, in some scanners at 250 ms. As a result, large ranges can be scanned in few seconds time, and thin slices (<1 mm thickness) can be obtained without significantly increasing the radiation exposure to the patient. Neighboring primary slices will be merged to generate slices in the 3–5 mm range, which are less degraded by image noise than the primary thin slices and are required for assessing soft tissue. The primary image stack is almost isotropic, that is, the voxels (volume elements) have similar diameters in all three dimensions. Therefore, sagittal or coronal images can be reconstructed from the primary data stack.

The speed of acquisition permits to obtain images during a defined phase of contrast enhancement following intravenous injection. Dynamic scanning to quantitatively assess the microcirculation is also possible but used less often, because repeated scans will cause significant radiation doses.

Radiation dose is an increasing issue even in oncology, since more than half of patients with cancer can be expected to become long-term survivors. CT now offers numerous options for dose reduction:

Dose modulation: The tube current needed to avoid significant image degradation by noise depends on the radiation absorption by the tissue in the gantry. The absorption is typically low for the neck (because it is slim) or the mid-chest (containing the lungs) and high for the shoulder region or the abdomen. For dose modulation along the body axis (in z direction), the image noise is measured in real time, and the tube current is adjusted in order to keep the noise in a target range. This can be combined with dose modulation during tube rotation, where the tube current is changed as the tube rotates around the patient. The rationale for this is that in a given area the absorption will depend on the direction of the beam in the transverse plane. For the shoulder region, for example, it will be lower for a sagittal than for a transversal beam direction, where the shoulders, clavicles, and vertebral bodies lie in one line and will all be passed by the same beam. Obviously, the tube current will not be based on image noise (because the image does not yet exist) but rather on the beam intensity directly at the detector.

Adjustment of tube voltage and dual-energy CT: The tube voltage determines the maximal beam energy and is around 120 kV in standard diagnostic protocols. However, the optimal voltage is always a tradeoff: The contrast between either bone (that contains calcium) or iodine-based contrast media and soft tissue will increase if the voltage is lowered to 80 kV or even 70 kV. At the same time the radiation dose will decrease significantly, as long as the tube current remains unchanged, and the image noise will increase. In body regions with little radiation absorption, the increase in image noise will be more than compensated by the gain in contrast, resulting in an increase in the contrast-to-noise ratio. Low-kV protocols can be used for angiographic protocols in the head, neck, chest, or abdomen, but also for nodule detection in the lungs. For abdominal CT, however, where the contrast resolution is at a premium, and the X-ray absorption is about the highest in the body, low-kV protocols still play less a role, except for children and very lean adults.

Postacquisition data processing: Users of darkroom software for private digital pictures know how image noise can to some degree be reduced without degrading contrast or resolution, and the same is possible for medical images. Nevertheless, the iterative reconstruction algorithms used on CT platforms do more than that. If applied to image data, they are a way of image postprocessing, but advanced algorithms are applied to raw data and are therefore an entirely different way of reconstructing images than conventional, filtered back-projection. They are powerful tools that should be used carefully, and can compensate for the increase in image noise caused by lowering tube current or voltage (**Fig. 1**). Many radiologists are still reluctant using them, because iteratively reconstructed images often have an unnatural, plastic-like appearance. Nevertheless, it will have to be learned that image aesthetics are at least not the sole criteria to wisely choose the scanning technique. This unconsidered, raw data processing also permits to reduce artifacts due to metal implants or highly concentrated contrast media, for example, in the subclavian or anonymous vein.

Dual-energy CT (DECT) is not a new development, but only in the past 10 years has it become routinely applicable. It consists of scanning the same slice with different tube voltages, fully or almost simultaneously. The way of accomplishing this differs by manufacturer—two tubes arranged in a 90 degree angle, rapid switching between voltages in a single tube, or separation of the components of the energy spectrum in different layers of the detector ring. The rationale is that tissues or substances in the body (typically soft tissue, fat, and either calcium or contrast medium) not only differ by X-ray absorption (which is the classical principle behind X-ray or CT imaging), but that they are different with regard to which part of the X-ray spectrum is more strongly absorbed. If the same region is passed by beams of different energy, or if two regions of the energy spectrum are retrospectively extracted, the contribution of two substances to the absorption may be calculated. This principle has been used since decades for dual-energy X-ray absorptiometry (DEXA), a method for measuring bone density. Here, the dual-energy principle was used to extract the contribution of bone and nonbone to the X-ray absorption. Since for DEXA, only a sagittal beam was used to scan the lumbar spine, the X-ray absorption

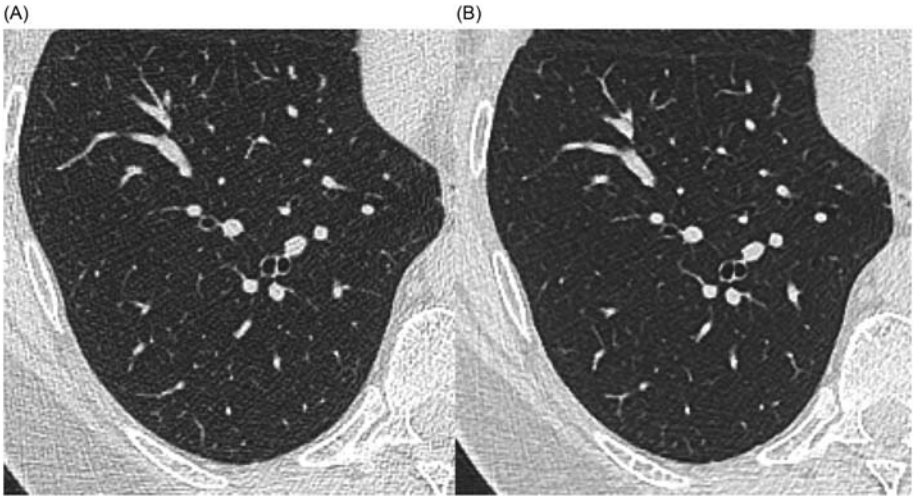

Fig. 1 CT of the lung reconstructed with standard filtered back projection (A) and raw data-based iterative reconstruction (B). Note the reduced noise artifacts on the iteratively reconstructed images. Iterative reconstruction can be used either to generate images with less noise, or to lower the tube current (and thereby the radiation dose to the patient), ending up with the same contrast-to-noise ratio as it would be with standard filtered back projection in combination with standard tube current.

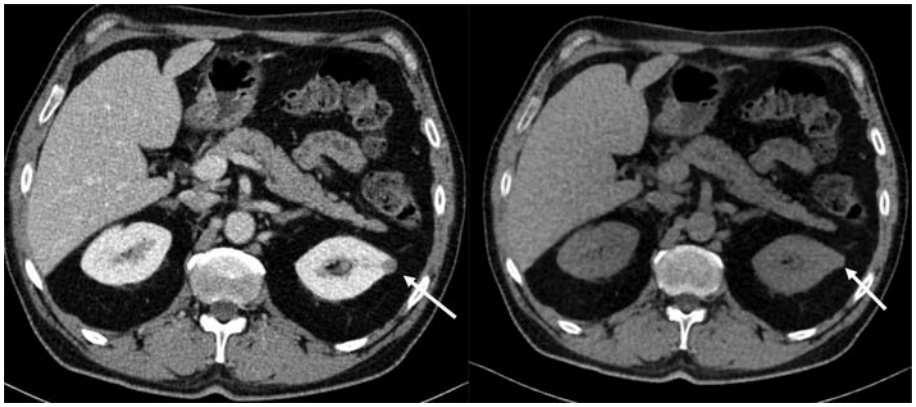

Fig. 2 Dual energy CT: CT of the kidneys with i.v. contrast medium (A) shows a small, hypodense lesion in the left kidney (*arrow*). Whether or not there is any contrast uptake in that lesion is crucial for discriminating a neoplasm from for example, a hemorrhagic cyst. Virtual native contrast (VNC) images (B) are retrospectively calculated from the simultaneously acquired Data obtained with different tube voltages, to exclude the attenuation caused by iodine. In this example, the density values on postcontrast and VNC images are identical, so that the suspicion for malignancy is low. Ultrasound confirmed a cyst.

by soft tissue and fat would otherwise have made bone density measurements impossible. In its first years, DECT has been used mainly for enhancing CT angiography, and only recently for oncological questions, particularly for assessing tissues such as bone marrow, where calcium, fat, and soft tissue are present in one voxel and cannot be resolved in space. A very practical, every-day application is calculating virtual native scans (Fig. 2). This means that only a postcontrast scan is obtained, and the dual-energy data are used to remove the attenuation caused by iodine. This obviates separate unenhanced scans and contributes to reducing the radiation dose. The logical continuation of DECT will be spectral CT, where each photon is measured for its energy. Today, only prototypes exist, and where the future clinical role of spectral CT is difficult to foresee.

Magnetic Resonance Imaging

Higher field strengths

For long, the field strengths were in the range from 0.2 T (particularly with open permanent magnets) to 1.5 T (closed superconducting magnets), with 1.5 T being most frequently used for oncological applications. 3 T magnets are now widely being used, mainly in hospitals or large outpatient facilities that operate them together with 1.5 T scanners. The advantages of 3 T over 1.5 T lies in a higher signal-to-noise ratio and a better quality of morphological images, which is of particular value in studies of the brain and spinal cord as well as in the pelvis. For prostate imaging at 1.5 T, for example, endorectal coils were used in the past to achieve

sufficient image quality, which made it an unpleasant procedure that was not happily accepted by patients. Since the introduction of 3 T scanners (and also fundamental improvements with 1.5 T ones), endorectal coils could be abandoned, which strongly increased the acceptance of the method. Nevertheless, increasing the field strength comes at a price. First, the forces to ferromagnetic implants are higher, and safety is even more an issue. Second, the radiofrequency that is transmitted by the body coil is 128 MHz instead of 64 MHz, and the heat deposition is higher than with 1.5 T, so sequence protocols need to be adapted not to exceed the specific absorption rate (SAR) limit. For both reasons, there are increasing compatibility issues with any kind of implants, be they stents, ports, shunts, bony implants with metal rods, and of course active medicinal products like insulin pumps or cochlea implants. Therefore, patients are expected to provide information about any implants already when making their appointment, so the clarification of compatibility issues (including inquiries, web searches, etc.) can be done beforehand.

Beyond safety issues, high field strengths may indeed degrade image quality because susceptibility artifacts are more pronounced than with lower field strengths. They will arise either in presence of metal or blood degradation products (where they may indeed improve the detection of small hemorrhages) or at tissue–air interfaces. Here, magnetic field inhomogeneities, if not compensated by "shimming" (geometrically adjustment of the magnetic field) the scanner, may, for example, cause difficulties in spectral fat suppression. Particularly in the lower neck and upper thoracic aperture such problems are common, because the geometry of the tissue–air interface (the skin) is so complex that it cannot be compensated by the shimming procedure, which is always rigid in all space directions.

Ultra-high field (UHF) MRI scanners (with e.g., 7 T or more) are so far only experimental. Technical challenges are paramount, some extrapolated from those with 3 T, some entirely new. For the brain, the results may nevertheless be stunning, but only as a result of meticulous refinements of the applied sequences. The potential of UHF MRI lies, for example, in the exploitation of susceptibility effects, imaging nonhydrogen nuclei, chemical characterization of tissue, all not yet ready for routine use. For the body trunk, the protocols are still in their infancy.

Whole-body MRI

Classically, MRI was designed for examining regions of the body, covering a field of view along the body axis (z direction) of about 40–50 cm. Modern scanners now come with an array of receive coils (the radiofrequency is transmitted by the "body coil" that is built into the scanner itself) that can cover the body all the way from the vertex to the feet. With this arrangement, the body can be scanned entirely, usually adding multiple coach positions to another and then composing for example, coronal or sagittal images to display the entire body (**Fig. 3**). Thanks to rapid MR sequences, this can be achieved in 30–45 min time, depending on which image contrasts are needed, and on whether i.v. contrast agents will be used. Obviously, this is a valuable tool for whole-body staging of metastatic disease or hematological neoplasms. Nevertheless, the images are complex, and the workload for the radiologist reading the entire body scan with several weightings and scan directions without missing relevant findings is much higher than with whole-body CT. Without a systematic workflow, relevant findings may easily be missed.

Functional imaging techniques

Dynamic contrast-enhanced MRI (DCE MRI) implies repetitive T1-weighted 3D image stacks being acquired over the same region with high temporal resolution before, during and after pump-controlled infusion of a gadolinium-based contrast agent, in order to assess tissue microcirculation. Using pharmacokinetic models, parameters can be extracted that reflect the relative blood volume in tissue, and the exchange rate of the contrast agent between the central and peripheral compartment, the latter consisting of both the terminal capillary intravascular as well as the extravascular space. The exchange rate is determined by both capillary perfusion and trans-capillary substrate exchange. DCE MRI is most frequently used for MR mammography and prostate imaging for discriminating benign from malignant lesions. Nevertheless, since spatial resolution is at a premium in both instances, the temporal resolution will not permit pharmacokinetic calculations. Therefore, the protocol may be termed multiphasic rather than dynamic, and instead of parameters, the shape of the time-intensity curve (e.g., continuously ascending, early peak followed by either a washout or a plateau) will be visually assessed. DCE MRI must be discriminated from dynamic susceptibility contrast MRI (DSC MRI). Here, a sharp contrast bolus is given, and T2*-weighted images are dynamically acquired to measure a drop in signal intensity which is induced by the presence of gadolinium inside and its absence outside the microvessels. Using indicator dilution models, the tissue perfusion can be calculated directly, provided there is no extravasation of the contrast agent. DCS MRI is standard of care for stroke imaging but can also be used for example, low-grade gliomas, where the blood–brain barrier is not disturbed.

With diffusion-weighted MR imaging (DWI), a series of magnetic gradients is applied to induce a signal loss in areas where extracellular water molecules are free to move randomly. The strength and duration of the gradients determine (expressed by the so-called B-value) the degree of diffusion-weighting. Since images are basically T2-weighted, the signal intensity of tissue depends on both its T2-signal intensity and diffusion restriction. Based on two or more signal intensities obtained with different B-values, the apparent diffusion coefficient (ADC) can be calculated, often represented in ADC maps generated from images obtained with different B-values (**Fig. 4**). More advanced techniques will account for the complexity of the interstitial space (e.g., diffusion kurtosis imaging) or the differential contribution of tissue perfusion and water diffusion to the signal (using, e.g., the intravoxel incoherent motion (IVIM) model; Le Bihan et al., 1988). Moreover, assessing the movement of water molecules separately for three dimensions may serve to assess the preferential direction of anisotropic structures, such as neural tracts.

As a rule, diffusion is restricted in tissues where cells are densely packed, as is often the case in malignant tumors (Chenevert et al., 2000). Although this is not specific, DWI has become an invaluable adjunct in oncologic imaging and can be used either to assist in the differential diagnosis of unclear lesions (e.g., in MRI of the prostate), or, in combination with a suppression of

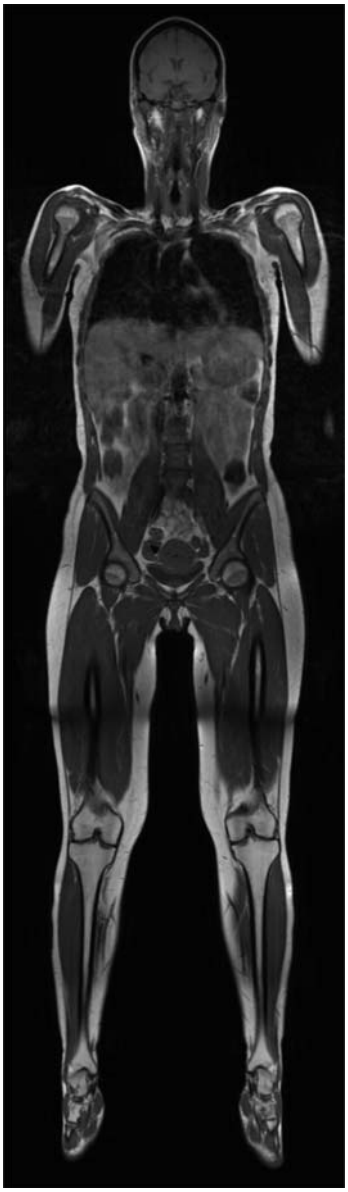

Fig. 3 Native whole-body MRI in a patient with asymptomatic multiple myeloma. Since the longitudinal field of view of clinical MRI scanners is typically 40–50 cm, image stacks are obtained stepwise, as the couch is advanced into the scanner, and then "stitched" together to generate, for example, coronal T1-weighted images as in this example. This patient has a typical, diffuse infiltration pattern with hypointensity of the bone marrow (owing to the replacement of fat by cell-dense tissue) in the vertebrae, the pelvis, the humeri and femora, sparing the epiphyses.

the background signals (from e.g., muscles or fat tissue), to generate whole-body overview images where only diffusion-restricted structures are displayed (**Figs. 9 and 12**). Such images resemble those from FDG-PET studies (sometimes they are called "poor man's PET") and are helpful to attract the reader's attention to suspicious finding, and then retrieve and further assess them on morphologic images.

Susceptibility-weighted MRI (SWI) uses sequences where signal voids arise in presences of differences in magnetic susceptibility, for example, in presence of contrast agents (in DSC MRI, see above), melanin, or blood degradation products. SWI sensitively detects microhemorrhages arising in brain metastases either spontaneously or as a result of treatment. Very typical intratumor susceptibility signals have been found in high-grade gliomas and helped to discriminate them from primary central nervous system lymphoma (Kickingereder et al., 2014).

Gadolinium chelates have been used as contrast agents in MRI since the 1980s. They are injected intravenously and are distributed in the intravascular and extracellular space (except for the brain, where they remain intravascular) and renally eliminated, like iodine-based contrast agents that are used for CT or fluoroscopy. One contrast agent (Gd-EOB-DTPA) is partly taken up by hepatocytes and excreted in the bile, and is therefore approved as a liver-specific contrast agent.

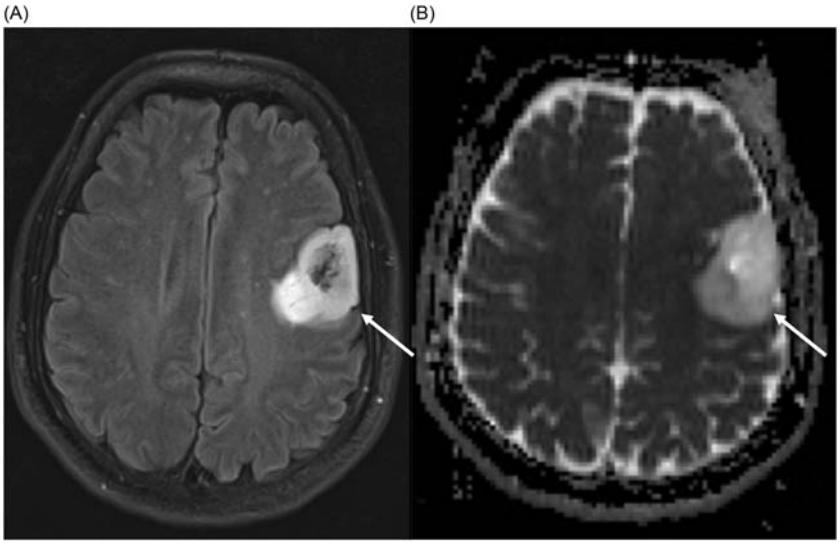

Fig. 4 Anaplastic astrocytoma in the left cerebral hemisphere, visible on a T2-weighted MR image with free water suppression (A) ("fluid-attenuated inversion recovery," FLAIR) and a parameter map showing the apparent diffusion coefficient ADC (B). The tumor is T2-hyperintense and shows increased water diffusion compared to the unaffected brain. Functional imaging techniques like diffusion-weighted imaging may assist in both differential diagnosis of suspicious tumors and in the delineation of the gross tumor volume for radiotherapy planning.

Gadolinium chelates are generally well tolerated, but risks are associated with free gadolinium being liberated from the chelator. In the 1990s, it became obvious that scleroderma-like skin changes (nephrogenic systemic fibrosis, NSF) occurred in patients with renal insufficiency who had received high or repeated doses of gadolinium chelates. Thanks to clear guidelines on the use and exclusion criteria for MRI contrast agents, hardly any NSF cases have occurred since. Only recently, however, gadolinium deposits were found in the dentate nucleus of the brain, visible as hyperintensity on T1-weighted, native images, and also proven in post mortem specimens, and have also to be expected elsewhere. There has been a clear association with both the cumulative amount of contrast agent given and also the type of chelators that have been used. Gadolinium is mainly released from linear chelators, whereas macrocyclic chelates are obviously so stable that the amount of liberated gadolinium is negligible. There is a clear move to using macrocyclic agents only, although no clinical sequelae of CNS deposits are known so far—and will be difficult to prove. The consequences for manufacturers may be serious, since some linear contrast agents that have been very successful until now will probably disappear from the market, except for the liver-specific Gd-EOB-DTPA, for which there is currently no macrocyclic alternative.

This in mind, there is continuing interest in techniques for tissue characterization, some being in use since long (e.g., spectroscopy, blood oxygen level-dependent (BOLD) neurofunctional MRI), others newly developed (MRI with hyperpolarized oxygen, chemical exchange saturation transfer (CEST) imaging). Mostly, they still require either specialized equipment such as ultra-high field scanners or an intense support by basic scientists or physicists, but some may evolve to become part of clinical protocols in future, as it has happened with diffusion-weighted imaging.

Ultrasound

Ultrasound (US) is one of the most underestimated imaging techniques, for numerous reasons, the most important ones being that it requires much skill and dexterity to be carried out, and that it will usually not yield volume-covering image stacks that can easily be reviewed by a second reader. It is sad to say that its being applied by numerous insufficiently educated users has damaged its reputation, triggering further, potentially costly and invasive measures. In the hand of a skilled and trained examiner, ultrasound is a powerful, versatile and fast imaging tool that is capable of solving many issues or at least guide further imaging studies. In the near field, with high frequencies, its spatial resolution is unsurpassed. Its contrast and resolution have been constantly improved over years, a kind of a "silent revolution".

The most striking development in the past years has been the introduction of ultrasound contrast media, along with specific contrast-specific imaging techniques. They consist of gas-filled microbubbles 1–10 μm in size with a soft shell that are suspended in water and injected intravenously. The most frequently used compound contains sulfur hexafluoride with a soft phospholipid shell. Originally, they were developed as signal enhancer for Doppler applications, but along with the official approval that became unusually protracted, the spectral and color Doppler techniques improved so substantially that the substances became almost obsolete for the originally intended use. However, the physics were deeply investigated, to reveal three different mechanisms of interaction between ultrasound waves and microbubbles, depending on the acoustic pressure: At very low intensities, the microbubbles act as simple scatterers and increase the echo intensity of the blood—as originally intended. At high intensities (i.e., those usually applied for B-mode and Doppler ultrasound), the positive and negative pressure forces cause the bubbles to rupture and emit

an acoustic signal, an event that is termed a "stimulated acoustic emission" (SAE). An SAE can be detected by the ultrasound probe and will be encoded either in a B-mode or in a color Doppler image. Thus, SAEs are a way to generate contrast-specific images, because their intensity is comparably high, but since they accompany the bubble's death, they are very short-lived. SAE imaging cannot be performed as a real-time examination, as would be desirable. At low intensities (about 10% of the usual output power), an interesting interaction occurs: Microbubbles have an own resonance frequency at which they expand and contract, dependent on their diameter. In case of resonance between transmitted ultrasound pulse and microbubble, this will vibrate at that frequency. As a happy coincidence, this is the case for microbubbles between 1 and 10 μm in diameter (i.e., that easily flow in the blood stream) and ultrasound in the usual diagnostic frequency range (between 1 and 20 MHz). The vibration is too low in amplitude to emit a signal, but the backscattered echo of the bubble will be distorted in shape when compared to the transmitted signal. In other terms, it will contain higher-frequency components, that is, second or higher harmonics. This is a peculiarity that not shared by backscatter from tissue and is now being used to separate echoes from microbubbles and tissue, using either high-pass frequency filters, sequences of inverted pulses, or both, and generate images that show the pattern of contrast enhancement. Although low-MI imaging (MI = mechanical index) does go along with some bubble destruction, enough bubbles will survive to enable real-time scanning for about 5 min.

Other than gadolinium- or iodine-based contrast media used for MRI or CT, ultrasound contrast agents remain strictly intravascular. Once the bubbles perish, the phospholipids are metabolized, and the gas is exhaled via the lungs. The absolute quantity of gas is in the range of few micro liter. Ultrasound contrast media are extremely well tolerated; allergic reactions may occur but are exceedingly rare, and so are nonallergic toxicities. They are contraindicated in advanced cardiac failure because fatalities had been observed in this context, but in absence of a plausible pathomechanism it can be assumed that these were coincident and caused by the disease itself rather than due to the compound.

The main application of contrast-enhanced ultrasound (CEUS) is to work up unclear liver lesions, where it has been shown to be diagnostically equivalent to CT or MRI (**Fig. 7**). Sonographic access provided, CEUS is the most sensitive method to detect even weak perfusion in tissue in anybody region.

Ultrasound is an exquisite method to guide a biopsy needle or an ablation device in real time and verify its correct placement, without exposing the physician to ionizing radiation as would be the case with fluoroscopic and sometimes CT guidance. It permits to directly visualize the progress of thermal ablation, and CEUS can be used to examine whether viable parts of the target have remained. If a lesion that was detected with CT or MRI is not visible in ultrasound, because it is for example, isoechogenic, the relevant image stack from the corresponding modality can be matched, using anatomical landmarks and an electronic positioning system attached to the transducer. The device is then guided via a split screen or an image overlay showing both the ultrasound and the CT or MRI images reconstructed in identical planes. Such systems are available either as additional equipment or readily implemented in high-end ultrasound machines. Such fusion-guided procedures are widely performed for example, liver or prostate lesions.

Current ultrasound research is focused on, for example, elastography, motion registration, or molecular imaging. Tissue elasticity can be assessed by analyzing the deformation of tissue upon movement of the transducer. More advanced techniques use a "push" pulse that causes tissue deformation and shear waves, both of which can be analyzed using ultra-fast techniques. Elastography may assist in detecting firm lesions that are isoechogenic to their surroundings, or in the differential diagnosis of otherwise unclear lesions, and is being used clinically for example, for assessing thyroid nodules or for detecting breast lesions. Current research is directed at using ultrasound for motion detection in the context of image-guided radiotherapy (IGRT). Ultrasound also offers possibilities for molecular imaging. Microbubbles can, for example, be attached to specific ligands or antibodies. The signal from stimulated acoustic emissions is so strong that every single bubble can be detected. However, such applications are limited to intravascular targets. Another approach is to make use of Bell's effect: Short laser pulses directed into the tissue will induce heat-induced, pulsatile tissue expansions and thereby the emission of an acoustic signal. Depending on whether monochromatic or spectral lasers are used, such optoacoustic imaging scan can be tailored to targets with known absorption peaks (e.g., melanin, oxygenated or de'oxygenated hemoglobin) or even dyes that are attached to specific ligands. Ultrasound for IGRT or molecular ultrasound imaging are still in their preclinical phase of development.

Positron Emission Tomography

The most important developments in positron emission tomography (PET) have been improvements of detector technology, the introduction of hybrid scanners and the development of specific tracers.

The principle of PET lies in the detection of two 511 keV photons emitted in opposing directions during an annihilation event from a collision of a positron (an antimatter particle emitted from isotopes like 18-fluorine, 11-carbon, or 68-gallium) and an electron. Positron-emitting isotopes are either chemically bound or chelated with substrates and are so used to map biochemical processes or specific markers in tissue. The main component of a PET scanner consists of a ring detector array that form its gantry. Whenever two coincident photons are registered, their source will be assumed to lie anywhere on the "line of response" connecting the two detector crystals. In modern "time of flight" scanners, a time lag between the two signals can be discriminated to better locate the annihilation event on the line of response and improve the spatial resolution. To quantify the tracer distribution in a 3D dataset, the raw data have to undergo a correction for attenuation and scatter. Attenuation correction is based on a transmission scan that would originally be acquired using a germanium source rotating around the patient.

Today, almost all PET cameras are part of PET/CT hybrid scanners. Only recently, PET/MR combinations are on the market, but only few of them have been installed so far. In the simplest case, the CT component will be used to obtain a low-dose transmission scan as the basis for the attenuation correction and as a map to locate the tracer distribution anatomically. However, the combination has the potential to fully exploit the diagnostic capabilities of both PET and contrast-enhanced multidetector spiral CT. The CT and PET scan are obtained after each other, and anatomical mismatches may be inevitable close to the diaphragm, in the abdominal viscera (in case of vivid peristalsis), or may occur in case of patient movements. Since the PET measurements take several minutes per bed position, the corresponding CT data cannot be acquired in a breathhold as would be done in normal CT examinations.

For PET/MR combinations, there are different solutions: (1) Shuttle systems with a PET/CT and an MRI scanners, with a couch system that can be docked to both machines, (2) two separate scanners (one MRI scanner and one PET camera) placed opposed to each other and connected by a common, rotatable couch table, (3) a PET detector insert inside a standard MRI gantry, and (4) a fully integrated, dedicated PET/MR hybrid machine. Besides technical challenges associated with such a construction (replacement of photomultipliers by photodiodes that do not interfere with the influences from the MRI scanner), specific issues need to be solved, since an MRI scan will obviously not contain radiodensity data as they would be needed for attenuation correction. Air and compact bone, for example, are both signal void on MRI scans. Therefore, one or several MRI sequences are used to create an attenuation map, using prior knowledge and empirical radiodensities. Furthermore, additional objects in the gantry like radiofrequency coils or ear protectors will not be contained in the obtained images but have to be accounted for. The developments in this regard are still ongoing.

Despite these challenges, PET/MR promises various improvements that are currently being explored. For various anatomical regions, MRI, not CT, is the morphologic imaging modality of choice—for example, the central nervous system, the pelvis, or parts of the musculoskeletal system—and is therefore preferable as an anatomic reference for the PET data. Fully integrated PET/MR scanners offer the possibility to measure PET and MRI data simultaneously and thereby perform a motion correction. This will improve both the anatomical location of the tracer uptake and also its quantification. Finally, PET/MR combines two highly versatile functional imaging modalities, which to compare is of high scientific interest.

The most widely used and best evaluated tracer is 18-fluorine-labeled deoxyglucose (FDG), a glucose analogue that is being used for staging and therapy monitoring of many tumors like lung cancer, lymphoma, malignant melanoma, multiple myeloma (**Fig. 13**), and others. Limitations arise from a high FDG uptake in for example, inflammatory lesions or healthy brain (which limits the value of FDG for imaging brain tumors) as well as a low FDG avidity in some common (prostate cancer) or less common (e.g., neuroendocrine) tumors. However, the past years have seen the development of additional tracers that are meanwhile being used clinically. The most important to mention are 68-Ga-DOTA-TOC (a somatostatin analogue taken up by neuroendocrine tumors or meningiomas), radiolabeled PSMA (prostate-specific membrane antigen) ligands for prostate carcinomas and its metastases, or amino acids for brain tumors. In addition to 11-C-methionin, 18-F-ethyl-tyrosine is now increasingly being used. In patient with advanced metastatic disease, DOTA-TOC or PSMA PET can serve to estimate whether sufficient dose may be achieved by radionuclide therapy, using the same substances as carriers.

Medical Image Computing

The era of films and lightboxes is definitely past. Even in private offices, images are stored and viewed digitally, and can be transferred to third parties for reading or consultation. There exist companies that have a team of radiologists on their payroll, whose task it is to read imaging studies performed anywhere on the globe. The availability of digital imaging data has triggered a plethora of applications designed to assist the reader, facilitate image handling and storage; some even promise to take over the radiologist's intellectual task, that is, to interpret the studies. Whether or not this may happen is mere speculation. Among numerous applications for analyzing, viewing and demonstrating images, two have immediate impact on imaging for cancer, automatic lung nodule detection and tools for response evaluation.

On plain X-ray films, even experienced readers will only detect nodules 1 cm in diameter or larger, and still miss many, because of overlying structures like ribs, vessels, etc. Therefore, CT is the method of choice for detecting lung nodules. Still, the presence of cross-sectioned vessels makes reading it a challenging task. One way of improving the conspicuity of lung nodules is to obtain thin primary slices (1 mm) and then create maximum intensity projections (MIP) in craniocaudal direction out image stacks of, for example, 10 primary slices. That way, vessel cross-sections will retain their strand-like or oval shape, whereas nodules will stand out as spherical structures.

Software solutions exist that analyze thin-section CT series of the lungs to find nodules in the lung and discriminate them from vessels. Some are implemented on the platforms of CT scanners and can be configured to add annotated images and transfer them to the PACS. Others will run on dedicated standalone workstations. Meanwhile, such software is reliable but will also yield false positive findings. So the user will have to cross-check the search results. Important to realize is that a nodule has to be surrounded by ventilated lung in order to be detected. Nodules with contact to the pleura, wedge-shaped sessile densities in the subpleural lung or masses with contact to the mediastinum will not be automatically detected, nor will be hilar masses.

Response evaluation

Clinical trials in oncology beyond phase I will often require an objective assessment of the tumor burden, usually based on imaging studies. Various evaluation criteria are being used, depending on the type of tumor and treatment, the most common ones being the Response Evaluation Criteria In Solid Tumors (RECIST), the International Working Group criteria for lymphomas, and the RANO

(Response Assessment in Neuro-Oncology) criteria for brain tumors. For immunotherapy, criteria have been adapted to better account for pseudoprogression or delayed response as may be encountered with this type of treatment. Apart from the RANO criteria, a response assessment in metastatic disease implies a heavy workload and requires a meticulous documentation to ensure that the index lesions that are followed are indeed identical. Originally, spreadsheets were used, but turned out to be impractical and error-prone. Software solutions that assist in this task are of invaluable help in any imaging facility involved in clinical trials. Such programs will use a dedicated, PACS-like user interface, provide a database for all measurements, measurement tools for diameters, areas, volumes, and suggest a timepoint response according to guideline's criteria. Additionally, they provide tools for measuring standardized uptake values in PET studies to support the response assessment.

It has to be said clearly that response criteria have been defined to support clinical trials, and so have been all related software tools. Neither were intended to serve as a basis for clinical decisions in routine oncology, and indeed they are too rigid for this purpose. Nevertheless, they are sometimes being used accompanying (rather than influencing) clinical decisions, as part of a quality assurance routine.

Imaging for Treatment Planning and Guidance

Both radiotherapy and percutaneous interventions use imaging for planning and guidance. For radiotherapy, the gross tumor volume (GTV) as well as the organs at risk will be defined using the imaging method and windowing on which the tumor is best to discriminate from its uninvolved surroundings, most often CT. Its advantage is that it is unaffected by spatial distortion that may accompany MRI measurements, but for MRI sequences are as well available that are corrected for spatial distortion. Image registration may be used to overlay CT, MRI, or PET images and improve the GTV delineation. PET in particular is a valuable adjunct to assess the presence of tumor in an otherwise unclear structure (i.e., a lymph node level), but for precise delineation of the GTV it should be used with caution, because the apparent borders between normal and abnormal can be strongly influenced by windowing.

Besides uncertainties regarding the true tumor extent and positioning (accounted for by the clinical and planning target volume), inter- and intra-fractional motion can cause underdosage to the tumor and overdosage to normal tissue. While issue of intra-fractional motion is still far from having been solved, movement or variations in positioning between treatment sessions can be detected by daily imaging in treatment position, using either an in-room CT or a cone-beam CT implemented in the linear accelerator, and corrected by for example, repositioning or alternating treatment plans (image-guided radiotherapy, IGRT). Hybrid installations of MRI scanners and linear accelerators, where image guidance would be feasible based on MR images are about to be installed, so that hardly any experiences exist in this regard. Nevertheless, MR guidance has been shown possible based on scans obtained in a standalone scanner, using a slim, custom-developed fixation device with integrated, MR-visible, stereotactic markers (Bostel et al., 2018).

Apart from endovascular interventions (e.g., chemoembolization), percutaneous biopsies or tumor ablations (using radiofrequency, microwaves, cryotherapy, or alcohol) are usually performed under ultrasound or CT guidance, depending on which method best shows the target and is best suited for its location—and is best mastered by the physician. MRI-guided interventions are only rarely performed, and if at all only in open scanners. For lesions that are best seen on MR images, image fusion is the best option. This has successfully been used for example, patients with elevated serum PSA and previous negative biopsies. Here, MRI including DWI and DCE MRI was used to locate suspicious areas in difficult to reach locations, and then to biopsy them under fusion ultrasound guidance.

Imaging the Primary Tumor

The choice of imaging techniques depends on the anatomical site to be imaged, available technology, and local expertise. Following the EU directive 97/43/EURATOM, several European countries, among them the United Kingdom and Germany, have published referral criteria (The Royal College of Radiologists, 2012; Strahlenschutzkommission, 2010) for imaging studies that have meanwhile undergone several revisions. Originally, they served radiation protection purposes, but have in the meantime evolved into up-to-date guidelines that are both scientifically founded and pragmatic in the context of availability and cost-effectiveness. Many of the recommendations in this article will follow the British and German referral criteria as well as the current oncological guidelines, for example, available from the German Arbeitsgemeinschaft Wissenschaftlicher und Medizinischer Fachgesellschaften (AWMF, www.awmf.org).

CNS Tumors

The annual mortality from primary CNS tumors is declining since the mid-1990s and in 2012 it was 2.89/100,000 (mortality) for women, and 4.43/100,000 for men (Becker and Wahrendorf, 1998). The treatment is multimodal and includes resection (if possible), radio- and chemotherapy as well as antiangiogenic, targeted or immune-stimulating agents.

The primary imaging modality for both primary brain intra- and extraaxial brain tumors as well as brain metastases is clearly MRI, including postcontrast T1-weighted sequences. Compared to CT, MRI is more sensitive and permits a better delineation of the gross tumor volume for radiotherapy planning. CT may be used whenever MRI is contraindicated and in addition to MRI in

radiotherapy planning or for detecting calcifications in oligodendrogliomas. T2-weighted sequences in MRI should include fluid-attenuated inversion recovery (FLAIR) sequences, where the signal from cerebrospinal fluid is suppressed (**Fig. 4**). FLAIR images are best suited to depict perifocal edema and low-grade components with an intact blood–brain barrier that will not take up contrast medium. Supplementary MRI investigations may be diffusion-weighted sequences (**Fig. 4**), dynamic susceptibility contrast (DSC) sequences or 1H spectroscopic imaging that may assist in depicting otherwise occult anaplastic foci in low-grade tumors and guide biopsies. 1H spectroscopy has proven valuable in irradiated patients for discriminating radiation-induced alterations from recurrences. In doubtful cases, amino acid PET/CT (using, e.g., 18-F-ethyl-tyrosine) may be used to assist in the delineation of vital foci, the choice of a biopsy site, and differentiation between treatment-induced changes and recurrences. 68-Ga-DOTA-TOC PET/CT is a specialized investigation for meningiomas and may aid in discriminating them from other meningeal tumors and in planning surgery or radiotherapy. In the follow-up of treated gliomas, MRI remains the primary investigation. Here FLAIR sequences play a pivotal role in detecting recurrences, since treatment with VEGF antibodies (e.g., Bevacizumab) may mask recurrences on contrast-enhanced images (Wen et al., 2010).

Head and Neck Tumors

Tumors of the upper aerodigestive tract are typically caused by chronic alcohol and tobacco consumption in combination, human papillomavirus (HPV) being another, independent risk factor. Apart from laryngeal cancer that causes hoarseness in early stages already, head and neck cancer in other locations may cause little or nonspecific symptoms, if at all. Often, a palpable metastatic lymph node will be the first clinical sign. The aggressiveness and metastatic pattern differ widely, depending on the site of the primary tumor. Whereas oropharyngeal cancer often presents with a pattern of rather few, morphologically unambiguous lymph node metastases, carcinoma of the hypopharynx rather may cause numerous micrometastases—which limits the diagnostic accuracy of lymph node imaging. The annual mortality (excluding laryngeal cancer) in 2012 was 5.34/100,000 in men, and 1.28/100,000 in women (Becker and Wahrendorf, 1998). Treatment is multimodal and includes chemoradiotherapy alone or preoperatively, resection where feasible, as well as targeted or immune-stimulating drugs, depending on the site of the primary tumor and the TNM stage.

For all palpable abnormalities in the neck or salivary glands, ultrasound is the immediate imaging modality of choice to verify the finding, learn which anatomical structure it belongs to, and, if necessary, to plan the next diagnostic steps. Often, the findings are typically benign, and no further measures are necessary.

For pharyngeal or laryngeal tumors the role of imaging is restricted to local and regional staging, whereas the diagnosis itself is made by endoscopy and biopsy. For assessing the depth of invasion, contrast-enhanced CT is accurate and reliable. MRI is the method of choice for nasopharyngeal tumors, but not for cancer of the oro- or hypopharynx or the larynx, where motions due to breathing and swallowing often degrade the image quality. For lymph node staging, CT and ultrasound are used in combination. The discrimination between metastatic and inflammatory lymph nodes (which often accompany head and neck cancer) remains a problem with all imaging modalities. Ultrasound, using high-frequency transducers (>14 MHz) has a superb spatial resolution, compared to CT and MRI, and allows to assess the normal intranodal anatomical structures (hilum, peripheral hyperplastic follicles, vascular architecture) as well as a lymph node's shape, and should be part of every regional staging for head and neck cancer. FDG PET/CT is of clear value for lymph node staging and being used depending on local standards and resources. It is an important adjunct in assessing the treatment response after neoadjuvant treatment prior to lymph node dissection. If used in radiation therapy planning for head and neck cancer, FDG PET/CT may help to significantly reduce the target volume in the majority of patients and thereby reduce radiation-induced toxicities (Wang et al., 2015).

Thyroid Tumors

Nodular goiters are common and endemic in areas with low iodine concentration in drinking water, and most nodules are benign. In fact, the annual incidence of thyroid carcinoma in 2014 was 3.7/100,000 in men, and 8.9/100,000 in women (Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. (GEKID), 2014), whereas the mortality in 2012 was 0.35/100,000 in men and 0.29/100,000 in women (Becker and Wahrendorf, 1998), which indicates that the cure rate is in the range of 90% or higher. This results from the fact that after thyroidectomy and modified neck dissection, the standard surgical procedure, radioiodine ablation using 131-J is highly effective in eradicating not only local remnants but also distant metastatic disease of follicular and papillary thyroid carcinoma. It will not work, however, if iodine is not (primarily in anaplastic or medullary thyroid carcinoma, MTC) or no longer be taken up, as may occur by dedifferentiation. Hence, the prognosis of anaplastic or secondarily dedifferentiated carcinoma is poor. For the same reason, distantly metastatic MTC is impossible to control definitely, but apart from highly aggressive variants, MTC will progress only slowly, over decades rather than years.

The primary task in the workup of thyroid nodules is to spare as many patients as possible invasive measures without missing the relatively few ones who have thyroid carcinoma, with the diagnostic tools ultrasound, 99-m-pertechnetate scintigraphy, cytology from fine needle aspiration, accompanied by serum levels for T3, T4, TSH, and calcitonin for the rare cases of medullary thyroid carcinoma. The tumor marker thyroglobulin is used only to screen for recurrences in patients post thyroidectomy and radioiodine ablation; in all other patients, its serum levels are of no diagnostic use. As a rule, hypoechogenic and scintigraphically cold nodules more than 10 mm in diameter should undergo fine needle aspiration, be followed up with ultrasound if the biopsy is benign, and rebiopsied in case of further growth. Recently, Tc-99m-MIBI scintigraphy has been used for nodules that are cold in pertechnetate

scintigraphy, and a persisting MIBI uptake after 120 min may be an additional indicator of malignancy. Still, under which circumstances MIBI scintigraphy should be performed remains to be determined. For preoperative nodal staging, additional ultrasound is the method of choice. Computed tomography plays almost no role for locoregional staging, since iodine-containing contrast agents are contraindicated. After thyroidectomy, radioiodine scintigraphy is the primary method for restaging, in combination with ultrasound. As long as any metastases continue to take up iodine, additional imaging studies will serve for morphologic workup of suspicious scintigraphic findings. Tumors that do not or no longer take up iodine (in case of secondary dedifferentiation, anaplastic, or medullary carcinoma), contrast-enhanced CT or MRI will be used in addition to ultrasound. FDG PET/CT is reserved for secondarily dedifferentiated carcinoma or anaplastic carcinoma.

Ten percentage of thyroid cancers are medullary carcinomas. Here, contrast-enhanced CT and ultrasound of the neck and abdomen are usual methods for staging as well as follow-up for patients who continue to have elevated serum levels for calcitonin or CEA. FDG PET/CT may be used in aggressive variants of the disease; slowly progressing tumors, the majority of cases, rather take up 18-F-DOPA, and PET/CT may be used to detect distant metastases. However, PET/CT studies for medullary carcinomas should be reserved for patients in whom positive findings would result in surgical resection. In late stages, this is only rarely the case.

Lung Tumors

For no other tumor, the etiology (smoking) is as well understood as for lung cancer, although nonsmokers with lung cancer are not infrequently seen, particularly at older age. There is now increasing awareness that the second-most important cause, exposition to radon (with an average annual dose of 1 mSv, depending on the geographic region), as it is being released for example, from building materials, is far from being under control. Lung cancer is a common and fatal disease, the annual mortality in 2012 being 32.08/100,000 in men and 14.5/100,000 in women (Becker and Wahrendorf, 1998).

The treatment depends on whether the patient has small-cell (SCLC) or nonsmall-cell lung cancer (NSCLC). SCLC is considered a systemic disease, even if no metastases are detected at staging. For NSCLC, surgical resection is restricted to patients without contralateral (N3) mediastinal and more distant metastases, often preceded by neoadjuvant chemo- and immunotherapy (in some centers, ipsilateral mediastinal (N2) nodal metastases already will rather trigger nonsurgical treatment). In all other stages, the patients are treated with chemoradiation, and here as well, targeted drugs and immune checkpoint inhibitors play an increasing role.

Many patients who are diagnosed with lung cancer have a plain chest X-ray as their first imaging study, being performed for more or less specific symptoms. In case of highly suspicious clinical symptoms (e.g., hemoptysis, dyspnea, recurrent nerve palsy) or X-ray findings (e.g., hilar mass, pneumonia irresponsive to antibiotics) and for staging the disease, cross-section imaging is needed. For diagnosing and staging lung cancer, contrast-enhanced CT remains the primary imaging modality of choice, supplemented by ultrasound of the abdomen, the neck, and the supraclavicular fossa. CT has to include the range between the lung apexes and both adrenal glands, but a complete scan of the abdomen in the portal phase of contrast enhancement will definitely make sense, considering that a baseline scan may be helpful to assess findings that are seen during the later course of the disease. In small cell lung cancer, a contrast-enhanced MRI of the brain is mandatory and may only be replaced by CT in case of contraindications to MRI.

MRI of the chest has no clear advantages over CT, with few exceptions. In tumors in the superior sulcus (Pancoast tumors), MRI, particularly in coronal orientation, is indicated to assess its depth of invasion into the cervical structures and its relationship to the cervicobrachial plexus. Moreover, MRI may be the only modality to delineate a tumor against a surrounding atelectasis, which may be important for the GTV or for response assessment.

Ultrasound of the chest may supplement CT in suspected chest wall invasion, particularly when MRI is unavailable. Endobronchial ultrasound is a specialized investigation and usually a guidance tool for transbronchial biopsies of suspicious lymph nodes.

FDG PET/CT is primarily indicated for characterization of singular pulmonary nodule undetermined in CT and for staging of nonsmall-cell lung cancer, which is highly FDG-avid in almost all cases, whenever radical surgery is intended. It may detect unsuspected mediastinal lymph node or distant metastases and identify patients who are no candidates for surgery. Those patients will usually undergo radiotherapy with selective nodal irradiation, and an available PET/CT study facilitates the delineation of the nodal GTV. If PET is unavailable, a bone scan may be used to screen for skeletal metastases. If they are suspected, their impact on bone stability needs to be assessed, often with X-ray films or CT. Of note, bone scintigraphy may be false negative (or cause a hard to detect "cold lesion") whenever there are large destructions of bone without reactively increased bone turnover. Pure bone marrow metastases may be negative as well, if they do not cause alterations to the mineralized bone.

There is an ongoing discussion on using low-dose CT for early detection of lung cancer in risk populations, that is, in current or former smokers (**Fig. 5**). Indeed, the largest prospective trial so far, the American National Lung Cancer Screening Trial (NLST) on more than 53,454 participants, comparing low-dose CT against chest X-ray, achieved a 20% reduction of lung cancer, and a 6.7% reduction in all-cause mortality in the screening group (Aberle et al., 2011). At least in some European trials, including the German LUSI trial (Becker et al., 2015), a similar mortality reduction was achieved, but due to smaller sample size, they still need to be meta-analyzed. The results from the largest European trial, NELSON from the Netherlands, are about to be published. While the hazards from radiation exposure are minimal (the effective dose is below 2 mSv and lies in the range of the annual dose from environmental sources), false positive findings are of concern, since they may trigger full-dose CT or PET/CT studies as well as invasive chest procedures. Furthermore, the issue of possible overdiagnosis and overtreatment (i.e., finding and treating lung cancer that, if left alone, would not have caused symptoms or been fatal) is unresolved. Since the NLST lacks a postscreening observation phase, other than some European trials, the question will remain open until the latter have been concluded. Upon a recent European position

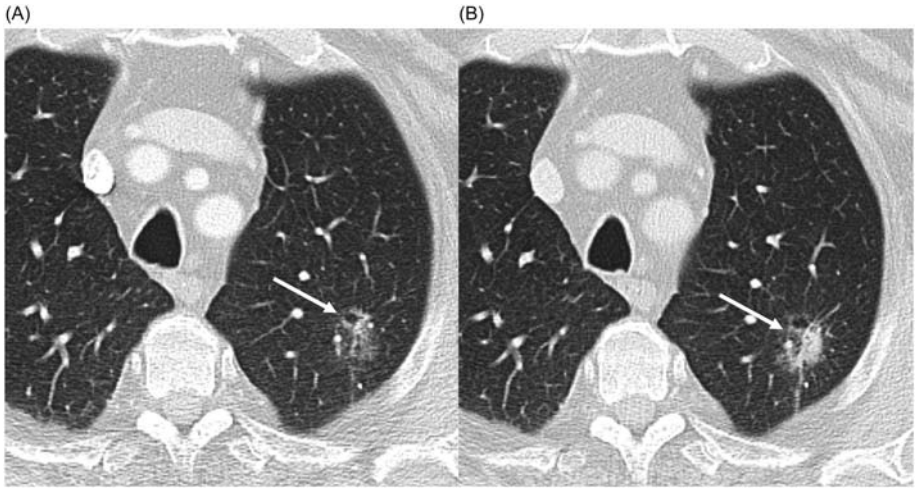

Fig. 5 Low-dose CT scans of the lung in a participant of the German "Lung Cancer Screening and Intervention" study (LUSI), 6 months apart. The initial scan (A) shows a ground-glass opacification (*arrow*) that at follow-up (B) has increased in size and density, and would now be termed "part-solid." Subsequent video-assisted thoracoscopy (VATS) with biopsy revealed lepidic adenocarcinoma with invasive foci.

statement calling for an implementation of lung cancer screening (Oudkerk et al., 2017), concerns have been raised that lung cancer were inadequate, arguing that smoking prevention would the more effective measure to reduce lung cancer mortality. In any case, it should be considered that quitters will remain at increased risk for the next 10–15 years.

Gastrointestinal Tumors

Barium-enhanced fluoroscopy, formerly the standard imaging method for tumors of the gastrointestinal tract, has become obsolete, except for few indications, like verifying or ruling out an esophageal stenosis as a cause of dysphagia, or examining the colon proximal to a stenosis that cannot be passed with the endoscope. Contrast-enhanced CT is now the imaging modality of choice, since due to the speed of acquisition peristalsis will no longer impair the image quality. MRI may be used as well, but is more susceptible to motion artifacts, and is inferior to CT in detecting lung metastases. It is, however, preferable in the aftercare for patients with a good long-term prognosis, to spare them a significant radiation dose that would otherwise accumulate over the years of follow-up. It can then be supplemented by native low-dose CT studies of the lung.

To assess the wall of hollow organs, some distension should be achieved, since nondistended or contracted bowel segments may easily mimic a mass. Most commonly, 1 to 1.5 L of water are administered either orally for the esophagus or stomach, or rectally for the large bowel, in combination with i.v. butylscopolamine—a technique commonly termed "Hydro-CT."

Esophageal cancer

Squamous cell and adenocarcinoma of the esophagus are different in many respects. Whereas squamous cell carcinoma may be located at any level, and its most important cause in the western world is tobacco and alcohol consumption, adenocarcinoma is most frequently induced by gastroesophageal reflux, intestinal metaplasia (Barrett's esophagus) being the typical precancerous change. The annual incidence for esophageal cancer in 2014 was 9.6/100,000 in men, and 2/100,000 in women (Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. (GEKID), 2014); the 2012 mortality was 4.78/100,000 in men, and 1.07/100,000 in women (Becker and Wahrendorf, 1998).

As with all gastrointestinal tumors, the diagnosis is routinely obtained by endoscopy and biopsy. Nevertheless, patients with dysphagia may in first instance have a barium swallow to verify whether a stenosis is present at all, and if so, where it is located. Not infrequently, esophageal carcinomas may grow mainly in the submucosa, so that mucosal abnormalities may not be very obvious at endoscopy. For staging, both contrast-enhanced CT and endoscopic ultrasound are primarily indicated. With endoscopic ultrasound (EUS), the layers of the esophageal wall can be nicely discriminated, and tumors can be diagnosed to either be confirmed to the mucosa and submucosa (T1), invade the muscular layer (T2), or breech the adventitia (T3 or T4). A discrimination between T3 and T4 (invasion of adjacent organs) is difficult, however, because EUS high-frequency probes are limited in penetration. Lymph nodes directly adjacent to the esophagus can be assessed and biopsied under endoscopic ultrasound guidance, if a suitable device is available. A precise discrimination of T stages lower than T4 may be of limited relevance for planning surgery, but is required prior to and after neoadjuvant therapy to assess the response to treatment. The strength of CT lies in the assessment of the overall extent of the tumor, a possible T4 stage with invasion into adjacent organs (e.g., trachea, left atrium, great vessels, thoracic vertebrae), and lymphatic and distant spread. CT is insensitive, however, in discriminating T1, T2, and T3 stages. Since both downstream and upstream lymph nodes may be involved, the neck, chest, and at least upper (if not the entire) abdomen has to be included. A postoperative CT study should be obtained as a baseline for follow up, because surgically induced alterations may

otherwise be hard to interpret and to discriminate from a recurrence. FDG PET/CT is not used routinely but in difficult cases, during staging as well as follow-up. If done, its CT part should be carried out as a full diagnostic protocol, including intravenous contrast, and not only in native, low-dose technique for attenuation correction and anatomical orientation.

Stomach cancer

For gastric cancer, the incidence and mortality have been constantly decreasing over the past decades, the 2012 mortality being 5.89/100,000 in men, and 3.14/100,000 in women, compared to almost 10-fold values around 1950 (Becker and Wahrendorf, 1998). Possible reasons among others are the consumption of fresh or refrigerated rather than nitrite-pickled food, or more effective treatment for chronic gastritis (including *Helicobacter*-associated disease). Nevertheless, gastric cancer is still a fatal disease once lymph node or distant metastases are present. According to the Laurén classification, tumors of the intestinal type are to be differentiated from diffuse tumors that lack adhesion molecules and may infiltrate the gastric wall in a single-cell pattern and extend far beyond what is evident clinically or at imaging. If resectable, it is treated by gastrectomy and lymph node dissection. Superficial tumors (T1a) may undergo endoscopic resection. If the resection has been radical, adjuvant chemotherapy is usually not given. In absence of distant disease, definite radiochemotherapy is a possible attempt at a cure. If distant metastases are present at the time of diagnosis, chemotherapy is indicated, and surgical procedures mainly serve to prevent symptoms, mainly obstruction.

The diagnosis is established by endoscopy and biopsy. To not miss a diffuse gastric carcinoma, special staining like PAS is needed to detect signet cells. For staging, hydro-CT and abdominal ultrasound, and endoscopic ultrasound are the imaging modalities of choice, CT mainly to detect a T3 or T4 stage, lymph node or distant metastases as well as a peritoneal spread and ascites. Nevertheless, a discrimination of an extension beyond the gastric wall (T3) may be difficult to discriminate from reactive inflammatory changes that often accompany gastric and also other gastrointestinal tumors, and lymph node metastases, especially those directly adjacent to the gastric wall, may be too small to appear suspicious or even be detected. Diffuse gastric carcinoma, not surprisingly, may show no circumscribed mass at all and be missed or grossly underestimated, and even a peritoneal dissemination may occur before a true gastric mass is apparent. CT has to cover the entire abdominal cavity, since peritoneal deposits may be present in the pelvis (a Krukenberg's tumor is an ovarian metastasis due to stomach cancer), and also the chest and infrahyoid neck, not to miss a Virchow's gland, a lymph node metastasis in the left venous angle. MRI is a special investigation to resolve open issues after CT and endoscopic ultrasound. FDG PET/CT is not routinely used for local staging but may be applied during aftercare for otherwise unclear lesions. Again, due to its single-cell infiltration pattern, diffuse gastric cancer will easily escape detection with PET/CT, and so will its metastases.

Cancer of the small bowel and appendix

Malignant tumors of the small bowel and appendix have in common that they are comparatively rare and usually inaccessible to endoscopy. If suspected clinically, contrast-enhanced hydro-CT in combination with abdominal ultrasound is usually the first imaging modality being used, but MRI is a good alternative, either in hydro technique as with CT, or, as a special investigation, with administration of a methyl-cellulose solution via a nasojejunal tube ("MR Sellink"), as it has been established for example, Crohn's disease. MR is especially useful if diffusion-weighted sequences are included that may highlight and draw the reader's attention to small lesions that would otherwise have escaped detection.

If PET/CT is being used, it should be carried out with CT as a full diagnostic, contrast-enhanced protocol. It should be considered that tumors of the small bowel or appendix not uncommonly have a neuroendocrine differentiation, in which case they may fail to take up FDG. 68-Ga-DOTA-TOC would then be a possible alternative.

Colorectal cancer

Colorectal cancer is among the three most common cancers and cancer-related causes of death in both sexes. Its mortality is on the decline, however. In 2012, it was 13.7/100,000 in men, and 8.2/100,000 in women (Becker and Wahrendorf, 1998). Its diagnosis is based on endoscopy and biopsy.

Colon and rectal cancer are fundamentally different regarding treatment protocols, and therefore also the required imaging procedures.

If no distant metastases are present, colon cancer is always treated by radical resection. The only imaging studies required preoperatively according to current guidelines are chest X-ray and abdominal ultrasound, to rule out lung or liver metastases. For rectal cancer, neoadjuvant as well as definite radiotherapy play a major role. As a consequence, thin-section MRI of the pelvis (CT in case of MRI contraindications) and endoscopic ultrasound are required to determine the distance of the tumor from the mesorectal fascia and diagnose possible perirectal lymph node metastases (**Fig. 6**). Their presence or absence is one criterion for the choice duration and fractionation of the neoadjuvant radiochemotherapy protocol. For both colon or rectal carcinoma, abdominal CT is indicated if there is suspicion of metastases, for example, at abdominal ultrasound. CT or MRI colonography are special investigations where the colon is inflated with air (or liquid endoluminal contrast agents with MRI). The tumor may so be seen as mural thickening and mass, and the depth of invasion into the wall or the surrounding be assessed. Additionally, 3D rendering virtual reality techniques can be used to generate colonoscopy-like views, allowing the user to "fly through" the lumen. The added value of CT or MRI colonography is obvious, but, considering the radiation dose, it should be reserved for resolving open questions that might influence clinical decisions, or rare cases where complete colonoscopy is not possible. Virtual colonoscopy may be used as an adjunct, but can neither replace CT or MR colonography or classical endoscopy, nor should it be used as a nonenhanced low-dose

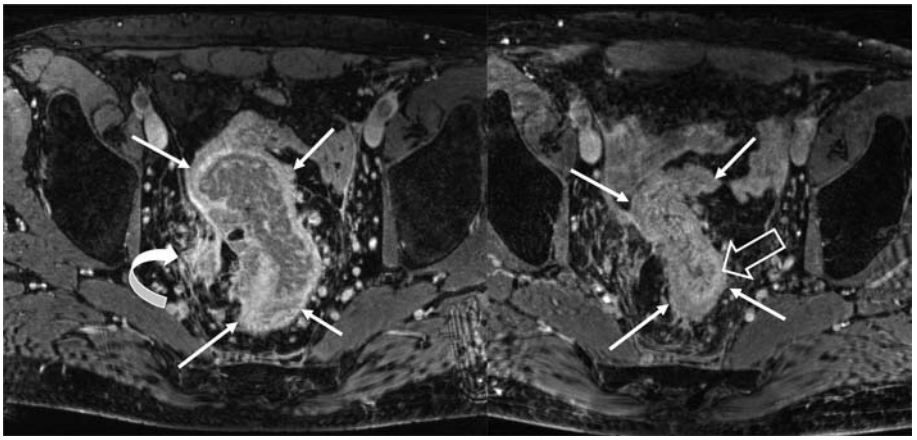

Fig. 6 Pelvic high-resolution MRI in a patient with locally advanced rectal cancer. On fat-suppressed, contrast-enhanced T1-weighted images at the time of diagnosis (A), there is a large rectal mass (*bold arrows*) with streaky infiltration into the mesorectal fat, and a broad extension to the mesorectal fascia (*curved arrow*), along with multiple enlarged lymph nodes within the mesorectum as well as along the pelvic wall. After neoadjuvant radiochemotherapy (B) there is marked remission of the tumor (*bold arrows*) as well as the extension to the mesorectal fascia and the lymph nodes. Note the recovery of the mural architecture in the rectal wall, the mucosa now being visible against the less enhancing submucosa (*open arrow*).

protocol for only visualizing the mucosal surface. Whether or not such techniques may in future be used as an alternative to colonoscopy for colon cancer screening is an open question. Currently, they clearly not being recommended for this purpose.

FDG PET/CT may have a peculiar role in colon cancer: Presacral tissue densities after primary treatment for rectal cancer often represent scar tissue, but their differential from presacral recurrences is often difficult. Here, a high uptake of FDG will point toward a recurrence as the cause. Furthermore, unlike in most other tumors, resection of metastases is an option in colorectal cancer, because there is a reasonable possibility that the visible metastases are the only ones. Whenever they are planned to be respected, a whole-body FDG PET/CT is a sensible choice to rule out additional metastases in other locations, that would prohibit such aggressive measures.

Hepatic Tumors

Focal hepatic lesions are an extremely common clinical problem, because they are frequent and also benign in most cases. The commonest tasks in which imaging studies are required are:

- To differentiate incidental liver lesions, usually detected by abdominal ultrasound,
- To stage for liver metastases in patients being diagnosed with for followed up for extrahepatic primary tumors,
- To diagnose and stage a suspected primary liver tumor.

Most incidentally detected liver lesions are benign (cysts, pseudotumors, hemangiomas, focal nodal hyperplasias, etc.). As a rule, the diagnosis of a simple cyst or a pseudotumor (e.g., focal sparing in fatty liver) should be made with ultrasound alone, without any further imaging or follow-up. Similarly, a lesion with the typical appearance of a hemangioma at ultrasound in a patient without a cancer history or risk factors should be definitely diagnosed with ultrasound, and have a single, final follow-up ultrasound for example, after 3 or 6 months. If one is unsure, it is better to refer the patient to someone more experienced with the technique than to order additional imaging studies. These are reserved for unclear cases, or for patients who have cancer in another location. If an additional CT or MRI would be performed anyhow (e.g., to stage a primary tumor in the abdomen), its protocol may be adapted in order to clarify the liver lesion. If not so, contrast-enhanced ultrasound (CEUS) would be first choice, because it implies no ionizing radiation, and in best case can be performed in the same session without leaving the patient in worries for longer time (**Fig. 7**). In large trials, CEUS has been found diagnostically equivalent to contrast-enhanced CT (Seitz et al., 2009; Strobel et al., 2009). If CEUS is unavailable or fails to resolve the issue, contrast-enhanced MRI including diffusion-weighted imaging is preferable over CT, at least in the interest of radiation protection. Furthermore, liver-specific contrast media are available in MRI, that may assist in differentiating liver lesions. In unclear cases, nuclear medicine studies (hepatobiliary or blood-pool scans) provide additional information to make the probable diagnosis.

When staging for liver metastases, the choice of the imaging method depends on the primary tumor and the a-priori risk for metastases. In many cases, CT or MRI of the liver will be included in the imaging protocol for primary staging, but should be supplemented by an abdominal ultrasound—if possible, after the CT or MRI, to help clarify unclear lesions, such as cysts or hemangiomas. It should be remembered that in cancer patients benign liver lesions are as frequent as in healthy persons.

Most primary liver tumors are either peripheral cholangio- or hepatocellular carcinomas (HCC). While HCC is common in Africa or the far east, following the endemic presence of hepatitis B virus and the exposure to aflatoxins, it is less common in Caucasians, mostly associated with alcoholic or nonalcoholic liver cirrhosis and/or chronic hepatitis B or C, or, less frequently, hereditary

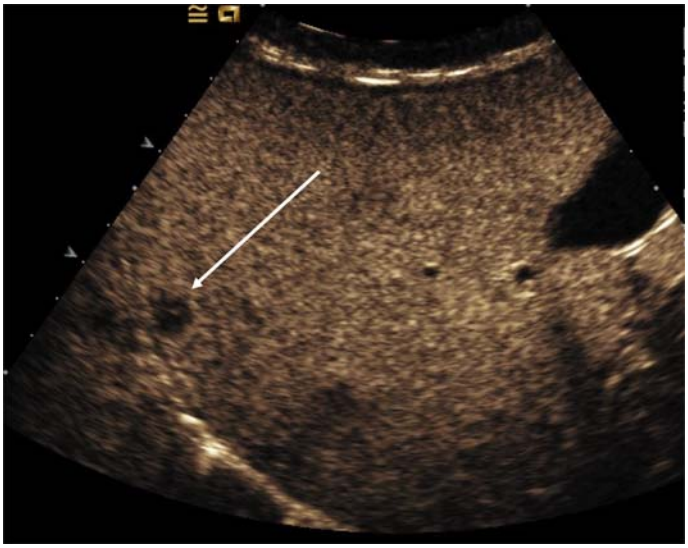

Fig. 7 Longitudinal section of the right liver lobe during contrast-enhanced ultrasound (CEUS) in a patient with malignant melanoma. Unenhanced B-mode ultrasound of the liver had been normal. In the portal phase of contrast enhancement, there is a hypoenhancing, subdiaphragmal nodule (*arrow*), that progressed at follow-up and disappeared after immunotherapy, and was therefore diagnosed as a liver metastasis. As a rule, CEUS is equally sensitive and specific to CT or MRI, sonographic access provided.

hemochromatosis. It is a highly vascularized tumor with a strong tendency to invade portal venous branches, giving it the typical pattern of a strong contrast enhancement in the arterial phase of injection (which is uncommon in cholangiocarcinoma and most liver metastases) and a contrast washout in later phases. The primary treatment, depending on the number and size of lesions as well on the remaining liver function, consists of resection, local radiofrequency tumor ablation, or, in eligible patients, liver transplantation. In transplant-eligible patients, chemoembolization via a supraselective angiographic catheter or radiofrequency ablation may serve to prevent further tumor growth until a donor organ becomes available ("bridging"). In clinical trials, photon or particle radiation therapy are being evaluated. In case of irresectability or recurrence, sorafenib is effective for palliation and prolonging survival.

Cholangiocarcinoma usually shows a strong fibroplasia with only scarce vessels in its center. Cholangiocarcinomas are best imaged and staged with MRI (CT, if MRI is contraindicated) in combination with abdominal ultrasound. Imaging studies should be used to guide the biopsy to the vital periphery and avoid the desmoplastic reaction in the tumor's center, where it may be false negative. Owing to the aggressiveness of the tumor, both the entire abdomen and the chest (using contrast-enhanced CT) should be imaged.

For HCC, valid imaging studies are crucial: The presence of a lesion with typical enhancement pattern in a single imaging modality in a patient at high risk is accepted as proof of HCC, without the need for a biopsy. The modality may be contrast-enhanced CT or MRI, or contrast-enhanced ultrasound, all of which are seen as equivalent. Only in unclear cases either an additional imaging modality or biopsy is needed. Imaging also serves to diagnose multifocal lesions as well as portal venous invasion, that may preclude surgical resection.

Pancreatic Tumors

Ductal adenocarcinoma of the pancreas is one of the most fatal cancers. Although it is not exceedingly frequent, it is the fourth-most common cancer-related cause of death in men and women, and its frequency is on the slow rise. Smoking is a risk factor, but on the whole, there is no clearly definable risk population, and no effective and acceptable measures are available for early detection. Radical surgery is the only chance for a cure. The role of imaging is to enable a diagnosis as early as possible, but since the symptoms are unspecific at the beginning, ultrasound is the only modality that can be recommended to be used generously—knowing that its effectiveness in detecting pancreatic cancer in a resectable stage is yet unproven. In unclear findings, suspicious biochemical findings, suspicious and persistent pain (especially a combination of epigastric and middle back pain) in combination with limited sonographic access, contrast-enhanced CT or MRI are to follow. CT is used most frequently, because it will reliably image the entire abdominal cavity, and the chest if needed. MRI is equally capable, but more expensive and less well tolerated by the patient. Whenever the pancreas is well visible at sonography, contrast-enhanced ultrasound is a reliable and safe procedure to prove or rule out a focal pancreatic lesion, because pancreatic adenocarcinoma is almost always hypovascular compared to the normal parenchyma. It would clearly deserve to be used more frequently and could so obviate CT or MRI studies for unclear sonographic findings.

Endocrine tumors of the pancreas are an entirely different entity and may be found in patients with type I multiple endocrine neoplasia. Whereas some produce insulin, glucagon, gastrin or vasoactive intestinal peptide (VIP) and are therefore being suspected

because of endocrine symptoms (e.g., hypoglycemia, recurrent peptic ulcers, or watery diarrhea), a significant portion will be non-secreting. The probability of a malignant variant depends on the endocrine type and is highest in gastrinomas. Their morphology is often different from adenocarcinoma; typically they will be sharply delineated and hypervascular. As in other locations in the body, endocrine tumors of the pancreas express somatostatin receptors and can thus be detected by somatostatin analogs, using for example, octreotide scintigraphy or 68-Ga-DOTA-TOC PET/CT.

Tumors of the Kidney, Ureters, and Bladder

Renal carcinoma is a less common cause of cancer-related death. Its mortality in 2012 was 5.3/100,000 in men, and 2.2/100,000 in women (Becker and Wahrendorf, 1998). Its growth rate is highly variable, and symptoms like flank pain, or gross hematuria usually indicate advanced local disease. Apart from rare disorders like von Hippel–Lindau disease, there are no known risk constellations. Although no screening program exists, the most renal tumors are nowadays found incidentally on occasion of an abdominal ultrasound for any reason. As a result, most patients with renal cancer will be definitely cured. Apart from metastases to the lymph nodes, lung, and bones, two findings are peculiar: Renal cell carcinomas tends to invade the renal vein, extending along the inferior vena cava until above the diaphragm. Furthermore, pancreatic metastases may occur, even as a late recurrence, long after primary therapy. The treatment is almost exclusively surgical, whenever possible by kidney-preserving procedures, otherwise by nephrectomy.

Two kinds of space-occupying lesions in the kidney are definitely benign—cysts and angiomyolipomas. Cysts can be confidently diagnosed at ultrasound if they exhibit typical features. This, however may be difficult in case of limited sonographic access, so that contrast-enhanced CT or MRI may be necessary for clarification. The other entity, angiomyolipoma, has a typical morphology at ultrasound (high echogenicity, sharp delineation), but as state of the art, a second imaging modality (usually native CT) is required to prove the presence of significant amounts of fat in the lesion.

As a rule, all focal renal lesions that are neither simple cysts nor angiomyolipoma are suspicious and should be respected, because the majority of them will be malignant, and no imaging modality is capable of excluding this.

Cancer of the renal pelvis, ureters, and bladder are entirely different clinically and in etiology. Typically, they are caused by chronic exposition to carcinogens, most importantly smoking, but also for example, aniline dyes. Therefore, as many as 50% of patients may have recurrences or second neoplasms of the urothelium at the time of diagnosis or in the later course. Hematuria is the leading symptom in 75% of patients. The treatment is mainly surgical, but superficial bladder tumors might be locally ablated via cystoscopy. In advanced bladder tumors not undergoing cystectomy, instillation of BCG has successfully been used to temporarily control the disease.

CT or MRI may be limited to the kidneys if only carried out to rule out a malignant lesion. Whenever the ultrasound findings are highly suspicious for malignancy, or gross hematuria is the chief symptom, the heart should be included, to detect a possible thrombus in the vena cava, as well as the entire abdomen including the bladder. Furthermore, the urine collecting system has to be assessed for possible neoplasms as a cause for hematuria. Therefore, scan timing in a renal CT or MRI protocol will differ from that in a standard abdominal one. A baseline native scan (either acquired prior to contrast infusion or, if DECT is being used, as a virtual native scan) is needed, to detect a weak contrast enhancement in a focal lesion. An arterial phase is optional and often omitted. The most important will be a scan in the phase when both renal cortex and medulla are enhanced, which is later than the usual portal phase, that is, 120 s post infusion. Finally, a CT urography, with contrast filling of the collecting system is obtained after 5 min. The same timing will apply if MRI is used instead of CT.

For bladder cancer, ultrasound as well as cystoscopy are the primary examinations, and contrast-enhanced MRI whenever invasion of the muscular layer or extension beyond the bladder are suspected. As a rule, the entire urine collecting system including renal calices and pelvis as well as ureters needs to be examined with CT or MRI urography, due to the high prevalence of simultaneous second neoplasms, if not plain X-ray films with retrograde contrast filling of the ureters or ureteroscopy are performed on occasion of cystoscopy.

Prostate Cancer

Prostate cancer is the most common malignant tumor in men. Although there has been a steep rise in incidence, its annual mortality is slowly declining, being about 11/100,000 in 2012, indicating that the rising incidence is mainly a screening artifact due to the frequent measurement of PSA in serum. This means that there is a significant risk of overdiagnosis and overtreatment, if not measures are taken to identify high-risk and low-risk cancers and manage them adequately. In this process, imaging plays a pivotal role, using four tools: Endorectal ultrasound-guided biopsies, MRI, MRI/ultrasound fusion biopsies, and PET/CT or PET/MRI with radiolabeled PSMA ligands.

First, patients with suspected prostate carcinoma will undergo systematic endorectal, ultrasound-guided biopsy and, if positive, nodal and skeletal imaging for staging, and then treatment: Radical prostatectomy, definite intensity-modified or particle radiotherapy, or, in case of metastases, antihormonal therapy. In case of negative biopsies despite elevated or increasing serum PSA levels, or to stage biopsy-proven prostate carcinoma for local extracapsular extension, multiparametric MRI is the method of choice. It combines T1- and T2-weighted imaging with diffusion-weighted imaging (including calculated maps for the apparent diffusion coefficient) and DCE MRI. 1H spectroscopy is no longer standard part of the protocol. Depending on the morphologic and functional features, possible lesions may be classified according to their probability of malignancy, using, for example, the PI-RADS that was published in its 2nd version in 2015 (American college of radiologists, 2015). Using commercial image fusion system,

suspicious lesions may be targeted and biopsied under ultrasound guidance from a perineal approach, although they may be invisible at ultrasound. This is particularly useful to reach suspicious lesions that may have been missed by standard systematic biopsies because of their location (e.g., ventrally).

The development of a PET ligand to the prostate-specific membrane antigen (PSMA) has been a revolution in prostate imaging, since it enables to locate metastases even in otherwise unsuspicious structures, like normal-size lymph nodes. The first available F18-labeled PSMA-compounds and the currently distributed tracers labeled with 68-gallium are excreted in the urine. The excreted tracer in the urinary bladder may hinder the detection of local recurrent tumors in close proximity to the bladder. However, 18-fluorine-labeled tracers that are not primarily renally excreted will soon be available. This might not only facilitate the detection of prostate carcinoma but also support the delineation of the gross tumor volume for radiotherapy planning. Optimally, for imaging the prostate itself using PSMA ligands, PET/MRI hybrid scanners would be preferable over PET/CT machines.

Tumors of the Uterus and Ovaries

Uterine tumors may be either endometrial adenocarcinoma or cervical squamous cell carcinoma, with an annual mortality of 2/100,000 and 1.9/100,000, respectively. They may be treated by resection or radiochemotherapy, depending on the local stage and presence or absence of nodal or distant metastases. Cervical carcinomas, if locally advanced or recurrent, can be devastating regarding quality of life. The diagnosis is typically made by either abrasion (corpus) or cone excision (cervix), supported by bimanual palpation and endovaginal or endorectal ultrasound for local staging. MRI is primarily indicated for staging both tumors locally, and should include the entire abdominal cavity to detect spread to retroperitoneal lymph nodes—that would be considered distant, not regional. The main target feature when imaging corpus tumors will be the proportion of the uterine wall occupied by the tumor. For cervical tumors, particular attention is paid to an infiltration of the parametria, the bladder, and the rectum, and the downwards extension into the vaginal wall. FDG PET/CT is a specialized investigation in cases where the presence or absence of metastases remains unclear.

Ovarian cancer accounts for 5/100,000 deaths per year and is often diagnosed in an advanced, incurable stage. Due to the high prevalence of benign, and mostly cystic masses, the confident exclusion of ovarian cancer may be highly challenging, endovaginal ultrasound being the primary imaging modality of choice. Here, the presence of solid or papillary components, thick septa or strong vascularization at color Doppler will argue in favor of malignancy. For MRI as a problem solver, recommendations have recently been released (Forstner et al., 2017), that pay particular attention to DCE MRI and diffusion-weighted imaging for discriminating unclear masses. Nevertheless, laparoscopy and biopsy are indicated early, not to miss the disease in a curable stage. Once the diagnosis of ovarian cancer has been made, staging has to be completed, preferably by abdominal ultrasound and CT of the chest and abdomen. Bone scans are usually not required, because bone metastases due to ovarian cancer are extremely rare.

Breast Cancer

Breast cancer is the commonest tumor in women and with an annual mortality of 16.1/100,000 also their leading cause of cancer death. Besides eradicating the disease, all treatment approaches strive at limiting resection as much as possible. This in mind, imaging is directed at enabling a diagnosis as early as possible, and at visualizing the true extent of the tumor inside the breast, in order to guide breast-conserving surgery. Mammography screening programs are in action in most countries, but despite rigorous quality assurance and training of the readers, only about 50% of all women who finally undergo biopsy for a screening-detected lesion will in fact have cancer.

Besides the introduction of digital mammography and constant improvement of ultrasound technology, three developments need mentioning that have significantly influenced detection and staging of breast cancer, or can be expected to do so: Digital tomosynthesis, DCE MRI, and diffusion-weighted MR imaging. Digital tomosynthesis is a technique where the breast remains fixed between the detector and the compression plate, and the tube is being moved over an angle of up to 50 degrees while the beam is on. From the data, stacks single slices (e.g., 1 mm thick) can be reconstructed, which permits an improved delineation of nodules and masses against other overlying structures. This is possible without significant radiation dose penalty, and additionally conventional mammography images can be extracted, whose quality is apparently not inferior to that of a normal mammogram (Zuley et al., 2014). DCE MRI has been investigated since the early 1990s, and has now become part or the routine. Its role as a problem solver for unclear lesions is limited, and biopsy under imaging guidance is clearly the best way toward a reliable diagnosis. Nevertheless, it contributes significantly to assessing the extent of even complex shaped tumors, multifocal or multicentric lesions, and even DCIS components are visible owing to their contrast uptake, especially if they are high-grade (**Fig. 8**). Other clear indications are breasts with implants (where neither mammography nor ultrasound make sense), and suspicious lesions post treatment, where scars have to be differentiated from residual or recurrent tumor. Diffusion-weighted images, finally, are difficult to obtain in good quality, owing to motion and magnetic field inhomogeneity along the complex-shaped tissue–air interface. Nevertheless, when fine-tuning and quality assurance are thoroughly carried out, the images will reliably highlight malignant foci against a signal-void background (**Fig. 9**). In a prospective study, an abbreviated (<10 min) protocol applied for women scheduled for biopsy due to screening-detected, suspicious lesions, diffusion-weighted imaging was sensitive and specific enough to possibly spare women without breast cancer a biopsy, with extremely few true cancers being missed (Bickelhaupt et al., 2015). Such protocols do not require any contrast agent and are short enough to be used on large scale. Whether or not they might be used instead of screening mammography in the future, is yet undetermined.

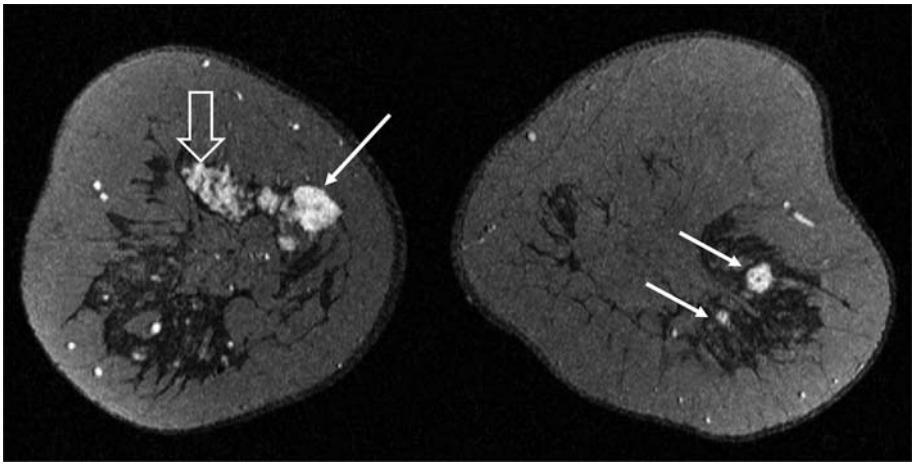

Fig. 8 Dynamic contrast-enhanced T1-weighted images in a patient with multifocal and bilateral breast cancer. Note the solid, circumscribed nodules (*bold arrows*) that correspond to invasive foci. Additionally, there is an extensive in-situ component that is visible as a less intense, inhomogeneous area of contrast enhancement with a segmental configuration (*open arrow*).

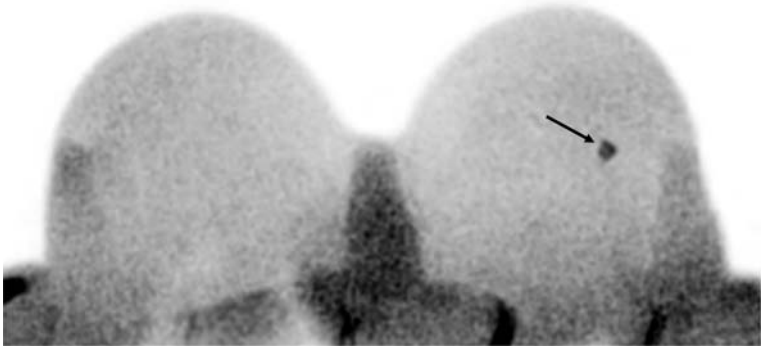

Fig. 9 Craniocaudal projection of axial diffusion-weighted images with background suppression in a participant of the German mammography screening program who had been recalled for a suspicious (BI-RADS 4) mammography finding in the left breast. The projection image permits to check the breasts for lesions with restricted diffusion at a single glance. In this case, the mammography finding corresponds to a lesion less than 10 mm in diameter that was histologically confirmed to be a ductal invasive carcinoma. Courtesy Dr. Sebastian Bickelhaupt, German Cancer Research Cencer (DKFZ), Heidelberg.

Hematologic and Lymphatic Neoplasms

Leukemia is being diagnosed based on blood and bone marrow samples. Other than myeloid ones, lymphatic leukemias are often accompanied by lymph node and sometimes extranodal masses that will be seen in imaging studies. In chronic lymphoid leukemia, which is in fact an indolent non-Hodgkin lymphoma, imaging is part of staging and follow-up. For malignant lymphomas, imaging of the neck, chest, and abdomen is mandatory to stage the disease and to assess its response to treatment (Cheson et al., 2007), usually by a combination of abdominal and cervical ultrasound and contrast-enhanced CT. In patients with a favorable long-term prognosis, particularly young ones, it is recommended to replace CT by MRI, at least for the abdomen. Furthermore, FDG PET/CT is recommended in guidelines for Hodgkin lymphoma and those non-Hodgkin lymphomas that are typically FDG-avid. It may supplement primary staging, but its most important role is to assess the presence or absence of viable lymphoma at an interim staging and/or after completion of the primary treatment. Especially when there was bulky disease at diagnosis, macroscopic residuals will persist after treatment that may or may not contain viable lymphoma tissue. Increased FDG uptake in these lesions almost invariable heralds a recurrence and also indicates an unfavorable prognosis. Furthermore, FDG PET/CT may be indicated whenever there is suspicion of recurrence during the later course.

Multiple myeloma, formally a non-Hodgkin lymphoma, is a monoclonal plasma cell disorder that arises primarily in the bone marrow and interacts with osteoclasts and osteoblasts in a specific fashion. It may cause destructions to mineralized bone, along with anemia, hypercalcemia, and renal damage, which are indications for systemic treatment. There are, however, asymptomatic precursor stages, namely monoclonal gammopathy of unclear significance (MGUS) and smoldering multiple myeloma (SMM), that are at risk for progression into a symptomatic stage, but do not yet require treatment. Together with biochemical and bone marrow diagnostics, imaging plays a pivotal role for decisions on treatment. For assessing the integrity of mineralized bone, the previously used plain X-ray skeletal survey are now being substituted by noncontrast whole-body CT, because the latter is more

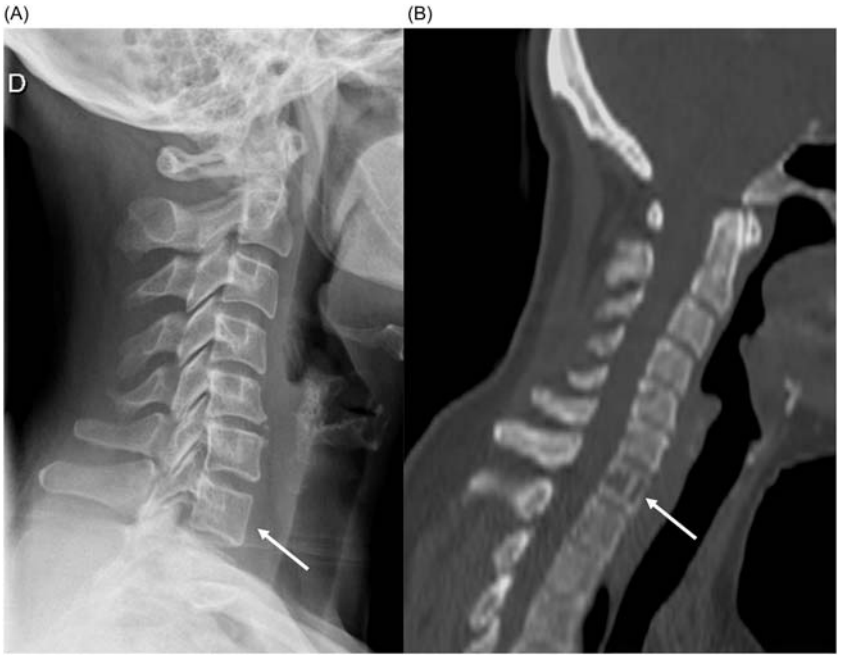

Fig. 10 Lateral X-ray plain film of the cervical spine (A) in a patient with smoldering multiple myeloma and so far without end organ damage. A sagittal reconstruction from an unenhanced, low-dose CT obtained within 4 weeks (B) shows a lytic bone lesion (*arrow*) that is invisible on plain films. The patient is therefore upstaged to symptomatic multiple myeloma, where systemic treatment is indicated.

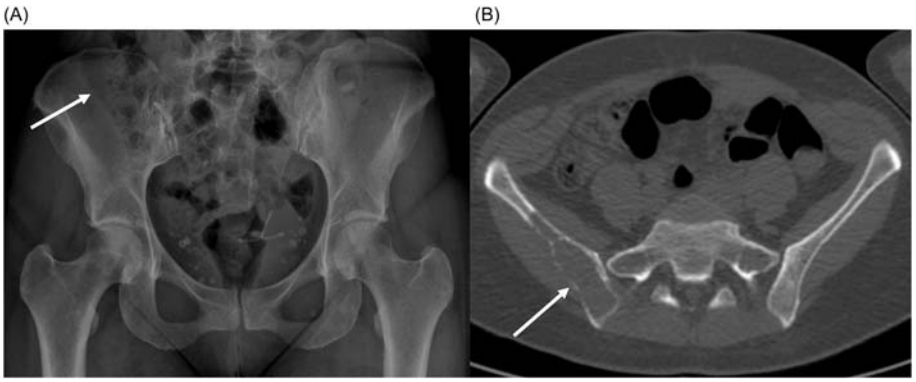

Fig. 11 Pelvic X-ray film (A) and CT (B) in a patient with multiple myeloma. On CT, a large destructive lesion in the iliac bone (*arrow*) is easily detected and was originally missed on plain films. Retrospectively, one can discriminate the vertical contour of the lesion (*arrow* in A), but it was obscured by overlying bowel gas.

sensitive and specific for bone destructions (**Figs. 10 and 11**), and its findings are of proven prognostic significance (Hillengass et al., 2017). For assessing the bone marrow and possible extraosseous myeloma foci, noncontrast whole-body MRI is now standard and is capable of detecting diffuse or focal bone marrow lesions that still have left the cortical and cancellous bone intact. According to the revised criteria of the International Myeloma Working Group, the presence of more than one focal bone marrow lesion is an indication to treatment, even if the mineralized bone remains intact (Rajkumar et al., 2014). FDG PET/CT is a valid modality for detecting focal myeloma infiltrates, but its sensitivity for diffuse bone marrow involvement is variable and probably dependent on the density of infiltration. If it is used, the CT data should be acquired using a full diagnostic protocol and enable a reliable assessment or the mineralized bone—and so to spare the patient an additional skeletal CT. Whole-body diffusion-weighted imaging is now a standard supplement to MRI in many centers and is extremely useful to facilitate the detection of diffuse and focal lesions, even in unusual locations (**Fig. 12**).

Tumors of the Bones and Soft Tissue

Primary bone and soft tissue malignancies are relatively rare tumors. The modality of choice to approach the diagnosis depends on the location. For primary bone tumors, diagnostic criteria applied to plain X-ray films have been established over many decades and

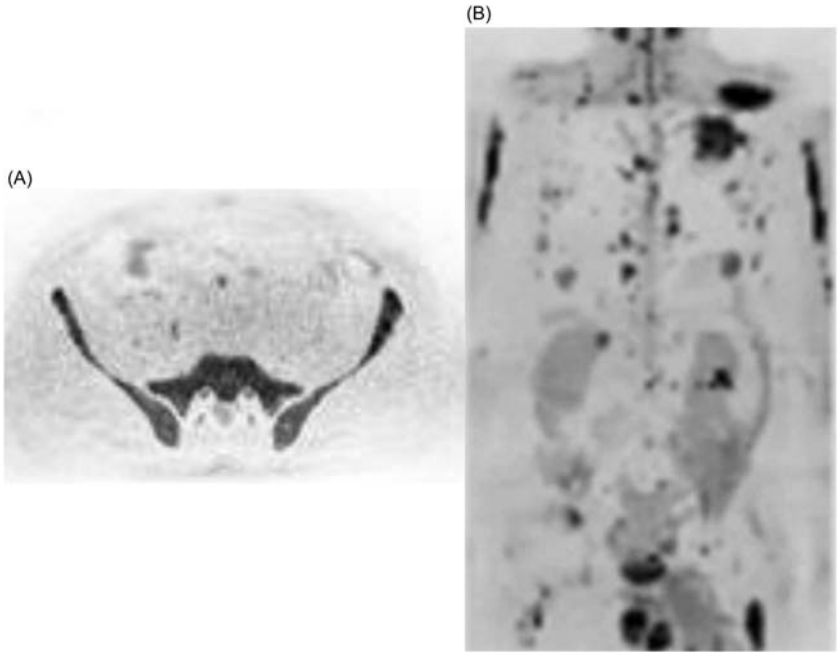

Fig. 12 Diffusion-weighed imaging (DWI) in plasma cell diseases. A transverse section at the level of the pelvis shows a diffuse infiltration pattern (A). A frontal projection image in a patient with a multifocal infiltration pattern, with lesions (B), for example, in the left scapula, left upper chest wall, both humeri, left femur, and numerous others.

remain valid. The first task of the reader is to identify tumor-like lesions where a biopsy should not be performed, because it is either unnecessary or even likely to yield falsely suspicious histologic findings that result in invasive measures ("do not touch lesions"). Of the remaining, some can be confidently diagnosed based on the plain film morphology, location, and patient's age, but in some the diagnosis will remain unclear until resection. Considering that such tumors are rare, and there are few radiologists who are truly experienced, it is strongly advised to seek an expert's opinion and to consult an orthopedic surgeon even before a biopsy is attempted. CT and MRI are valuable adjuncts for making the diagnosis, but their most important role is to delineate the true extent of the tumor, beyond what is visible on plain films.

For primary soft tissue tumors, MRI, CT, or ultrasound again serve to identify typically benign entities (e.g., lipoma), and to identify the organ or compartment of origin. If not the entity is evident from the lesion's imaging morphology (e.g., liposarcoma) or location, the contribution of imaging, including PET/CT, to making the diagnosis is limited. Such tumors will be respected, taking care of their integrity, in order not to contaminate the operation bed. Following up an unclear soft tissue mass is definitely inadequate.

Imaging Distant Disease

Usually, regional lymph nodes will be assessed when imaging the primary tumor, as outlined above, and some protocols also account for the most common distant metastases. In special situations, imaging is mainly dedicated to detecting distant disease. The most important applications are:

- Whole-body imaging: PET/CT is a valid method for lymph node as well as distant metastases, using FDG, PSMA ligands, DOTA-TOC or other tracers, depending on the histology. Diffusion-weighted MRI is sensitive for distant as well as lymph node metastases, but in the latter it is not specific for malignant involvement. Like metastases, reactively altered lymph nodes have a high signal. For brain metastases, neither FDG PET/CT nor diffusion imaging are valid, because of the brain's high FDG uptake and physiologic diffusion restriction.
- Detecting lung metastases: The modality of choice is CT. If for any reason no information about soft tissue is needed, it can be performed without contrast medium and in low-dose technique, which will keep the effective dose below 2 mSv, usually even lower.
- Detecting lymph node metastases: MRI contrast media dedicated to lymph node imaging have been on the market, but have been unsuccessful there and meanwhile withdrawn. For superficial lymph nodes, high-frequency ultrasound (14 MHz or higher) is clearly the best and cheapest method, but requires a skilled and experienced examiner.
- Detecting liver metastases: Usually, the liver is included in the primary imaging protocol. If examined alone, contrast-enhanced ultrasound is the first method to suggest, because it is sensitive, specific, and cost-effective. Alternatives are contrast-enhanced CT

or MRI including diffusion-weighted sequences. FDG PET/CT may be limited in value because of the moderately high background uptake of the liver itself.

- Detecting CNS metastases: The method of choice is clearly contrast-enhanced MRI, which is superior to all competing modalities. If meningeal spread is suspected, longitudinal, contrast-enhanced images of the entire spinal canal are added.
- Detecting skeletal metastases: 99m-Tc-Bisphosphonate bone scan continues to be the standard but has shortcomings in highly lytic lesions (e.g., due to thyroid or renal cancer) because the bony structures are either completely destroyed or the metastases induce no reactively increased bone turnover. The same may apply to pure bone marrow deposits. PET/CT with NaF-18 is an alternative to bone scans whenever 99m-Tc is unavailable but has the same limitations.
- Cancer of unknown primary (CUP) is a special indication. Usually, the origin of the resected tumorous tissue will be tried to discriminate from the pathological workup of the biopsy. If this is unsuccessful, the imaging methods are tried to be wisely chosen, along with for example, an endoscopic search. PET/CT is often included but may be unsuccessful if the tracer is not being taken up, or the assumed primary tumor is very small, as for example, in malignant melanoma. By the same token, in widespread disease with a high tumor load, it will be difficult to discriminate the metastases from the tumor of origin.

Response Monitoring

A tumor's response to treatment is generally attributed to a reduction in size, which is the sole criterion in almost all formal response criteria, including those for immune therapy. Powerful software tools are clinically approved that support physicians in formal response assessment in clinical trials. However, reduction in size occurs relatively late in the course of the treatment, with few exceptions like some lymphomas, where one can assume that treatment-induced apoptosis rather than necrosis is the predominant response mechanism. Therefore, functional features have been and are being explored to mirror or predict treatment response. The best evaluated one is the FDG uptake in PET/CT (Fig. 13), which in many tumors has been shown to be reduced much earlier than any changes in tumor size become apparent. The enhancement of contrast agents, that can easily be picked up in

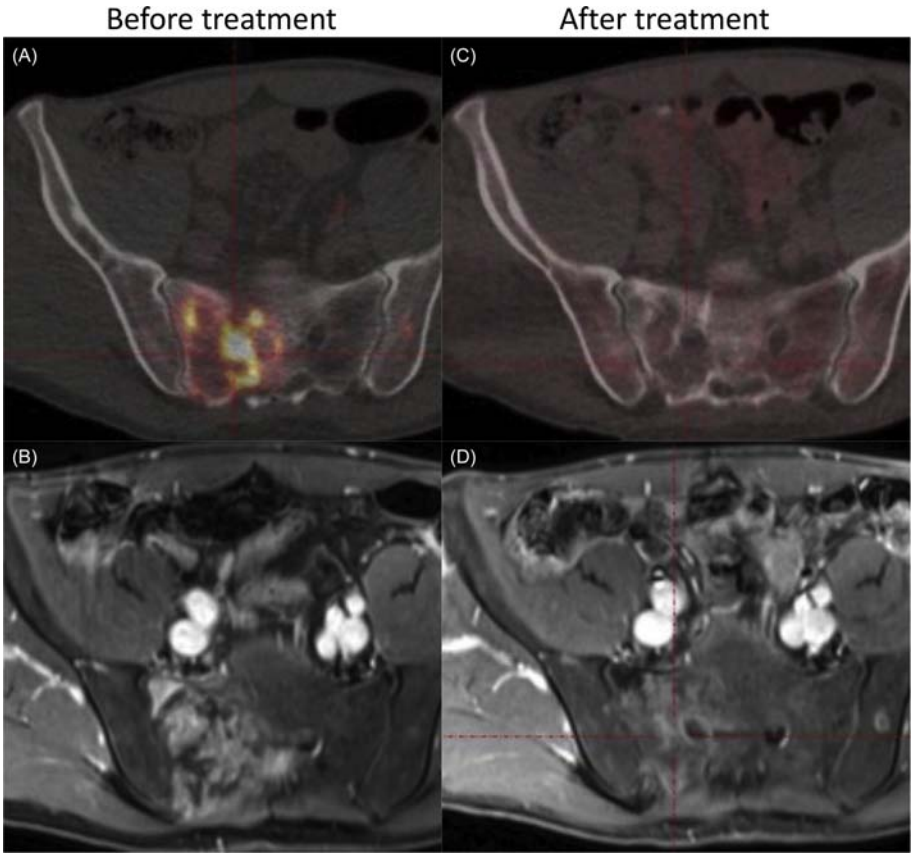

Fig. 13 FDG-PET/CT (A and C) and contrast-enhanced, fat-suppressed T1-weighted MRI (B and D) in a patient with a large, destructive myeloma infiltrate in the right sacral bone, obtained before (A and B) and after (C and D) high-dose chemotherapy with stem cell support. Initially, there is intense contrast enhancement and marked FDG uptake, both being markedly reduced after treatment. Courtesy of Dr. Bettina Beuthien-Baumann, Heidelberg.

contrast-enhanced ultrasound, CT or MRI, or can be formally quantified in dynamic MRI, is another criterion and is for example, being used for gastrointestinal stroma tumors (GIST), or hepatocellular carcinomas. Changes in diffusion-weighted imaging are further, possible criteria that are being investigated. Nevertheless, the current state is that the disappearance or non-progression of lesions remains the chief response criterion, and that functional interim tests for deciding upon a preterm change in treatment regimen are rarely in action, with rare exceptions. In this context, FDG-PET/CT is being used in malignant lymphoma for response assessment after primary chemotherapy, and increasingly for interim staging during treatment. Whether or not these studies will contribute to decisions regarding the therapy regime, is still open.

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References

Aberle, D.R., Adams, A.M., Berg, C.D., Black, W.C., Clapp, J.D., Fagerstrom, R.M., Gareen, I.F., Gatsonis, C., Marcus, P.M., Sicks, J.D., 2011. Reduced lung-cancer mortality with low-dose computed tomographic screening. The New England Journal of Medicine 365, 395–409.

American college of radiologists (2015) PI-RADS. Prostate imaging—reporting and data system Version 2. https://www.acr.org/-/media/ACR/Files/RADS/Pi-RADS/PIRADS-V2.pdf?la=en.

Becker, N., Wahrendorf, J., 1998. Krebsatlas der Bundesrepublik Deutschland 1981–1990. Springer, Berlin/Heidelberg/New York.

Becker, N., Motsch, E., Gross, M.L., Eigentopf, A., Heussel, C.P., Dienemann, H., Schnabel, P.A., Eichinger, M., Optazaite, D.E., Puderbach, M., Wielputz, M., Kauczor, H.U., Tremper, J., Delorme, S., 2015. Randomised study on early detection of lung cancer with MSCT in Germany: Results of the first 3 years of follow-up after randomisation. Journal of Thoracic Oncology 10, 890–896.

Bickelhaupt, S., Laun, F.B., Tesdorff, J., Lederer, W., Daniel, H., Stieber, A., Delorme, S., Schlemmer, H.P., 2015. Fast and noninvasive characterization of suspicious lesions detected at breast Cancer X-ray screening: Capability of diffusion-weighted MR imaging with MIPs. Radiology 278, 689–697.

Bostel, T., Pfaffenberger, A., Delorme, S., Dreher, C., Echner, G., Haering, P., Lang, C., Splinter, M., Laun, F., Müller, M., Jäkel, O., Debus, J., Huber, P.E., Sterzing, F., Nicolay, N.H., 2018. Prospective feasibility analysis of a novel off-line approach for MR-guided radiotherapy. Strahlentherapie und Onkologie.

Chenevert, T.L., Stegman, L.D., Taylor, J.M., Robertson, P.L., Greenberg, H.S., Rehemtulla, A., Ross, B.D., 2000. Diffusion magnetic resonance imaging: An early surrogate marker of therapeutic efficacy in brain tumors. Journal of the National Cancer Institute 92, 2029–2036.

Cheson, B.D., Pfistner, B., Juweid, M.E., Gascoyne, R.D., Specht, L., Horning, S.J., Coiffier, B., Fisher, R.I., Hagenbeek, A., Zucca, E., Rosen, S.T., Stroobants, S., Lister, T.A., Hoppe, R.T., Dreyling, M., Tobinai, K., Vose, J.M., Connors, J.M., Federico, M., Diehl, V., 2007. Revised response criteria for malignant lymphoma. Journal of Clinical Oncology 25, 579–586.

Forstner, R., Thomassin-Naggara, I., Cunha, T.M., Kinkel, K., Masselli, G., Kubik-Huch, R., Spencer, J.A., Rockall, A., 2017. ESUR recommendations for MR imaging of the sonographically indeterminate adnexal mass: An update. European Radiology 27, 2248–2257.

Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. (GEKID) (2014) Krebsatlas der Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. (GEKID). www.gekid.de.

Hillengass, J., Moulopoulos, L.A., Delorme, S., Koutoulidis, V., Mosebach, J., Hielscher, T., Drake, M., Rajkumar, S.V., Oestergaard, B., Abildgaard, N., Hinge, M., Plesner, T., Suehara, Y., Matsue, K., Withofs, N., Caers, J., Waage, A., Goldschmidt, H., Dimopoulos, M.A., Lentzsch, S., Durie, B., Terpos, E., 2017. Whole-body computed tomography versus conventional skeletal survey in patients with multiple myeloma: A study of the International Myeloma Working Group. Blood Cancer Journal 7, e599.

Kalender, W.A., Seissler, W., Klotz, E., Vock, P., 1990. Spiral volumetric CT with single-breath-hold technique, continuous transport, and continuous scanner rotation. Radiology 176, 181–183.

Kickingereder, P., Wiestler, B., Sahm, F., Heiland, S., Roethke, M., Schlemmer, H.P., Wick, W., Bendszus, M., Radbruch, A., 2014. Primary central nervous system lymphoma and atypical glioblastoma: Multiparametric differentiation by using diffusion-, perfusion-, and susceptibility-weighted MR imaging. Radiology 272, 843–850.

Le Bihan, D., Breton, E., Lallemand, D., Aubin, M.L., Vignaud, J., Laval-Jeantet, M., 1988. Separation of diffusion and perfusion in intravoxel incoherent motion MR imaging. Radiology 168, 497–505.

Oudkerk, M., Devaraj, A., Vliegenthart, R., Henzler, T., Prosch, H., Heussel, C.P., Bastarrika, G., Sverzellati, N., Mascalchi, M., Delorme, S., Baldwin, D.R., Callister, M.E., Becker, N., Heuvelmans, M.A., Rzyman, W., Infante, M.V., Pastorino, U., Pedersen, J.H., Paci, E., Duffy, S.W., de Koning, H., Field, J.K., 2017. European position statement on lung cancer screening. The Lancet Oncology 18, e754–e766.

Rajkumar, S.V., Dimopoulos, M.A., Palumbo, A., Blade, J., Merlini, G., Mateos, M.V., Kumar, S., Hillengass, J., Kastritis, E., Richardson, P., Landgren, O., Paiva, B., Dispenzieri, A., Weiss, B., LeLeu, X., Zweegman, S., Lonial, S., Rosinol, L., Zamagni, E., Jagannath, S., Sezer, O., Kristinsson, S.Y., Caers, J., Usmani, S.Z., Lahuerta, J.J., Johnsen, H.E., Beksac, M., Cavo, M., Goldschmidt, H., Terpos, E., Kyle, R.A., Anderson, K.C., Durie, B.G., Miguel, J.F., 2014. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. The Lancet Oncology 15, e538–e548.

Seitz, K., Strobel, D., Bernatik, T., Blank, W., Friedrich-Rust, M., Herbay, A., Dietrich, C.F., Strunk, H., Kratzer, W., Schuler, A., 2009. Contrast-enhanced ultrasound (CEUS) for the characterization of focal liver lesions—Prospective comparison in clinical practice: CEUS vs. CT (DEGUM multicenter trial). Ultraschall in der Medizin 30, 383–389.

Strahlenschutzkommission (2010) Orientierungshilfe für bildgebende Untersuchungen. Empfehlungen der Strahlenschutzkommission http://www.ssk.de/SharedDocs/Beratungsergebnisse_PDF/2008/Orientierungshilfe.pdf?__blob=publicationFile.

Strobel, D., Seitz, K., Blank, W., Schuler, A., Dietrich, C.F., von Herbay, A., Friedrich-Rust, M., Bernatik, T., 2009. Tumor-specific vascularization pattern of liver metastasis, hepatocellular carcinoma, hemangioma and focal nodular hyperplasia in the differential diagnosis of 1,349 liver lesions in contrast-enhanced ultrasound (CEUS). Ultraschall in der Medizin 30, 376–382.

The Royal College of Radiologists, 2012. iRefer. Making the best use of clinical radiology. The Royal College of Radiologists, London.

Wang, J., Zheng, J., Tang, T., Zhu, F., Yao, Y., Xu, J., Wang, A.Z., Zhang, L., 2015. A randomized pilot trial comparing position emission tomography (PET)-guided dose escalation radiotherapy to conventional radiotherapy in chemoradiotherapy treatment of locally advanced nasopharyngeal carcinoma. PLoS One 10, e0124018.

Wen, P.Y., Macdonald, D.R., Reardon, D.A., Cloughesy, T.F., Sorensen, A.G., Galanis, E., Degroot, J., Wick, W., Gilbert, M.R., Lassman, A.B., Tsien, C., Mikkelsen, T., Wong, E.T., Chamberlain, M.C., Stupp, R., Lamborn, K.R., Vogelbaum, M.A., van den Bent, M.J., Chang, S.M., 2010. Updated response assessment criteria for high-grade gliomas: Response assessment in neuro-oncology working group. Journal of Clinical Oncology 28, 1963–1972.

Zuley, M.L., Guo, B., Catullo, V.J., Chough, D.M., Kelly, A.E., Lu, A.H., Rathfon, G.Y., Lee Spangler, M., Sumkin, J.H., Wallace, L.P., Bandos, A.I., 2014. Comparison of two-dimensional synthesized mammograms versus original digital mammograms alone and in combination with tomosynthesis images. Radiology 271, 664–671.

Opisthorchis viverrini, *Clonorchis sinensis*, and Cholangiocarcinoma

Catherine de Martel and Martyn Plummer, International Agency for Research on Cancer, Lyon, France

Opisthorchis viverrini (O. viverrini) and *Clonorchis sinensis (C. sinensis)* are trematode (flatworm or fluke) parasites belonging to different species, but members of the same family, Opisthorchiidae. They are found in specific endemic areas in Eastern Asia and the Russian Federation. Because these flukes establish chronic infection within intrahepatic bile ducts, they are also commonly called "liver flukes". Both species are similar in morphology, life cycle, and mode of transmission. After years of chronic infection, the damage caused by the parasites may lead to the development of the bile duct cancer cholangiocarcinoma (CCA), which is the second most common primary liver cancer after hepatocellular carcinoma and bears a very poor prognosis. In 1994, the International Agency for Research on Cancer (IARC) classified *O. viverrini* as carcinogenic to humans (group 1 in the scale used by IARC to assess strength of evidence); *C. sinensis* was only classified as probably carcinogen to humans (group 2A) due to the limited number of human studies published at that time. In 2009 however, new data had become available and allowed the classification of *C. sinensis* in group 1 as well, with sufficient evidence in humans to conclude that both parasites cause CCA in endemic regions.

Geographical Distribution and Burden of Infection

Within areas of Eastern Asia and the Russian Federation, the geographic pattern of liver fluke infection is very uneven (Fig. 1), but high rates are more likely seen in rural than urban environments, especially in wetlands and agricultural areas. Prevalence of liver

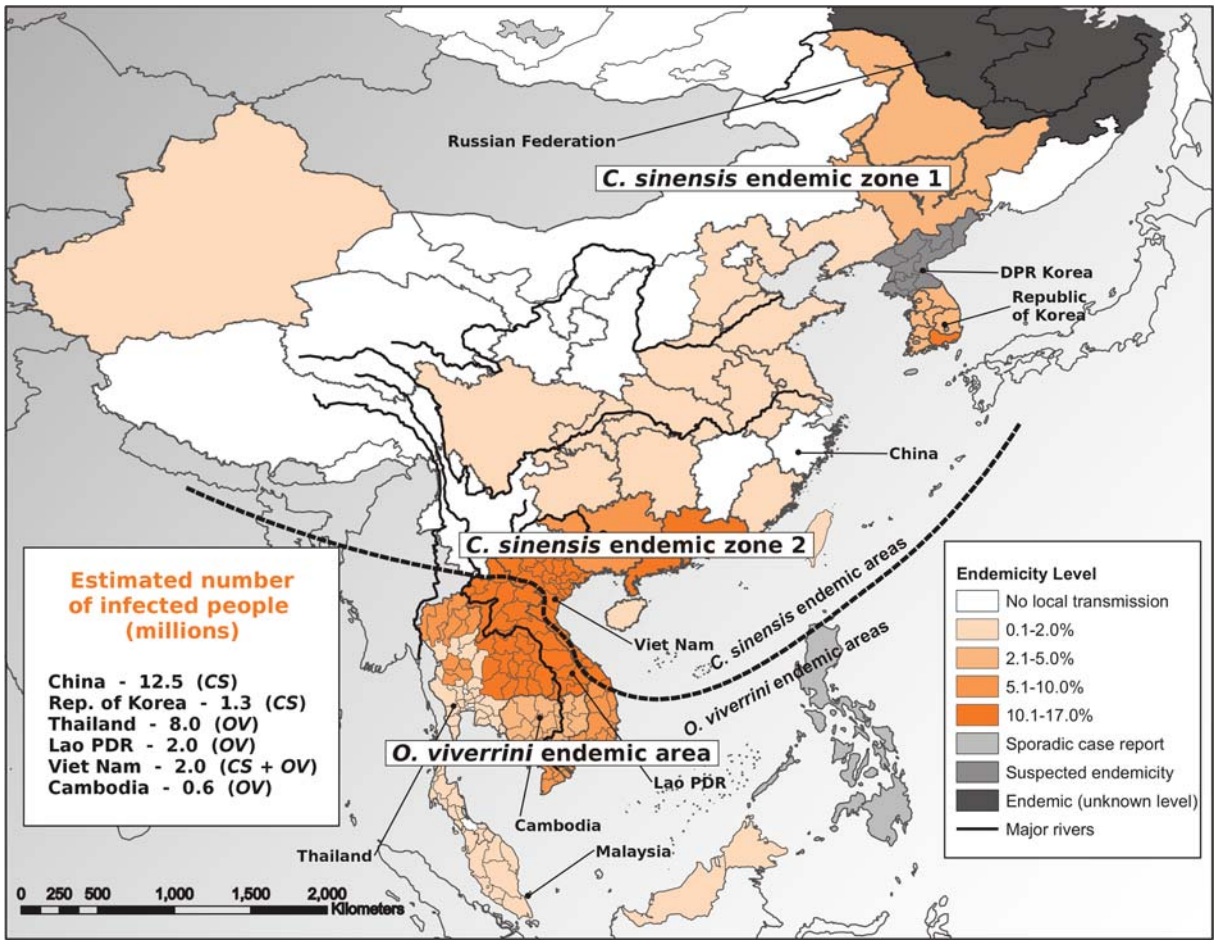

Fig. 1 Prevalence of liver flukes in Eastern Asia. Modified from *Opisthorchis Viverrini* and *Clonorchis Sinensis*. *IARC Monograph*, **100B**, 345. http://monographs.iarc.fr/ENG/Monographs/vol100B/index.php. Courtesy of Dr. Song Liang, Department of Environmental and Global Health, College of Public Health and Health Professions, University of Florida, USA, who did the art work based on data provided by the Working Group.

fluke infection depends on the distribution of fresh water, specific intermediary hosts (snail and fish), and local customs of eating traditional dishes made of raw or undercooked fish. Men are more frequently infected with liver flukes than women, especially in areas where *C. sinensis* infection is endemic: in these areas, the prevalence sex ratio (M/W) is around 2.0 whereas it is only around 1.2 for *O. viverrini* infection. Precise estimates of the number of infected people are extremely difficult to obtain as epidemiological studies are few and patchy, and the disease occurrence shows high variation within small geographical areas. For example, the results of field surveys in Kampong Chan province, Cambodia for the prevalence of *O. viverrini* showed that endemic villages (population prevalence of 30%) and non-endemic villages (population prevalence of 0%) coexisted within a distance of only 9 km, whereas the raw freshwater fish consumption rates were very high in all villages. Nationwide surveys prove very difficult to undertake, until the complex human and environmental factors that influence the distribution of liver fluke infection in endemic areas are fully understood.

C. sinensis

The world burden of *C. sinensis* infection is much higher than that of *O. viverrini*, as the parasite is found in a much wider area. *C. sinensis* is endemic in Eastern Asia, namely in China, the Republic of Korea, northern Viet Nam and eastern parts of the Russia Federation (see **Fig. 1**). It is also probably endemic in the Democratic People's Republic of Korea, and possibly in Malaysia. Although *C. sinensis* was highly prevalent in Japan decades ago, it is now extremely rarely seen there. Rare cases have also been reported in some other countries or areas as a result of tourism or immigration. In Viet Nam, *C. sinensis* is endemic in northern provinces, whereas *O. viverrini* is detected in central provinces.

Estimates of the total number of people infected with *C. sinensis* in the world have risen from 7 million in the 1990s to 15 million in 2004. This increase is due to several factors: population growth in major endemic areas during this period, the expansion of freshwater fish aquaculture, and better performing food distribution networks. One should add to these factors the difficulty for the elderly to modify their cultural habit of consuming undercooked or raw fish, while the young also become progressively accustomed to this habit. More recent estimates advance a total number varying between 12.5 and 35 million infected people worldwide, acknowledging that these numbers might only reflect the tip of the iceberg.

China alone bears the main burden of *C. sinensis* infection (over 80%). The parasite is found in 25 provinces but the population prevalence varies considerably, ranging from sporadic in some provinces (0.1% in Shandong or Sichuan provinces) to highly prevalent in others (70% in some areas from Guangdong province). The two main endemic areas are situated in the Southeast (Guangxi and Guangdong provinces) and in the Northeast (Heilongjiang province) (see **Fig. 1**). Note that some areas such as Hong Kong or Shanghai are not endemic for the parasite, but infections may still be acquired there—as in other non-endemic areas of China—by eating raw fish transported from endemic areas.

O. viverrini

O. viverrini is highly endemic in the northeastern region of Thailand, in Cambodia, in Southern regions of the Lao People's Democratic Republic, and in central Viet Nam (see **Fig. 1**). The number of people infected in these countries is estimated to be around 8 million in Thailand and 2 million in the Lao People's Democratic Republic. No national prevalence data are available in Viet Nam, but several hotspots for the infection have been recognized in the center of the country, including Nam Dinh and Ninh Binh provinces, with population prevalence of around 30%. Similarly, four Cambodian provinces (Takaev, Kandal, Kampong Cham, and Kampong Thom) have been identified as endemic areas of *O. viverrini* infection in a study conducted between 2006 and 2012 in five provinces, but nationwide surveys are still lacking, and other endemic area are yet to be precisely described.

Life Cycle of the Parasites

As food-borne trematodes, *O. viverrini* and *C. sinensis* have similar life cycles (see **Fig. 2**). People that have a habit of eating raw or uncooked freshwater fish constitute the final hosts for the worms, but so do a wide range of wild or domestic fish-eating animals such as dogs, pigs and cats. The elaborate life cycle of the parasite involves two separate intermediate hosts living in fresh water.

Adult liver flukes may live in their final hosts for years: in absence of treatment, their lifespan in humans can be as long as 25–30 years. Laid eggs are produced in the biliary tract by hermaphrodite adult worms (up to 4000 eggs per worm per day) and then eliminated with feces. If eggs happen to fall into freshwater reservoirs, they may then be ingested by specific aquatic snails, which constitute the primary intermediate hosts; eggs hatch into the snail as miracidiae, that is, ciliated larvae, and after a 90 days cycle of reproduction, cercariae, that is, free swimming larvae, escape from the snail into the water and adhere to the flesh of susceptible fish species, secondary intermediate hosts. Cercariae penetrate the subcutaneous tissues and muscle of the fish and slowly mature as metacercariae, its final larval form, within 45 days. The life cycle ends when definitive hosts (i.e., humans or other mammals) catch and eat raw or undercooked infected fish. Through gastric acidic digestion, the metacercariae separate from the fish, excyst in the duodenum of the host and migrate into intrahepatic bile ducts where they develop into adult worms.

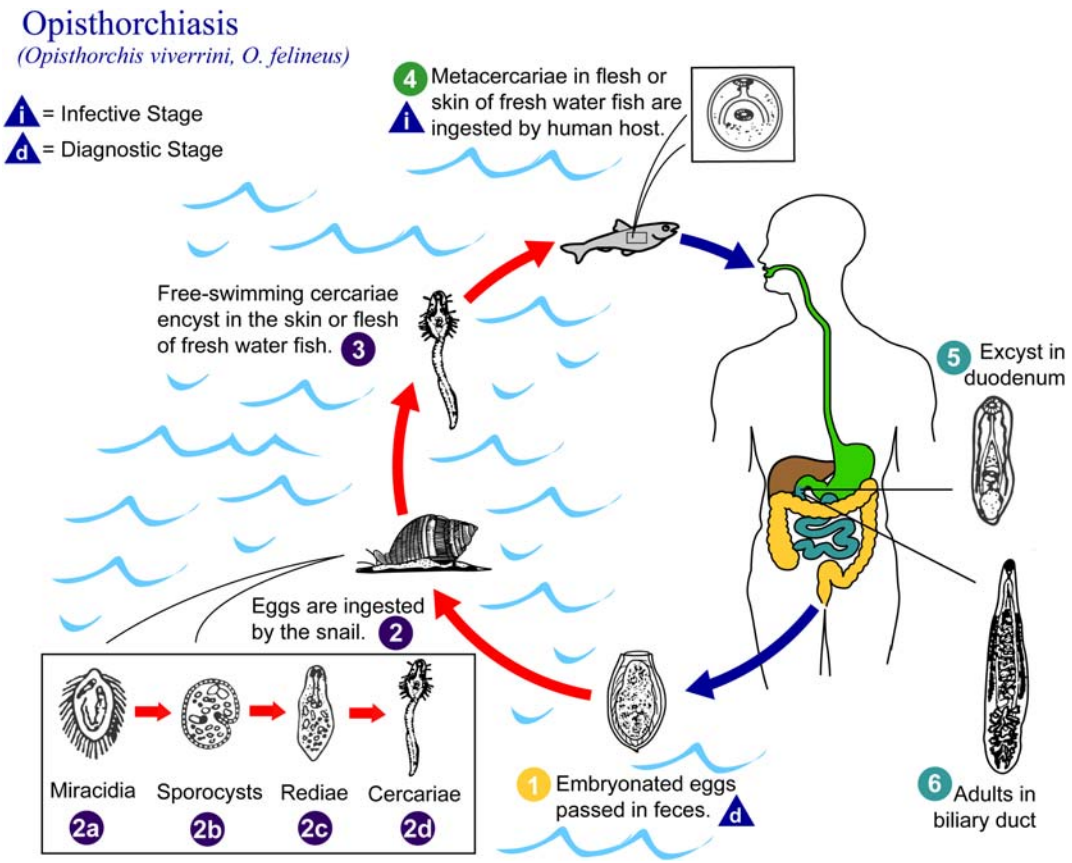

Fig. 2 Life cycle of *O. viverrini* and *C. sinensis*. From Centers for Disease Control and Prevention. https://www.cdc.gov/dpdx/opisthorchiasis/index.html and https://www.cdc.gov/dpdx/clonorchiasis/index.html. Courtesy of Alexander J. da Silva, PhD, and Melanie Moser.

Clinical Symptoms and Complications

O. viverrini and *C. sinensis* share the same clinical manifestations. The variations described in different geographical areas are more likely to reflect the duration and intensity of infection or the nutrition and comorbidity affecting the host than differences due to the species.

Clinical symptoms are related to the intensity of the infection. Infections involving < 100 adult worms are usually asymptomatic, but even in heavily infected hosts, only 10% show clinical manifestations. Common symptoms are unspecific and intermittent and may include asthenia, insomnia, pain in the right upper abdominal quadrant, nausea, anorexia, headache, dizziness, weight loss and diarrhea. If undertaken, abdominal ultrasound examination may show an enlarged gallbladder, and the presence of sludge and/or gallstones. Periductal fibrosis seen as periportal echoes along the intrahepatic biliary trees is also a common ultrasonographic feature in chronic liver fluke infections. In children, heavy infection may lead to slower growth or developmental retardation.

A small percentage of patients might develop complications, ranging from cholecystis, cholangitis, and pancreatitis, to cholangiocarcinoma. More typical symptoms of these complications may include fever, jaundice, hepatomegaly and liver tenderness.

Liver Flukes and Cholangiocarcinoma

The most severe complication of chronic liver fluke infection is the development of CCA. Many pathways could be implicated as the pathogenesis of the infection encompasses several factors. These include mechanical injuries of the biliary ducts by the suckers of the feeding worms; biliary obstruction by the adult parasites leading in turn to stasis and subsequent bacterial infections, including possibly colonization by *Helicobacter* species; chronic inflammation and excessive immune response from the host; and carcinogenic actions of the parasite's metabolic products such as Ov-GRN-1 secreted by *O. viverrini*, which can stimulate angiogenesis, suppress apoptosis, and promote tumor invasion.

Epidemiological studies (including correlation ecological studies, case series, and case control studies), animal studies (mostly in hamsters), and mechanistic evidence strongly indicate that liver flukes are causally related to CCA. Various attempts to quantify the

number of CCA cases attributable to liver flukes in Asia range from 1300 cases to 7000. All the authors who tried this exercise acknowledged that the estimate they provided was most probably well under the true number.

Pooled relative risks (RRs) from the available case control studies were estimated to range from 4 to 8 for both parasites. However, the RR of cholangiocarcinoma might be substantially underestimated by some methods of exposure assessment (i.e., detection of eggs in feces, or patient history self-testimony), both in the population, and in case control studies. Some evidence for this underestimate comes from the contrast between analytical and descriptive epidemiology. For example, the RR between 4 and 8 for *C. sinensis* from pooled case control studies is unable to account for the 60-fold variation in cholangiocarcinoma incidences between geographical regions. It is therefore probable that all statistical methods are underestimating the true burden with currently available data.

Diagnosis of Liver Flukes Infection

Liver fluke infection is suspected on the anamnestic description of recurrent clinical symptoms and raw fish consumption; blood cell count showing an elevated count of eosinophils—white cells whose function is to neutralize invading microbes, primarily parasites, and dilatation of the peripheral intrahepatic bile ducts shown by ultrasound, computed tomography or magnetic resonance imaging scans. Confirmation of diagnosis relies on different types of diagnostic techniques. In most areas, the diagnosis is mainly based on detection of eggs in feces by microscopy, usually using the Kato Katz technique. Trained parasitologists are however needed to make the identification. Even then, because the eggs are similar in size and are all oval and operculated, *C. sinensis* eggs cannot sometimes be differentiated from other Heterophyidae (e.g., *Haplorchis*, *Heterophyes*, etc.) or Opisthorchiidae eggs. The World Health Organization (WHO) has recommended the Kato Katz technique in areas with moderate to high endemicity (i.e., where the proportion of infected individuals is $>10\%$–50%). Where the prevalence is lower, the low specificity of this technique makes it less appropriate; more specific tools should be used, such as immunodiagnostic techniques and molecular methods. Of note, because endemic areas overlap with areas of high prevalence of hepatitis B virus infection, which shares some of its unspecific symptoms, liver fluke infection might be misdiagnosed as viral hepatitis, and coinfection of the flukes and the virus frequently happen in the same individuals. Whether liver flukes and hepatitis B virus co-infection have a synergistic effect on the development of CCA or hepatocellular carcinoma is plausible but has been little studied to date.

Individual Treatment and Population Control

Currently, the major strategies for community prevention and control encompasses fecal examination and treatment of individual cases with praziquantel, a highly effective broad-spectrum trematocidal and custodial drug. The recommended dose is 25 mg/kg three times daily for 2–3 days. A single dose of 40 mg/day has been used in preventive chemotherapy programs and resulted in 95% cure rate of light infections, but only 89% and 69% in intermediate and heavy infections, respectively.

For the purposes of public health control, WHO recommends carrying out community screening and diagnosis at the district level, and implementing preventive chemotherapy with praziquantel at a dosage of 40 mg/kg, single administration, as indicated in Table 1.

When treatment is delivered, information, education, and communication (IEC) should be given to the population to enhance sustainability by avoiding constant reinfection. Education may be a real challenge in areas where raw fish consumption is a strong tradition, or where people wrongly believe that drinking alcohol or consuming spicy food can prevent infection. No vaccine candidate has yet shown efficacy in animal models, and the control of human disease through vaccination is not an option in the near future. Environmental control is equally challenging and may encompass many measures including prevention of fecal contamination of fresh water, or, in the future, innovative techniques such as GIS-Based spatial identification of risk areas for liver flukes, or oral vaccination of the secondary intermediate hosts (fish) using probiotics.

Table 1 WHO recommendations for community diagnosis

District	*Prevalence of infection in the sample population*[a]	*Action to be taken*	
		Type of intervention and target population	*Interval of re-treatment*
High risk	≥ 20	Universal treatment (MDA)—Treat all individuals in the district	12 months
		Option 1: universal treatment (MDA)—Treat all individuals in the district	24 months
Low risk	<20	*Option 2*: targeted treatment—Treat all individuals in the district who report habitually eating raw fish	12 months

[a]The Kato-Katz thick smear technique should be used.Abbreviation: MDA, mass drug administration.

From http://www.who.int/foodborne_trematode_infections/clonorchiasis/clonorchiasis_more/en/ and http://www.who.int/foodborne_trematode_infections/opisthorchiasis/viverrini_more/en/.

See also: Cancer Disparities. Cancer-Related Inflammation in Tumor Progression.

Further Reading

Fürst, T., Keiser, J., Utzinger, J., 2012. Global burden of human food-borne trematodiasis: A systematic review and meta-analysis. The Lancet Infectious Diseases 12, 2010–2021.
IARC, 2012. *Opisthorchis viverrini* and *Clonorchis sinensis*. In biological agents. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 100B, 342–370. http://monographs.iarc.fr/ENG/Monographs/vol100B/index.php.
Lai, D.H., Hong, X.K., Su, B.X., et al., 2016. Current status of *Clonorchis sinensis* and clonorchiasis in China. Transactions of the Royal Society of Tropical Medicine and Hygiene 110, 21–27.
Plummer, M., de Martel, C., Franceschi, S., 2017. Global burden of cancers attributable to liver flukes. The Lancet Global Health 5, e140.
Qian, M.B., Utzinger, J., Keiser, J., Zhou, X.N., 2016. Clonorchiasis. The Lancet 387, 800–810.

Relevant Website

http://www.who.int/foodborne_trematode_infections/en/—WHO webpage on foodborne infection.

Oral and Oropharyngeal Cancer: Pathology and Genetics

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Abbreviation
HPV Human papilloma virus

Oral Squamous Cell Carcinoma

Definition

A carcinoma with squamous differentiation arising from the mucosal epithelium. Recognized subtypes are: verrrucous carcinoma, basaloid squamous cell carcinoma, papillary squamous cell carcinoma, spindle cell squamous cell carcinoma, adenosquamous carcinoma and lymphoepithelial carcinoma. Subtypes only will be discussed regarding their pathologic features as they are with a few exceptions morphological variants without any proven relationship with other clinicopathological variables.

Burden

Squamous cell carcinoma represents $>90\%$ of cancers in the oral cavity. With respect to epidemiology, specific geographical regions must be considered separately, because there is a marked variation in incidence. A high incidence of oral cancer is found in southern Asia with age-standardized incidence rates of >10 cases per 100,000 population per year. Worldwide, oral cancer incidence is higher among males (5.5 cases per 100,000 population per year) than females (2.5 cases per 100,000). Most oral cancers occur in patients aged 50–70 years.

Risk Factors

Smoking is by far the most important cause of oral cancer. Alcohol consumption interacts synergistically with smoking, resulting in a more than additive risk. In some parts of the world, the use of areca nut whether or not mixed with other substances represents an important risk factor. In contrast to the oropharynx, oral cavity squamous cancers only rarely harbor high-risk HPV.

Pathology

Most cancers of the oral cavity and mobile tongue are moderately or well differentiated; poorly differentiated squamous cell carcinoma is less frequent. Well-differentiated OSCC is characterized by nests, cords, and islands of large cells with pink cytoplasm, prominent intercellular bridges, and round nuclei, which may not be obviously hyperchromatic. Dyskeratotic cells and squamous pearls are prominent. Well-differentiated tumors tend to invade in large islands, whereas less-differentiated tumors tend to have small islands and dispersed individual cells at the invasive front (Figs. 1 and 2).

Pathology of Subtypes

Verrucous Carcinoma

Verrucous carcinoma consists of blunt projections and invaginations of well-differentiated squamous epithelium, composed of one to several layers of basal cells and an expanded layer of spinous cells that lack cytological atypia. There is marked surface keratinization. Mitoses are rare and confined to the basal cell layer, and there are no abnormal mitoses. Verrucous carcinoma invades the stroma with a well-defined pushing border (Fig. 3). Verrucous carcinoma has a better prognosis than conventional oral squamous cell carcinoma.

Basaloid Squamous Cell Carcinoma

Basaloid squamous cell carcinoma consists of rounded nests with smooth borders and peripheral palisading. Gland-like foci with basophilic myxoid or mucoid material are common and mimic true gland formation. A variable degree of nuclear pleomorphism is present, and high mitotic activity, apoptosis, and necrosis are common. Also, comedonecrosis may be present. Stromal hyalinization is characteristic; it can be linear between and around nests and nodular within nests. Keratinization occurs abruptly adjacent to

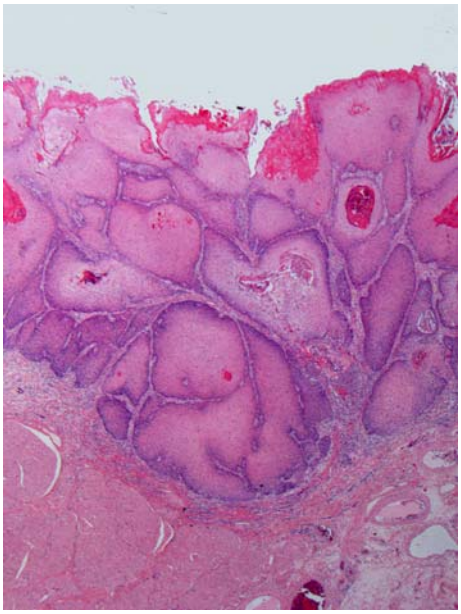

Fig. 1 Well-differentiated OSCC arises from the surface epithelium and invades the submucosal tissues as large keratinizing tumor nests.

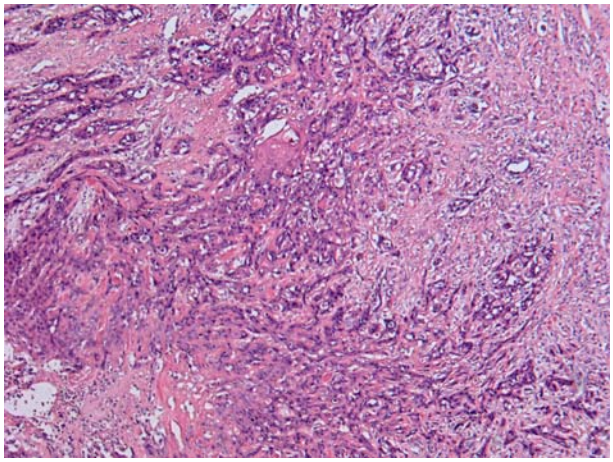

Fig. 2 Poorly-differentiated OSCC lacks significant keratinization and irregularly infiltrates the stroma as small cords or individual cells.

basaloid cells (Fig. 4). Whether basaloid squamous cell carcinoma has a worse prognosis than conventional squamous cell carcinoma is an issue that has not yet been definitely settled.

Papillary Squamous Cell Carcinoma

Papillary squamous cell carcinoma is characterized by a papillary growth pattern, with thin fibrovascular cores covered with malignant epithelial cells (Fig. 5). Two types of surface epithelium are described: one resembles high-grade keratinizing epithelial dysplasia, and in the other, the epithelial cells are immature and basaloid, with no evidence of maturation or keratinization. This type of squamous cell carcinoma has a better prognosis than conventional squamous cell carcinoma, primarily due to low-stage presentation, with a low metastatic potential.

Spindle Cell Squamous Cell Carcinoma

Spindle cell squamous cell carcinoma typically grows as an exophytic mass with a predominantly ulcerated surface, sometimes containing remnants of dysplastic squamous epithelium and frequently showing areas of transition from squamous cells to malignant spindled or pleomorphic tumor cells with hypercellularity, necrosis, atypical mitotic figures, and hyperchromatic nuclei (Fig. 6). Some cases exhibit heterologous mesenchymal differentiation in the form of malignant bone, cartilage, or skeletal muscle.

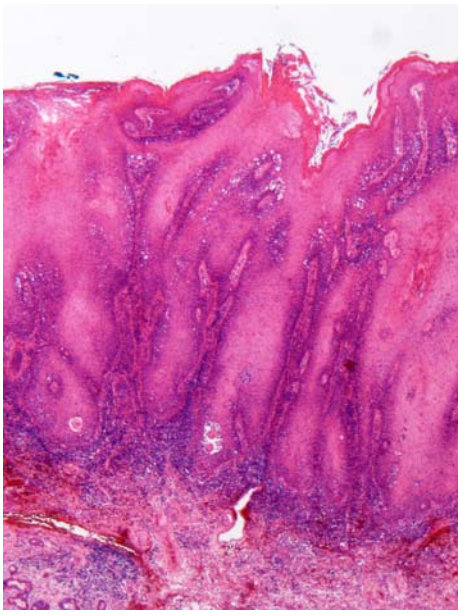

Fig. 3 Verrucous carcinoma exhibits prominent surface keratinization and a broad, pushing pattern of stromal invasion.

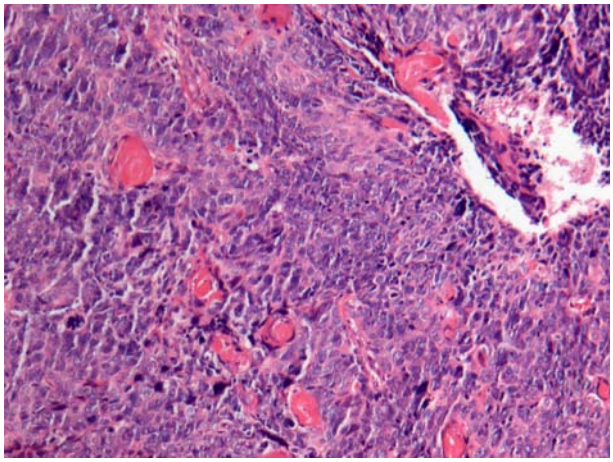

Fig. 4 Basaloid squamous cell carcinoma consists of basaloid cells with high nuclear:cytoplasmic ratios demonstrating abrupt keratinization.

Occasionally, spindle cell squamous cell carcinoma may have deceptively bland appearance, mimicking reactive myofibroblastic proliferation or granulation tissue. The prognosis of this subtype does not differ from conventional squamous cell carcinoma.

Adenosquamous Carcinoma

Adenosquamous carcinoma shows both squamous and glandular differentiation. The adenocarcinoma consists of cribriform and tubuloglandular structures and tends is mainly restricted to the deeper parts of the tumor (Fig. 7). This subtype is rare in the oral cavity, with larynx and hypopharynx being more common sites. Prognosis is worse than for conventional squamous cell carcinoma.

Lymphoepithelial Carcinoma

Lymphoepithelial carcinoma is a squamous cell carcinoma subtype morphologically similar to non-keratinizing nasopharyngeal carcinoma, undifferentiated subtype. The tumor is characterized by large cells with round to oval vesicular nuclei, and large central nucleoli. The neoplastic cells generally have scant amphophilic or eosinophilic cytoplasm. Unlike in the nasopharynx, lymphoepithelial carcinoma in the oral cavity appears to be unrelated to EBV. Lack of data on outcome precludes any firm statements.

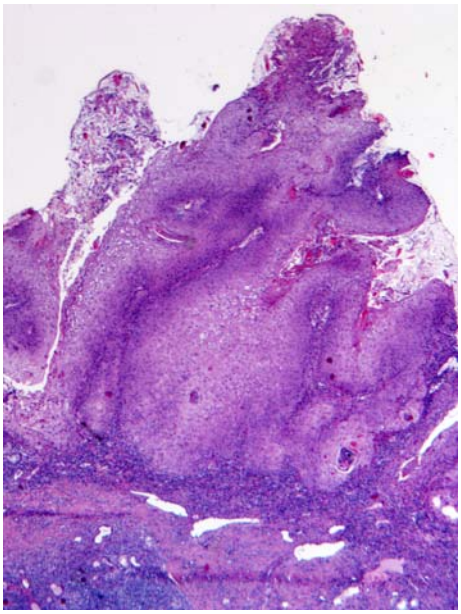

Fig. 5 Papillary squamous cell carcinoma grows as exophytic fronds of malignant squamous epithelium surrounding fibrovascular cores.

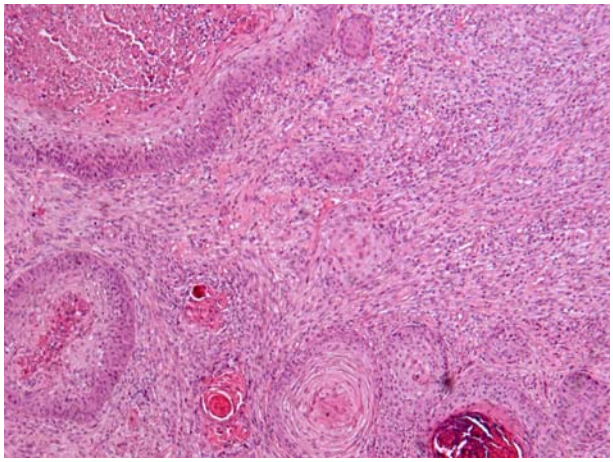

Fig. 6 Spindle cell squamous cell carcinoma has a component of conventional squamous cell carcinoma and a separate component of undifferentiated malignant spindle cells.

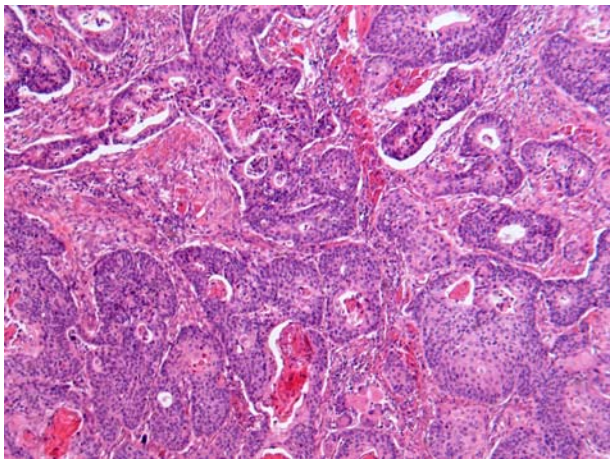

Fig. 7 Adenosquamous carcinoma demonstrates clear-cut glandular differentiation, particularly in the deeper portions of the tumor.

Molecular Pathology and Genetics

Most OSCCs are genetically unstable with chromosomal loss at 3p, 8p, 9p, 17p and gains at 3q and 11q. These changes may extend for some distance from the clinical lesion, underpinning the clinical phenomenon of field cancerization. Genes reported to have a role in OSCC (e.g., *TP53*, *CDKN2A*, *PTEN*, *HRAS*, *PIK3CA*, and *NOTCH1*) are mutated with sufficient frequency to support their potential role as drivers in cancer development.

Microenvironment Including Immune Response

Data regarding the immune microenvironment of oral squamous cell carcinoma is limited. A few studies have reported a subset of these cancers expressing the immune checkpoint receptor PD-L1.

Staging and Grading

Most cases of oral squamous cell carcinoma are moderately differentiated. There is no clear relationship between grading and prognosis. There are some indications however that evaluation of the invasive tumor front may have some prognostic relevance, with growth in tiny strands being worse than invasion in a pushing border. Staging is done conforming the most recent UICC guidelines (8th edition).

Prognostic and Predictive Biomarkers

An important prognostic factor is the status of surgical resection margins. Resection margins clear of tumor are associated with a lower recurrence rate and a better survival. An adequate margin of resection has not been precisely defined, but for oral squamous cell carcinoma margins of 5 mm are generally believed to be adequate.

Other significant prognostic factors are tumor size, nodal status, and distant metastasis. Histological risk factors associated with a worse prognosis include a non-cohesive growth pattern at the invasion front, perineural and lymphovascular invasion, bone invasion, and tumor thickness >4 mm. High-grade dysplasia at a mucosal margin correlates with local recurrence and second primary tumors. Extracapsular spread from metastasis in the neck, two or more positive nodes, and involvement of lower neck worsen prognosis. Conventional histological grading correlates poorly with clinical outcome.

Prospective Vision

Current research on oral squamous cell carcinoma has included work on improving early detection through screening saliva or serum for biomarkers, refining risk progression models particularly for low-stage cancers that behave unpredictably, and investigating novel treatment strategies like targeted therapies and immune checkpoint inhibition to improve a prognosis that has largely remained unchanged for decades.

Oropharyngeal Squamous Cell Carcinoma

Definition

Oropharyngeal squamous cell carcinoma is an epidemiologically, pathologically, and clinically distinct form of head and neck squamous cell carcinoma associated with high-risk HPV.

Burden

The incidence of oropharyngeal carcinoma has risen over the past three decades. Most patients are middle-aged males.

Risk Factors

Oropharyngeal squamous cell carcinoma is caused by high-risk HPV, with type 16 responsible for $>90\%$ of all cases. Oral sex is an established risk factor for oral HPV infection.

Pathology

HPV associated oropharyngeal squamous cell carcinoma generally exhibits a distinctive non-keratinizing morphology (Fig. 8). The tumor originates in the crypt epithelium and grows beneath the surface epithelial lining as nests and lobules. Tumor nests are often embedded in lymphoid stroma, and may be penetrated by lymphoid cells. Similar to squamous cell cancer of the oral cavity, the

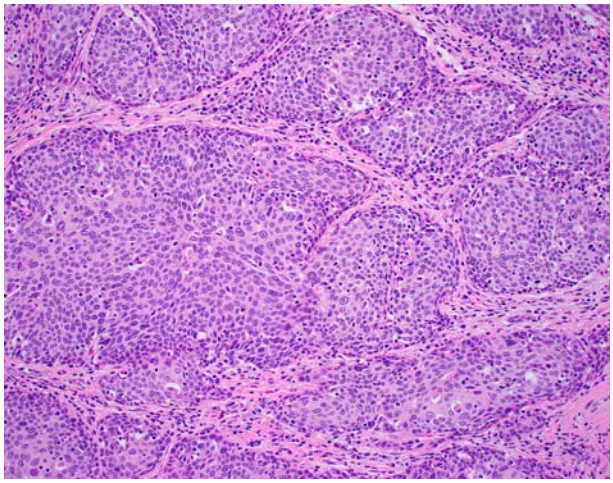

Fig. 8 HPV associated oropharyngeal squamous cell carcinoma grows as lobules of primitive cells with high nuclear:cytoplasmic ratios and limited keratinization.

morphological spectrum includes variants with papillary, adenosquamous, lymphoepithelioma-like, and spindle cell features. The clinical behavior of these morphological variants is the same as for the cases showing the typical histomorphology.

Molecular Pathology and Genetics

HPV oncoproteins E6 and E7 inactivate p53 and RB by targeting them for protein degradation, thus disabling the protective effects of these tumor suppressor genes. Oropharyngeal HPV-driven cancer has a gentic profile that differs from smoking-driven squamous cell carcinomas, with fewer mutations overall and fewer *TP53* mutations, but more frequent *PIC3CA* mutation or amplification and occasional *TRAF3* mutations not seen in HPV-unrelated tumors.

Microenvironment Including Immune Response

HPV associated oropharyngeal carcinomas arise from the specialized tonsillar crypt epithelium. These crypts have a distinct and complex immune environment that is not fully understood. As one important example, the immune checkpoint receptor PD-L1 is selectively expressed in the tonsillar crypt epithelium, seemingly creating an immune-privileged site where the effector function of virus-specific T cells is downmodulated, facilitating immune evasion by HPV.

Staging and Grading

Histologic grading is not currently advocated. Staging is done conforming the most recent UICC guidelines (8th edition).

Prognostic and Predictive Biomarkers

HPV associated oropharyngeal squamous cell carcinoma shows a better prognosis than the HPV negative tumors seen at this site but more commonly in the oral cavity. This improved prognosis is diminished in smokers with HPV associated oropharyngeal squamous cell carcinoma.

Prospective Vision

Future research directions in HPV associated squamous cell carcinoma research include early detection (made challenging by a lack of pre-malignant epithelial changes), novel treatment strategies like de-escalation radiation therapy or immunotherapy, and the long-term effects of routine HPV vaccination on the incidence of these carcinomas.

See also: Cancers of the Oral Cavity: Diagnosis and Treatment. Nasopharyngeal Carcinoma: Diagnosis and Treatment.

Further Reading

Brierly, J.D., Gospodarowicz, M.K., Wittekind, C. (Eds.), 2017. TNM classification of malignant tumours, 8th edn. Wiley-Blackwell, Chichester.

Gale, N., Zidar, N., Cardesa, A. and Nadal, A. (2016). Benign and potentially malignant lesions of the squamous epithelium and squamous cell carcinoma. In: Cardesa A., Slootweg P.J., Gale N. and Franchi A. (eds.) Pathology of the head and neck. 2nd edn, pp. 1–48. Heidelberg: Springer.

Lydiatt, W.M., Patel, S.G., O'Sullivan, B., et al., 2017journal. Head and neck cancers – major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA: a Cancer Journal for Clinicians 67, 122–137.

Lyford-Pyke, S., Peng, S., Young, G.D., et al., 2013journal. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Research 73, 1733–1741.

Ramos-García, P., Ruiz-Ávila, I., Gil-Montoya, J.A., et al., 2017journal. Relevance of chromosomal band 11q13 in oral carcinogenesis: an update of current knowledge. Oral Oncology 72, 7–16.

Rivera, C., Oliveira, A.K., Costa, R.A.P., et al., 2017journal. Prognostic biomarkers in oral squamous cell carcinoma: a systematic review. Oral Oncology 72, 38–47.

Sloan, P., Gale, N., Hunter, K., et al., 2017book. Tumours of the oral cavity and mobile tongue. In: El-Naggar, A.K., Chan, J.K.C., Grandis, J.R., Takata, T., Slootweg, P.J. (Eds.), WHO classification of head and neck tumours, 4th edn. Lyon, IARC, pp. 105–131.

The Cancer Genome Atlas Network, 2015journal. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 517, 576–582.

Westra, W.H., Boy, S., el-Mofty, S.K., et al., 2017book. Tumours of the oropharynx (base of tongue, tonsils, adenoids). In: El-Naggar, A.K., Chan, J.K.C., Grandis, J.R., Takata, T., Slootweg, P.J. (Eds.), WHO classification of head and neck tumors, 4th edn. Lyon, IARC, pp. 133–146.

Oral Cavity Cancer: Diagnosis and Treatment

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Glossary

Alveolar ridge One of the two jaw ridges either on the roof of the mouth between the upper teeth and the hard palate or on the bottom of the mouth behind the lower teeth.
Erythroplakia Chronic, red, generally asymptomatic lesion or patch on the mucosal surface that cannot be attributed to a traumatic, vascular, or inflammatory cause.
Extranodal extension Extension of metastatic carcinoma from within a lymph node through the fibrous capsule and into the surrounding connective tissue.
Interstitial brachytherapy Radioactive material inserted directly into body tissue.
Leukoplakia A white patch or plaque on the mucosal surface that cannot be rubbed off and may represent hyperplastic or dysplastic tumors.
Retromolar trigone A paired triangular area situated posterior to the third mandibular molars on the left and right sides of the oral cavity.

Nomenclature

ABS American Brachytherapy Society
AIDS Acquired immunodeficiency syndrome
AJCC American Joint Committee on Cancer
ARO Arbeitsgemeinschaft Radiologische Onkologie (i.e., Association of Radiological Oncology)
ASCO American Society of Clinical Oncology
CI Confidence interval
CT Computed tomography
EGFR Epidermal growth factor receptor
EORTC European Organization for Research and Treatment of Cancer
ESTRO European Society for Therapeutic Radiology and Oncology
GEC Groupe Européen de Curiethérapie (i.e., European Brachytherapy Group)
GITR Glucocorticoid-induced tumor necrosis factor receptor-related protein
GORTEC Groupe d'Oncologie Radiothérapie Tête et Cou (i.e., Radiotherapy Oncology Group Head and Neck)
Gray (Gy) Joule per kilogram
HDR High dose rate
HIGRT Hypofractionated image-guided radiation therapy (HIGRT)
HPV Human papillomavirus
HR Hazard ratio
HSV Herpes simplex virus
IDO Indoleamine-pyrrole 2,3-dioxygenase
IGRT Image-guided radiotherapy
IMRT Intensity modulated radiotherapy
KIR Killer-cell immunoglobulin-like receptor
MACH-NC Meta-analysis of chemotherapy in head and neck cancer
MARCH Meta-analysis of radiotherapy in carcinoma of the head and neck
MRI Magnetic resonance imaging
NCCN National Comprehensive Cancer Network
NCDB National Cancer Database
NRG National Clinical Trials Network group created through the coordinated efforts of the National Surgical Adjuvant Breast and Bowel Project (*N*SABP), the Radiation Therapy Oncology Group (*R*TOG), and the Gynecologic Oncology Group (*G*OG)
OCAT Oral Cavity Adjuvant Therapy trial
PD-1 Programmed cell death protein-1

PET Positron emission tomography
PORT Postoperative radiotherapy
RTOG Radiation Therapy Oncology Group
SABR Stereotactic ablative radiotherapy
SBRT Stereotactic body radiation therapy
SEER Surveillance Epidemiology and End Results
TIGIT T-cell immunoreceptor with Ig and ITIM domains
VMAT Volumetric modulated arc therapy

Introduction

The oral cavity consists of the oral vestibule and oral cavity proper. Its primary function is to serve as the entrance of the alimentary tract and to initiate the digestive process. It also serves as a secondary respiratory conduit, a site of sound modification for the production of speech, and a chemosensory organ. The development of a primary malignancy within the oral cavity as well as subsequent cancer treatment can affect these normal functions.

While cancers of the oral cavity only account for approximately 2% of all new cancer diagnoses in the United States, it is the most common site of all head and neck cancers (Siegel et al., 2017). In India, oral cavity cancer is among the three most common malignancies, accounting for over 30% of all reported malignancies in the country due to the high prevalence of chewing and smoking various carcinogens (Sankaranarayanan et al., 2005; Coelho, 2012). Surveillance Epidemiology and End Results (SEER) program data estimate 32,670 cases of cancer of the oral tongue, mouth, and oral cavity for 2017 in the United States (Siegel et al., 2017). An estimated 300,400 new cases and 145,400 deaths from cancer of the oral cavity and lip occurred worldwide in 2012 (Torre et al., 2015). Oral cavity cancer incidence rates worldwide have increased in many countries with tobacco epidemics that are currently peaking, but incidence rates have declined significantly among both males and females in areas where tobacco use peaked previously (Warnakulasuriya, 2009; Chaturvedi et al., 2013; Franceschi et al., 2000; Simard et al., 2014). Incidence rates for oral cancer sites related to human papillomavirus (HPV) infections are increasing in some countries, hypothesized to be in part due to changes in oral sexual behavior (Chung et al., 2014; **Fig. 1**).

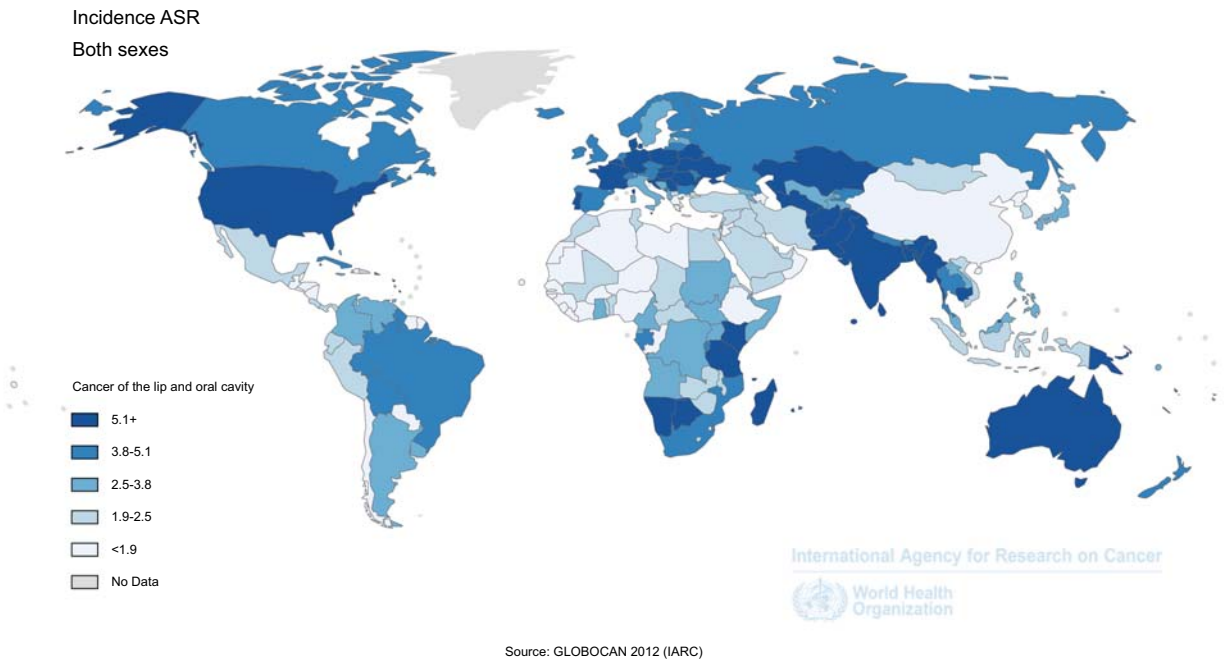

Fig. 1 Incidence of oral cavity cancer by country. Reproduced with permission from Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D. and Bray, F. (2013). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; Available from: http://globocan.iarc.fr, accessed on 24 Nov 2017.

Subsites within the oral cavity from which a primary malignant neoplasm can develop include the lips, oral tongue, floor of the mouth, hard palate, retromolar trigone, alveolar ridge, buccal mucosa, and very rarely the teeth. Classification of tumors by subsite is useful because patterns of spread, specific treatment, and clinical outcomes vary for each specific subsite.

Anatomy

The oral cavity begins anteriorly at the junction of the skin and vermilion border of the lips. The roof, or superior portion, of the oral cavity extends posteriorly to the junction between the hard and soft palate. The floor, or inferior portion, of the oral cavity extends posteriorly to the V-shaped sulcus terminalis. Other than the mandibular periosteum, there is no other finite fascial plane to inhibit a tumor's extension and invasion in the oral cavity. The specific anatomic subsites of the oral cavity are outlined in the following sections (Fig. 2).

Lips

The lips are the anterior most aspect of the oral vestibule. The lips begin externally at the well demarcated transitional zone between the facial skin and the vermillion surface of the lips known as the vermilion border. The vermillion surface is separated into an outer darker dry vermilion and an interior pale wet vermilion. The upper and lower lips connect to one another by the labial commissure. The orbicularis oris muscle comprises the substance of the lips and is innervated by the buccal branches of the facial nerve. Upper lip sensation is provided by the infraorbital nerve, which is a branch of the maxillary division of the trigeminal nerve, whereas lower lip sensation is provided by the mental nerve, which is a branch of the mandibular division of the trigeminal nerve. The first echelon draining lymphatics of the lower lip are the submental, submandibular, subdigastric, and rarely facial nodes. Mental foramen adenopathy is rare and indicates mental nerve involvement. The first echelon draining lymphatics of the upper lip are the submandibular nodes and occasionally preauricular, parotid, and buccal nodes. The second echelon draining lymphatics for both lips are the upper anterior cervical nodes, though occasionally direct spread may occur.

Oral Tongue

Only the anterior two-thirds of the tongue are considered part of the oral cavity. The terminal sulcus, situated in a V-shaped configuration immediately posterior to the circumvallate papillae, demarcates the division between the oral tongue (i.e., anterior two-thirds) and the base of tongue (i.e., posterior one-third). The oral tongue can be subdivided into four anatomic areas: the tip, lateral borders, dorsal surface, and ventral surface. The fibrous septum divides the tongue into right and left halves. The oral tongue mucosa covers paired intrinsic and extrinsic muscles. There are four paired intrinsic muscles which alter the shape of the tongue during speech and swallowing: superior longitudinal, inferior longitudinal, vertical, and transverse muscles. There are three paired extrinsic muscles of the oral tongue which allow for protrusion, retraction, and side-to-side movement: genioglossus, hyoglossus, and styloglossus. Motor innervation is provided entirely by the paired hypoglossal nerves. General sensation of the anterior two-thirds of

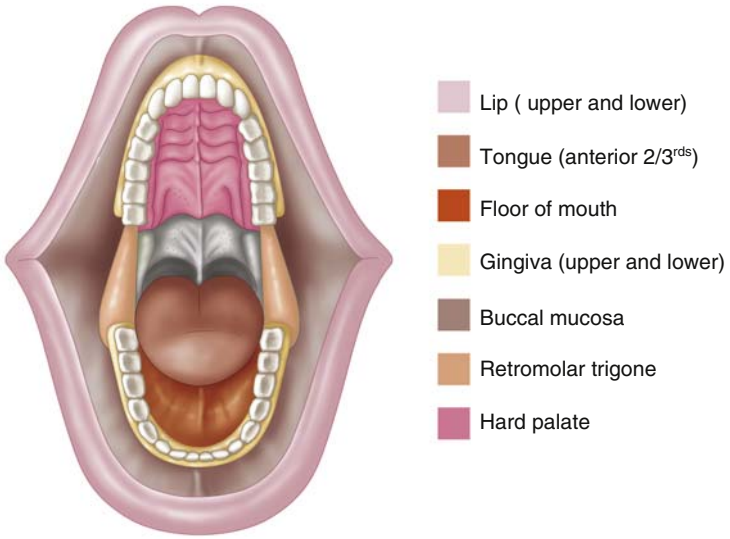

Fig. 2 Anatomic sites of the oral cavity. From Montero, P. H. and Patel, S. G. (2015). Cancer of the oral cavity. *Surgical Oncology Clinics of North America* **24**(3), 491–508.

the tongue is supplied by the lingual nerve, a branch of the mandibular division of the trigeminal nerve. Taste fibers from the anterior two-thirds of the tongue travel in the chorda tympani branch of the facial nerve. The glossopharyngeal nerve provides both sensation and taste to the circumvallate papillae and posterior third of the tongue. The blood supply to the oral tongue is primarily via the lingual artery, with primary drainage into the internal jugular vein via the lingual veins. The first echelon draining lymphatics for the anterior portion of the oral tongue are the submandibular, subdigastric, and anterior middle cervical nodes, with the tip of the tongue also draining to the submental nodes. The first echelon draining lymphatics for the posterior portion of the oral tongue are the submandibular and subdigastric nodes. Typically anterior tumors tend to spread lower in the neck than posterior tumors. Additionally, tumors located on the lateral third of each half of the oral tongue tend to spread ipsilaterally, whereas tumors located more medially tend to spread bilaterally. Skip metastases to inferior cervical lymph nodes have also been commonly reported for anterior oral tongue cancers (Byers et al., 1997; Woolgar, 1997; **Fig. 3**).

Floor of Mouth

The floor of mouth is bounded anteriorly and laterally by the lower gingiva, medially by the oral tongue, and posteriorly at the insertion of the anterior tonsillar pillar into the oral tongue. The mylohyoid, genioglossus, and geniohyoid muscles comprise the muscular floor of the oral cavity. This region is divided into right and left halves by the lingual frenulum and contains the ostia of the submandibular and sublingual salivary glands. The sublingual glands lie immediately beneath the floor of mouth mucosa and are separated by the genioglossus and geniohyoid muscles. The submandibular glands lie on the external surface of the mylohyoid muscle near its insertion to the mandible and terminate at Wharton's (submandibular) ducts, which are approximately 5 cm long and course between the sublingual gland and genioglossus muscle before exiting near the anterior floor of mouth at midline bilaterally. Motor innervation is provided by the hypoglossal nerves, whereas sensory innervation is provided by the lingual nerve. The first echelon draining lymphatics are the submandibular and subdigastric nodes. Lymphatic spread to the submental nodes is rare. The second echelon draining lymphatics are the middle and lower anterior cervical nodes.

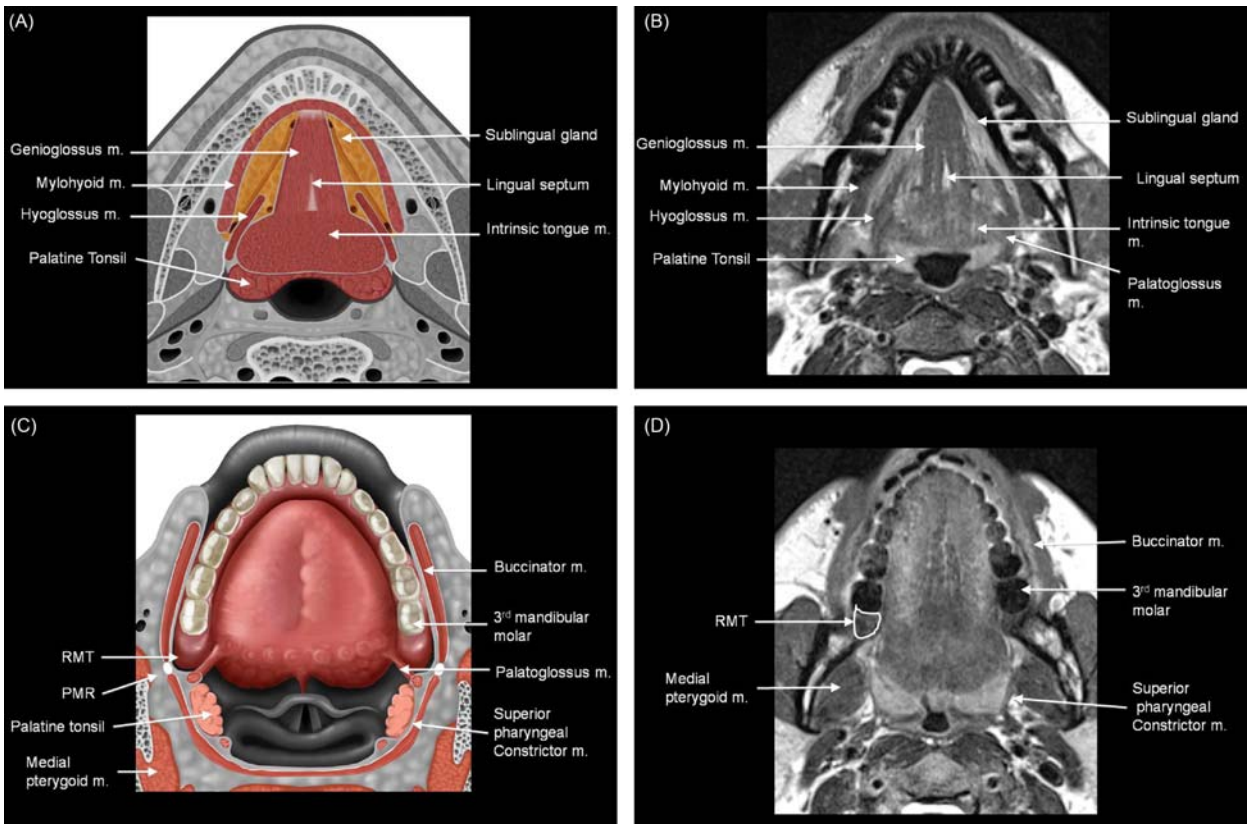

Fig. 3 Axial magnetic resonance images of normal oral cavity anatomy. (A) Axial illustration at the level of the floor of mouth demonstrating the extrinsic tongue muscles and sublingual space. (B) Axial T2-weighted image at the level of the floor of mouth. (C) Axial illustration at the level of the oral tongue demonstrating the oral tongue and retromolar trigone (RMT). PMR, pterygomandibular raphe (facial band connecting the buccinator and superior constrictor muscles). (D) Axial T2-weighted image at the level of the oral tongue. From Aiken, A. H. (2013). Pitfalls in the staging of cancer of oral cavity cancer. *Neuroimaging Clinics of North America* **23**(1), 27–45.

Hard Palate

The hard palate is the roof of the oral cavity and separates this site from the nasal cavity. It extends posteriorly and medially from the maxillary alveolar ridge to the posterior edge of the palatine bone, creating a semilunar arch shape. The bony structure is formed by the palatine processes of the maxilla and the horizontal plates of the palatine bones. Centrally, the periosteum is covered by a firmly attached mucosa, while peripherally there is a minimal submucosa containing vasculature, which may result in an increased risk of spread. The hard palate is continuous with the soft palate posteriorly, which is excluded from the oral cavity proper. Vascular supply is provided by the greater palatine artery, which anastomoses with the nasopalatine artery. Veins from the hard palate terminate in the pterygoid venous plexus. The hard palate is innervated by the greater palatine and nasopalatine nerves, two branches of the maxillary division of the trigeminal nerve, both of which initially pass through the pterygopalatine ganglion. The paired greater palatine arteries and nerves, along with secretomotor fibers to the salivary glands on the posterior hard palate, enter the oral cavity via the greater palatine foramen located medial to the third molar. The nasopalatine nerve descends through the incisive foramen to supply the most anterior parts of the hard palate. The first echelon draining lymphatics are the submandibular or upper deep cervical nodes. The midline hard palate may drain bilaterally. Retropharyngeal nodes may rarely be involved (Fig. 4).

Alveolar Ridge

The superior (maxillary) and inferior (mandibular) alveolar ridges include the alveolar processes and overlying mucosa. The mucosa covering the inferior alveolar ridge extends from the lower gingivobuccal sulcus to the floor of the mouth, and further extends posteriorly to the ascending ramus of the mandible. The mucosa covering the superior alveolar ridge extends from the upper gingivobuccal sulcus to the junction of the hard palate, and further extends posteriorly to the upper end of the pterygopalatine arch. General sensory innervation of the maxillary and mandibular gingiva is from branches of the maxillary and mandibular divisions of the trigeminal nerve, respectively. The lymphatic drainage of the alveolar ridges is to the submental, submandibular, upper deep jugular, and retropharyngeal nodes.

Teeth

Teeth extend from within the alveolar ridges and erupt from the gingiva. Teeth are divided into four quadrants using descriptors of right versus left and mandibular versus maxillary. In adults, each quadrant has two incisors, one canine, two premolars, and three molars going from medial to lateral. Going from outside to inside in cross-section, teeth are made of enamel, centrum, dentine, and pulp—the latter containing the vessels and nerves. The mandibular teeth are innervated by the inferior alveolar nerves, whereas the maxillary teeth are innervated by the superior alveolar nerves. The first echelon draining lymphatics are the submandibular nodes, except for the incisors which drain directly to submental nodes. Odontogenic tumors are exceedingly rare and are typically only locally invasive. They will not be discussed further in this article (Fig. 5).

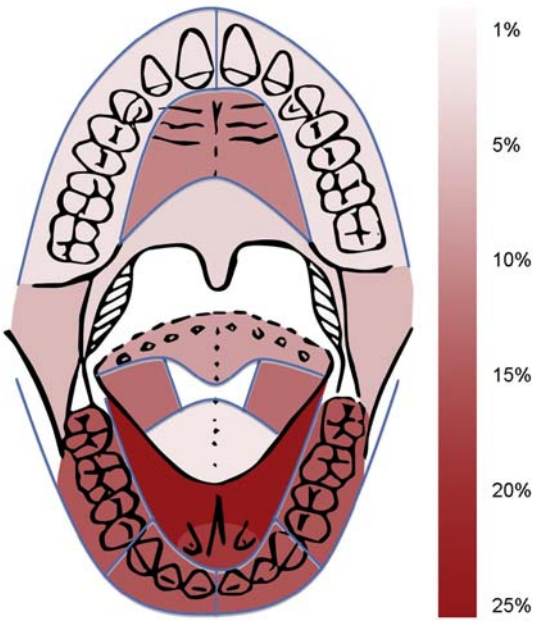

Fig. 4 Anatomical map to illustrate the distribution of all oral squamous cell carcinomas. From Sundermann, B. V., Uhlmann, L., Hoffmann, J., Freier, K., and Thiele, O. C. (2018). The localization and risk factors of squamous cell carcinoma in the oral cavity: A study of 1501 cases. *Journal of Cranio-Maxillofacial Surgery* **46**(2), 177–182.

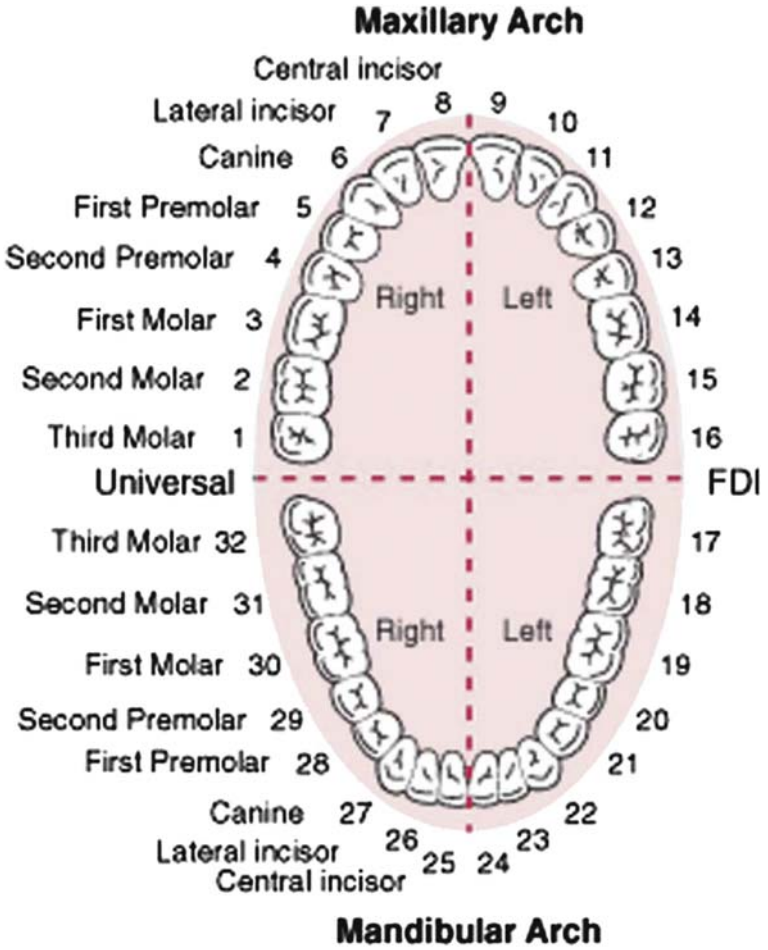

Fig. 5 Universal numbering system for permanent teeth as recommended by the Federation Dentaire Internationale. From Madani, M., Berardi, T., and Stoopler, E. T. (2014). Anatomic and examination considerations of the oral cavity. *Medical Clinics of North America* **98**(6), 1225–1238.

Retromolar Trigone

The retromolar trigone, as the name suggests, is a paired triangular area situated posterior to the third mandibular molars on the left and right sides of the oral cavity. The base of each triangle is formed by the third mandibular molar, and the apex is in continuity with the maxillary tuberosity located posterior to the third maxillary molars. The retromolar trigone is bounded laterally by the gingival buccal sulcus and medially by the anterior tonsillar pillar. Branches of the mandibular division of the trigeminal nerve provide sensory innervation to this area. The predominant lymphatic drainage from the retromolar trigone is to the upper deep jugular nodes, though there may be some drainage into periparotid and retropharyngeal nodes.

Buccal Mucosa

The buccal mucosa is comprised of the mucosal surfaces of the cheeks and lips, which form the anterolateral boundaries of the oral vestibule. It is contiguous with the mucosa that lines the floor of mouth and alveolar ridges. There are approximately 800–1000 minor salivary glands located throughout the buccal mucosa as well as other parts of the contiguous oral mucosa. The ostia of Stensen's (parotid) ducts are located within the buccal mucosa at the parotid papillae across from the second maxillary molars. Innervation is predominantly supplied by the buccal nerve, a branch of the mandibular division of the trigeminal nerve, but the superior portion is also innervated by the superior labial branches of the infraorbital nerve. Lymphatic drainage of the buccal mucosa is complex. The inferior and medial parts usually drain to submandibular nodes, whereas the lateral and upper parts may drain to parotid, buccinator, or preauricular nodes.

Epidemiology and Etiology

The epidemiology of oral cavity cancer strongly reflects exposure to various environmental agents. The most frequently cited risk factor for oral cavity cancer is tobacco, including smoking cigarettes, cigars, or pipes or using smokeless tobacco (Danaei et al.,

2005). Concomitant alcohol consumption acts synergistically and greatly increases the risk of developing an oral malignancy (Hashibe et al., 2009). Betel nuts, commonly chewed in Southeast Asia and India, also have high tumorigenic potential in the oral cavity (Coelho, 2012). Other reported risk factors for oral cavity cancer include gutka, supari, bidi, paan, vitamin A deficiency, and chronic irritation from poor oral hygiene or syphilis (Mahapatra et al., 2015; Chinn & Myers, 2015). High-risk strains of HPV, a sexually transmitted infection, are believed to be contributing to a steady increase in a subset of neoplasms originating from the oral cavity, particularly in patients younger than the historical average (Chocolatewala & Chaturvedi, 2009; Patel et al., 2011). For lip cancer, in particular, ultraviolet radiation is an established risk factor (Perea-Milla Lopez et al., 2003). Rarely, oral cavity cancer may occur as a secondary malignancy many years after prior radiotherapy to the head and neck. In the absence of these known risk factors, certain genetic syndromes such as Fanconi anemia and dyskeratosis congenita have strong associations with the development of oral cavity cancer (Prime et al., 2001).

The lip is the most prevalent site of oral cancer in several geographical areas worldwide, accounting for 25%–60% of all oral cavity cancer cases (Antoniades et al., 1995; Moore et al., 1999). Host and environmental factors associated with lip cancer include fair skin, cumulative and early sunlight exposure, skin reaction to sun exposure, outdoor occupations, genetic predisposition, immunosuppression, low socioeconomic status, and possibly viruses such as the Herpes simplex virus (HSV) and HPV (Perea-Milla Lopez et al., 2003). The lower lip, which is far more frequently affected than the upper lip, receives more direct exposure to solar ultraviolet radiation which causes DNA damage (**Fig. 6**).

The oral tongue is the second most common anatomic subsite of oral cavity cancer. The remaining anatomic subsites, in descending order of frequency among over 3,300 *de novo* oral cavity cancers treated at a single United States cancer center from 1970 to 1999, include the floor of mouth, retromolar trigone, alveolar ridge, palate, and lastly buccal mucosa (Chen & Myers, 2001). The median age at diagnosis of these malignancies is around age 60, and they are more common in males. Although rare in the United States, carcinoma of the buccal mucosa is the most common malignancy of the oral cavity in Southeast Asia because of the widespread use of betel nut.

About one in every 50 malignancies diagnosed in the United States originates in the oral cavity (Siegel et al., 2017). At a similar rate, nearly one in every 50 malignancies diagnosed in the United States is believed to be caused by HPV (Siegel et al., 2017; Klopp et al., 2014). Avoidance of the previously described known carcinogens and optimal skin protection from ultraviolet radiation has led to a decrease in the incidence of oral cavity cancer in many parts of the world (Chaturvedi et al., 2013; Franceschi et al., 2000). Prevention of most HPV-associated malignancies is thought to be possible by HPV vaccines (Kurdgelashvili et al., 2013). Since mid-2006, HPV vaccination has been recommended for females aged 11–12 years and through 26 years if not previously vaccinated (Markowitz et al., 2016). HPV vaccination is now routinely recommended at age 11 or 12 regardless of sex and recommended for males aged 13 through 21 years if not previously vaccinated (HPV Vaccine Recommendations, 2017). Rapid reductions of upwards of 90% of HPV 6/11/16/18 infections and genital warts were demonstrated after introduction of the quadrivalent HPV vaccination programs in young women in Australia, Europe, North America, and New Zealand (Garland et al., 2016). HPV-associated cancer rates are subsequently expected to decline and the full public health potential is not yet realized because carcinogenesis after HPV infection may require several decades to become manifest.

However, benefits of HPV vaccination may have less of an impact on oral cavity cancers than other HPV-related malignancies. A meta-analysis of 17 studies demonstrated a weaker association between HPV and cancer of the oral cavity compared to cancer of the oropharynx (Hobbs et al., 2006). After testing archival tissue from 2670 patients diagnosed between 1993 and 2005, HPV DNA was detected in 32% of oral cavity cancers in contrast to 90%–99% of invasive and in situ cervical cancers and 70% of oropharyngeal cancers (Saraiya et al., 2015). A meta-analysis reported a lower prevalence of HPV in oral cancer at 24% (Combes & Franceschi, 2014). Despite these HPV DNA prevalence rates for oral cavity cancers, recent international studies suggest that HPV rarely plays a driving role in oncogenesis because HPV mRNA or p16 is detected in as few as 3%–5% of oral cavity cancers (Castellsague et al., 2014). Nevertheless, certain oral cavity subsites such as the floor of mouth and tongue seem to be predominantly infected by HPV (Hübbers & Akgül, 2015). Therefore, the clinical significance that HPV vaccines will have on oral cavity cancers in particular remains to be seen.

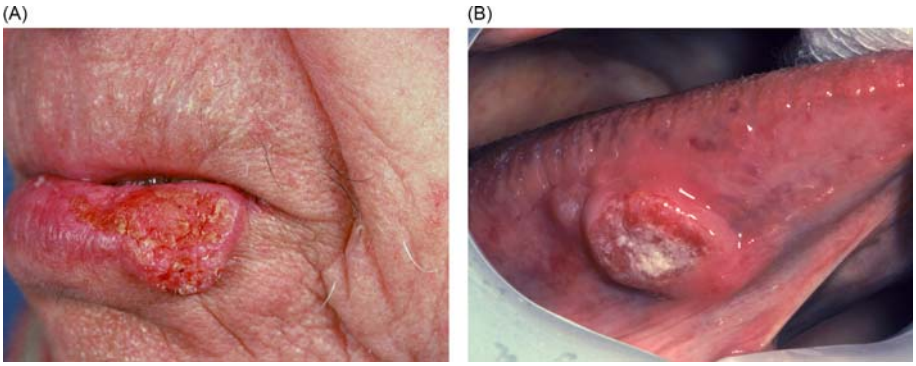

Fig. 6 (A) Oral squamous cell carcinoma of the inferior lip. (B) Oral squamous cell carcinoma of the right ventral tongue. Images appears with permission from VisualDx.

Most public health efforts have focused on prevention of oral cavity cancer by minimizing exposure to known carcinogens rather than screening. Increased regulation and public education on the harmful effects of tobacco, for example, has led to decreasing rates of smoking in many developed countries (Warnakulasuriya, 2009; Chaturvedi et al., 2013; Franceschi et al., 2000; Simard et al., 2014). There is no routine screening for cancer of the oral cavity, but special attention should be given to those at high risk based on the before-mentioned risk factors. In a regional United States oral cancer detection program in the Boston area, the diagnosis of early-stage disease increased from 20% to 33% over a 3-year period when investigators stressed the importance of routine oral examination. Furthermore, screening for and identification of leukoplakia may lead to not only earlier oral cavity cancer detection, but also reduced mortality based on a SEER analysis (Yanik et al., 2015). This was confirmed by an oral visual inspection program in India which screened over 87,000 individuals with a reduced mortality rate ratio of 0.79 in those who screened positive (Sankaranarayanan et al., 2005).

Patients with oral cavity cancer are at an elevated risk of developing a second primary cancer, likely because of field cancerization (Day & Blot, 1992). In a meta-analysis of over 40,000 patients with head and neck cancer, patients with carcinoma of the oral cavity had the highest rate of second primary cancers (Haughey et al., 1992). A retrospective review of 851 patients with head and neck cancer between 1978 and 1990 identified an 18% probability of developing a second metachronous malignancy at 5 years for patients with oral cavity cancer (Schwartz et al., 1994).

Natural History

Premalignant Lesions

Actinic keratosis is the earliest clinically recognizable manifestation of lip cancer. Leukoplakia and erythroplakia are common gross clinical descriptors of benign, premalignant, and malignant lesions seen throughout the intraoral cavity. Leukoplakia, or a white patch or plaque that cannot be rubbed off, may represent hyperplastic tumors or dysplastic tumors (Mehanna et al., 2009; **Fig. 7**). Hyperplastic tumors spontaneously regress 30%–40% of the time and have a low risk for malignant transformation. In contrast, dysplastic tumors rarely spontaneously regress and have a higher risk for malignant transformation. Microscopic features of dysplastic leukoplakias include abnormal orientation of cells, nuclear hyperchromatism, increased mitosis, and a high nuclear-cytoplasmic ratio. Erythroplakias, or bright red velvety plaques, rarely spontaneously regress and have a higher malignant transformation rate than leukoplakias (Shafer & Waldron, 1975). Oral homogeneous erythroplakias have been histopathologically documented to show 51% invasive carcinoma, 40% carcinoma in situ, and 9% mild or moderate dysplasia (Reichart & Philipsen, 2005). Another premalignant disorder prominent in Southeast Asia due to betel nut chewing is oral submucous fibrosis (Wollina et al., 2015; **Fig. 8**).

Pathology

Squamous cell carcinoma is the predominant histopathologic type of cancer in the oral cavity. Oral squamous cell carcinoma can present as several histologic variants including basaloid, verrucous, papillary, adenoid, acantholytic, cuniculatum, pseudoglandular, spindle cell, sarcomatoid, and adenosquamous (Pathak et al., 2014). Verrucous carcinomas are typically low grade, slow growing, and rarely metastasize despite the tendency to invade deep tissues (Chen & Myers, 2001; Candau-Alvarez et al., 2014). The buccal mucosa is the most common site for verrucous carcinoma. Basaloid was once believed to be a more aggressive variant, but the largest reported series of basaloid squamous cell carcinoma of the oral cavity to date demonstrates that this histology carries a comparable prognosis to conventional-type oral squamous cell carcinoma (Fritsch et al., 2014). Spindle cell carcinoma, on the

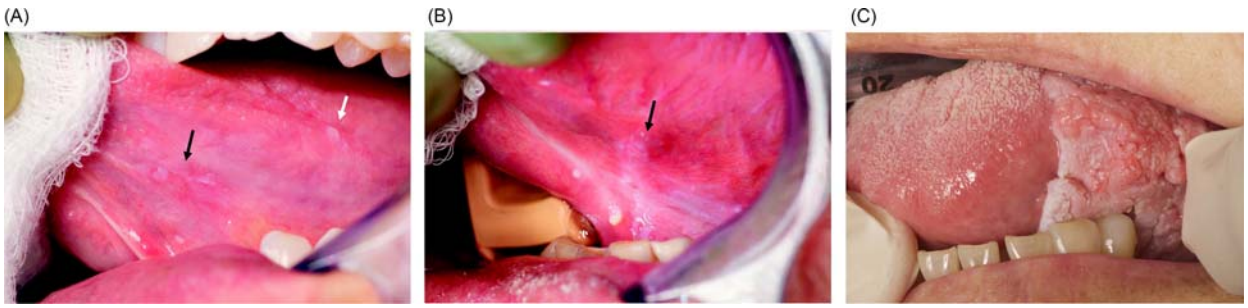

Fig. 7 Clinical examples of leukoplakia and proliferative verrucous leukoplakia. (A) A 36-year-old nonsmoking woman with multifocal leukoplakia of the left lateral tongue and left ventral tongue. Excisional biopsy demonstrated moderate dysplasia of the lateral tongue lesion (*white arrow*) and mild dysplasia of the ventral tongue lesion (*black arrow*). (B) A 52-year-old man with 30-pack-year smoking history and heterogeneous leukoplakia of the left floor of mouth. Excisional biopsy demonstrated an area of carcinoma in situ at the superior aspect (*black arrow*) of the biopsy. (C) A 52-year-old woman with a history of proliferative verrucous leukoplakia of the left lateral tongue. A deep excisional biopsy at the time of this picture demonstrated an invasive component consistent with a verrucous carcinoma ultimately requiring left partial glossectomy. From Bewley, A. F. and Farwell, D. G. (2017). Oral leukoplakia and oral cavity squamous cell carcinoma. *Clinics in Dermatology* **35**(5), 461–467.

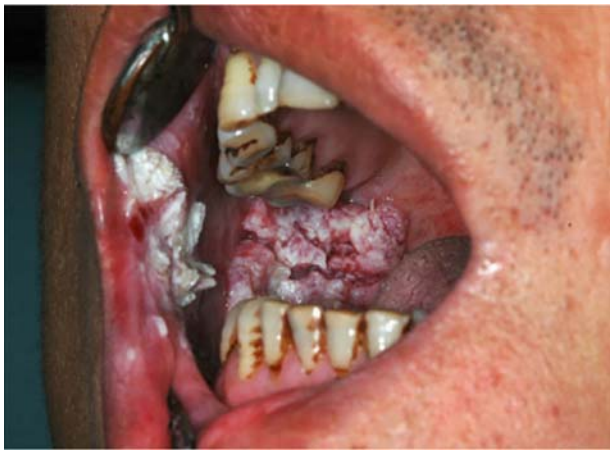

Fig. 8 Oral submucous fibrosis associated with oral cancer of the right buccal mucosa. From Jian, X., Huang, X., Liu, D., Xu, P., and Liu, X. (2013). Analysis of clinical symptoms and signs in cases of oral submucous fibrosis which have transformed into squamous cell carcinoma. *Open Journal of Stomatology* **3**, 235–240.

other hand, accounts for $<1\%$ of all tumors of the oral cavity and tends to be aggressive, with reported median overall survival times of <1–2 years (Ellis & Corio, 1980; Su et al., 2006).

Nonsquamous histologies cumulatively represent $<10\%$ of all oral cavity neoplasms (Daley & Darling, 2003). Examples of these nonsquamous histologies are listed in **Table 1**. The most common nonsquamous histology of the lip is basal cell carcinoma. Excluding the lip, malignancies of the minor salivary glands are the next most common type of oral cavity tumors behind squamous cell carcinoma. These tend to occur on the hard palate, and adenoid cystic carcinoma is the most common histology (Weber et al., 1989).

The third most common type of malignancy of the intraoral cavity is lymphoma. Most lymphomas in the head and neck arise not in the oral cavity, but rather in Waldeyer's ring which comprises the tonsils, base of tongue, and nasopharynx (Guevara-Canales et al., 2013). The oral cavity is the primary site of approximately 2%–4% of all extranodal lymphomas, and this location is more common in patients with acquired immunodeficiency syndrome (AIDS) than the general population (Kobler et al., 2005; Silva et al., 2016). Another neoplasm frequently seen in the oral cavity among AIDS patients is Kaposi's sarcoma (Fatahzadeh & Schwartz, 2013).

Table 1 Nonsquamous cell malignant tumors of the oral cavity

Cells or tissue of origin	*Cancer name*	*Usual age group*	*Prognosis*
Melanocytes	Melanoma	Adults	Very poor
Salivary glands	Many types	Adults	Good to poor
Fibroblasts	Fibrosarcoma	Young adults	Intermediate to poor
	Malignant fibrous histiocytoma	Older adults	Intermediate to poor
Myofibroblasts	Myofibrosarcoma	Adults	Intermediate to poor
Fat cells	Liposarcoma	Adults	Intermediate to poor
Skeletal muscle	Rhabdomyosarcoma	Children and adolescents	Intermediate
Smooth muscle	Leiomyosarcoma	Adults	Poor
Peripheral nerves	Neurosarcoma	Young adults	Very poor to poor
	Malignant granular cell tumour	Adults	Poor
Synovial cells	Synovial sarcoma	Adolescents and young adults	Poor
Endothelial cells	Angiosarcoma	Elderly people	Very poor
	AIDS-related Kaposi's sarcoma	Young adults	Poor
	Classic Kaposi's sarcoma	Elderly men	Good
Lymphocytes	Lymphoma	Adults and children	Good, intermediate or poor
Plasma cells	Plasmacytoma	Adults	Good to intermediate
	Multiple myeloma	Older adults	Poor to very poor
Bone cells	Osteosarcoma	Children and young adults	Intermediate to poor
	Parosteal osteosarcoma	Children and adults	Good
Cartilage cells	Chondrosarcoma	Adults	Intermediate
Odontogenic cells	Various carcinomas and sarcomas	Adults	Poor
Metastatic tumours	Breast, lung, prostate, kidney, thyroid	Adults	Very poor
	Leukemic infiltrate	Children and adults	Variable

From Daley, T. and Darling, M. (2003). Nonsquamous cell malignant tumours of the oral cavity: An overview. *Journal of the Canadian Dental Association* **69**(9), 577–582.

Other malignant histologies presenting in the oral cavity include adenocarcinomas, melanoma, ameloblastoma, plasmacytoma, multiple myeloma, teratoma, various sarcomas, and metastasis from other primary tumors (Gholizadeh et al., 2016; Gupta et al., 2012; Kolekar et al., 2016; Shah et al., 2010; Benoist & van Looij, 2013). Mucosal melanomas generally have a worse prognosis than cutaneous melanomas. Fortunately, melanoma of the oral cavity represents $<1\%$ of all melanomas (Smyth et al., 1993).

Important histopathologic prognostic factors include depth of invasion, worst pattern of invasion, perineural invasion, lymphovascular invasion, and extranodal extension (ENE). Greatest depth of invasion is measured from the horizon of the basement membrane closest to the intact squamous mucosa and dropping a "plumb line" from the horizon. Worst pattern of invasion score 5 is when satellite dispersion is ≥ 1 mm from neighboring satellites, and is significantly predictive of poor outcome. Perineural invasion and lymphovascular invasion should both be subclassified as either intratumoral or extratumoral, as well as focal or multifocal. Histopathologic designations of ENE include none (i.e., not present), microscopic (i.e., ≤ 2 mm of ENE), and macroscopic (i.e., >2 mm or gross ENE).

Patterns of Spread

About 90% of all squamous cell carcinomas of the lip start on the sun-exposed lower lip vermilion (Fitzpatrick, 1984). Basal cell carcinomas, alternatively, are more common on the upper lip. The commissures are rarely sites of disease origin. The disease may spread along the lip to involve the buccal mucosa or spread deeply to involve the gingiva, mandible, and in advanced cases the mental nerve (Baker & Krause, 1980). Lymphatic invasion and nodal positivity occurs in about 5%–10% of lip cancer cases overall. This rate is higher for bulkier and more advanced tumors. Draining lymphatics and therefore sites of nodal spread for upper versus lower lip tumors, as well as all other intraoral primary tumors, are specified in the prior Anatomy section (**Fig. 9**).

Squamous cell carcinomas of the oral tongue tend to originate on the lateral and ventral surfaces of the tongue. Most lateral tumors tend to be located on the middle and posterior thirds of the oral tongue. Dorsal tumors are rare and tend to occur in the posterior midline. Tumors on the tip of the tongue are usually diagnosed early. Local invasion into surrounding structures generally occurs late, but when it occurs, it can spread anteriorly or laterally into the floor of mouth and mandible; posteriorly into the glossotonsillar sulcus, retromolar trigone, tonsillar pillars, and base of tongue; and deep into the oral tongue musculature. Clinical nodal positivity ranges from 15% to 80%, the highest risk of nodal metastases among all oral cancer subsites, and is dependent on the size of the primary tumor and depth of invasion (Rusthoven et al., 2008; Almangush et al., 2015). For patients with clinically positive nodes, the risk of occult contralateral nodal disease is about 30%. Cancers of the anterior oral tongue have been documented as being able to skip levels II–III and spread directly to level IV (Byers et al., 1997; Woolgar, 1997).

Carcinomas of the floor of mouth, in contrast to oral tongue cancer, generally invade into surrounding structures early. About 90% of tumors start within 2 cm of the anterior midline floor. The tumors spread anteriorly into the lower gingiva and lower lip; posteriorly into muscles at the root of the tongue; laterally into the gingiva and along the mandibular periosteum or through the cortex in advanced stages; and deep into the genioglossus, geniohyoid, and sublingual gland. The mylohyoid acts as a barrier to deeper extension, until the tumor is more advanced, such that disease spreads along the muscle, escapes the oral cavity, and emerges in the neck near the angle of the mandible mimicking an enlarged lymph node. Lymphatic invasion and clinical nodal positivity range from 10% to 55% and are directly correlated to T-stage. For patients with clinically positive nodes, the risk of occult contralateral nodal disease is about 50%.

Most gingival tumors, roughly 80%, arise on the lower gingiva. Carcinomas of the alveolar ridge and retromolar trigone tend to invade bone early. Tumors of the lower alveolar ridge and retromolar trigone also have a high propensity of lymph node metastasis, second only to oral tongue carcinomas among all oral cavity subsites. Tumors of the inferior alveolar ridge may access the mandibular canal and the inferior alveolar nerve, whereas tumors of the superior alveolar ridge may invade into the maxillary antrum or floor of the nose. Tumors of the superior alveolus are less likely to metastasize to the neck than tumors of the inferior alveolus.

Carcinomas of the buccal mucosa commonly arise on the lateral walls, adjacent to the lower molars. One route of progression is into the gingivobuccal gutters, and ultimately invasion of the gingiva and bone can arise. Alternatively, they can invade the buccinator muscle, extend to the buccal fat pad, and invade subcutaneous tissues. More advanced tumors can even go on to involve the parotid gland and facial nerve. The likelihood of clinical nodal positivity at the time of diagnosis is approximately 10%–30%, and the risk of subclinical disease is between 5% and 15%. Bilateral neck involvement is rare for this subsite. The risk of nodal involvement increases to 60% for more advanced stage tumors (i.e., T4).

The hard palate has a relatively dense mucoperiosteum that is relatively resistant to invasion from hard palate carcinomas. Carcinomas of the hard palate can invade superiorly into the nasal cavity through the incisive fossa where the primary and secondary palates are fused. Furthermore, these tumors may invade into the surrounding gingiva and maxillary alveolar process, posteriorly into the soft palate, and posterolaterally through the greater palatine foramina into the pterygopalatine fossa which communicates with the skull base. The rate of nodal involvement at the time of diagnosis is about 13%–24% with a risk of subclinical involvement of 22% (**Fig. 10**).

The superior deep jugular nodes are the most frequently involved sites of lymphatic metastasis by cancers of the oral cavity. Approximately 25% of patients with carcinoma of the oral cavity will have occult nodal metastases. Contralateral metastases are more common in tumors that approach or cross the midline. In general, cervical lymph node involvement from oral cavity primary sites is predictable and orderly, spreading first to upper, then middle, and subsequently lower cervical nodes.

Hematogenous invasion is generally a rare initial presentation for oral cavity cancer. The lungs are the most common site of distant metastases; skeletal and hepatic metastases occur less often. Mediastinal lymph node metastases are considered distant

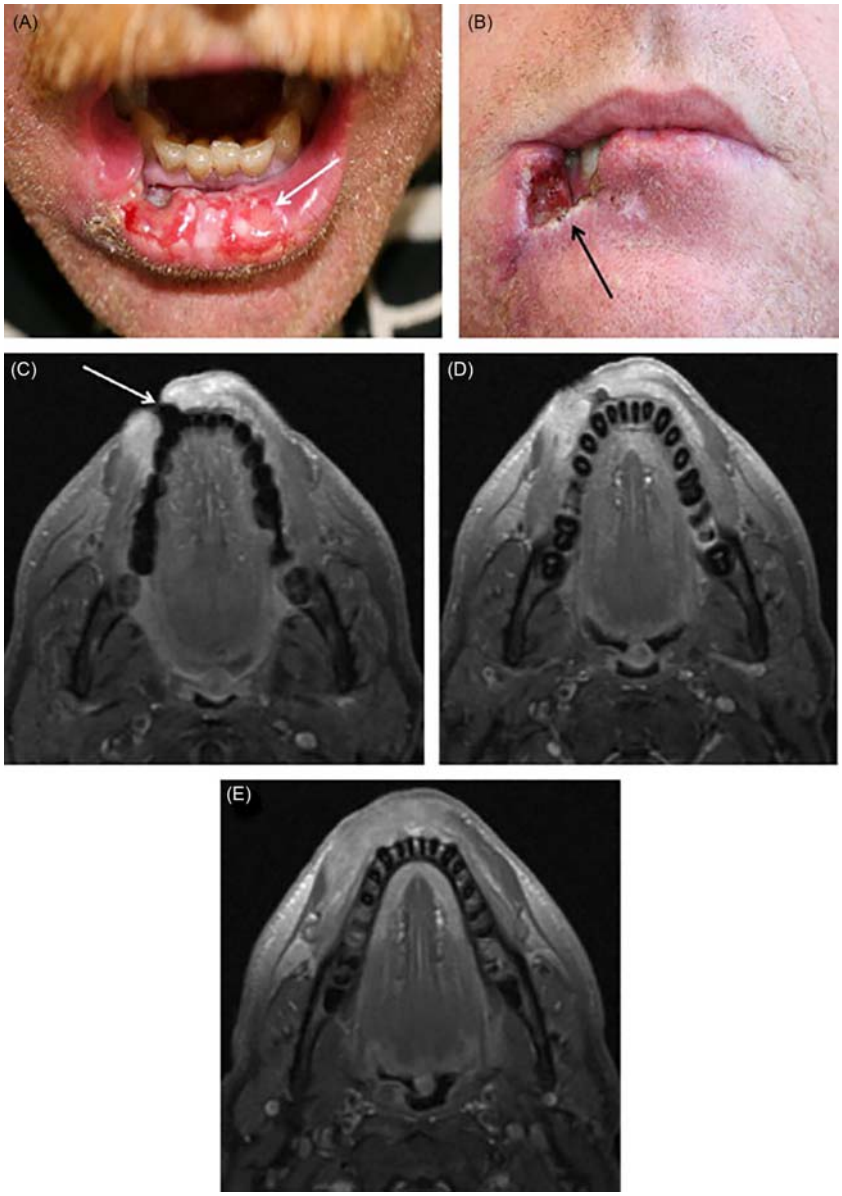

Fig. 9 Lip carcinoma. (A, B) Photographs show extensive ulceration and infiltration with erythroleukoplakia of the right lower lip and labial mucosa. Extension across the midline is seen intraorally (*white arrow* in A) and ulceration with breakdown of the skin is seen extraorally (*black arrow* in B). (C, D, E) Axial postcontrast T1-weighted images from superior to inferior show an enhancing mass extending from the skin surface to the buccal surface of the mandible, without evidence of bone invasion. Note the area of ulceration superiorly (*arrow* in C). From Hagiwara, M., Nusbaum, A., Schmidt, B. L. (2012). MR assessment of oral cavity carcinomas. *Magnetic Resonance Imaging Clinics of North America* **20**(3), 473–494.

metastases, except level VII or anterior superior mediastinal lymph nodes cephalad of the innominate artery. An increased risk of distant metastasis is associated with a higher burden of lymph node involvement, advanced primary tumor stage, and recurrent disease.

Presentation and Diagnosis

Clinical Presentation

Although the oral cavity is an anatomic region readily accessible to visual inspection and palpation, many patients present with advanced-stage disease because of vague and initially painless symptoms. About 30%–40% of patients with cancer of the oral cavity harbor cervical lymph node metastases at the time of diagnosis.

The most common presenting symptom for cancer of the oral cavity is a nonhealing lesion. Similarly, persistent pain in the mouth that does not resolve is also very common. Other presenting signs and symptoms include persistent leukoplakia or

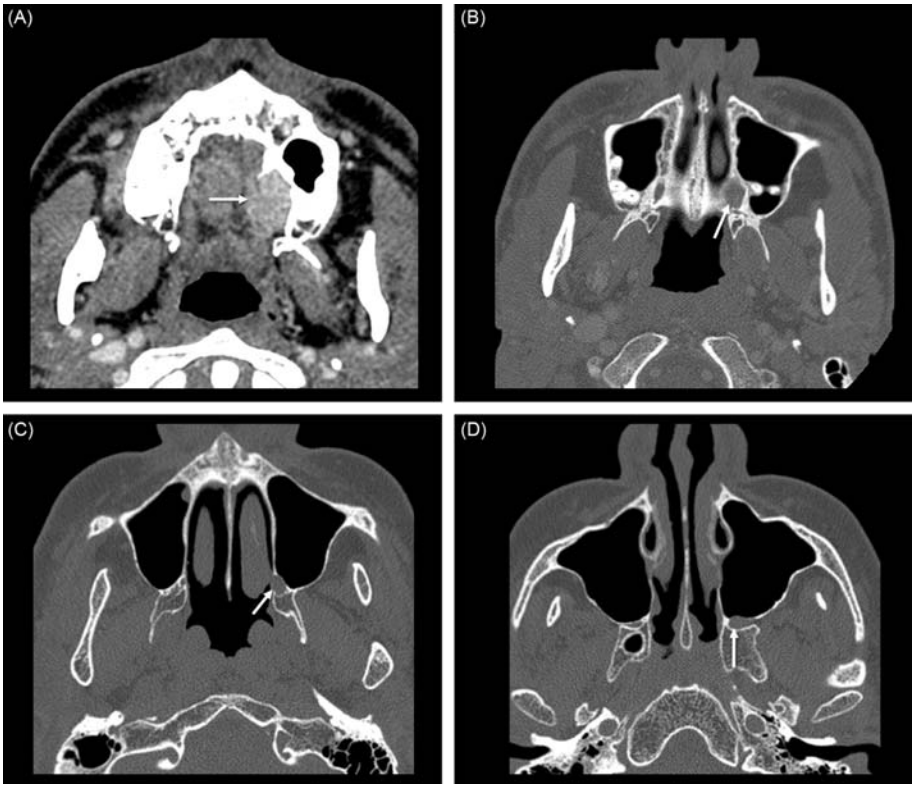

Fig. 10 Left hard palate mucoepidermoid carcinoma. (A) Axial contrast-enhanced CT shows 2-cm enhancing mass with fairly well-circumscribed borders (*arrow*) in the left hard palate. Well-circumscribed borders are common in minor salivary gland malignancies, making it difficult to differentiate them from benign minor salivary gland tumors. (B, C) Bone window from axial CT images (at the level of the hard palate (B) and just above) show asymmetric widening of the left greater palatine foramen (*arrow*), compatible with perineural tumor extension. (D) Bone window from axial CT images shows extension of abnormal widening to the left pterygopalatine fossa (*arrow*). An MR image is needed to assess for proximal intracranial extension to determine if this tumor was resectable. From Aiken, A. H. (2013). Pitfalls in the staging of cancer of oral cavity cancer. *Neuroimaging Clinics of North America* **23**(1), 27–45.

erythema, difficulty chewing or swallowing, limited tongue mobility, bleeding, odynophagia, trismus, constant halitosis, globus sensation, otalgia, loose teeth, ill-fitting or uncomfortable dentures, voice changes, weight loss, numbness, and persistent or enlarging masses in the mouth and/or neck despite antibiotics.

Otalgia suggests involvement of cranial nerves V, VII, IX, or X—though the auriculotemporal branch of the mandibular division of the trigeminal nerve is the most commonly involved in oral cavity primary tumors (Scarbrough et al., 2003). Facial numbness may suggest involvement of the trigeminal nerve. Hypoesthesia of the lower lip and teeth is often from malignant penetration of the mandible and perineural spread along the inferior alveolar nerve. The presence of trismus may indicate invasion into the pterygoid musculature.

The differential diagnosis of lip cancer includes actinic cheilitis, leukoplakia, herpes simplex, syphilitic chancre, keratoacanthoma, hemangioma, and fibroma. The differential diagnosis for an oral tongue cancer includes granular cell myoblastoma, pyogenic granuloma, and hypertrophied papillae. The differential diagnosis for other intraoral cancers includes leukoplakia, candidiasis, and lichen planus.

Diagnostic Evaluation

Patients who present with cancer of the oral cavity should undergo a comprehensive history and physical examination. A detailed history of present illness should specifically evaluate for any of the aforementioned symptoms and identify carcinogenic risk factors, such as tobacco, alcohol, or betel nut use. A complete past medical history should be performed to take into account comorbid illnesses when creating an ideal treatment plan. A family medical history should be obtained to evaluate for inheritable genetic mutations. A full review of systems should be performed to evaluate for concurrent illnesses or systemic manifestations of the disease.

A meticulous physical examination of the head and neck should be performed, with particular focus on the oral cavity. This typically begins with a full inspection of the oral cavity in good lighting. Bimanual palpation of the oral cavity can help assess extent of induration, fixed tissues, and bony involvement. A thorough palpation of the neck is important to assess regional nodal disease.

Evaluation of other areas of the aerodigestive tract for synchronous primaries and functional deficits can be performed by indirect pharyngolaryngoscopy using small mirrors and/or direct pharyngolaryngoscopy using a flexible fiberoptic scope.

The next important step in diagnostic evaluation is biopsy to confirm malignancy. Histopathologic sampling can be performed by fine needle aspiration with or without ultrasound guidance, core needle biopsy, incisional biopsy, or exfoliative cytology. Immunohistochemistry staining can be performed on core needle and incisional biopsy specimens. Tumor HPV status can be determined using various testing platforms, including HPV DNA in situ hybridization, HPV RNA in situ hybridization, HPV polymerase chain reaction, and p16 immunochemistry (Duncan et al., 2013). The immunohistochemical staining for p16 is inexpensive, has near universal availability, and is relatively straightforward to interpret (Singhi & Westra, 2010; Lydiatt et al., 2017; **Fig. 11**).

Imaging modalities that may be used for staging, treatment planning, and posttreatment monitoring include X-ray, ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). A chest CT is helpful to exclude lung metastases or identify a synchronous primary lung cancer, especially in patients with a significant smoking history. A head and neck CT, ideally with IV contrast, is the most commonly used modality to determine the extent of soft-tissue and bony involvement as well as to identify occult disease in the neck. The "puffed-cheek" maneuver during CT scanning may be helpful to better visualize and assess extent of invasion for buccal and gingival mucosa primary tumors (Weissman & Carrau, 2001). If CT scanning is not available, then panoramic radiographs can be used to demonstrate mandibular invasion. MRI may be used in cases of contrast allergy, poorly visualized lesions on CT such as from significant dental artifact, to establish the extent of primary tumor, and evaluating for perineural spread. Ultrasound may be used to screen for enlarged lymph nodes that are not clinically detectable, and in experienced hands the accuracy of ultrasound when combined with fine needle aspiration may be superior to CT or MRI for staging the neck (van den Brekel et al., 1993). PET-CT is highly sensitive in staging of patients with squamous cell carcinoma of the head and neck, but it is insensitive to evaluation of patients with glandular tumors, low volume tumors, and cystic metastases (Escott, 2013; Johnson & Branstetter, 2014). A prospective cohort study demonstrated that PET-CT based staging had a significantly higher detection rate of distant metastasis or synchronous cancer compared to other combined modalities (Rohde et al., 2017).

The presence of ENE, which is associated with worse outcome, is most commonly diagnosed pathologically, but it may also be identified on physical exam and imaging. Clinically, ENE may be diagnosed by the presence of a matted mass of nodes, involvement of overlying skin or adjacent soft tissues, or clinical signs of nerve invasion. Radiographically, ENE may be diagnosed by an indistinct nodal margin, irregular nodal capsular enhancement, or infiltration into adjacent fat or muscle.

Basic blood tests such as complete blood count, comprehensive metabolic panel, and liver function tests are useful to determine overall health and underlying organ function. These can diagnose anemia, malnutrition, kidney disease, or liver disease. Occasionally basic labs may also suggest metastatic disease to the liver or bone. These labs in combination with complete past medical history can also guide appropriate treatment, such as surgery or preferred systemic agent based on predicted treatment-related toxicity. Other diagnostic tests to consider in certain patients include electrocardiogram and pulmonary function tests prior to surgery. Candidates for radiation therapy should ideally undergo dental evaluation prior to initiation of treatment. Additionally, many patients benefit from multidisciplinary referrals to nutritionists and speech therapists.

Staging

Historically, cancers of the oral cavity were predominately staged based on the size of the primary tumor; invasion into surrounding structures; the size, laterality, and number of nodal metastases; and the presence or absence of distant metastatic disease. The American Joint Committee on Cancer (AJCC) 8th Edition introduced two new parameters in oral cavity staging: depth of invasion and

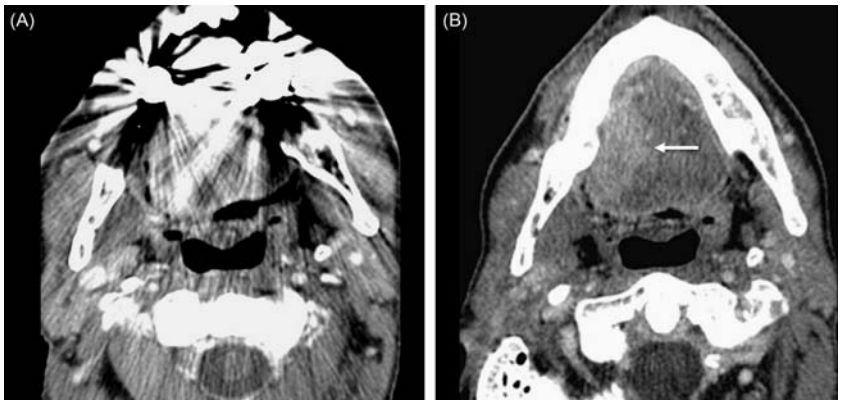

Fig. 11 Usefulness of reangled images to visualize squamous cell carcinoma of the right oral tongue. (A) Axial CT through the oral cavity is nondiagnostic because of dental amalgam and extensive streak artifact. (B) Delayed and reangled axial contrast-enhanced CT image shows a large enhancing right oral tongue mass (*arrow*), effacing the right sublingual space. From Aiken, A. H. (2013). Pitfalls in the staging of cancer of oral cavity cancer. *Neuroimaging Clinics of North America* **23** (1), 27–45.

ENE. Effectively, depth of invasion will increase the T category by 1 for each 5 mm of tumor depth until ≥10 mm. Pathological presence of ENE will increase the nodal category by 1 to either N2a or N3b depending on the size, laterality, and quantity of nodes involved.

Management

General Management

Treatment goals for cancer of the oral cavity include eradication of malignancy, preservation or restoration of form and function, avoidance or minimization of treatment sequelae, and prevention of second cancers. The ideal treatment to achieve these goals, whether as single modality or in combination, depends on various disease-, treatment-, provider-, and patient-related factors. Examples of these respective factors include tumor stage and location; potential treatment morbidity and convenience; multidisciplinary team expertise and available technology; and patient performance status and comorbid disease. A multidisciplinary approach is paramount in the optimal management of patients with cancer of the oral cavity. Multidisciplinary evaluation prior to treatment disposition helps to ensure consensus recommendations are made and to facilitate interdisciplinary coordination of care.

Early stage oral cavity tumors (e.g., T1-T2, N0-N1) are generally managed with single modality locoregional treatment consisting of surgery or radiation therapy. Surgery is generally preferred, but radiation therapy is appropriate when overall health or functional status precludes an operation, when there is an anticipated poor functional or cosmetic outcome, or simply when a patient declines surgery. For patients with locally advanced oral cavity tumors (e.g., T3-T4, N2-N3), upfront surgery followed by adjuvant radiation therapy with or without concurrent chemotherapy is generally preferred. However, in patients unable to undergo surgical resection, definitive chemoradiotherapy has been shown to be a reasonable option.

Prognostic and Predictive Factors

A series of nomograms and retrospective analyses have identified factors associated with recurrence and survival. One such nomogram predicts preoperative prognosis in patients with oral cavity squamous cell carcinoma and was designed after reviewing demographic, host, and tumor characteristics of 1617 patients treated primarily with surgery at a single-institution tertiary care cancer center between 1985 and 2009 (Montero et al., 2014). The most influential predictors of both recurrence and cancer-specific mortality probability were tumor size, nodal status, subsite, and bone invasion. A retrospective analysis of a single United States institution's 35-year experience treating 230 primary invasive squamous cell carcinomas of the oral cavity with surgery and postoperative radiotherapy (PORT) demonstrated that the following variables were significant on multivariate analysis for locoregional control: positive margins, vascular invasion, perineural invasion, extracapsular extension, and T stage (Hinerman et al., 2004).

One of the consistently reported prognostic factors for oral cavity cancers is depth of primary tumor invasion, which is associated with occult cervical metastases, especially in oral tongue cancer. Although the oral cavity subsites, study designs, and techniques for measuring depth of invasion varied significantly, a review of >50 studies identified tumor thickness/depth of invasion as predictive of nodal involvement and survival (Pentenero et al., 2005). A multicenter international study of patients with T1-T2, N0 oral tongue squamous cell carcinoma identified depth of invasion and worst pattern of invasion as strong predictors for locoregional recurrence and cancer-related mortality (Almangush et al., 2015). Consequently, the authors proposed that multimodality treatment should be utilized for early-stage oral tongue carcinomas with depth of invasion ≥4 mm or with a growth pattern characterized by small cell islands or satellites. This was supported by a meta-analysis, which analyzed sixteen studies consisting of 1136 patients with oral cavity squamous cell carcinoma to assess the predictive value of tumor thickness for cervical lymph node involvement (Huang et al., 2009). The optimal tumor thickness cutoff point was <4 mm, yielding a negative predictive value of 95.5% for cervical lymph node involvement.

Additionally, the pattern of pathologically involved cervical lymph nodes is associated with patient outcomes. An analysis of prognostic factors in 255 patients with oral cavity squamous cell carcinoma with ENE identified level IV and V nodal metastases and tumor depth ≥12 mm as independent predictors of 5-year survival (Liao et al., 2011; **Fig. 12**). These factors divided patients into three prognostic groups. In the low-risk group (no level IV/V metastases and tumor depth <12 mm), intermediate-risk group (no level IV/V metastases and tumor depth ≥12 mm), and high-risk group (level IV/V metastases present), the 5-year overall survival rates were 50%, 28%, and 10%, respectively.

Surgery

The overall health and functional status of the patient are important determinants in choosing between surgical and nonsurgical approaches. Surgery is generally preferred as definitive treatment of the primary tumor because it is expeditious, convenient, and offers pathologic information to guide treatment of the neck and adjuvant therapy. General principles of surgery include adequate exposure, wide negative margins (e.g., 1–2 cm mucosal margins and 1 cm bone margins), proximal transection of clinically-involved nerves, evaluation of the neck (elective vs. therapeutic), and tracheostomy if postoperative edema and airway obstruction are anticipated (**Fig. 13**).

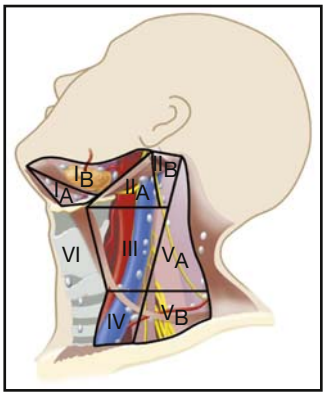

Fig. 12 Cervical lymph node levels. From Montero, P. H. and Patel, S. G. (2015). Cancer of the oral cavity. *Surgical Oncology Clinics of North America* **24**(3), 491–508.

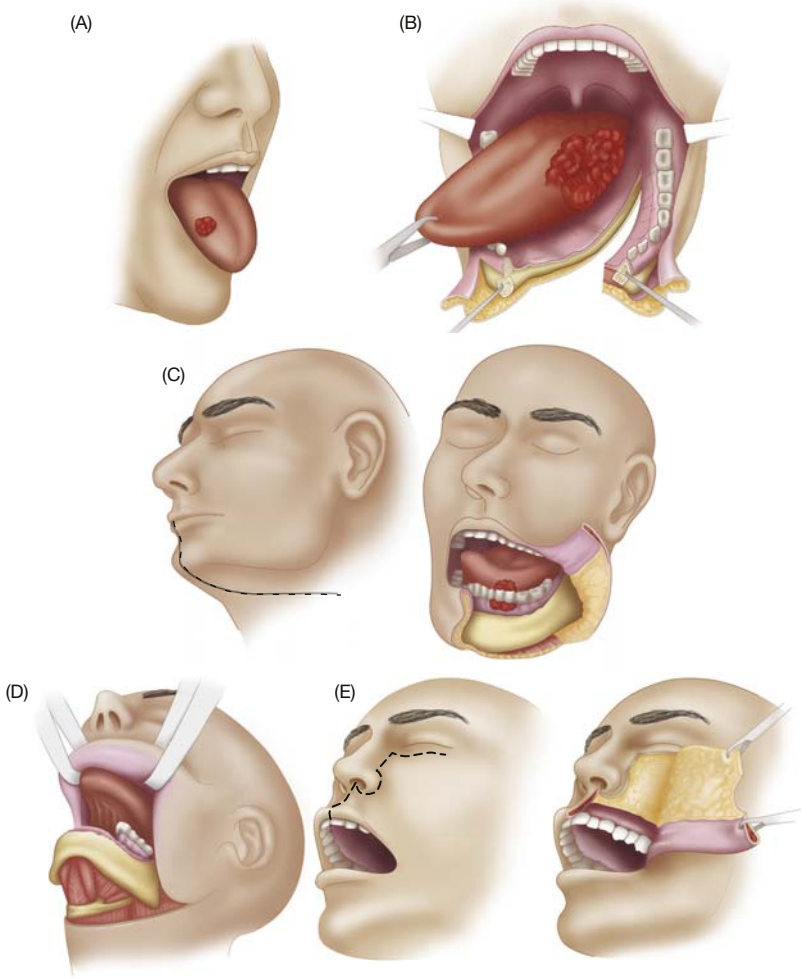

Fig. 13 Various surgical approaches: (A) peroral, (B) mandibulotomy, (C) lower cheek flap, (D) visor flap, and (E) upper cheek flap. From Montero, P. H., Patel, S. G. (2015). Cancer of the oral cavity. *Surgical Oncology Clinics of North America* **24**(3), 491–508.

Surgical management of the primary tumor

For early-stage lesions of the oral cavity, a transoral method of approach to resection generally provides adequate exposure to obtain negative margins. For advanced or poorly visualized tumors, a mandibulotomy or pull-through technique is often utilized for floor of mouth, tongue, and mandibular pathology while a Weber-Ferguson incision or mid-face degloving may be necessary for

maxillary or palatal lesions. When compared to open approaches, transoral approaches lead to shorter hospitalizations, and decreased tracheotomy and PEG dependence (Moore et al., 2012; White et al., 2010). Of note, most of the functional outcomes research on transoral versus traditional approaches pertains to oropharyngeal carcinoma but can be extrapolated to the oral cavity.

For lip cancers, surgical excision with intraoperative margin analysis with immediate reconstruction is the preferred method of treatment. For lesions involving up to one-third of the lip, excision with primary closure is generally performed. If the proposed excision involves up to two-thirds of the lip, local flaps are required for reconstruction. In general, regardless of the reconstruction needed (e.g., Abbe flap, Estlander flap if the oral commissure is involved, Karapandzic flap, radial forearm free tissue transfer with or without palmaris tendon suspension, etc.), wide local excision is indicated.

For cancers that involve the alveolar ridge, resection of a portion of the mandible is sometimes indicated (Genden et al., 2005). Given that the mucosa is densely adherent to the underlying periosteum, it is not uncommon to see bony erosion. While mandibular invasion is associated with decreased overall survival, medullary space invasion (rather than cortical invasion) is associated with decreased disease-specific survival and overall survival (Li et al., 2017). A marginal mandibulectomy, or rim resection of only the alveolar process or the lingual cortex of the mandible, is oncologically safe as long as the tumor abuts or only superficially invades the mandible (Chen et al., 2011; Patel et al., 2008). Physical exam findings (such as fixation and paresthesias) and imaging (CT or MRI) should be used to determine extent of surgical resection preoperatively (Van Cann et al., 2008; Nomura et al., 2005). In dentulous patients, in additional to erosion and infiltration of the bone, the tumor can migrate through the dental socket into the mandible, especially in the setting of dental extractions (Genden et al., 2005). When there is clinical or radiographic evidence of medullary space involvement, a segmental mandibulectomy is indicated. Of particular importance when determining extent of surgery is the status of the mental/inferior alveolar nerve. Clinical or radiographic findings that are concerning for involvement will require a more aggressive resection to obtain microscopically uninvolved bone, marrow, and nerve margins (**Fig. 14**).

Wide local excision (i.e., partial glossectomy) is the treatment of choice for small tumors confined to the tongue without significant posterior extension. In more advanced cases (e.g., deep extension to the extrinsic tongue muscles or involvement of the floor of mouth), a more invasive approach to the tumor is necessary to provide negative margins. The extent of resection (i.e., amount of oral tongue and floor of mouth mucosa) and the approach needed play a significant role in the reconstruction. Small transoral resections of the oral tongue can be closed primarily or left open to heal through secondary intention. Larger defects can be reconstructed with a split-thickness autograft if there is limited floor of mouth involvement. If significant portions of the floor of mouth are involved, one must be concerned about tethering and the associated functional consequences such as dysarthria and dysphagia. If a significant amount of floor of mouth mucosa is resected, local, regional, or free flap reconstruction with thin pliable tissue is necessary to preserve tongue mobility. Regional flaps like the submental island flap can be used for reconstruction without effecting oncologic safety (Kramer et al., 2015; Rigby & Hayden, 2014; Howard et al., 2014).

Cancer of the buccal mucosa can generally be excised via transoral approach. The buccinator muscle can be resected along with the primary lesion to provide an appropriate deep margin. Larger and more advanced tumors may require a more extensive ablation (e.g., resection of the pterygoids, subcutaneous fat and overlying skin with a resultant through-and-through defect, etc.) and reconstruction.

Small, early-stage lesions of the hard palate can also be removed through a wide local excision with the periosteum as an appropriate deep margin. If the periosteum or the underlying bone is involved, similar to an alveolar ridge lesion, resection of the bone via a maxillectomy is necessary. An inferior or subtotal maxillectomy may be necessary to obtain negative margins. If the nasal cavity or maxillary sinus is entered as a consequence of tumor ablation, the amount of connection will dictate reconstruction. Obturators, local flaps, or free flap reconstruction (bony vs. soft tissue) are all viable options with differing risks and benefits (**Fig. 15**).

Advances in reconstructive surgery, together with improvements in our understanding of the physiology of swallowing and in speech rehabilitation, have helped to reduce the morbidity associated with surgery in the oral cavity. Surgical reconstruction may be

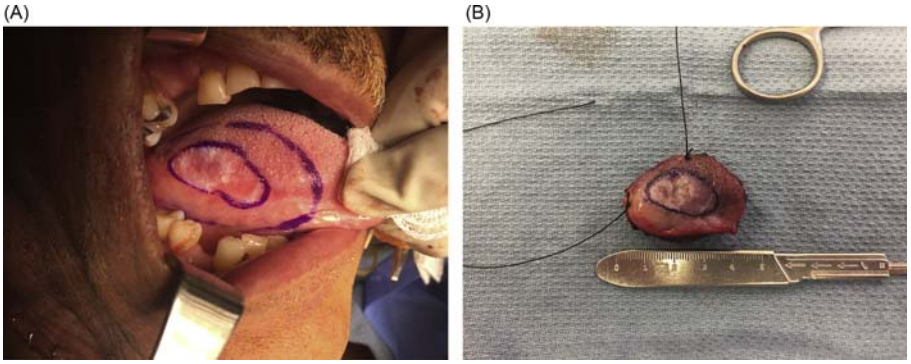

Fig. 14 (A) cT1 squamous cell carcinoma of the right lateral tongue, marked circumferentially with a 1-cm margin. (B) Same specimen after resection; the greatest degree of margin shrinkage is noted near the floor of the mouth. From Shapiro, M. and Salama, A. (2017). Margin analysis: Squamous cell carcinoma of the oral cavity. *Oral and Maxillofacial Surgery Clinics of North America* **29**(3), 259–267.

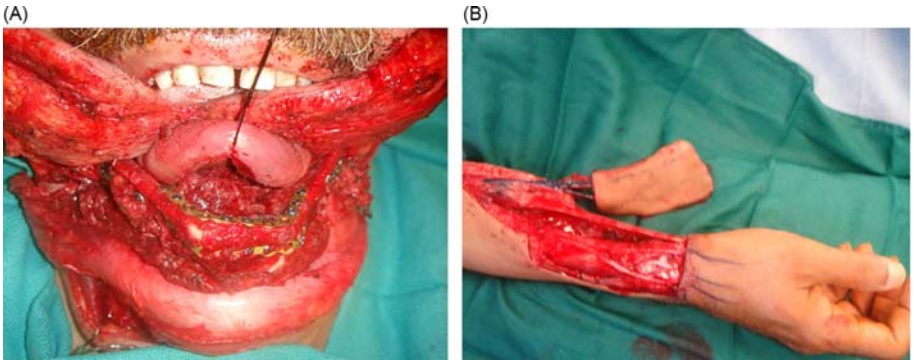

Fig. 15 Fibular (left) and radial forearm (right) free flaps are two common flaps used in oral cavity reconstruction after major resections. From Montero, P. H. and Patel, S. G. (2015). Cancer of the oral cavity. *Surgical Oncology Clinics of North America* **24**(3), 491–508.

performed after confirmation of complete resection with negative surgical margins. The reconstructive ladder ranging from primary closure or secondary intention to free tissue transfer must be employed with special consideration to long term swallowing, speech, and breathing. Small surgical defects of the oral tongue may not require reconstruction and therefore are often allowed to heal by secondary intention while a similarly sized defect of the floor of mouth could lead to debilitating tethering, dysphagia, and dysarthria. Larger or complex defects may be reconstructed by a combination of primary closure, split-thickness skin graft, vascularized cutaneous free flap, regional myocutaneous flap, or microvascular free flap (Urken et al., 1994). Reconstruction of a segmental defect of the mandible is a complex topic that is beyond the scope of this article (**Fig. 16**).

Surgical management of the neck

More than 90% of occult metastatic disease from oral cavity cancer involves nodal groups I–III (submental, submandibular, upper, and middle jugulodigastric nodes), thus a selective neck dissection removing these levels (formerly known as a supraomohyoid neck dissection) is oncologically sound, especially in the clinically N0 neck. Elective supraomohyoid neck dissection has been shown to improve survival rates compared to primary tumor resection alone in early stages of oral squamous cell carcinoma (Brazilian Head and Neck Cancer Study Group, 1998; Kligerman et al., 1994; D'Cruz et al., 2015). In particular, patients with T1-T2, N0 oral tongue cancer with tumor thickness >4 mm had significant improvement in disease-specific survival with elective neck dissection compared to observation. Because of the survival advantage and the poor salvage rate, elective neck dissection has been the standard of care (Abu-Ghanem et al., 2016).

In general, elective neck dissection in the clinically N0 neck is indicated when there is at least a 20% chance of lymph node involvement. The lower lip has a low risk of occult metastasis and therefore does not require elective neck dissection (Ozturk et al., 2015; Gary et al., 1995). A systematic review and meta-analysis demonstrated that the overall rate of cervical metastasis from a maxillary squamous cell carcinoma was 31%, with increasing risk as T-stage advances (Zhang and Peng, 2016). Patients with a primary tumor that involves the oral tongue, floor of mouth, mandibular alveolar ridge, or buccal mucosa present with an increased risk of occult cervical metastasis and should therefore be considered for an elective neck dissection (Capote et al., 2007; Fasunla et al., 2011). Based on lymphatic drainage pathways, midline tumors should be treated with bilateral neck dissections.

Recently, results were published on the first 500 patients enrolled on a prospective, randomized controlled trial evaluating the effect of elective node dissection (i.e., ipsilateral neck dissection with the primary surgery) versus therapeutic node dissection (i.e., neck dissection following nodal relapse) on survival in patients with lateralized T1-T2 squamous cell carcinomas of the oral cavity

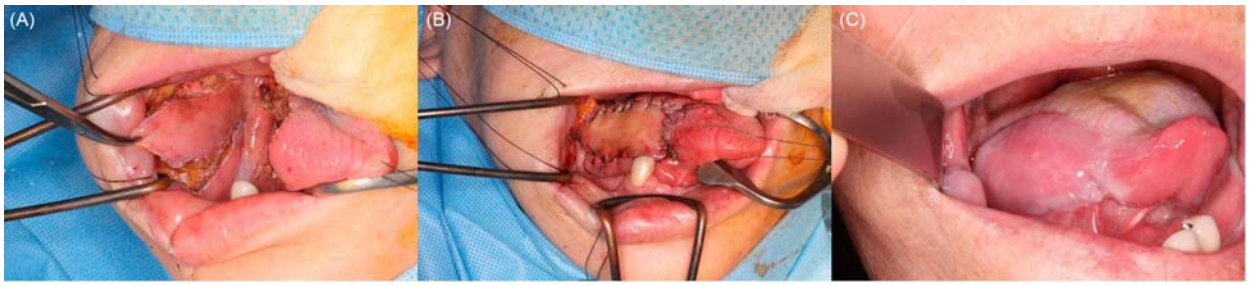

Fig. 16 After partial glossectomy, a posterior-based buccinator myomucosal flap (based on buccal artery) was harvested (A) and sutured to the residual tongue (B). Photograph taken 6 months postoperatively reveals healthy condition of the transplanted flap without shrinkage (C). From Ahn, D., Lee, G. J., and Sohn, J. H. (2017). Reconstruction of oral cavity defect using versatile buccinator myomucosal flaps in the treatment of cT2–3, N0 oral cavity squamous cell carcinoma: Feasibility, morbidity, and functional/oncological outcomes. *Oral Oncology* **75**, 95–99.

(D'Cruz et al., 2015). After a median follow up of 39 months, elective neck dissection resulted in higher rates of 3-year overall and disease-free survival than therapeutic neck dissection with similar rates of adverse events in the elective-surgery and therapeutic-surgery groups (6.6% and 3.6%, respectively).

A systematic review and meta-analysis comparing elective neck dissection with observation in patients with clinically node-negative, early-stage T1-T2 oral tongue squamous cell carcinoma showed that elective neck dissection can significantly reduce the rate of regional nodal recurrence and improve disease-specific survival in this population (Abu-Ghanem et al., 2016). A meta-analysis on selective versus comprehensive neck dissection in oral squamous cell carcinoma patients with clinically node-positive necks showed that selective neck dissection achieves similar regional control, disease-specific survival, and overall survival compared with comprehensive neck dissection (Liang et al., 2015).

Overall, there is increasing evidence that elective neck dissection in oral cavity carcinoma, even early stage, reduces regional recurrence rates, disease specific survival, and improves overall survival compared to observation alone (D'Cruz et al., 2015; Fasunla et al., 2011) (**Table 2**).

Sentinel lymph node biopsy is a less invasive procedure than neck dissection which is routinely used for other nonoral cavity sites of primary malignancy. A German prospective consecutive cohort analysis first correlated sentinel lymph node positivity with survival in early oral cavity and oropharyngeal cancer (Broglie et al., 2013). The authors found that the 3-year overall survival in patients with a negative sentinel lymph node was 98% compared to 71% in sentinel lymph node positive patients. Similar differences were seen in disease-specific survival (95% vs. 76%) and disease-free survival (98% vs. 73%). In addition, they identified correlation between the presence of isolated tumor cells or micrometastases and survival. Other authors have reported the sensitivity and negative predictive value of sentinel lymph node biopsy for oral cancer staging as 71% and 94%, respectively (Rigual et al., 2013). Further research to validate these findings may lead to changes in patterns of care based on the utility of the sentinel lymph node biopsy.

Adjuvant Therapy Following Definitive Surgical Resection

Following surgical resection, patients with pathologic features such as advanced primary T stage (T3-T4), lymphovascular space invasion, perineural invasion, positive margins, multiple pathologically involved cervical lymph nodes, and ENE have been shown to be at high-risk for locoregional recurrence (Cooper et al., 1998). PORT, frequently in conjunction with chemotherapy, results in a decreased risk of locoregional relapse for these patients (Lavaf et al., 2008; Maccomb, 1957). A nomogram constructed using data from 590 patients treated at a United States cancer center and validated using data from 417 patients treated at a Brazilian hospital can be used to help decide adjuvant treatment; however, limitations of the nomogram include the lack of important pathologic variables including ENE, perineural invasion, and lymphovascular space invasion, as well as the nonuniform use of radiation therapy between the construction and validation cohorts (39.5% vs. 60.4%) (Gross et al., 2008). More recently this group has created and validated an online nomogram using data from >1400 patients to estimate locoregional failure-free survival with and without PORT (Wang et al., 2013). Factors predictive of a locoregional control benefit from PORT include high N stage, low T stage, positive margins, young age, and female sex.

Adjuvant radiotherapy is preferred over preoperative radiotherapy based on Radiation Therapy Oncology Group (RTOG) 7303, which demonstrated that PORT provided superior locoregional control (70% vs. 58%) when compared to preoperative radiotherapy in patients with advanced head and neck cancer. Overall survival was unaffected by the timing (Tupchong et al., 1991). PORT provides the advantage of avoiding delay in implementation of surgical resection and obtaining complete pathologic staging of the tumor. However, postoperative wound complications may delay the initiation of PORT, and the regional hypoxia that can accompany the postoperative state may diminish the effectiveness of PORT compared to that achievable under conditions of full oxygenation.

Table 2 Types of neck dissections

	Lymph nodes excised	*Other structures excised*	*Structures preserved*
Radical neck dissection	Levels I–V	Sternocleidomastoid muscle, internal jugular vein, spinal accessory nerve, submandibular gland	
Modified radical neck dissection type I	Levels I–V	Sternocleidomastoid muscle, internal jugular vein, submandibular gland	Spinal accessory nerve
Modified radical neck dissection type II	Levels I–V	Internal jugular vein, submandibular gland	Sternocleidomastoid muscle, spinal accessory nerve
Modified radical neck dissection type III	Levels I–V	Submandibular gland	Sternocleidomastoid muscle, internal jugular vein, spinal accessory nerve
Supraomohyoid neck dissection	Levels I–III	Submandibular gland	Sternocleidomastoid muscle, internal jugular vein, spinal accessory nerve

From Montero, P. H., Patel, S. G. (2015). Cancer of the oral cavity. *Surgical Oncology Clinics of North America* **24**(3), 491–508.

Overall treatment time has been shown to be a critical factor in tumor control for cancers of the head and neck, including those of the oral cavity. Improved outcomes have been demonstrated when all radiation therapy is completed within 40 days for oral tongue cancer (Mendenhall et al., 1989). For patients with advanced head and neck cancer treated with combined modality therapy, the overall time from surgery to the completion of radiation therapy has been shown to be inversely related to prognosis. Multiple phase III prospective trials have demonstrated that the cumulative duration of combined modality therapy drives locoregional control and survival (Ang et al., 2001; Rosenthal et al., 2017). Significantly improved outcomes have been reported when the treatment package time was <85 days, and the treatment package time had a greater impact on locoregional control and survival than the dose of radiation administered (Rosenthal et al., 2017). High-risk patients who had a prolonged delay between surgery and PORT had an improved outcome with an accelerated fractionation schedule compared with those irradiated once daily (Ang et al., 2001).

Adjuvant chemoradiotherapy

Cisplatin-based chemotherapy combined with PORT has been compared to PORT alone for medically fit head and neck cancer patients in multiple randomized studies. These studies demonstrated statistically significant (Bernier et al., 2004; Bachaud et al., 1996; Fietkau et al., 2006) or strong statistical trends (Cooper et al., 2004) for improved locoregional control and disease-free survival with the addition of chemotherapy to PORT. Two of these studies demonstrated a statistically significant improvement in overall survival (Bernier et al., 2004; Noronha et al., 2018), while the others have shown numerically improved but not statistically significant survival improvements (Fietkau et al., 2006; Cooper et al., 2004). Of note, at least one quarter of patients in both the European Organization for Research and Treatment of Cancer (EORTC) 22931 and RTOG 9501 studies had oral cavity cancer, 26% and 27% respectively. A pooled analysis of the latter two trials demonstrated improved overall survival with the addition of concurrent chemotherapy to PORT for patients with positive margins and/or ENE (Bernier et al., 2005).

More recently, the final results of the Oral Cavity Adjuvant Therapy (OCAT) trial comparing multiple adjuvant treatment strategies (conventionally fractionated radiation with five fractions per week vs. altered fractionation with six fractions per week vs. conventional chemoradiation with weekly cisplatin 30 mg/m^2) in patients with resectable high-risk, locally advanced oral cavity cancer (stage III/IV and at least ENE, involvement of 2 or more regional lymph nodes, microscopically positive margins, extensive soft tissue or skin involvement requiring major reconstruction, perineural invasion, or lymphovascular space invasion) were presented (Laskar et al., 2016). No significant difference in locoregional control, disease-free survival, or overall survival was noted between the treatment arms. Planned subgroup analysis showed that chemoradiotherapy with weekly cisplatin resulted in improved locoregional control, disease-free survival, and overall survival compared to conventional radiation for patients with T3-T4, N2-N3 disease with ENE. There was no apparent difference in toxicity or radiotherapy compliance among the three regimens. The results of this trial have to be interpreted carefully, as most of the patients developed oral cavity cancer from betel nut chewing. Therefore, it is not clear if these results can be extrapolated to other areas where oral cavity cancer typically results from other risk factors. Also, a majority of the patients on the trial had gingiva/buccal tumors, so it is not clear if these results can be extrapolated to patients with oral tongue or floor of mouth cancers (**Fig. 17**). Additionally, the chemotherapy regimen utilized (30 mg/m^2 of weekly cisplatin) is considered to be suboptimal (see below), which may account for the lack of difference in outcomes between treatment arms.

Concurrent chemotherapy regimens for adjuvant chemoradiotherapy

Many consider bolus cisplatin (100 mg/m^2) given every 3 weeks with radiation therapy to be the standard of care for cancers of the head and neck; however, the optimal chemotherapy schedule is unknown. Bolus cisplatin (100 mg/m^2) schedules were tested in the previously mentioned RTOG 9501 and EORTC 22931 studies (Bernier et al., 2004; Cooper et al., 2004). A French randomized trial (Bachaud et al., 1996) utilized 50 mg weekly cisplatin, while the ARO 96-3 trial (Fietkau et al., 2006) employed cisplatin 20 mg/m^2 and 5-fluorouracil 600 mg/m^2 on days 1–5 and 29–33. A phase III randomized trial comparing the use of bolus cisplatin (100 mg/m^2) to weekly cisplatin (40 mg/m^2) with concurrent adjuvant radiation therapy in patients with high-risk (ENE, positive surgical margins, or pN2-N3) squamous cell carcinoma of the oral cavity demonstrated no difference in overall or locoregional recurrence free survival at 1 year (Tsan et al., 2012). A recently reported study in patients with head and neck cancer ($>90\%$ with oral cavity cancer) receiving adjuvant or definitive concurrent chemoradiotherapy compared cisplatin delivered 30 mg/m^2 weekly versus 100 mg/m^2 every 3 weeks (Noronha et al., 2018). Bolus cisplatin (100 mg/m^2) was found to improve locoregional control, which was the primary endpoint; however, many consider the weekly cisplatin regimen of 30 mg/m^2 utilized in this trial to be suboptimal.

The role of carboplatin-based chemotherapy regimens given concurrently with PORT versus PORT alone remains poorly defined. Randomized studies have been attempted, but unfortunately these studies were closed early before their accrual goals were met. Preliminary results revealed no significant benefit in locoregional control, disease-free survival, or overall survival from combining carboplatin with PORT (Expert Panel on Radiation Oncology-Head and Neck et al., 2011).

RTOG 0234 is a phase II trial of high-risk postoperative patients (positive margin, ENE, and/or ≥ 2 pathologically involved cervical nodes) randomized to PORT in combination with cetuximab (400 mg/m^2 loading dose followed by 250 mg/m^2 weekly) and weekly docetaxel (15 mg/m^2) or to PORT with cetuximab (400 mg/m^2 loading dose followed by 250 mg/m^2 weekly) and weekly cisplatin (30 mg/m^2) (Harari et al., 2014). Published results from this study compared favourably to outcomes seen in similar patients treated with PORT and cisplatin (100 mg/m^2 every 3 weeks) on RTOG 9501. It should be noted that almost half of the patients enrolled on RTOG 0234 (46.8%) had cancers of the oral cavity. Based on these findings, RTOG 1216,

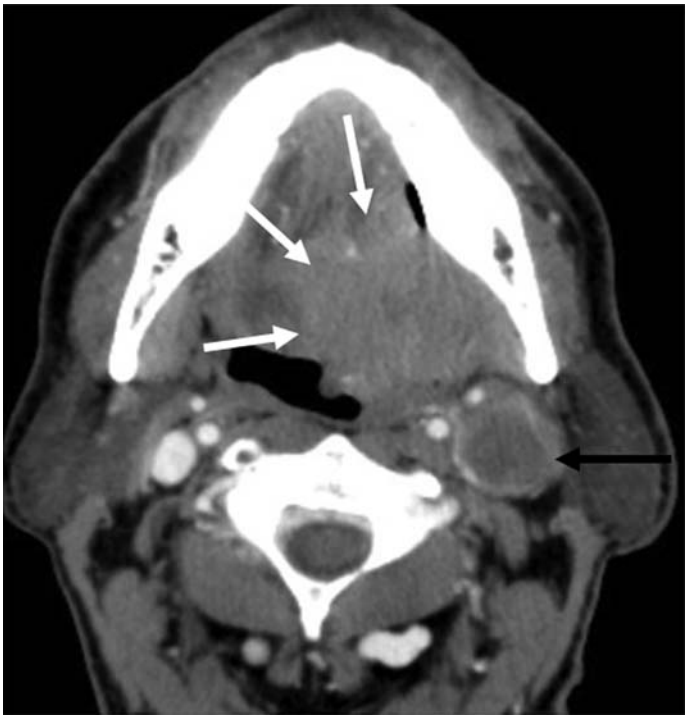

Fig. 17 Contrast-enhanced CT axial image in a 55-year-old male smoker with squamous cell carcinoma of the tongue demonstrates an enhancing soft tissue mass involving the left oral tongue (*white arrows*) with extension to the midline. There is also a large necrotic-appearing left level 2 lymph node (*black arrow*) consistent with nodal metastasis. From Meesa, I. R., Srinivasan, A. (2015). Imaging of the oral cavity. *Radiologic Clinics of North America* **53**(1), 99–114.

a randomized phase II/III trial, was designed to determine the effectiveness of docetaxel with or without cetuximab relative to cisplatin in the setting of adjuvant PORT.

Currently, no randomized phase III data exist regarding the use of other chemotherapy agents in the postoperative setting for head and neck cancer. As a result, many consider cisplatin 100 mg/m^2 every 3 weeks to be the standard.

Adjuvant radiotherapy dose, volume, and modality

The optimal adjuvant radiotherapy dose is not well established. Most of the randomized studies which demonstrated a benefit of concurrent chemoradiotherapy in the adjuvant setting utilized doses of 60–66 Gy in 2 Gy daily fractions to high-risk areas (primary tumor bed with a positive margin, or nodal regions with ENE) and doses of 50–54 Gy in 2 Gy fractions to areas at risk for microscopic involvement. In three of the four randomized studies investigating adjuvant chemoradiotherapy, doses $>$65 Gy were prescribed to high-risk areas (Bernier et al., 2004; Bachaud et al., 1996; Fietkau et al., 2006), while the other study, RTOG 9501, utilized a dose of 60 Gy with or without an optional 6 Gy boost (Cooper et al., 2004). Despite the fact that little evidence exists to support the higher PORT doses used in these randomized trials over the doses established from PORT-alone dose-finding studies (63 Gy for ENE and 57.6 Gy for all other areas), we recommend similar dosing schedules as the randomized trials given that they were associated with significant overall survival benefits in patients with ENE and positive margins.

Frequently, the radiotherapy treatment volume used in the adjuvant setting for head and neck cancer encompasses the primary tumor site and the bilateral cervical lymph node regions, yet it is unclear whether both always need to be included. For patients with completely resected primary tumors and negative margins whose only indication for PORT is pathologic cervical lymphadenopathy, some clinicians would recommend therapy only to the neck. Similarly, for patients who have undergone a comprehensive neck dissection with pathologically uninvolved cervical lymph nodes and have only a positive margin in the primary tumor bed as their sole indication for treatment, clinicians may recommend PORT to the tumor bed only. Another setting where a less comprehensive radiation volume may be recommended is for patients with well-lateralized primary tumors where the reported patterns of progression suggest that this may be appropriate (Expert Panel on Radiation Oncology-Head and Neck et al., 2011).

Intensity modulated radiation therapy (IMRT) is the recommended treatment modality for head and neck cancers whenever high-dose, curative-intent irradiation is administered, whether definitively or adjuvantly (Mell et al., 2005). The benefits of IMRT are further discussed later in this article in the IMRT subsection of Definitive Radiation Therapy.

Definitive Radiation Therapy

Historically, the oral cavity has been divided into subsites, as the location of the primary tumor would determine the radiation modalities that could be utilized to deliver dose. As external beam radiotherapy delivery techniques have improved, these distinctions have become less clinically relevant. For early stage oral cavity cancer, radiotherapy alone can result in excellent local control and survival rates; however, local control decreases with more advanced tumor stage (**Fig. 18**).

The optimal dose fractionation schedule for patients receiving definitive radiotherapy alone for oral cavity cancer has not yet been established. Randomized data (Beitler et al., 2014; Overgaard et al., 2003; Overgaard et al., 2010) and meta-analyses (Budach et al., 2006; Bourhis et al., 2006) support an overall survival benefit with the use of accelerated fractionation or hyperfractionated radiotherapy, such that some form of altered fractionation should be utilized with treating oral cavity cancer patients with radiotherapy alone. One multi-institutional, international, randomized study comparing an accelerated regimen of 66–70 Gy delivered in 2 Gy daily fractions 6 days per week to the same dose delivered 5 days per week found improved locoregional control (42% vs. 30%, $p = .004$), disease-free survival (50% vs. 40%, $p = .03$), and a trend toward improved overall survival (35% vs. 28%, $p = .07$) with accelerated fractionation (Overgaard et al., 2010). When analysed with respect to HPV status, accelerated fractionated radiotherapy resulted in improved local control for both p16-positive (hazard ratio [HR] 0.56 [95% confidence interval (CI) 0.33–0.96]) and p16-negative tumors (HR 0.77 [95% CI 0.60–0.99]) (Lassen et al., 2011). However, when a more intensive accelerated regimen of 1.8 Gy twice daily to 59.4 Gy was compared to 70 Gy in 2 Gy fractions in stage III/IV head and neck cancer patients, no statistically significant benefits were seen in terms of locoregional control or overall survival (Poulsen et al., 2001). It is unknown if the lack of benefit seen was due to the regimen used, or due to inclusion criteria as the benefit for acceleration in some randomized studies was less significant for those with stage IV disease as well as those with a larger nodal disease burden (Overgaard et al., 2010).

The meta-analysis of radiotherapy in carcinoma of the head and neck (MARCH) Collaborative Group pooled 15 randomized studies (including 6515 patients, 13% with oral cavity cancer) and compared standard fractionation to either accelerated or hyperfractionated radiotherapy. Altered fractionation schedules were associated with an 8.5% absolute reduction in local failure and a 3.4% absolute improvement in 5-year overall survival. Further analysis demonstrated no significant interaction of tumor site on overall survival or tumor control. Heterogeneity in the patients included on the accelerated and hyperfractionated trials did not allow for direct comparisons, but patients treated with a hyperfractionated radiotherapy regimen were noted to have an 8.2% absolute improvement in overall survival at 5 years compared to only a 2% absolute improvement in those treated with an accelerated fractionation schedule (Bourhis et al., 2006). This meta-analysis was updated recently with increased patient numbers and follow up, and it confirmed the earlier finding that altered fractionation was associated with an improvement in overall survival, with an absolute benefit of 1.2% at 10 years (Lacas et al., 2017). This benefit also appeared to be limited to patients receiving hyperfractionated therapy, with an absolute benefit in overall survival of 3.9% at 10 years.

RTOG 9003 compared standard fractionation (70 Gy in 2 Gy/day fractions), hyperfractionation (81.6 Gy in 1.2 Gy/fraction, twice-daily), split-course accelerated fractionation (67.2 Gy in 1.6 Gy/fraction twice-daily with a 2 week rest after 38.4 Gy), and accelerated fractionation with a concomitant boost (72 Gy in 1.8 Gy/fraction for 14 fractions followed by 1.8 Gy morning and 1.5 Gy afternoon boost to gross disease) for patients with cancers of the head and neck, excluding only nasopharynx; however, only about 10% of patients had oral cavity cancer (Fu et al., 2000). In the initial report, locoregional control was improved with hyperfractionation and accelerated concomitant boost as compared to the standard fractionation. A trend toward improved disease-free survival for patients treated with hyperfractionation (37.6% vs. 31.7%, $p = .067$) or accelerated concomitant boost (39.3% vs. 31.7%, $p = .054$) was also noted. With longer follow up, patients receiving a hyperfractionated regimen demonstrated

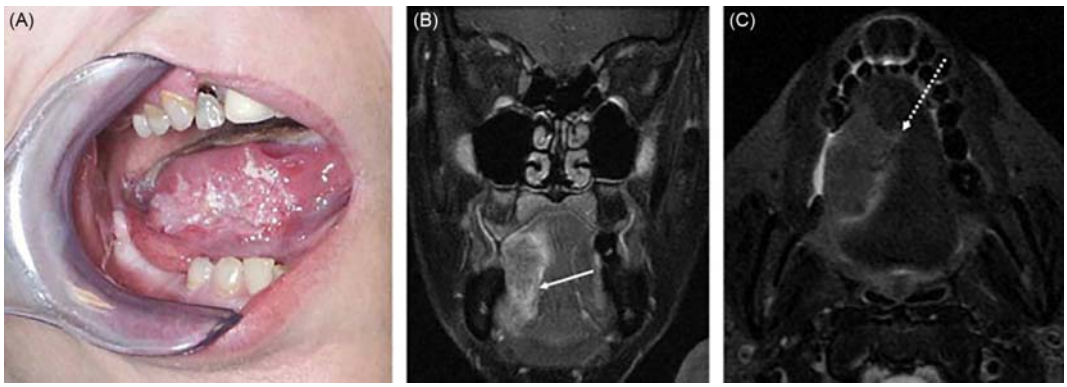

Fig. 18 Right lateral tongue squamous cell carcinoma crossing midline, with involvement of the genioglossus muscles. (A) Photograph shows an extensive area of mucosal irregularity and leukoplakia involving the right lateral tongue. The patient was unable to move the tongue due to tumor infiltration and pain. Coronal T1-weighted postcontrast fat-saturated (B) and axial STIR (C) images show a T2-hyperintense enhancing mass in the right tongue. The coronal image shows inferior extension into the floor of mouth/sublingual space (*solid white arrow* in B) and medial involvement of the genioglossus muscle. The axial image shows extension across the midline (*dashed arrow* in C). From Hagiwara, M., Nusbaum, A., and Schmidt, B. L. (2012). MR assessment of oral cavity carcinomas. *Magnetic Resonance Imaging Clinics of North America* **20**(3), 473–494.

improved overall survival (HR 0.81, p = .05) when patients were censored at 5 years (Beitler et al., 2014). Hyperfractionated patients also exhibited less late toxicity when compared to accelerated fractionated patients. Most notably, in patients who were disease-free at 5 years, only 4.8% of hyperfractionated patients had feeding tubes versus 13.0% of patients receiving accelerated fractionation with concomitant boost. It is difficult to determine the clinical significance of these toxicity data today since all of patients in the RTOG study were treated with parallel-opposed fields and the blocking techniques were rudimentary with only minimal sparing of the parotid glands and pharyngeal constrictor muscles. Currently, contemporary IMRT and image-guided radiotherapy (IGRT) techniques allow for increased sparing of these structures, which likely significantly reduces late treatment-related morbidity.

Intensity modulated radiotherapy

Intensity modulated radiotherapy (IMRT) has been widely adopted for the treatment of head and neck cancers (Mell et al., 2005). Retrospective single institution (Studer et al., 2007; Studer et al., 2012) and SEER-Medicare (Beadle et al., 2014) analyses have shown significant improvements in local control and cause-specific survival, respectively, with the use of IMRT in oral cavity cancer patients. Helical tomotherapy and volumetric modulated arc therapy (VMAT) are advanced forms of IMRT. While theoretical consideration of second cancers exists with IMRT (Hall, 2006), this technology has the ability to minimize normal organ exposure to radiation. This is particularly important for oral cavity cancer patients as pharyngeal constrictor dose is associated with dysphagia (Eisbruch et al., 2004; Caudell et al., 2010) and dose to the parotid, submandibular, and minor salivary glands are associated with xerostomia (Nutting et al., 2011). In addition, IMRT has the potential to decrease acute dermatitis. A prospective, randomized trial demonstrated the beneficial impact of IMRT on the reduction of xerostomia in patients with cancer of the oropharynx or hypopharynx, using the late effects of normal tissues scale, unstimulated and sodium citrate stimulated parotid saliva from each parotid orifice and the floor of mouth, and patient-reported quality of life (Nutting et al., 2011; **Fig. 19**).

Brachytherapy

Brachytherapy is the placement of radioactive materials in close proximity to or inside of tumors and was developed prior to IMRT or the use of concurrent chemotherapy. It allowed practitioners to deliver a tumoricidal dose of radiation to tumors in the head and neck while minimizing mandibular dose. Improved external beam radiotherapy planning and delivery techniques as well as a recognition that the incidence of osteoradionecrosis resulting from brachytherapy is significantly underreported, has resulted in a substantial decrease in the utilization brachytherapy for oral cavity tumors. Historically, brachytherapy's role in oral cavity cancer has been to boost the dose to gross disease following external beam radiotherapy. Typically, catheters are implanted under general anesthesia in the operating room, and as such oral cavity brachytherapy is a highly operator-dependent procedure (**Fig. 20**). Given the historically poor locoregional control rates for oral cavity tumors treated with external beam radiotherapy alone, intensifying the treatment with brachytherapy makes logical sense. Higher rates of locoregional control have been achieved using an integrated treatment approach of external beam radiotherapy directed at the primary and bilateral neck, followed by a brachytherapy boost (Harrison, 1997).

Complications of brachytherapy for oral cavity tumors can include mucosal ulceration, soft tissue necrosis, and osteoradionecrosis of the mandible (YamazakI et al., 2013). The risk of complications appears to be related to the technique of implantation, but may reach as high as 30%. Quality of life analyses comparing a combined regimen of brachytherapy plus external beam radiotherapy to surgery followed by PORT favored the combination of brachytherapy and external beam radiotherapy, suggesting that in experienced hands this is a reasonable treatment method for head and neck carcinoma (Levendag et al., 2004). There is limited information regarding the use of brachytherapy and chemotherapy concurrently, and as such this approach should be avoided outside of a formal trial setting.

Brachytherapy guidelines

The American Brachytherapy Society (ABS) has published guidelines for the use of high dose rate (HDR) brachytherapy for head and neck cancer, including specific guidelines for oral cavity tumors (Nag et al., 2001). The ABS panel members advise that whenever possible, a spacer be constructed and inserted at the time of the HDR treatment to reduce doses to normal tissues such as the mandible and mucosa of the palate. The relative risk of normal tissue injury for lesions near the mandible, such as the floor of mouth, is greater than for oral tongue, where the bone is not contiguous with the target volume. The interval between external beam radiotherapy and brachytherapy should be minimized (within 1–2 weeks, depending on the degree of recovery from mucositis), and the total duration of combined radiation therapy should be kept as short as possible (ideally <8 weeks) to minimize tumor cell repopulation. Expert panel evidence, as well as single institution series, recommend external beam radiotherapy doses of 40–50 Gy to the primary tumor (or 45–60 Gy when the neck is treated for microscopic disease) followed by a HDR brachytherapy boost of 2.7–3.0 Gy per fraction for 6–7 doses with locoregional control of implanted tumors reaching 80%. Similar to the ABS, the European Brachytherapy Group (GEC) and European Society for Therapeutic Radiology and Oncology (ESTRO) developed joint guidelines for the use of brachytherapy in the treatment of head and neck carcinoma based on consensus recommendations given the lack of randomized trial data (Mazeron et al., 2009; Kovács et al., 2017). For oral cavity tumors, these guidelines recommend 46–50 Gy of external beam radiotherapy followed by a 15–30 Gy low dose rate brachytherapy boost or equivalent HDR dose. In order to minimize overall treatment time, HDR brachytherapy is often administered as two fractions per day separated by at least six hours.

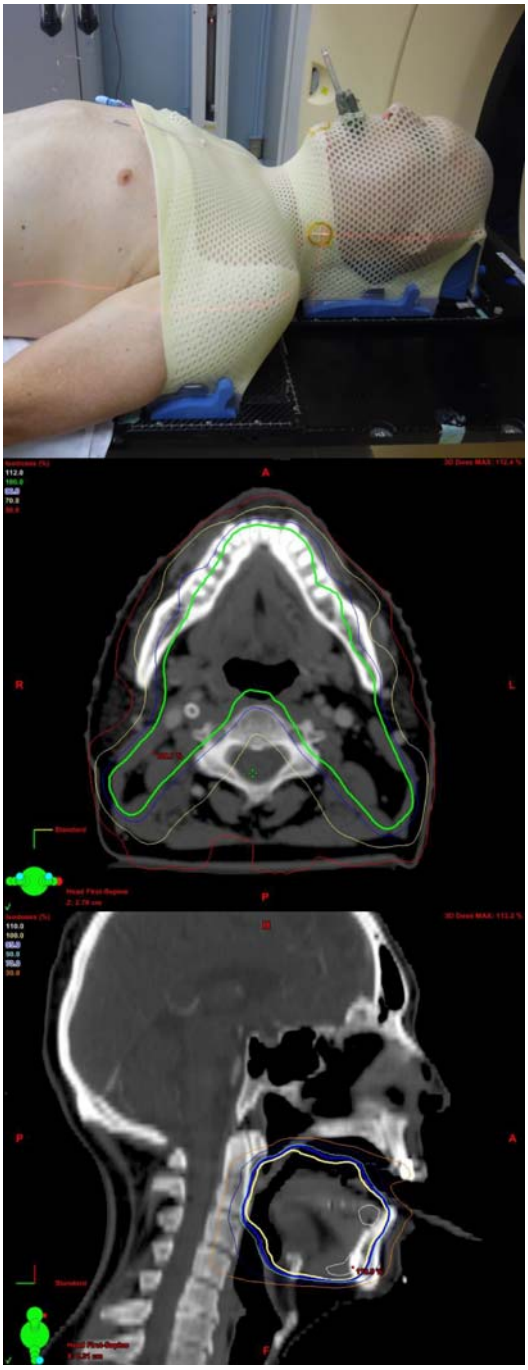

Fig. 19 (Top) CT simulation for radiation treatment planning with patient in supine position, immobilized with five point customized thermoplastic face mask covering the shoulders and a bite block in place to depress the tongue away from minor salivary glands. (Middle) Axial CT image showing the isodose lines for the intensity-modulated primary radiation fields encompassing the high-risk elective nodal regions and the primary tumor. (Bottom) Sagittal CT image showing the isodose lines for the intensity-modulated boost radiation fields to treat the primary tumor.

Concurrent chemoradiotherapy for locoregionally advanced oral cavity cancer

Although surgical resection followed by adjuvant radiation with or without chemotherapy is preferred for patients with large infiltrative primary tumors of the oral cavity, definitive concurrent chemoradiotherapy has been shown to be a reasonable option for patients who are unable to undergo surgical resection. In addition, surgical resection may be possible but not recommended for certain patients given the associated surgical morbidity and likely necessity of adjuvant chemoradiotherapy, which has a similar toxicity profile to definitive intent chemoradiotherapy (Fig. 21).

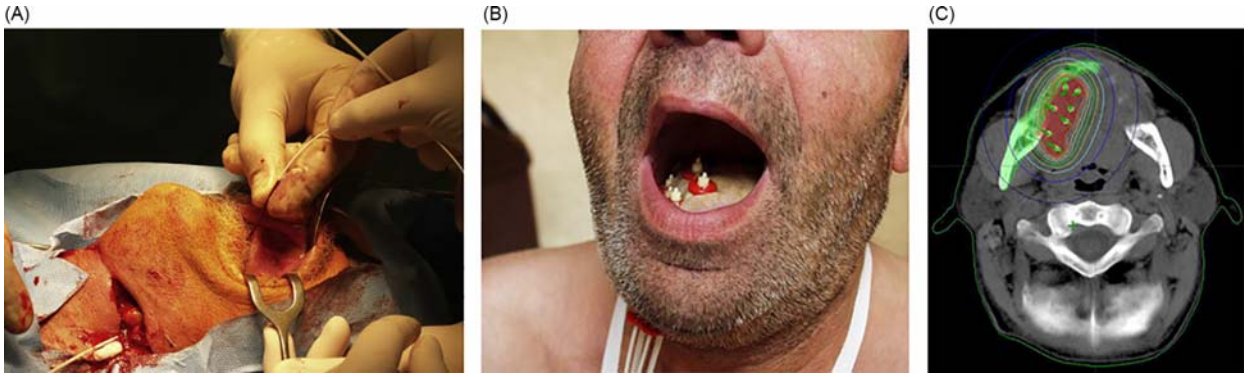

Fig. 20 (A) Technique of afterloading catheters insertion. (B) Implantation of afterloading catheters. (C) CT-based planning of dose distribution. From Tuček L, Petera J, Sirák I, Vošmik M, Doležalová H, Brokešová S, Hodek M, Kašaová L, Paluska P. (2011) Hyperfractionated high-dose rate brachytherapy in the treatment of oral tongue cancer. *Reports of Practical Oncology and Radiotherapy* **16**(6), 243–247.

Multiple studies have demonstrated a benefit with concurrent chemoradiotherapy in the definitive treatment of head and neck cancer, including oral cavity cancer (Adelstein et al., 2003; Brizel et al., 1998; Wendt et al., 1998). However, the use of concurrent chemoradiotherapy for patients with locoregionally advanced oral cavity cancer without distant metastasis is based primarily on the results of the meta-analysis of chemotherapy in head and neck cancer (MACH-NC), which demonstrated a 6.5% absolute improvement in overall survival at 5 years from the use of concurrent chemoradiotherapy compared to radiotherapy alone (Pignon et al., 2009). However, the beneficial effects of chemotherapy appeared to decrease with age. Patients with age >70 years did not derive a significant benefit, likely because more advanced age is associated with increasing frailty and more competing risks of mortality. Subgroup analysis demonstrated the benefit of adding chemotherapy to locoregional treatment alone for patients with oral cavity cancer, with a HR of 0.87 (95% CI 0.80–0.93) and an absolute improvement in 5-year overall survival of 5.1% (Blanchard et al., 2011). While this meta-analysis did not show a significant interaction between chemotherapy timing (adjuvant, neoadjuvant, vs. concomitant) and survival for oral cavity primaries, this may only be a consequence of a lack of power.

There are no randomized trials of chemoradiotherapy versus radiotherapy alone in the definitive treatment of patients with oral cavity cancer. A multiinstitution retrospective analysis investigated the outcomes of patients with locally advanced oral cavity cancer treated with definitive chemoradiotherapy on protocols between 1994 and 2008 and demonstrated 5-year overall and progression-free survival rates of 66% and 67%, respectively (Stenson et al., 2010). This cohort was updated recently to include patients treated up to 2014 and demonstrated 5-year overall and progression-free survival rates of 63% and 59%, respectively (Melotek et al., 2016). Five-year locoregional and distant control were reported as 79% and 87%, respectively. Long-term toxicities of this regimen were acceptable, with crude rates of osteoradionecrosis and sustained need for a feeding tube of 19% and 14%, respectively. A separate study from the same institution reported the outcomes of patients with oral cavity cancer treated on protocol with chemoradiotherapy using IMRT and demonstrated estimated 5-year locoregional progression-free survival, disease-free survival, and overall survival of 71%, 76%, and 76%, respectively (Pederson et al., 2011). By contrast, another single institution series of oral cavity cancer patients treated with definitive chemoradiation between 1990 and 2011 reported worse outcomes with a 3-year disease-specific survival of 38% and actuarial 5-year overall survival of 15% (Scher et al., 2015). A recent National Cancer Database (NCDB) analysis reported a 3-year overall survival of 38% for patients with locally advanced oral cavity squamous cell carcinoma treated with definitive concurrent chemoradiotherapy (Spiotto et al., 2017). Notably, patients treated with chemoradiotherapy were more likely to be older than 60 years old, have more comorbidities, and have more advanced primary tumor and/or nodal stage compared to patients treated surgically with PORT.

Concurrent chemoradiotherapy regimens

The associated toxicities of concurrent chemoradiotherapy have stimulated the search for the optimal chemotherapy regimen. Cisplatin given every 3 weeks at 100 mg/m^2 is often cited as the standard regimen. For oropharyngeal cancer, the Groupe d'Oncologie Radiothérapie Tête et Cou (GORTEC) randomized study demonstrated an overall survival advantage using a carboplatin/5-fluorouracil regimen specifically chosen to avoid cisplatin-related tinnitus, renal dysfunction, and emesis (Calais et al., 1999). Randomized studies comparing alternative cisplatin dosing schedules (such as 30–40 mg/m^2 weekly or 20 mg/m^2/day on days 1–5 and 22–26) to bolus cisplatin or to other chemoradiotherapy platforms have been conducted in nasopharyngeal cancer patients (Lee et al., 2016) or in the postoperative oral cavity setting (Tsan et al., 2012). However, multiple randomized studies that compared radiotherapy alone to concurrent chemoradiotherapy using nonbolus cisplatin schedules including daily cisplatin (6 mg/m^2/day) (Jeremic et al., 2000), weekly cisplatin (40 mg/m^2) (Sharma et al., 2010), or cisplatin (20 mg/m^2/day) given on days 1–5 repeated every 3 weeks (Huguenin et al., 2004) had comparable outcomes to bolus cisplatin. As previously mentioned, a recently reported study in patients with head and neck cancer (>90% with oral cavity cancer) receiving adjuvant or definitive concurrent chemoradiotherapy compared cisplatin delivered 30 mg/m^2 weekly versus 100 mg/m^2 every 3 weeks (Noronha et al.,

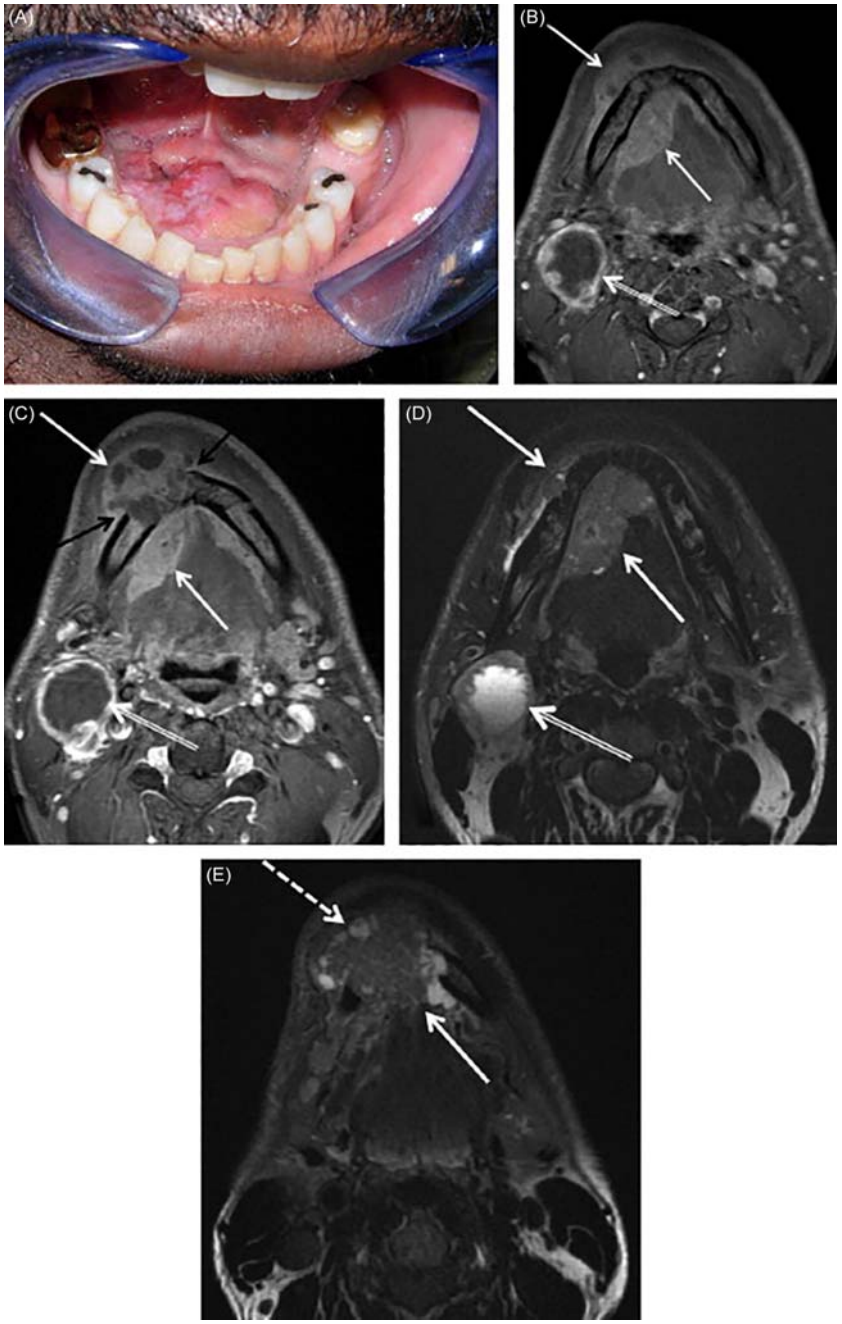

Fig. 21 T4a floor of mouth squamous cell carcinoma with bone invasion and necrotic right level II nodal metastasis. (A) Photograph shows a large exophytic and ulcerated mass in the right floor of mouth extending across the midline. (B, C) Axial postcontrast fat-saturated T1-weighted images from superior to inferior show a large heterogeneously enhancing mass with areas of necrosis in the right floor of mouth (*solid white arrows*) extending across the midline anteriorly. Gross tumor invasion of the mandible with erosion through the outer cortex is seen (*solid black arrows* in C). No evidence was seen on imaging of perineural invasion in the inferior alveolar canal. A large necrotic right level II node is seen posteriorly (*double-lined white arrows*). (D, E) T2-weighted fat-saturated images from superior to inferior similarly show a mass in the floor of mouth (*solid arrows*) extending across the midline, with anterior extension through the mandible into the submental region (*dashed white arrow* in E). Note the low T2 signal intensity of most of the mass, indicating hypercellularity. The necrotic right level II node (*double lined white arrow* in D) is also seen. From Hagiwara, M., Nusbaum, A., Schmidt, B. L. (2012). MR assessment of oral cavity carcinomas. *Magnetic Resonance Imaging Clinics of North America* **20**(3), 473–494.

2018). Bolus cisplatin (100 mg/m^2) was found to improve the primary endpoint of locoregional control; however, many consider the weekly cisplatin regimen of 30 mg/m^2 to be suboptimal. National Comprehensive Cancer Network (NCCN) guidelines recommend cisplatin 40 mg/m^2 if a weekly regimen is utilized, but high-dose cisplatin (100 mg/m^2 every 3 weeks for three cycles) remains the preferred regimen.

Concurrent chemotherapy and accelerated radiotherapy

Two recently reported randomized studies showed that there was no benefit to accelerated radiotherapy over conventionally fractionated radiotherapy when delivered with concurrent platinum-based chemotherapy. GORTEC 99-02 randomized locoregionally advanced head and neck cancer patients to either very accelerated radiotherapy 64.8 Gy (1.8 Gy twice daily); 70 Gy (2 Gy daily over 7 weeks) with concurrent carboplatin 70 mg/m^2 and 5-fluorouracil 600 mg/m^2/day on days 1–4, 22–25, and 43–46; or 70 Gy (2 Gy daily to 40 Gy, then 1.5 Gy twice daily) with carboplatin 70 mg/m^2 and 5-fluorouracil 600 mg/m^2/day on days 1–4 and 29–33. Outcomes in both chemoradiotherapy arms were similar, and both were superior to very accelerated radiotherapy with lower rates of acute toxicity and percutaneous gastrostomy tube placement during therapy and at 5 years (Bourhis et al., 2012).

Similarly, RTOG 0129 randomized patients to 72 Gy accelerated concomitant boost radiotherapy with two cycles of cisplatin 100 mg/m^2 or to 70 Gy daily radiotherapy with three cycles of cisplatin 100 mg/m^2. Again, there was no benefit to accelerated chemoradiotherapy seen in 8-year overall survival (accelerated 48% vs. conventional 48%), locoregional progression (37% vs. 39%) or worst grade 3–5 toxicity (38% vs. 37%) (Nguyen-Tan et al., 2014). Interestingly, on an unplanned post-hoc analysis, it appeared that standard fractionation patients who received one cycle of chemotherapy had worse outcomes than those who received the two or three cycles of chemotherapy. The value of acceleration via the use of a simultaneous integrated boost, a commonly used approach for IMRT delivery, must therefore be questioned when concurrent chemotherapy is being used as part of the treatment regimen.

Acute toxicities of chemoradiotherapy

The use of concurrent chemoradiotherapy in patients with oral cavity cancer is a life-changing event. Toxicities may include fatigue, nausea, emesis, thickened secretions, xerostomia, mucositis, dysphagia, odynophagia, alopecia, dermatitis, anemia, neutropenia, hoarseness, Lhermitte's syndrome, and infection.

Dysphagia is perhaps the most difficult acute and late complication. Prior to any treatment, oral cavity cancer patients are less likely to be affected by tumor-induced dysphagia than those with other cancers of the head and neck (Stenson et al., 2000). A retrospective review of prospectively collected data demonstrated that head and neck cancer patients with more advanced tumor stage (T3-T4) treated with chemoradiotherapy manifested an improvement in swallowing function that outweighed treatment-related toxicity, likely due to reduction in tumor volume (Salama et al., 2008). The study also noted a trend for worse post-chemoradiotherapy swallowing function in patients with increasing age, whereas patients with good performance status were less likely to have worse swallowing function.

Since dysphagia can significantly impact nutritional status, early therapeutic interventions with swallowing exercises to strengthen the pharyngeal musculature are recommended. Patients should be instructed to swallow as large a volume as possible during and after treatment, and perform exercises shown to improve swallowing ability (Rosenthal et al., 2006). Prospectively, dysphagia has been associated with exceeding specific dosimetric thresholds to the pharyngeal constrictors or laryngeal apparatus when treating other disease sites, which should be taken into consideration during radiotherapy planning, as discussed further below (Eisbruch et al., 2011).

Late toxicities of chemoradiotherapy

Late toxicities of chemoradiotherapy for patients with oral cavity cancer can include dysphagia, fibrosis, osteoradionecrosis, trismus, xerostomia, dental caries, feeding tube dependence, hearing loss, kidney damage, and neuropathy. The RTOG reported late toxicities following chemoradiotherapy based on a pooled analysis of 230 locally advanced head and neck cancer patients treated on three prospective chemoradiotherapy trials (Machtay et al., 2008). Patients with older age and increased tumor stage (T3-T4) were more likely to experience late toxicity, as well as those who underwent a posttreatment neck dissection. When late toxicity was analyzed by primary tumor site, patients with oropharyngeal and oral cavity primary tumors were statistically less likely to experience late toxicity; however, it should be noted that only 5% of patients had an oral cavity primary.

The incidence of late chemoradiotherapy toxicities are illustrated in single and multiinstitution experiences. For example, one multiinstitution retrospective analysis reported that only 7.8% of oral cavity cancer patients were dependent on gastric tube feedings for all caloric intake after a median follow up of 3.25 years post-chemoradiotherapy, while the remainder of patients were able to maintain their weight and nutrition by oral intake alone (Stenson et al., 2010). The investigators also reported that 18.4% of patients developed osteoradionecrosis requiring surgical intervention, with over half requiring only one procedure to achieve satisfactory results. These results were found to be similar in an updated analysis including patients treated up to 2014 with a median follow up of 5.7 years, with no difference in the rate of osteoradionecrosis across treatment decades (Melotek et al., 2016). In a separate analysis of patients with oral cavity cancer treated with chemoradiotherapy utilizing IMRT, it was noted that although a majority of patients required feeding tube placement at some point during the course of therapy, $<15\%$ had a feeding tube at the time of last follow up (Pederson et al., 2011). This study also showed an acceptable rate of osteoradionecrosis, 14%, after a median follow up of 60 months (**Table 3**).

Deintensification of therapy for HPV-positive oral cavity cancer patients

Given better outcomes of HPV-related oropharyngeal cancer patients, in combination with the known acute and late effects of surgery, chemotherapy, and radiotherapy, many are investigating strategies to maintain high rates of tumor control and survival while deintensifying curative intent treatment in locoregionally advanced patients. However, because of the weaker association between HPV and cancer of the oral cavity as discussed previously in the Epidemiology and Etiology section, deintensification

Table 3 Selected modern series reporting rates of osteoradionecrosis

Study	*No.*	*Primary*	*RT technique*	*Median follow-up (months)*	*ORN incidence (%)*	*Risk factors/comments*
Tsai et al. (2013)	402	T1-T2 oropharynx	3D-CRT (17%) IMRT (83%)	31	7.5	Higher V50 and V60 associated with ORN
Gevorgyan et al. (2013)	1575	Head and neck	3D-CRT IMRT	26	0.84	3D-CRT (vs. IMRT) predictive of severity of ORN
Duarte et al. (2014)	158	Head and neck	3D-CRT (63%) IMRT (37%)	NR	6.3	3D-CRT (10.1%) vs. IMRT (0%) ORN rate
Chen et al. (2016)	1692	Oral	IMRT	36.8 (mean)	6.2	Primary site (floor of mouth, buccal mucosa, retromolar trigone, or gum), segmental mandibulectomy, total dose $>$75 Gy associated with ORN
Maesschalck et al. (2016)	234	Oropharynx	3D-CRT (62%) IMRT (38%)	3D-CRT: 4.9 years IMRT: 3.2 years	11	No difference in ORN rate between 3D-CRT and IMRT
De Felice et al. (2016)	653	Head and neck	3D-CRT (11%) IMRT (89%)	NR	5.5	Smoking associated with ORN persistence, no dosimetric factors associated with ORN
Raguse et al. (2016)	149	Head and neck	3D-CRT (70%) IMRT (30%)	41	25.5	Any comorbidity, pre-RT mandibular surgery, poor oral hygiene, and insufficient dentoalveolar surgery associated with ORN
Studer et al. (2016)	531	Oral cavity, salivary gland, mesopharynx	IMRT	38	7	Marginal or periosteal bone resection associated with ORN
Owosho et al. (2017)	1023	Oral cavity, oropharynx	IMRT	52.5	4.3	Poor periodontal status, history of alcohol use and radiation dose associated with ORN

Abbreviations: ORN, osteoradionecrosis; 3D-CRT, 3-dimensional conformal radiotherapy; IMRT, intensity-modulated radiotherapy; NR, not reported.

From Moon, D. H., Moon, S. H., Wang, K., Weissler, M. C., and Chera, B. S. (2017). Incidence of, and risk factors for, mandibular osteoradionecrosis in patients with oral cavity and oropharynx cancers. *Oral Oncology* **72**, 98–103.

of therapy for oral cavity tumors is not an active area of research. Furthermore, in contrast to findings for oropharyngeal cancer, some studies suggest worse prognosis with HPV-positive oral cavity cancer compared to HPV-negative disease (Lee et al., 2012; Duray et al., 2012).

Systemic Therapy

The use of chemotherapy to treat head and neck cancer has evolved from its initial role only in the recurrent or metastatic setting to standard use in the definitive treatment setting. After nearly six decades of clinical research, a wealth of data demonstrate the benefit of concurrent chemotherapy administration in the definitive treatment of head and neck cancer with radiotherapy as summarized previously. For locoregionally advanced disease, the utility of neoadjuvant cytotoxic chemotherapy has been evaluated, as well as the safety and efficacy of targeted systemic agents and concomitant radiotherapy with or without chemotherapy.

Induction chemotherapy prior to definitive local therapy

The use of neoadjuvant chemotherapy prior to surgical resection or radiotherapy for oral cavity cancer patients has been tested in multiple randomized studies. In particular, one trial investigated the use of induction chemotherapy (cisplatin and 5-fluorouracil) followed by surgery with or without adjuvant radiation therapy (for high-risk patients only) in locally advanced resectable oral cavity squamous cell carcinoma and demonstrated no benefit in overall survival between the treatment arms at 5 years (Licitra et al., 2003). More recently, a randomized phase III trial evaluating the use of induction chemotherapy (docetaxel, cisplatin, and 5-fluorouracil) in locally advanced resectable oral cavity squamous cell carcinoma demonstrated no significant benefit in overall or disease-free survival from the addition of preoperative chemotherapy to surgery and adjuvant radiotherapy (Zhong et al., 2013). However, post-hoc analysis indicated that patients in the experimental arm who had a clinical response or $\leq$10% viable tumor cells on pathology demonstrated improved locoregional and distant control, as well as increased overall survival.

Whether induction chemotherapy prior to concurrent chemoradiotherapy improves survival when compared to chemoradiotherapy alone was an area of active study advocated by some given that distant metastases are frequently a site of first failure for patients with locoregionally advanced head and neck cancer (Brockstein et al., 2004). However, mature results from multiple randomized studies did not demonstrate improved survival with induction chemotherapy followed by concurrent chemoradiotherapy (Cohen et al., 2014; Haddad et al., 2013). It is unknown if the lack of demonstrated survival benefit was due to the therapy itself or the fact that these studies were initiated prior to robust knowledge of the behavior of HPV-associated oropharyngeal cancers. Therefore, inclusion of these patients with more favorable outcomes were not accounted for in the study design and may complicate interpretation of these studies.

Targeted agents and radiotherapy

Epidermal growth factor receptor (EGFR) is over-expressed in nearly 80%–90% of cases of head and neck squamous cell carcinoma and correlates with poor prognosis and radiation resistance. Preclinical evidence showed that blocking EGFR restores radiation sensitivity and enhances cytotoxicity, which led to clinical trials evaluating this class of agents. Cetuximab was approved in combination with radiation for the treatment of locally advanced head and neck squamous cell carcinoma based on a single randomized study comparing radiotherapy 70–76.8 Gy with or without weekly cetuximab (loading dose of 400 mg/m^2 followed by 250 mg/m^2). Combination therapy improved locoregional control, disease-free survival, and overall survival; however, this study did not include oral cavity cancer patients (Bonner et al., 2006; Bonner et al., 2010). Based upon lower-level evidence, there is expert consensus that cetuximab is appropriate in oral cavity cancer (category 2B recommendation). Updated analyses show that the addition of cetuximab for head and neck cancer remains beneficial regardless of p16 status (Rosenthal et al., 2016), and the presence of a prominent rash is a prognostic factor (Bonner et al., 2010). Consideration should be given to the use of altered radiation fractionation with cetuximab since improved overall survival was seen in patients who were treated with accelerated concomitant boost and hyperfractionated radiotherapy.

Cetuximab should be used with caution in specific geographic areas (particularly the southeastern United States) where severe anaphylactic reactions can occur. These are mediated by an immunoglobulin E response to the galactose-alpha-1,3-galactose oligosaccharide found on the Fab portion of the cetuximab heavy chain (Chung et al., 2008). The fully humanized monoclonal antibody to EGFR, panitumumab, has a much lower rate of severe allergic reactions, but no level-1 evidence exists to support equivalent efficacy to cetuximab for head and neck cancer. Other than geographic limitations, there is no consensus as to which patient populations are better served by concurrent chemotherapy versus concurrent cetuximab with radiation therapy. Certain patient factors, such as renal dysfunction or overall poor functional status, prompt physicians to utilize cetuximab preferentially over cisplatin with radiotherapy, but no level-1 evidence supports these as indications. Of note, those same medical conditions were exclusion criteria for the randomized trial that demonstrated cetuximab's benefit over radiation therapy alone (**Fig. 22**).

Targeted agents in combination with cytotoxins and radiotherapy

A meta-analysis of several phase II trials and retrospective studies comparing radiation therapy with concurrent cetuximab versus concurrent cisplatin found better survival and locoregional control in patients treated with radiation and cisplatin (Petrelli et al., 2014). RTOG 0522, which notably did not include oral cavity cancer patients, failed to show benefit of the combination of cetuximab with chemoradiotherapy over standard chemoradiotherapy, and the addition of cetuximab was associated with more grade

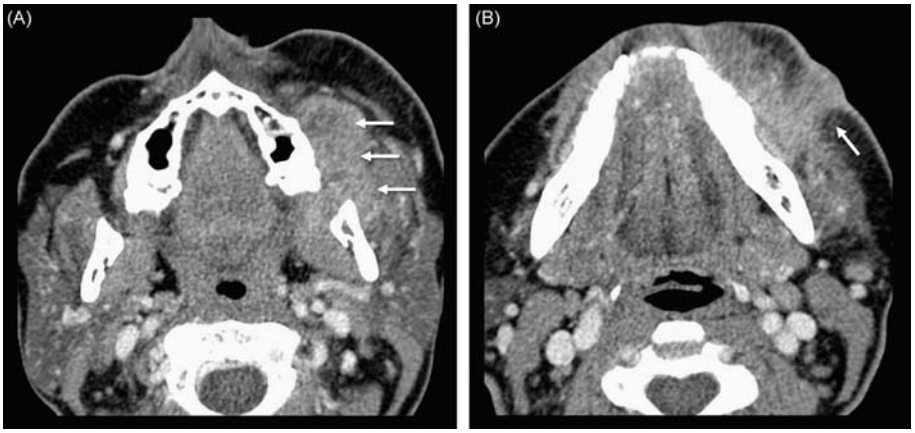

Fig. 22 Squamous cell carcinoma of the left buccal mucosa, T4a. (A) Axial contrast-enhanced CT shows large left buccal mass (*arrows*) extending to maxillary RMT. (B) Axial contrast-enhanced CT (inferior to A) shows inferior and lateral extension to involve the skin (*arrow*). Involvement of the skin is critical to note, because the tumor is upstaged to T4a. From Aiken, A. H. (2013). Pitfalls in the staging of cancer of oral cavity cancer. *Neuroimaging Clinics of North America* **23**(1), 27–45.

3–4 toxicity (Ang et al., 2014). More recently, the published results of the GORTEC 2007-01 phase III randomized trial which included oral cavity cancer patients demonstrated that three cycles of concomitant carboplatin 70 mg/m^2/day and 5-fluoruracil 600 mg/m^2/day on days 1–4 with cetuximab-radiotherapy markedly improved progression-free survival and locoregional control with a nonsignificant gain in overall survival compared to cetuximab-radiotherapy without chemotherapy (Tao et al., 2018). To date the triple drug combination of cetuximab, carboplatin, and 5-fluouracil has not been compared to cisplatin alone. Therefore, single agent platinum-based chemoradiotherapy remains the standard of care for locally advanced squamous cell carcinoma of the head and neck.

Oral cavity cancer patients have also been included in studies evaluating the addition of bevacizumab to chemoradiotherapy. The addition of bevacizumab to 5-fluorouracil, hydroxyurea, and twice daily radiotherapy did not confer any benefit in a study of locoregionally advanced head and neck cancer patients, of which oral cavity was the most common primary site (31%) (Salama et al., 2011). Bevacizumab has also been integrated with erlotinib (synchronous dual inhibition of VEGF and EGFR) and cisplatin (33 mg/m^2 cisplatin on days 1–3 of weeks 1 and 5) together with 1.25 Gy twice-daily radiotherapy to 70 Gy. Fifteen percent of patients had oral cavity primary tumors. At a median follow-up of 46 months, 3-year locoregional control and overall survival were promising compared to historical series at 86% and 85%, respectively (Yoo et al., 2012). Soft tissue and osteoradionecrosis occurred in both series, and careful attention should be paid to the results of ongoing studies integrating bevacizumab to multiple chemoradiotherapy platforms for locoregionally advanced head and neck cancer patients including those with oral cavity tumors.

Posttreatment Management and Surveillance

Following definitive therapy for oral cavity cancer, patients should be seen regularly for clinical evaluation. Initial posttreatment follow up varies based on the duration and severity of acute treatment-related toxicities in order to provide appropriate supportive care and ensure adequate nutrition. Typically patients are seen every 1–3 months for the first year, then every 2–6 months for the second year, then every 4–8 months for years 3 through 5, and then every 12 months thereafter.

At each of these visits, a detailed history, thorough physical examination of the head and neck region, and direct fiberoptic laryngoscopy (if possible) should be performed. Periodic blood tests may be ordered to assess for adequate posttreatment organ function, such as the thyroid following neck irradiation or kidneys following cisplatin. Carotid ultrasounds may also be ordered to screen for accelerated carotid atherosclerosis causing hemodynamically significant stenosis following neck irradiation, although there is no consensus on the optimal timing. An annual low-dose CT scan should be performed for patients that meet lung cancer screening criteria based on smoking history. Additional imaging studies may be obtained if the clinical history or physical exam is concerning for any changes that may indicate tumor recurrence. Patients should also be encouraged to undergo regular dental evaluation, including dental cleaning and topical fluoride treatment. If dental extraction or other surgical intervention is planned within the irradiated field, then discussion with the radiation oncologist should be initiated for consideration of referral for hyperbaric oxygen treatment.

After definitive radiotherapy or chemoradiotherapy, follow-up imaging should be performed within the first three months of treatment completion in patients with node-positive presentations. In the neck, a radiographic complete response on CT is defined as nonenhancing, nonnecrotic nodal tissue <1.5 cm in size and is associated with 100% long-term disease control in the neck (Liauw et al., 2006). Surveillance CT imaging of the primary site does not add additional information to physical or fiberoptic examination and therefore should not be performed unless clinically indicated (Sullivan et al., 2011).

PET-CT is more often used as the sole imaging modality following the completion of radiotherapy to assess nodal response. A prospective analysis of over 100 node-positive head and neck cancer patients followed with PET-CT at around 12 weeks posttherapy and again 4 weeks later (if equivocal or greater residual activity was present on the 12 week scan) treated with neck dissection for metabolic persistence helped to clarify the role of PET-CT scan following chemoradiotherapy. Irrespective of residual CT abnormalities in the neck, a negative neck on the PET scan at 12 weeks, defined as the absence of metabolic activity, was associated with no isolated nodal progression (Porceddu et al., 2011). The negative predictive value of PET was 98.1% (95% CI 93.2–99.8) compared to 96.8% with CT (95% CI 88.8–99.6), and false positive readings were seen in only 1.8% of PET-CT scans compared to 38% of CT scans, yielding a positive predictive value of 77.8% for PET-CT and 14% for CT. Long-term follow-up of this patient cohort found that PET-CT-directed posttherapy neck management adequately spared >90% of patients from a neck dissection, with only one patient experiencing a neck recurrence after initial metabolic resolution (Sjovall et al., 2015). Of note, these patients also had a contrast enhanced CT scan performed 6 weeks after completion of treatment, and all patients with a negative contrast-enhanced CT scan were subsequently found to have a negative PET. Therefore, 12-week PET imaging may not be necessary for patients who have had a prior negative posttreatment contrast-enhanced CT scan.

More recently, a randomized study of 564 patients compared the utility of post-chemoradiotherapy PET-CT-based neck surveillance versus a planned neck dissection in patients with clinical AJCC 7th Edition N2 or N3 disease. Two-year overall survival was 84.9% (95% CI 80.7–89.1) for patients undergoing PET-CT-based neck surveillance compared to 81.5% (95% CI 76.9–86.3) in the group receiving a planned neck dissection. No differences were noted in the 2-year locoregional control rates between groups, with 91.9% (95% CI 88.5–95.3) control in the PET-CT surveillance group and 91.4% (95% CI 87.8–95.0) control in the planned neck dissection group. Additionally, PET-CT-based surveillance was more cost effective, saving an average of $2190 per patient. For these reasons, PET-CT-based surveillance of head and neck cancer patients should be considered standard post chemoradiotherapy (Mehanna et al., 2016).

Approximately 90% of tumor recurrences will occur within the first 18 months following treatment. Patients without evidence of disease two or more years after treatment are at greater risk of developing a secondary cancer than recurrence of their original disease. Patients who continue to use tobacco and alcohol are at the highest risk. Should a second primary head and neck cancer occur, identification at an early stage is paramount in order to achieve the best cure rate with the least invasive means of treatment. Therefore, close follow-up is essential in patients with cancers of the oral cavity (**Fig. 23**).

Treatment of Recurrent and Metastatic Oral Cavity Cancer

The appropriate management of metastatic or recurrent oral cavity cancer depends largely on the extent of disease, the prior therapy administered, and whether recurrence is local, regional, or both. If there is distant disease recurrence, systemic therapy approaches will likely assume primary importance. In the case of small recurrences at the primary site for patients treated with primary excision only, further excision with or without PORT is often recommended. For larger recurrences in patients who received radiation as part of their initial management, the rate of surgical salvage is quite low; however, in some cases, further resection may be considered for palliation or as an attempt at curative treatment, particularly in the setting of a clinical trial. Reirradiation is possible, but with the potential for increased toxicity. Systemic therapy and palliative care are also treatment options for this group of patients. The risks and benefits of each treatment should be discussed with the individual patient.

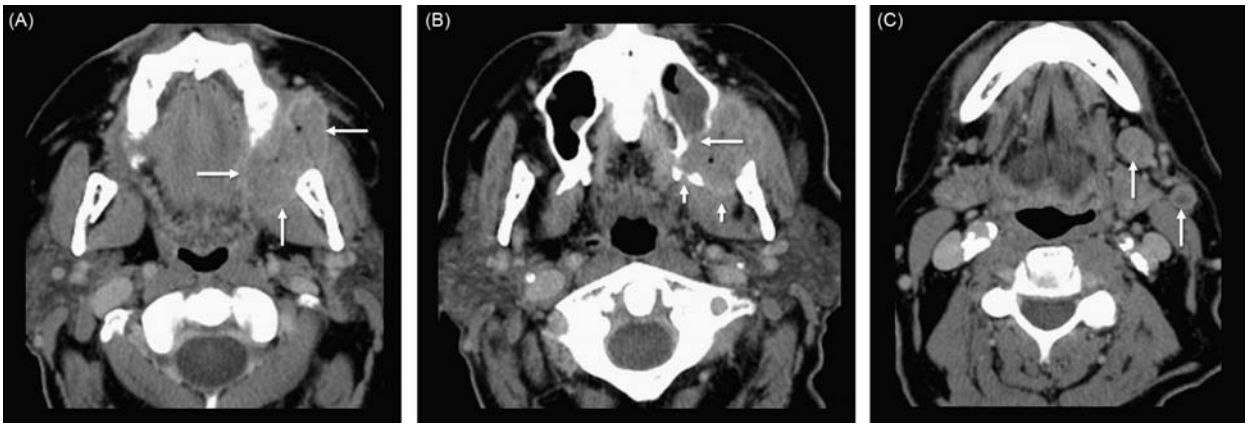

Fig. 23 Squamous cell carcinoma of the left retromolar trigone (RMT), T4b N2b. (A) Axial contrast-enhanced CT demonstrates large tumor centered in the left RMT (*arrows*). (B) Axial contrast-enhanced CT (cranial to A) shows obvious invasion of the posterior maxilla involving the maxillary sinus (*long arrow*) from tumor growing superiorly along the pterygomandibular raphe. Tumor is therefore upstaged to T4a. Also note tumor extension to medial pterygoid plate and masticator space (*short arrows*), resulting in overall T4b stage. (C) Axial contrast-enhanced CT shows metastatic level I and II nodes (*arrows*). Some have central low density, a very specific sign for metastasis. From Aiken, A. H. (2013). Pitfalls in the staging of cancer of oral cavity cancer. *Neuroimaging Clinics of North America* **23**(1), 27–45.

Systemic therapy for recurrent and metastatic oral cavity cancer

The standard of care for patients with recurrent or metastatic oral cavity or oropharyngeal cancer is systemic therapy with platinum-based chemotherapy. Phase III trials have investigated cisplatin (with or without 5-fluorouracil or methotrexate), 5-fluorouracil, single-agent methotrexate, and carboplatin with 5-flurouracil (Forastiere et al., 1992; Anon, 1990; Jacobs et al., 1992). Many drugs, in addition to platinum agents, 5-fluorouracil, and methotrexate, have been shown in phase II trials to exhibit single agent activity such as paclitaxel (Forastiere et al., 1998), docetaxel (Dreyfuss et al., 1996), gemcitabine (Catimel et al., 1994), ifosfamide (Sandler et al., 1998), vinorelbine (Saxman et al., 1998), pemetrexed (Pivot et al., 2001), capecitabine (Martinez-Trufero et al., 2010), and irinotecan (Murphy, 2005). Single agent cisplatin (100 mg/m^2) has been shown to improve overall survival compared to best-supportive care (Morton et al., 1985) and superior to single agent methotrexate in a phase III randomized trial (Anon, 1990). Many randomized trials have tried to improve survival with combination cisplatin-based regimens. Cisplatin (100 mg/m^2) with 5-fluorouracil (1000 mg/m^2/day) demonstrated improved response rates (32% vs. 17%, p = .035), but did not improve median survival (5.7 months) over cisplatin alone (Jacobs et al., 1992). Similar results were seen when cisplatin/5-fluorouracil, carboplatin/5-fluorouracil, and methotrexate alone were compared in a randomized phase III trial. Cisplatin/5-fluorouracil and carboplatin/5-fluorouracil demonstrated increased response rates (32% and 21%, respectively) compared to methotrexate alone (10%), but neither regimen improved median survival (6.6 and 5.6 months, respectively) compared to methotrexate alone (5.0 months) (Forastiere et al., 1992). Combination treatments were associated with worse toxicity. Similar response rates and survival have been seen with cisplatin/paclitaxel versus cisplatin/5-fluorouracil, providing a more easily administered treatment option for patients (Gibson et al., 2005).

Adding agents targeting EGFR to platinum-based systemic therapy has been shown to improve overall survival compared to platinum-based systemic therapy alone in a phase III study. The EXTREME study randomized recurrent and metastatic head and neck cancer patients to cisplatin 100 mg/m^2 on day 1 or carboplatin AUC = 5 on day 1 along with 5-fluorouracil 1000 mg/m^2/day on days 1–4 every 3 weeks, with or without cetuximab 250 mg/m^2 (following a loading dose of 400 mg/m^2) that was continued until disease progression or patient intolerance (Vermorken et al., 2008). The addition of cetuximab to chemotherapy resulted in an improvement in median overall survival from 7.4 to 10.1 months and an improvement in median progression-free survival from 3.3 to 5.6 months. Currently there are no phase III trials to suggest that addition of tyrosine kinase inhibitors (including gefitinib or erlotinib) to platinum-based therapy provides a survival benefit. Ongoing studies are investigating the role of bevacizumab and other targeted agents in combination with chemotherapy.

For patients with platinum-refractory recurrent and metastatic head and neck cancer, immune checkpoint inhibitors targeting the programmed cell death protein-1 (PD-1) pathway have significantly changed second-line therapy. The Checkmate 141 study randomized 361 patients (48.5% oral cavity cancers) to the anti-PD-1 antibody nivolumab 3 mg/kg every 2 weeks or investigator choice of 40–60 mg/m^2 methotrexate, 30–40 mg/m^2 docetaxel, or 250 mg/m^2 cetuximab (after a loading dose of 400 mg/m^2). Patients receiving nivolumab had longer median (7.5 vs. 5.1 months) and 1-year overall survival (36% vs. 16.6%, HR 0.70 [95% CI 0.51–0.96], p = .01) (Ferris et al., 2016). Similarly, the phase III KEYNOTE-040 trial randomized 495 patients to receive the anti-PD-1 antibody pembrolizumab 200 mg every 3 weeks or investigator choice of methotrexate, docetaxel or cetuximab. On intention to treat analysis, median overall survival was 8.4 months with pembrolizumab versus 7.1 months in the control arm (HR 0.81 [95% CI 0.66–0.99], one-sided p = .0204) (Cohen et al., 2017).

Reirradiation for locoregionally confined recurrent or second primary disease

For patients with locoregionally confined recurrent disease of the oral cavity, surgical resection is recommended, although it is only possible in a small percentage of patients (Taussky et al., 2005). In patients who are not surgical candidates (Choe et al., 2011) or following surgery in those with high-risk pathologic features (Janot et al., 2008), a second course of full dose radiotherapy with concurrent chemotherapy results in long-term survival in approximately 20% of patients. Patients able to undergo a surgical resection prior to reirradiation, as well as those not previously treated with chemotherapy and treated to higher doses, have been shown to have improved outcomes. Because of the high risk of toxicity (approximately 20% carotid rupture rate and 15% fatal toxicity), patients undergoing a second course of concurrent chemoradio therapy should be managed at experienced centers.

It remains unclear whether chemotherapy alone or chemotherapy and reirradiation is the optimal therapy for these patients, as a phase III comparison of these modalities failed to accrue patients. A nomogram (with concordance index of 0.68) exists to predict 2-year locoregional control following fractionated reirradiation for recurrent head and neck cancer using data from 257 patients treated at a single United States institution (Riaz et al., 2014). The criteria used included stage, site, organ dysfunction, surgical salvage, and radiotherapy dose. In this nomogram, the oral cavity subsite was associated with worse outcomes compared to reirradiation of other head and neck sites.

Recently, the use of hypofractionated image-guided radiation therapy (HIGRT), otherwise known as stereotactic body radiation therapy (SBRT) or stereotactic ablative radiotherapy (SABR), has been associated with high rates of treated tumor control in previously irradiated head and neck cancer patients with locoregionally-confined recurrences (Baliga et al., 2017). HIGRT is an attractive treatment option, as it is generally delivered over 1–2 weeks, as compared to 5–7 weeks of concurrent chemoradiation, and it has been safely combined with cetuximab (Vargo et al., 2015). However, similar to chemotherapy with conventionally fractionated reirradiation, high-grade late toxicities including carotid blowout have been reported, with patients with recurrent or second primary tumors arising in the larynx/hypopharynx being more likely to have high-grade late toxicity following reirradiation with HIGRT. The ability to offer HIGRT is limited by the size and location of the recurrent tumor. Furthermore, reirradiation with HIGRT should only be delivered by those with experience with this technique due to concerns for toxicity.

Prospective Vision

An area of active investigation for oral cavity cancer is identifying better systemic agents and determining appropriate timing of newer systemic therapies relative to surgery and radiotherapy. Several ongoing clinical trials are evaluating the safety and efficacy of immune checkpoint inhibitors in head and neck cancer. The use of immunotherapy will likely evolve from its current role in the recurrent and metastatic setting to earlier use in initial combined modality definitive treatment. Other clinical trials are evaluating the use of various combinatorial approaches of immune checkpoint inhibitors with immune modulators of regulatory T cells or natural killer cells (e.g., indoleamine-pyrrole 2,3-dioxygenase [IDO], glucocorticoid-induced tumor necrosis factor receptor-related protein [GITR], T-cell immunoreceptor with Ig and ITIM domains [TIGIT], killer-cell immunoglobulin-like receptor [KIR], etc.). IDO inhibitors in particular have demonstrated early promise in improving response rates to PD-1 inhibitors and are currently in ongoing clinical trials. Clinical studies inducing HPV-induced immunogenicity in HPV-positive disease are also being conducted through HPV vaccination trials. Additional clinical trials investigating radiosensitizers, proteasome inhibitors, and targeted therapies are also being conducted. Altered fractionation and adaptive radiation planning are two other areas of active investigation to identify methods of achieving better locoregional control and survival with reduced toxicity. Many prospective randomized trials are implementing various pharmaceutical interventions and oral care protocols to prevent or minimize mucositis and/or xerostomia. Additionally, chemoprevention to delay or prevent oral cavity cancer is an area of ongoing study. Many clinical questions remain to be answered, and significant advances in the coming years will likely translate into improved outcomes for patients with oral cavity cancer.

See also: Oral and Oropharyngeal Cancer: Pathology and Genetics.

References

Abu-Ghanem, S., Yehuda, M., Carmel, N., et al., 2016. Elective neck dissection vs observation in early-stage squamous cell carcinoma of the oral tongue with no clinically apparent lymph node metastasis in the neck: A systematic review and meta-analysis. JAMA Otolaryngology. Head & Neck Surgery 142, 857–865.

Adelstein, D.J., Li, Y., Adams, G.L., et al., 2003. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. Journal of Clinical Oncology 21, 92–98.

Almangush, A., Bello, I.O., Coletta, R.D., et al., 2015. For early-stage oral tongue cancer, depth of invasion and worst pattern of invasion are the strongest pathological predictors for locoregional recurrence and mortality. Virchows Archiv 467, 39–46.

Ang, K.K., Trotti, A., Brown, B.W., et al., 2001. Randomized trial addressing risk features and time factors of surgery plus radiotherapy in advanced head-and-neck cancer. International Journal of Radiation Oncology Biology Physics 51, 571–578.

Ang, K.K., Zhang, Q., Rosenthal, D.I., et al., 2014. Randomized phase III trial of concurrent accelerated radiation plus cisplatin with or without cetuximab for stage III to IV head and neck carcinoma: RTOG 0522. Journal of Clinical Oncology 32, 2940–2950.

Anon, 1990. A phase III randomised trial of cisplatinum, methotrextate, cisplatinum + methotrexate and cisplatinum + 5-FU in end stage squamous carcinoma of the head and neck. Liverpool Head and Neck Oncology Group. British Journal of Cancer 61, 311–315.

Antoniades, D.Z., Styanidis, K., Papanayotou, P., Trigonidis, G., 1995. Squamous cell carcinoma of the lips in a northern Greek population. Evaluation of prognostic factors on 5-year survival rate. European Journal of Cancer Part B, Oral Oncology 31 (B(5)), 333–339.

Bachaud, J.M., Cohen-Jonathan, E., Alzieu, C., David, J.M., Serrano, E., Daly-Schveitzer, N., 1996. Combined postoperative radiotherapy and weekly cisplatin infusion for locally advanced head and neck carcinoma: Final report of a randomized trial. International Journal of Radiation Oncology, Biology, Physics 36, 999–1004.

Baker, S.R., Krause, C.J., 1980. Carcinoma of the lip. The Laryngoscope 90, 19–27.

Baliga, S., Kabarriti, R., Ohri, N., et al., 2017. Stereotactic body radiotherapy for recurrent head and neck cancer: A critical review. Head & Neck 39, 595–601.

Beadle, B.M., Liao, K.-P., Elting, L.S., et al., 2014. Improved survival using intensity-modulated radiation therapy in head and neck cancers: A SEER-Medicare analysis. Cancer 120, 702–710.

Beitler, J.J., Zhang, Q., Fu, K.K., et al., 2014. Final results of local-regional control and late toxicity of RTOG 9003: A randomized trial of altered fractionation radiation for locally advanced head and neck cancer. International Journal of Radiation Oncology, Biology, Physics 89, 13–20.

Benoist, L.B.L., van Looij, M.A.J., 2013. Melanoma of the oral cavity. New England Journal of Medicine 368, e14.

Bernier, J., Domenge, C., Ozsahin, M., et al., 2004. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. The New England Journal of Medicine 350, 1945–1952.

Bernier, J., Cooper, J.S., Pajak, T.F., et al., 2005. Defining risk levels in locally advanced head and neck cancers: A comparative analysis of concurrent postoperative radiation plus chemotherapy trials of the EORTC (#22931) and RTOG (# 9501). Head & Neck 27, 843–850.

Blanchard, P., Baujat, B., Holostenco, V., et al., 2011. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): A comprehensive analysis by tumour site. Radiotherapy and Oncology 100, 33–40.

Bonner, J.A., Harari, P.M., Giralt, J., et al., 2006. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. The New England Journal of Medicine 354, 567–578.

Bonner, J.A., Harari, P.M., Giralt, J., et al., 2010. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. The Lancet Oncology 11, 21–28.

Bourhis, J., Overgaard, J., Audry, H., et al., 2006. Hyperfractionated or accelerated radiotherapy in head and neck cancer: A meta-analysis. Lancet 368, 843–854.

Bourhis, J., Sire, C., Graff, P., et al., 2012. Concomitant chemoradiotherapy versus acceleration of radiotherapy with or without concomitant chemotherapy in locally advanced head and neck carcinoma (GORTEC 99-02): An open-label phase 3 randomised trial. The Lancet Oncology 13, 145–153.

Brazilian Head and Neck Cancer Study Group, 1998. Results of a prospective trial on elective modified radical classical versus supraomohyoid neck dissection in the management of oral squamous carcinoma. The American Journal of Surgery 176, 422–427.

Brizel, D.M., Albers, M.E., Fisher, S.R., et al., 1998. Hyperfractionated irradiation with or without concurrent chemotherapy for locally advanced head and neck cancer. The New England Journal of Medicine 338, 1798–1804.

Brockstein, B., Haraf, D.J., Rademaker, A.W., et al., 2004. Patterns of failure, prognostic factors and survival in locoregionally advanced head and neck cancer treated with concomitant chemoradiotherapy: A 9-year, 337-patient, multi-institutional experience. Annals of Oncology 15, 1179–1186.
Broglie, M.A., Haerle, S.K., Huber, G.F., Haile, S.R., Stoeckli, S.J., 2013. Occult metastases detected by sentinel node biopsy in patients with early oral and oropharyngeal squamous cell carcinomas: Impact on survival. Head & Neck 35, 660–666.
Budach, W., Hehr, T., Budach, V., Belka, C., Dietz, K., 2006. A meta-analysis of hyperfractionated and accelerated radiotherapy and combined chemotherapy and radiotherapy regimens in unresected locally advanced squamous cell carcinoma of the head and neck. BMC Cancer 6, 28.
Byers, R.M., Weber, R.S., Andrews, T., McGill, D., Kare, R., Wolf, P., 1997. Frequency and therapeutic implications of "skip metastases" in the neck from squamous carcinoma of the oral tongue. Head & Neck 19, 14–19.
Calais, G., Alfonsi, M., Bardet, E., et al., 1999. Randomized trial of radiation therapy versus concomitant chemotherapy and radiation therapy for advanced-stage oropharynx carcinoma. Journal of the National Cancer Institute 91, 2081–2086.
Candau-Alvarez, A., Dean-Ferrer, A., Alamillos-Granados, F.J., et al., 2014. Verrucous carcinoma of the oral mucosa: An epidemiological and follow-up study of patients treated with surgery in 5 last years. Medicina Oral, Patología Oral y Cirugía Bucal 19, e506–e511.
Capote, A., Escorial, V., Muñoz-Guerra, M.F., Rodríguez-Campo, F.J., Gamallo, C., Naval, L., 2007. Elective neck dissection in early-stage oral squamous cell carcinoma—Does it influence recurrence and survival? Head & Neck 29, 3–11.
Castellsague, X., Alemany, L., Holzinger, D., 2014. In: *In:* Estimation of the HPV etiological fraction in over 4,000 head and neck cancers worldwide. International Human Papillomavirus Conference. Seattle, WA2014.
Catimel, G., Vermorken, J.B., Clavel, M., et al., 1994. A phase II study of Gemcitabine (LY 188011) in patients with advanced squamous cell carcinoma of the head and neck. EORTC Early Clinical Trials Group. Annals of Oncology 5, 543–547.
Caudell, J.J., Schaner, P.E., Desmond, R.A., Meredith, R.F., Spencer, S.A., Bonner, J.A., 2010. Dosimetric factors associated with long-term dysphagia after definitive radiotherapy for squamous cell carcinoma of the head and neck. International Journal of Radiation Oncology, Biology, Physics 76, 403–409.
Chaturvedi, A.K., Anderson, W.F., Lortet-Tieulent, J., et al., 2013. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. Journal of Clinical Oncology 31, 4550–4559.
Chen, A.Y., Myers, J.N., 2001. Cancer of the oral cavity. Disease-a-Month 47, 275–361.
Chen, Y.-L., Kuo, S.-W., Fang, K.-H., Hao, S.-P., 2011. Prognostic impact of marginal mandibulectomy in the presence of superficial bone invasion and the nononcologic outcome. Head & Neck 33, 708–713.
Chen, J.-A., et al., 2016. Osteoradionecrosis of mandible bone in patients with oral cancer-associated factors and treatment outcomes. Head & Neck 38, 762–768.
Chinn, S.B., Myers, J.N., 2015. Oral cavity carcinoma: Current management, controversies, and future directions. Journal of Clinical Oncology 33, 3269–3276.
Chocolatewala, N., Chaturvedi, P., 2009. Role of human papilloma virus in the oral carcinogenesis: An Indian perspective. Journal of Cancer Research and Therapeutics 5, 71–77.
Choe, K.S., Haraf, D.J., Solanki, A., et al., 2011. Prior chemoradiotherapy adversely impacts outcomes of recurrent and second primary head and neck cancer treated with concurrent chemotherapy and reirradiation. Cancer 117, 4671–4678.
Chung, C.H., Mirakhur, B., Chan, E., et al., 2008. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. The New England Journal of Medicine 358, 1109–1117.
Chung, C.H., Bagheri, A., D'Souza, G., 2014. Epidemiology of oral human papillomavirus infection. Oral Oncology 50, 364–369.
Coelho, K.R., 2012. Challenges of the oral cancer burden in India. Journal of Cancer Epidemiology 2012, 701932.
Cohen, E.E., Karrison, T.G., Kocherginsky, M., et al., 2014. Phase III randomized trial of induction chemotherapy in patients with N2 or N3 locally advanced head and neck cancer. Journal of Clinical Oncology 32, 2735–2743.
Cohen, E.E., Harrington, K.J., Le Tourneau, C., et al., 2017. LBA45_PRPembrolizumab (pembro) vs standard of care (SOC) for recurrent or metastatic head and neck squamous cell carcinoma (R/M HNSCC): Phase 3 KEYNOTE-040 trial. Annals of Oncology 28 mdx440.040.
Combes, J.-D., Franceschi, S., 2014. Role of human papillomavirus in non-oropharyngeal head and neck cancers. Oral Oncology 50, 370–379.
Cooper, J.S., Pajak, T.F., Forastiere, A., et al., 1998. Precisely defining high-risk operable head and neck tumors based on RTOG #85-03 and #88-24: Targets for postoperative radiochemotherapy? Head & Neck 20, 588–594.
Cooper, J.S., Pajak, T.F., Forastiere, A.A., et al., 2004. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. The New England Journal of Medicine 350, 1937–1944.
D'Cruz, A.K., Vaish, R., Kapre, N., et al., 2015. Elective versus therapeutic neck dissection in node-negative oral cancer. New England Journal of Medicine 373, 521–529.
Daley, T., Darling, M., 2003. Nonsquamous cell malignant tumours of the oral cavity: An overview. Journal of the Canadian Dental Association 69, 577–582.
Danaei, G., Vander Hoorn, S., Lopez, A.D., Murray, C.J.L., Ezzati, M., 2005. Causes of cancer in the world: Comparative risk assessment of nine behavioural and environmental risk factors. The Lancet 366, 1784–1793.
Day, G.L., Blot, W.J., 1992. Second primary tumors in patients with oral cancer. Cancer 70, 14–19.
De Felice, F., et al., 2016. Osteoradionecrosis following treatment for head and neck cancer and the effect of radiotherapy dosimetry: The Guy's and St Thomas' Head and Neck Cancer Unit experience. Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology 122, 28–34.
Duarte, V.M., et al., 2014. Comparison of dental health of patients with head and neck cancer receiving IMRT vs conventional radiation. Journal of Otolaryngology Head & Neck Surgery 150, 81–86.
van den Brekel, M.W.M., Castelijns, J.A., Stel, H.V., Golding, R.P., Meyer, C.J.L., Snow, G.B., 1993. Modern imaging techniques and ultrasound-guided aspiration cytology for the assessment of neck node metastases: A prospective comparative study. European Archives of Oto-Rhino-Laryngology 250, 11–17.
Dreyfuss, A.I., Clark, J.R., Norris, C.M., et al., 1996. Docetaxel: An active drug for squamous cell carcinoma of the head and neck. Journal of Clinical Oncology 14, 1672–1678.
Duncan, L.D., Winkler, M., Carlson, E.R., Heidel, R.E., Kang, E., Webb, D., 2013. p16 immunohistochemistry can be used to detect human papillomavirus in oral cavity squamous cell carcinoma. Journal of Oral and Maxillofacial Surgery 71, 1367–1375.
Duray, A., Descamps, G., Decaestecker, C., et al., 2012. Human papillomavirus DNA strongly correlates with a poorer prognosis in oral cavity carcinoma. The Laryngoscope 122, 1558–1565.
Eisbruch, A., Schwartz, M., Rasch, C., et al., 2004. Dysphagia and aspiration after chemoradiotherapy for head-and-neck cancer: Which anatomic structures are affected and can they be spared by IMRT? International Journal of Radiation Oncology, Biology, Physics 60, 1425–1439.
Eisbruch, A., Kim, H.M., Feng, F.Y., et al., 2011. Chemo-IMRT of oropharyngeal cancer aiming to reduce dysphagia: Swallowing organs late complication probabilities and dosimetric correlates. International Journal of Radiation Oncology, Biology, Physics 81, e93–e99.
Ellis, G.L., Corio, R.L., 1980. Spindle cell carcinoma of the oral cavity. A clinicopathologic assessment of fifty-nine cases. Oral Surgery, Oral Medicine, and Oral Pathology 50, 523–533.
Escott, E.J., 2013. Role of positron emission tomography/computed tomography (PET/CT) in head and neck cancer. Radiologic Clinics 51, 881–893.
Expert Panel on Radiation Oncology-Head and Neck, Salama, J.K., Saba, N., et al., 2011. ACR appropriateness criteria® adjuvant therapy for resected squamous cell carcinoma of the head and neck. Oral Oncology 47, 554–559.
Fasunla, A.J., Greene, B.H., Timmesfeld, N., Wiegand, S., Werner, J.A., Sesterhenn, A.M., 2011. A meta-analysis of the randomized controlled trials on elective neck dissection versus therapeutic neck dissection in oral cavity cancers with clinically node-negative neck. Oral Oncology 47, 320–324.
Fatahzadeh, M., Schwartz, R.A., 2013. Oral Kaposi's sarcoma: A review and update. International Journal of Dermatology 52, 666–672.

Ferris, R.L., Blumenschein Jr., G., Fayette, J., et al., 2016. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. The New England Journal of Medicine 375, 1856–1867.
Fietkau, R., Lautenschläger, C., Sauer, R., et al., 2006. Postoperative concurrent radiochemotherapy versus radiotherapy in high-risk SCCA of the head and neck: Results of the German phase III trial ARO 96-3. Journal of Clinical Oncology 24, 5507.
Fitzpatrick, P.J., 1984. Cancer of the lip. The Journal of Otolaryngology 13, 32–36.
Forastiere, A.A., Metch, B., Schuller, D.E., et al., 1992. Randomized comparison of cisplatin plus fluorouracil and carboplatin plus fluorouracil versus methotrexate in advanced squamous-cell carcinoma of the head and neck: A Southwest Oncology Group study. Journal of Clinical Oncology 10, 1245–1251.
Forastiere, A.A., Shank, D., Neuberg, D., SGt, T., DeConti, R.C., Adams, G., 1998. Final report of a phase II evaluation of paclitaxel in patients with advanced squamous cell carcinoma of the head and neck: An Eastern Cooperative Oncology Group trial (PA390). Cancer 82, 2270–2274.
Franceschi, S., Bidoli, E., Herrero, R., Muñoz, N., 2000. Comparison of cancers of the oral cavity and pharynx worldwide: Etiological clues. Oral Oncology 36, 106–115.
Fritsch, V.A., Gerry, D.R., Lentsch, E.J., 2014. Basaloid squamous cell carcinoma of the oral cavity: An analysis of 92 cases. The Laryngoscope 124, 1573–1578.
Fu, K.K., Pajak, T.F., Trotti, A., et al., 2000. A Radiation Therapy Oncology Group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation radiotherapy for head and neck squamous cell carcinomas: First report of RTOG 9003. International Journal of Radiation Oncology, Biology, Physics 48, 7–16.
Garland, S.M., Kjaer, S.K., Muñoz, N., et al., 2016. Impact and effectiveness of the quadrivalent human papillomavirus vaccine: A systematic review of 10 years of real-world experience. Clinical Infectious Diseases 63, 519–527.
Gary, E., Raskob, C., Robert, P., Zitsch, I., Park, C.W., Renner, G.J., Rea, J.L., 1995. Outcome analysis for lip carcinoma. Otolaryngology-Head and Neck Surgery 113, 589–596.
Genden, E.M., Rinaldo, A., Jacobson, A., et al., 2005. Management of mandibular invasion: When is a marginal mandibulectomy appropriate? Oral Oncology 41, 776–782.
Gevorgyan, A., et al., 2013. Osteoradionecrosis of the mandible: A case series at a single institution. Journal of Otolaryngology Head & Neck Surgery 42, 46.
Gholizadeh, N., Mehdipour, M., Rohani, B., Esmaeili, V., 2016. Extramedullary plasmacytoma of the oral cavity in a young man: A case report. Journal of Dentistry 17, 155–158.
Gibson, M.K., Li, Y., Murphy, B., et al., 2005. Randomized phase III evaluation of cisplatin plus fluorouracil versus cisplatin plus paclitaxel in advanced head and neck cancer (E1395): An intergroup trial of the Eastern Cooperative Oncology Group. Journal of Clinical Oncology 23, 3562–3567.
Gross, N.D., Patel, S.G., Carvalho, A.L., et al., 2008. Nomogram for deciding adjuvant treatment after surgery for oral cavity squamous cell carcinoma. Head & Neck 30, 1352–1360.
Guevara-Canales, J.O., Morales-Vadillo, R., Sacsaquispe-Contreras, S.J., et al., 2013. Malignant lymphoma of the oral cavity and the maxillofacial region: Overall survival prognostic factors. Medicina Oral, Patología Oral y Cirugía Bucal 18, e619–e626.
Gupta, S., Singh, S., Gill, M., Goyal, R., Hasija, S., Sen, R., 2012. Teratoma of the tongue. Journal of Surgical Case Reports 2012, 6.
Haddad, R., O'Neill, A., Rabinowits, G., et al., 2013. Induction chemotherapy followed by concurrent chemoradiotherapy (sequential chemoradiotherapy) versus concurrent chemoradiotherapy alone in locally advanced head and neck cancer (PARADIGM): A randomised phase 3 trial. The Lancet Oncology 14, 257–264.
Hall, E.J., 2006. Intensity-modulated radiation therapy, protons, and the risk of second cancers. International Journal of Radiation Oncology, Biology, Physics 65, 1–7.
Harari, P.M., Harris, J., Kies, M.S., et al., 2014. Postoperative chemoradiotherapy and cetuximab for high-risk squamous cell carcinoma of the head and neck: Radiation Therapy Oncology Group RTOG-0234. Journal of Clinical Oncology 32, 2486–2495.
Harrison, L.B., 1997. Applications of brachytherapy in head and neck cancer. Seminars in Surgical Oncology 13, 177–184.
Hashibe, M., Brennan, P., Chuang, S.C., et al., 2009. Interaction between tobacco and alcohol use and the risk of head and neck cancer: Pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Cancer Epidemiology Biomarkers & Prevention 18, 541–550.
Haughey, B.H., Arfken, C.L., Gates, G.A., Harvey, J., 1992. Meta-analysis of second malignant tumors in head and neck cancer: The case for an endoscopic screening protocol. The Annals of Otology, Rhinology, and Laryngology 101, 105–112.
Hinerman, R.W., Mendenhall, W.M., Morris, C.G., Amdur, R.J., Werning, J.W., Villaret, D.B., 2004. Postoperative irradiation for squamous cell carcinoma of the oral cavity: 35-year experience. Head & Neck 26, 984–994.
Hobbs, C.G.L., Sterne, J.A.C., Bailey, M., Heyderman, R.S., Birchall, M.A., Thomas, S.J., 2006. Human papillomavirus and head and neck cancer: A systematic review and meta-analysis. Clinical Otolaryngology 31, 259–266.
Howard, B.E., Nagel, T.H., Donald, C.B., Hinni, M.L., Hayden, R.E., 2014. Oncologic safety of the submental flap for reconstruction in oral cavity malignancies. Otolaryngology-Head and Neck Surgery 150, 558–562.
HPV Vaccine Recommendations. 2017 Centers for Disease Control and Prevention. Accessed 28 Dec 2017 at: https://www.cdc.gov/vaccines/vpd/hpv/hcp/recommendations.html.)
Huang, S.H., Hwang, D., Lockwood, G., Goldstein, D.P., O'Sullivan, B., 2009. Predictive value of tumor thickness for cervical lymph-node involvement in squamous cell carcinoma of the oral cavity. Cancer 115, 1489–1497.
Hübbers, C.U., Akgül, B., 2015. HPV and cancer of the oral cavity. Virulence 6, 244–248.
Huguenin, P., Beer, K.T., Allal, A., et al., 2004. Concomitant cisplatin significantly improves locoregional control in advanced head and neck cancers treated with hyperfractionated radiotherapy. Journal of Clinical Oncology 22, 4665–4673.
Jacobs, C., Lyman, G., Velez-García, E., et al., 1992. A phase III randomized study comparing cisplatin and fluorouracil as single agents and in combination for advanced squamous cell carcinoma of the head and neck. Journal of Clinical Oncology 10, 257–263.
Janot, F., de Raucourt, D., Benhamou, E., et al., 2008. Randomized trial of postoperative reirradiation combined with chemotherapy after salvage surgery compared with salvage surgery alone in head and neck carcinoma. Journal of Clinical Oncology 26, 5518–5523.
Jeremic, B., Shibamoto, Y., Milicic, B., et al., 2000. Hyperfractionated radiation therapy with or without concurrent low-dose daily cisplatin in locally advanced squamous cell carcinoma of the head and neck: a prospective randomized trial. Journal of Clinical Oncology 18, 1458–1464.
Johnson, J.T., Branstetter, B.F., 2014. PET/CT in head and neck oncology: State-of-the-art 2013. The Laryngoscope 124, 913–915.
Kligerman, J., Lima, R.A., Soares, J.R., et al., 1994. Supraomohyoid neck dissection in the treatment of T1/T2 squamous cell carcinoma of oral cavity. The American Journal of Surgery 168, 391–394.
Klopp, A., Smith, B.D., Alektiar, K., et al., 2014. The role of postoperative radiation therapy for endometrial cancer: Executive Summary of an American Society for Radiation Oncology evidence-based guideline. Practical Radiation Oncology 4, 137–144.
Kobler, P., Borcic, J., Zore, I.F., Nola, M., Sertic, D., 2005. Primary non-Hodgkin's lymphoma of the oral cavity. Oral Oncology Extra 41, 12–14.
Kolekar, S.S., Chincholi, T., Nangare, N., Patankar, R., 2016. Oral teratoma. International Journal of Applied and Basic Medical Research 6, 54–56.
Kovács, G., Martinez-Monge, R., Budrukkar, A., et al., 2017. GEC-ESTRO ACROP recommendations for head & neck brachytherapy in squamous cell carcinomas: 1st update—Improvement by cross sectional imaging based treatment planning and stepping source technology. Radiotherapy and Oncology 122, 248–254.
Kramer, F.-J., Böhrnsen, F., Moser, N., Schliephake, H., 2015. The submental island flap for the treatment of intraoral tumor-related defects: No effect on recurrence rates. Oral Oncology 51, 668–673.
Kurdgelashvili, G., Dores, G.M., Srour, S.A., Chaturvedi, A.K., Huycke, M.M., Devesa, S.S., 2013. Incidence of potentially human papillomavirus–related neoplasms in the United States, 1978 to 2007. Cancer 119, 2291–2299.
Lacas, B., Bourhis, J., Overgaard, J., et al., 2017. Role of radiotherapy fractionation in head and neck cancers (MARCH): An updated meta-analysis. The Lancet Oncology 18, 1221–1237.

Laskar, S.G., Chaukar, D., Deshpande, M., et al., 2016. Phase III randomized trial of surgery followed by conventional radiotherapy (5 fr/Wk) (Arm A) vs concurrent chemo-radiotherapy (Arm B) vs accelerated radiotherapy (6fr/Wk) (Arm C) in locally advanced, stage III and IV, resectable, squamous cell carcinoma of oral cavity- oral cavity adjuvant therapy (OCAT): Final results (NCT00193843). Journal of Clinical Oncology 34, 6004.

Lassen, P., Eriksen, J.G., Krogdahl, A., et al., 2011. The influence of HPV-associated p16-expression on accelerated fractionated radiotherapy in head and neck cancer: Evaluation of the randomised DAHANCA 6&7 trial. Radiotherapy and Oncology 100, 49–55.

Lavaf, A., Genden, E.M., Cesaretti, J.A., Packer, S., Kao, J., 2008. Adjuvant radiotherapy improves overall survival for patients with lymph node-positive head and neck squamous cell carcinoma. Cancer 112, 535–543.

Lee, J.Y., Sun, J.-M., Oh, D.R., et al., 2016. Comparison of weekly versus triweekly cisplatin delivered concurrently with radiation therapy in patients with locally advanced nasopharyngeal cancer: A multicenter randomized phase II trial (KCSG-HN10-02). Radiotherapy and Oncology 118, 244–250.

Lee, L.-A., Huang, C.-G., Liao, C.-T., et al., 2012. Human papillomavirus-16 infection in advanced oral cavity cancer patients is related to an increased risk of distant metastases and poor survival. PLoS One 7, e40767.

Levendag, P., Nijdam, W., Noever, I., et al., 2004. Brachytherapy versus surgery in carcinoma of tonsillar fossa and/or soft palate: Late adverse sequelae and performance status: Can we be more selective and obtain better tissue sparing? International Journal of Radiation Oncology, Biology, Physics 59, 713–724.

Li, C., Lin, J., Men, Y., Yang, W., Mi, F., Li, L., 2017. Does medullary versus cortical invasion of the mandible affect prognosis in patients with oral squamous cell carcinoma? Journal of Oral and Maxillofacial Surgery 75, 403–415.

Liang, L., Zhang, T., Kong, Q., Liang, J., Liao, G., 2015. A meta-analysis on selective versus comprehensive neck dissection in oral squamous cell carcinoma patients with clinically node-positive neck. Oral Oncology 51, 1076–1081.

Liao, C.-T., Lee, L.-Y., Huang, S.-F., et al., 2011. Outcome analysis of patients with oral cavity cancer and extracapsular spread in neck lymph nodes. International Journal of Radiation Oncology Biology Physics 81, 930–937.

Liauw, S.L., Mancuso, A.A., Amdur, R.J., et al., 2006. Postradiotherapy neck dissection for lymph node-positive head and neck cancer: The use of computed tomography to manage the neck. Journal of Clinical Oncology 24, 1421–1427.

Licitra, L., Grandi, C., Guzzo, M., et al., 2003. Primary chemotherapy in resectable oral cavity squamous cell cancer: A randomized controlled trial. Journal of Clinical Oncology 21, 327–333.

Lydiatt, W.M., Patel, S.G., O'Sullivan, B., et al., 2017. Head and neck cancers—Major changes in the American Joint Committee on cancer eighth edition cancer staging manual. CA: a Cancer Journal for Clinicians 67, 122–137.

Maccomb, W.F.G., 1957. Planned combination of surgery and radiation in treatment of advanced primary head and neck cancers. The American Journal of Roentgenology, Radium Therapy, and Nuclear Medicine 77, 397–414.

Machtay, M., Moughan, J., Trotti, A., et al., 2008. Factors associated with severe late toxicity after concurrent chemoradiation for locally advanced head and neck cancer: An RTOG analysis. Journal of Clinical Oncology 26, 3582–3589.

Maesschalck, T.D., et al., 2016. Comparison of the incidence of osteoradionecrosis with conventional radiotherapy and intensity-modulated radiotherapy. Head Neck 38, 1695–1702.

Mahapatra, S., Kamath, R., Shetty, B., Binu, V., 2015. Risk of oral cancer associated with gutka and other tobacco products: A hospital-based case-control study. Journal of Cancer Research and Therapeutics 11, 199–203.

Markowitz, L.E., Liu, G., Hariri, S., Steinau, M., Dunne, E.F., Unger, E.R., 2016. Prevalence of HPV after introduction of the vaccination program in the United States. Pediatrics 137 (3), 1–9.

Martinez-Trufero, J., Isla, D., Adansa, J.C., et al., 2010. Phase II study of capecitabine as palliative treatment for patients with recurrent and metastatic squamous head and neck cancer after previous platinum-based treatment. British Journal of Cancer 102, 1687–1691.

Mazeron, J.-J., Ardiet, J.-M., Haie-Méder, C., et al., 2009. GEC-ESTRO recommendations for brachytherapy for head and neck squamous cell carcinomas. Radiotherapy and Oncology 91, 150–156.

Mehanna, H., Wong, W.L., McConkey, C.C., et al., 2016. PET-CT surveillance versus neck dissection in advanced head and neck cancer. The New England Journal of Medicine 374, 1444–1454.

Mehanna, H.M., Rattay, T., Smith, J., McConkey, C.C., 2009. Treatment and follow-up of oral dysplasia—A systematic review and meta-analysis. Head & Neck 31, 1600–1609.

Mell, L.K., Mehrotra, A.K., Mundt, A.J., 2005. Intensity-modulated radiation therapy use in the U.S., 2004. Cancer 104, 1296–1303.

Melotek, J.M., Brisson, R.J., Villaflor, V.M., et al., 2016. Definitive chemoradiation therapy for advanced oral cavity cancer: A 20-year experience. International Journal of Radiation Oncology Biology Physics 96, S84.

Mendenhall, W.M., Parsons, J.T., Stringer, S.P., Cassisi, N.J., Million, R.R., 1989. T2 oral tongue carcinoma treated with radiotherapy: Analysis of local control and complications. Radiotherapy and Oncology 16, 275–281.

Montero, P.H., Yu, C., Palmer, F.L., et al., 2014. Nomograms for preoperative prediction of prognosis in patients with oral cavity squamous cell carcinoma. Cancer 120, 214–221.

Moore, E.J., Olsen, S.M., Laborde, R.R., et al., 2012. Long-term functional and oncologic results of transoral robotic surgery for oropharyngeal squamous cell carcinoma. Mayo Clinic Proceedings 87, 219–225.

Moore, S.R., Johnson, N.W., Pierce, A.M., Wilson, D.F., 1999. The epidemiology of lip cancer: A review of global incidence and aetiology. Oral Diseases 5, 185–195.

Morton, R.P., Rugman, F., Dorman, E.B., et al., 1985. Cisplatinum and bleomycin for advanced or recurrent squamous cell carcinoma of the head and neck: A randomised factorial phase III controlled trial. Cancer Chemotherapy and Pharmacology 15, 283–289.

Murphy, B.A., 2005. Topoisomerases in the treatment of metastatic or recurrent squamous carcinoma of the head and neck. Expert Opinion on Pharmacotherapy 6, 85–92.

Nag, S., Cano, E.R., Demanes, D.J., Puthawala, A.A., Vikram, B., American Brachytherapy, S., 2001. The American Brachytherapy Society recommendations for high-dose-rate brachytherapy for head-and-neck carcinoma. International Journal of Radiation Oncology, Biology, Physics 50, 1190–1198.

Nguyen-Tan, P.F., Zhang, Q., Ang, K.K., et al., 2014. Randomized phase III trial to test accelerated versus standard fractionation in combination with concurrent cisplatin for head and neck carcinomas in the Radiation Therapy Oncology Group 0129 trial: Long-term report of efficacy and toxicity. Journal of Clinical Oncology 32, 3858–3866.

Nomura, T., Shibahara, T., Cui, N.-H., Noma, H., 2005. Patterns of mandibular invasion by gingival squamous cell carcinoma. Journal of Oral and Maxillofacial Surgery 63, 1489–1493.

Noronha, V., Joshi, A., Patil, V., et al., 2018. Once-a-week versus once-every-3-weeks cisplatin chemoradiation for locally advanced head and neck cancer: A phase III randomized noninferiority trial. Journal of Clinical Oncology 36, 1064–1072.

Nutting, C.M., Morden, J.P., Harrington, K.J., et al., 2011. Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): A phase 3 multicentre randomised controlled trial. The Lancet Oncology 12, 127–136.

Overgaard, J., Hansen, H.S., Specht, L., et al., 2003. Five compared with six fractions per week of conventional radiotherapy of squamous-cell carcinoma of head and neck: DAHANCA 6 and 7 randomised controlled trial. Lancet 362, 933–940.

Overgaard, J., Mohanti, B.K., Begum, N., et al., 2010. Five versus six fractions of radiotherapy per week for squamous-cell carcinoma of the head and neck (IAEA-ACC study): A randomised, multicentre trial. The Lancet Oncology 11, 553–560.

Owosho, A.A., et al., 2017. The prevalence and risk factors associated with osteoradionecrosis of the jaw in oral and oropharyngeal cancer patients treated with intensity-modulated radiation therapy (IMRT): The memorial sloan kettering cancer center experience. Oral Oncology 64, 44–51.

Ozturk, K., Gode, S., Erdogan, U., Akyildiz, S., Apaydin, F., 2015. Squamous cell carcinoma of the lip: Survival analysis with long-term follow-up. European Archives of Oto-Rhino-Laryngology 272, 3545–3550.
Patel, R.S., Dirven, R., Clark, J.R., Swinson, B.D., Gao, K., O'Brien, C.J., 2008. The prognostic impact of extent of bone invasion and extent of bone resection in oral carcinoma. The Laryngoscope 118, 780–785.
Patel, S.C., Carpenter, W.R., Tyree, S., et al., 2011. Increasing incidence of oral tongue squamous cell carcinoma in young white women, age 18 to 44 years. Journal of Clinical Oncology 29, 1488–1494.
Pathak, J., Swain, N., Patel, S., Poonja, L.S., 2014. Histopathological variants of oral squamous cell carcinoma-institutional case reports. Journal of Oral and Maxillofacial Pathology 18, 143–145.
Pederson, A.W., Salama, J.K., Witt, M.E., et al., 2011. Concurrent chemotherapy and intensity-modulated radiotherapy for organ preservation of locoregionally advanced oral cavity cancer. American Journal of Clinical Oncology 34, 356–361.
Pentenero, M., Gandolfo, S., Carrozzo, M., 2005. Importance of tumor thickness and depth of invasion in nodal involvement and prognosis of oral squamous cell carcinoma: A review of the literature. Head & Neck 27, 1080–1091.
Perea-Milla Lopez, E., Minarro-del Moral, R.M., Martinez-Garcia, C., et al., 2003. Lifestyles, environmental and phenotypic factors associated with lip cancer: A case-control study in southern Spain. British Journal of Cancer 88, 1702–1707.
Petrelli, F., Coinu, A., Riboldi, V., et al., 2014. Concomitant platinum-based chemotherapy or cetuximab with radiotherapy for locally advanced head and neck cancer: A systematic review and meta-analysis of published studies. Oral Oncology 50, 1041–1048.
Pignon, J.P., le Maitre, A., Maillard, E., Bourhis, J., MACH-NC Collaborative Group, 2009. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): An update on 93 randomised trials and 17,346 patients. Radiotherapy and Oncology 92, 4–14.
Pivot, X., Raymond, E., Laguerre, B., et al., 2001. Pemetrexed disodium in recurrent locally advanced or metastatic squamous cell carcinoma of the head and neck. British Journal of Cancer 85, 649–655.
Porceddu, S.V., Pryor, D.I., Burmeister, E., et al., 2011. Results of a prospective study of positron emission tomography-directed management of residual nodal abnormalities in node-positive head and neck cancer after definitive radiotherapy with or without systemic therapy. Head & Neck 33, 1675–1682.
Poulsen, M.G., Denham, J.W., Peters, L.J., et al., 2001. A randomised trial of accelerated and conventional radiotherapy for stage III and IV squamous carcinoma of the head and neck: A Trans-Tasman Radiation Oncology Group Study. Radiotherapy and Oncology 60, 113–122.
Prime, S.S., Thakker, N.S., Pring, M., Guest, P.G., Paterson, I.C., 2001. A review of inherited cancer syndromes and their relevance to oral squamous cell carcinoma. Oral Oncology 37, 1–16.
Raguse, J.-D., et al., 2016. Patient and treatment-related risk factors for osteoradionecrosis of the jaw in patients with head and neck cancer. Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology 121, 215–221 e1.
Reichart, P.A., Philipsen, H.P., 2005. Oral erythroplakia—A review. Oral Oncology 41, 551–561.
Riaz, N., Hong, J.C., Sherman, E.J., et al., 2014. A nomogram to predict loco-regional control after re-irradiation for head and neck cancer. Radiotherapy and Oncology 111, 382–387.
Rigby, M.H., Hayden, R.E., 2014. Regional flaps: A move to simpler reconstructive options in the head and neck. Current Opinion in Otolaryngology & Head and Neck Surgery 22, 401–406.
Rigual, N., Loree, T., Frustino, J., et al., 2013. Sentinel node biopsy in lieu of neck dissection for staging oral cancer. JAMA Otolaryngology. Head & Neck Surgery 139, 779–782.
Rohde, M., Nielsen, A.L., Johansen, J., et al., 2017. Head-to-head comparison of chest X-ray/head and neck MRI, chest CT/head and neck MRI, and 18F-FDG PET/CT for detection of distant metastases and synchronous cancer in oral, pharyngeal, and laryngeal cancer. Journal of Nuclear Medicine 58, 1919–1924.
Rosenthal, D.I., Lewin, J.S., Eisbruch, A., 2006. Prevention and treatment of dysphagia and aspiration after chemoradiation for head and neck cancer. Journal of Clinical Oncology 24, 2636–2643.
Rosenthal, D.I., Harari, P.M., Giralt, J., et al., 2016. Association of human papillomavirus and p16 status with outcomes in the IMCL-9815 Phase III registration trial for patients with locoregionally advanced oropharyngeal squamous cell carcinoma of the head and neck treated with radiotherapy with or without cetuximab. Journal of Clinical Oncology 34, 1300–1308.
Rosenthal, D.I., Mohamed, A.S.R., Garden, A.S., et al., 2017. Final report of a prospective randomized trial to evaluate the dose–response relationship for postoperative radiation therapy and pathologic risk groups in patients with head and neck cancer. International Journal of Radiation Oncology Biology Physics 98, 1002–1011.
Rusthoven, K., Ballonoff, A., Raben, D., Chen, C., 2008. Poor prognosis in patients with stage I and II oral tongue squamous cell carcinoma. Cancer 112, 345–351.
Salama, J.K., Stenson, K.M., List, M.A., et al., 2008. Characteristics associated with swallowing changes after concurrent chemotherapy and radiotherapy in patients with head and neck cancer. Archives of Otolaryngology—Head & Neck Surgery 134, 1060–1065.
Salama, J.K., Haraf, D.J., Stenson, K.M., et al., 2011. A randomized phase II study of 5-fluorouracil, hydroxyurea, and twice-daily radiotherapy compared with bevacizumab plus 5-fluorouracil, hydroxyurea, and twice-daily radiotherapy for intermediate-stage and T4N0-1 head and neck cancers. Annals of Oncology 22, 2304–2309.
Sandler, A., Saxman, S., Bandealy, M., et al., 1998. Ifosfamide in the treatment of advanced or recurrent squamous cell carcinoma of the head and neck: A phase II Hoosier Oncology Group trial. American Journal of Clinical Oncology 21, 195–197.
Sankaranarayanan, R., Ramadas, K., Thomas, G., et al., 2005. Effect of screening on oral cancer mortality in Kerala, India: A cluster-randomised controlled trial. The Lancet 365, 1927–1933.
Saraiya, M., Unger, E.R., Thompson, T.D., et al., 2015. US Assessment of HPV Types in Cancers: Implications for current and 9-valent HPV vaccines. Journal of the National Cancer Institute 107, djv086.
Saxman, S., Mann, B., Canfield, V., Loehrer, P., Vokes, E., 1998. A phase II trial of vinorelbine in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. American Journal of Clinical Oncology 21, 398–400.
Scarbrough, T.J., Day, T.A., Williams, T.E., Hardin, J.H., Aguero, E.G., Thomas, C.R.J., 2003. Referred otalgia in head and neck cancer: A unifying schema. American Journal of Clinical Oncology 26, e157–e162.
Scher, E.D., Romesser, P.B., Chen, C., et al., 2015. Definitive chemoradiation for primary oral cavity carcinoma: A single institution experience. Oral Oncology 51, 709–715.
Schwartz, L.H., Ozsahin, M., Zhang, G.N., et al., 1994. Synchronous and metachronous head and neck carcinomas. Cancer 74, 1933–1938.
Shafer, W.G., Waldron, C.A., 1975. Erythroplakia of the oral cavity. Cancer 36, 1021–1028.
Shah, A., Ali, A., Latoo, S., Ahmad, I., 2010. Multiple myeloma presenting as gingival mass. Journal of Maxillofacial and Oral Surgery 9, 209–212.
Sharma, A., Mohanti, B.K., Thakar, A., Bahadur, S., Bhasker, S., 2010. Concomitant chemoradiation versus radical radiotherapy in advanced squamous cell carcinoma of oropharynx and nasopharynx using weekly cisplatin: A phase II randomized trial. Annals of Oncology 21, 2272–2277.
Siegel, R.L., Miller, K.D., Jemal, A., 2017. Cancer statistics, 2017. CA: A Cancer Journal for Clinicians 67, 7–30.
Silva, T.D.B., Ferreira, C.B.T., Leite, G.B., de Menezes Pontes, J.R., Antunes, H.S., 2016. Oral manifestations of lymphoma: A systematic review. ecancermedicalscience 10, 665.
Simard, E.P., Torre, L.A., Jemal, A., 2014. International trends in head and neck cancer incidence rates: Differences by country, sex and anatomic site. Oral Oncology 50, 387–403.
Singhi, A.D., Westra, W.H., 2010. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer 116, 2166–2173.
Sjovall, J., Chua, B., Pryor, D., et al., 2015. Long-term results of positron emission tomography-directed management of the neck in node-positive head and neck cancer after organ preservation therapy. Oral Oncology 51, 260–266.

Smyth, A.G., Ward-Booth, R.P., To EWH, et al., 1993. Malignant melanoma of the oral cavity—An increasing clinical diagnosis? British Journal of Oral and Maxillofacial Surgery 31, 230–235.
Spiotto, M.T., Jefferson, G., Wenig, B., Markiewicz, M., Weichselbaum, R.R., Koshy, M., 2017. Differences in survival with surgery and postoperative radiotherapy compared with definitive chemoradiotherapy for oral cavity cancer: A national cancer database analysis. JAMA Otolaryngology. Head & Neck Surgery 143, 691–699.
Stenson, K.M., MacCracken, E., List, M., et al., 2000. Swallowing function in patients with head and neck cancer prior to treatment. Archives of Otolaryngology – Head & Neck Surgery 126, 371–377.
Stenson, K.M., Kunnavakkam, R., Cohen, E.E., et al., 2010. Chemoradiation for patients with advanced oral cavity cancer. Laryngoscope 120, 93–99.
Studer, G., Zwahlen, R.A., Graetz, K.W., Davis, B.J., Glanzmann, C., 2007. IMRT in oral cavity cancer. Radiation Oncology 2.
Studer, G., Brown, M., Bredell, M., et al., 2012. Follow up after IMRT in oral cavity cancer: Update. Radiation Oncology 7, 84.
Studer, G., Bredell, M., Studer, S., Huber, G., Glanzmann, C., 2016. Risk profile for osteoradionecrosis of the mandible in the IMRT era. Strahlentherapie und Onkologie: Organ der Deutschen Röntgengesellschaf 192, 32–39.
Su, H.-H., Chu, S.-T., Hou, Y.-Y., Chang, K.-P., Chen, C.-J., 2006. Spindle cell carcinoma of the oral cavity and oropharynx: Factors affecting outcome. Journal of the Chinese Medical Association 69, 478–483.
Sullivan, B.P., Parks, K.A., Dean, N.R., Rosenthal, E.L., Carroll, W.R., Magnuson, J.S., 2011. Utility of CT surveillance for primary site recurrence of squamous cell carcinoma of the head and neck. Head & Neck 33, 1547–1550.
Tao, Y., Auperin, A., Sire, C., et al., 2018. Improved outcome by adding concurrent chemotherapy to cetuximab and radiotherapy for locally advanced head and neck carcinomas: Results of the GORTEC 2007-01 phase III randomized trial. Journal of Clinical Oncology.
Taussky, D., Dulguerov, P., Allal, A.S., 2005. Salvage surgery after radical accelerated radiotherapy with concomitant boost technique for head and neck carcinomas. Head & Neck 27, 182–186.
Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., Jemal, A., 2015. Global cancer statistics, 2012. CA: A Cancer Journal for Clinicians 65, 87–108.
Tsai, C.J., et al., 2013. Osteoradionecrosis and radiation dose to the mandible in patients with oropharyngeal cancer. International Journal of Radiation Oncology Biology Physics 85, 415–420.
Tsan, D.-L., Lin, C.-Y., Kang, C.-J., et al., 2012. The comparison between weekly and three-weekly cisplatin delivered concurrently with radiotherapy for patients with postoperative high-risk squamous cell carcinoma of the oral cavity. Radiation Oncology 7, 215.
Tupchong, L., Scott, C.B., Blitzer, P.H., et al., 1991. Randomized study of preoperative versus postoperative radiation therapy in advanced head and neck carcinoma: Long-term follow-up of RTOG study 73-03. International Journal of Radiation Oncology, Biology, Physics 20, 21–28.
Urken, M.L., Moscoso, J.F., Lawson, W., Biller, H.F., 1994. A systematic approach to functional reconstruction of the oral cavity following partial and total glossectomy. Archives of Otolaryngology – Head & Neck Surgery 120, 589–601.
Van Cann, E.M., Koole, R., Oyen, W.J.G., et al., 2008. Assessment of mandibular invasion of squamous cell carcinoma by various modes of imaging: Constructing a diagnostic algorithm. International Journal of Oral and Maxillofacial Surgery 37, 535–541.
Vargo, J.A., Ferris, R.L., Ohr, J., et al., 2015. A prospective phase 2 trial of reirradiation with stereotactic body radiation therapy plus cetuximab in patients with previously irradiated recurrent squamous cell carcinoma of the head and neck. International Journal of Radiation Oncology, Biology, Physics 91, 480–488.
Vermorken, J.B., Mesia, R., Rivera, F., et al., 2008. Platinum-based chemotherapy plus cetuximab in head and neck cancer. The New England Journal of Medicine 359, 1116–1127.
Wang, S.J., Patel, S.G., Shah, J.P., et al., 2013. An oral cavity carcinoma nomogram to predict benefit of adjuvant radiotherapy. JAMA Otolaryngology. Head & Neck Surgery 139, 554–559.
Warnakulasuriya, S., 2009. Global epidemiology of oral and oropharyngeal cancer. Oral Oncology 45, 309–316.
Weber, R.S., Guillamondegui, O.M., Byers, R.M., Palmer, J.M., El-Naggar, A., McNeese, M.D., 1989. Minor salivary gland tumors of the lip and buccal mucosa. The Laryngoscope 99, 6-9.
Weissman, J.L., Carrau, R.L., 2001. Puffed-cheek CT improves evaluation of the oral cavity. American Journal of Neuroradiology 22, 741–744.
Wendt, T.G., Grabenbauer, G.G., Rodel, C.M., et al., 1998. Simultaneous radiochemotherapy versus radiotherapy alone in advanced head and neck cancer: A randomized multicenter study. Journal of Clinical Oncology 16, 1318–1324.
White, H.N., Moore, E.J., Rosenthal, E.L., et al., 2010. Transoral robotic-assisted surgery for head and neck squamous cell carcinoma: One- and 2-year survival analysis. Archives of Otolaryngology – Head & Neck Surgery 136, 1248–1252.
Wollina, U., Verma, S.B., Ali, F.M., Patil, K., 2015. Oral submucous fibrosis: An update. Clinical, Cosmetic and Investigational Dermatology 8, 193–204.
Woolgar, J.A., 1997. Detailed topography of cervical lymph-node metastases from oral squamous cell carcinoma. International Journal of Oral and Maxillofacial Surgery 26, 3–9.
Yamazakl, H., Yoshida, K., Yoshioka, Y., et al., 2013. High dose rate brachytherapy for oral cancer. Journal of Radiation Research 54, 1–17.
Yanik, E.L., Katki, H.A., Silverberg, M.J., Manos, M.M., Engels, E.A., Chaturvedi, A.K., 2015. Leukoplakia, oral cavity cancer risk, and cancer survival in the U.S. elderly. Cancer Prevention Research 8, 857–863.
Yoo, D.S., Kirkpatrick, J.P., Craciunescu, O., et al., 2012. Prospective trial of synchronous bevacizumab, erlotinib, and concurrent chemoradiation in locally advanced head and neck cancer. Clinical Cancer Research 18, 1404–1414.
Zhang, W.-B., Peng, X., 2016. Cervical metastases of oral maxillary squamous cell carcinoma: A systematic review and meta-analysis. Head & Neck 38, E2335–E2342.
Zhong, L.-P., Zhang, C.-P., Ren, G.-X., et al., 2013. Randomized phase III trial of induction chemotherapy with docetaxel, cisplatin, and fluorouracil followed by surgery versus up-front surgery in locally advanced resectable oral squamous cell carcinoma. Journal of Clinical Oncology 31, 744–751.

Further Reading

Bourhis, J., Sun, S., Sire, C., et al., 2016. Cetuximab-radiotherapy versus cetuximab-radiotherapy plus concurrent chemotherapy in patients with N0-N2a squamous cell carcinoma of the head and neck (SCCHN): Results of the GORTEC 2007-01 phase III randomized trial. Journal of Clinical Oncology 34, 6003.
Chi, A.C., Day, T.A., Neville, B.W., 2015. Oral cavity and oropharyngeal squamous cell carcinoma—An update. CA: A Cancer Journal for Clinicians 65 (5), 401–421.
Chinn, S.B., Myers, J.N., 2015. Oral cavity carcinoma: Current management, controversies, and future directions. Journal of Clinical Oncology 33 (29), 3269–3276.
Diaz EM, Kies MS, Sturgis EM, et al. Neoplasms of the head and neck. In: Kufe DW, Bast RC, Hait WN, et al. Holland-Frei Cancer Medicine. London: BC Decker 2006 (pg. 1131-1178).
Furness, S., Glenny, A.M., Worthington, H.V., Pavitt, S., Oliver, R., Clarkson, J.E., Macluskey, M., Chan, K.K., Conway, D.I., 2011. Interventions for the treatment of oral cavity and oropharyngeal cancer: Chemotherapy. Cochrane Database of Systematic Reviews 13 (4), CD006386.
Harris CM, Ghali GE, Holmes JD, Dierks EJ, Montes DM. Peterson's principles of oral and maxillofacial surgery. Shelton, CT: PMPH-USA 3rd edn, Chapters 31–33 (pg. 677-742).
Harrison, L.B., Sessions, R.B., Kies, M.S., 2014. Head and neck cancer: A multidisciplinary approach, 4th edn. LWW, Philadelphia, PA. Chapters 16 pp. 335–373; Chapter 23 pp. 635–671.
Lydiatt, W.M., Patel, S.G., O'Sullivan, B., Brandwein, M.S., Ridge, J.A., Migliacci, J.C., Loomis, A.M., Shah, J.P., 2017. Head and Neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. CA: A Cancer Journal for Clinicians 67 (2), 122–137.

Manon RR, Myers JN, Skinner HD, Harari PM. Perez and Brady's principles and practice of radiation oncology. Philadelphia, PA: LWW 6th edn, Chapter 44 (pg. 795-816).
Meyers JN, Hanna EY, Myers EN. Cancer of the head and neck. Philadelphia, PA: LWW 5th edn. 2016, Chapters 12–13 (pg. 317-346).
Trotta, B.M., Pease, C.S., Rasamny, J.J., Raghavan, P., Mukherjee, S., 2011. Oral cavity and oropharyngeal squamous cell cancer: Key imaging findings for staging and treatment planning. Radiographics 31 (2), 339–354.

Relevant Websites

https://www.nccn.org/professionals/physician_gls/pdf/head-and-neck.pdf - National Comprehensive Cancer Network.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3473765/pdf/bjr-84-944.pdf - Law CP, Chandra RV, Hoang JK, Phal PM. Imaging the oral cavity: key concepts for the radiologist. The British Journal of Radiology. 2011;84(1006):944-957. https://doi.org/10.1259/bjr/70520972.
https://www.cdc.gov/vaccines/vpd/hpv/hcp/recommendations.html - Centers for Disease Control and Prevention. HPV Vaccine Recommendations.
https://oncohemakey.com/oral-cavity-4/ - Oncohema Key. Oral Cavity.
https://www.nidcr.nih.gov/oralhealth/Topics/OralCancer/DetectingOralCancer.htm - National Institute of Dental and Craniofacial Research. Oral Cancer.

Ovarian Cancer: Diagnosis and Treatment

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Abbreviations

BSO Bilateral salpingo-oorphorectomy
CEA Carcinoembryonic antigen
CT Computed tomography
EOC Epithelial ovarian cancer
FDA US Food and Drug Administration
FDG-PET Positron emission tomography with fluorodeoxyglucose
FNA Fine-needle aspiration
MRI Magnetic resonance imaging
NCCN US National Comprehensive Cancer Network
PARP Poly (ADP-ribose) polymerase
PET Positron emission tomography
ROMA Risk of Ovarian Malignancy Algorithm
RT Radiotherapy
TAH Total abdominal hysterectomy
USO Unilateral salpingo-oorphorectomy
VEGF Vascular endothelial growth factor
VEGFR Vascular endothelial growth factor receptor

Definition

Ovarian neoplasms comprise several distinct histopathological entities. The most common of them is ovarian carcinoma, a malignant epithelial tumor originating from the ovaries (epithelial ovarian cancer, EOC), which accounts for approximately 90% of all ovarian cancer cases. This entry does not specifically cover other, less frequent, ovarian cancer types. However, the symptoms and the diagnostic considerations for most of them are similar to those of EOC.

Presentation and Diagnosis

Early ovarian cancer of any type is usually asymptomatic or associated with minimal nonspecific symptoms. Advanced epithelial ovarian carcinoma (EOC) manifests with a wide spectrum of vague nonspecific symptoms, such as abdominal discomfort and/or bloating, irregular menstruations or abnormal vaginal bleeding, difficulty eating and/or early satiety. Increased abdominal size and pelvic pain have been shown to be independently associated with the disease. Urinary symptoms (urinary urgency or increased frequency) may occur as a result of the pressure of the tumor mass on the bladder. A swollen leg due to venous thrombosis is also common. Gastrointestinal symptoms, like nausea and vomiting, constipation, and diarrhea, are usually associated with later stages of the disease, with tiredness and weight loss which follow. Ovarian or pelvic mass and/or ascites may be found at physical examination in patients with more advanced disease.

Given the nonspecific symptoms, ovarian cancer is usually detected at advanced stages. At the same time, several trials have shown that screening using the existing methods does not decrease mortality from ovarian cancer, while it may lead to overtreatment and complications associated with an unnecessary surgery. Therefore, many clinical guidelines and regulatory bodies (like the US Food and Drug Administration (FDA)) recommend against screening the general population for ovarian cancer, be it by transvaginal ultrasound examination, by measuring serum CA-125 levels, or by using any of the ovarian cancer tests currently available on the market. However, any pelvic mass in a woman over 1 year after menopause as well as the above-mentioned symptoms without any diagnosed source of malignancy in a woman of any age should be treated as a suspected ovarian cancer and warrant ultrasound examination and other studies as indicated.

Ultrasound is typically used for the initial evaluation of a pelvic mass and/or in case of symptoms suggesting a possible ovarian cancer. Abdominal/pelvic magnetic resonance imaging (MRI) may be useful to determine the malignant potential of lesions indeterminate by ultrasound. Otherwise, positron emission tomography with fluorodeoxyglucose (FDG-PET) combined with computed

tomography (CT) may be useful to this effect. Abdominal/pelvic CT is useful to assess for metastases but has less value than ultrasound or MRI for evaluating the ovarian mass. However, CT is useful for diagnosing cystic teratomas and cystadenomas. Both MRI and CT should be performed with contrast, for example gadolinium. Chest radiography or CT is often performed to exclude pleural effusions or pulmonary spread. In patients with gastrointestinal symptoms, a gastrointestinal tract workup may additionally be indicated.

Premenopausal women often present with benign cysts, many of which regress spontaneously. Masses that are smaller than 8 cm in premenopausal women and show cystic nature on ultrasound are likely to be benign lesions. They should regress after 2 months of oral contraceptives. For putative cysts which do not regress after 2 months as well as for all other lesions, a tissue-based diagnosis should be made.

The definitive diagnosis of most ovarian cancers is made based on a pathological analysis of a biopsy or a surgical specimen (pre-, intra-, or postoperatively). When a highly suspicious isolated adnexal or pelvic mass is present, surgical removal of an intact ovary is recommended to avoid tumor spillage and upstaging. Fine-needle aspiration (FNA) or percutaneous biopsy for diagnosing presumed early-stage ovarian cancer should be avoided if possible to prevent rupturing the cyst and spilling the malignant cells into the peritoneal cavity. However, FNA may be necessary in patients with diffuse carcinomatosis or in those with bulky tumors who are not candidates for surgery. Mucinous histology may indicate a metastasis of a gastrointestinal tumor and should prompt an evaluation of the gastrointestinal tract. A metastasis from the breast may be ruled out with the help of mammography.

Several serum markers have been suggested as potentially useful in the diagnostic workup for epithelial ovarian cancer, in particular CA-125, carcinoembryonic antigen (CEA), CA 19-9, and human epididymis 4 protein (HE-4). A very elevated CEA-to-CA-125 ratio should raise suspicion for a possible gastrointestinal malignancy. The use of serum CA-125 and HE-4 levels has been approved by FDA as part of the risk of ovarian malignancy algorithm (ROMA) which estimates the risk of finding a malignancy at surgery in women presenting with a pelvic mass. However, this test is rather an aid helping primary care physicians to identify patients who should be referred to a gynecologic oncologist than a real diagnostic tool and its use for diagnosing pelvic masses is not recommended by the expert panel of the US National Comprehensive Cancer Network (NCCN). Still, some serum markers, if initially elevated, may be useful to monitor the response to treatment in patients with epithelial ovarian tumors.

Management and Therapy

The most common primary treatment for presumed ovarian cancer is surgery, often followed by systemic chemotherapy. Primary Fallopian tube and peritoneal cancers are managed in the same way as epithelial ovarian cancers.

The surgery should consist of a surgical exploration and comprehensive surgical staging, and debulking (i.e., removing the primary tumor and as much of the metastatic disease as possible, also called cytoreductive surgery). The recommended surgical approach for all patients is total abdominal hysterectomy (TAH) with bilateral salpingo-oophorectomy (BSO). In selected patients with lowest-stage low-risk disease who wish to preserve fertility, a unilateral salpingo-oophorectomy (USO) which preserves the contralateral ovary and the uterus may be considered. However, in some cases, BSO may be necessary. If macroscopic disease is visible outside the ovary, all visible tumor should be removed. This may require extensive surgery, including bowel resection and/or appendectomy, partial gastrectomy, stripping of the diaphragm or other peritoneal surfaces, liver resection, distal pancreatectomy, omentectomy, splenectomy, cholecystectomy, partial cystectomy and/or ureteroneocystostomy. Suspicious and/or enlarged lymph nodes are also dissected whenever possible. It has been shown that systematic lymphadenectomy in patients with advanced ovarian cancer is associated with increased overall survival. Using minimally invasive techniques may be considered in patients with early-stage disease, while many surgeons tend to use open laparotomy in patients with more widespread disease. A comprehensive surgical staging including peritoneal cytology and multiple peritoneal biopsies is important in all cases to rule out the presence of occult higher-stage disease.

Surgery alone is sufficient in a small proportion of select ovarian cancer patients with low-stage and low-risk disease. A majority of patients, however, receive adjuvant chemotherapy following the primary surgery. Platinum (carboplatin or cisplatin) with paclitaxel is recommended for most patients, with intravenous or intraperitoneal administration. Dose-dense paclitaxel with carboplatin may also be considered. Patients with low-volume residual disease after surgical debulking are candidates for intraperitoneal therapy.

Patients who are not candidates for optimal frontline debulking understood as removing all visible disease (R0) may be considered for neoadjuvant chemotherapy followed by interval debulking and adjuvant chemotherapy. Even though in general the best outcomes are obtained with debulking performed as part of the primary treatment, neoadjuvant chemotherapy with interval debulking has been shown to be noninferior to upfront debulking in patients with bulky high-stage (IIIC–IV) disease. Therefore, it may be considered as an alternative in these patients, in particular in those with important comorbidities which may make them poor candidates for initial surgery. A prior histological confirmation of the ovarian cancer diagnosis (by FNA, core biopsy, or paracentesis) and a careful evaluation by a gynecologic oncologist are necessary. The optimal strategies to select patients best suited for primary cytoreduction versus neoadjuvant chemotherapy are still under investigation. In particular, scoring systems based on diagnostic laparoscopy and preoperative imaging are being evaluated to this effect.

Intravenous taxane/carboplatin and liposomal doxorubicin/carboplatin regimens are recommended for both neoadjuvant chemotherapy and adjuvant chemotherapy after interval debulking. The standard combined intraperitoneal/intravenous regimen of paclitaxel with cisplatin may also be used as adjuvant therapy following interval debulking. For nonsurgical patients with poor performance status, advanced disease (stage IV) and important comorbidities, as well as for elderly patients, single-agent platinum regimens may be more appropriate.

Radiotherapy (RT) is not recommended for the primary treatment of patients with EOC. Even though some studies have reported that it may provide a treatment benefit in patients with advanced cancer and/or small-volume residual disease present after primary surgery, it is usually not acceptable due to severe toxicities. However, localized RT is an option for palliative treatment to control symptoms in selected patients with advanced recurrent disease.

Ovarian cancer has a high response rate to the frontline treatment. However, advanced EOC recurs in over 75% of patients and has a very poor prognosis. Recurrence may be identified clinically, biochemically (rising serum CA-125 levels) and/or with imaging. The treatment options depend on the primary treatment and the length of the recurrence-free period after completing the primary therapy.

Patients with recurrent ovarian cancer are often retreated with multiple courses of chemotherapy. The disease which recurred after over 6 months without progression following a platinum-based chemotherapy (classified as platinum-sensitive disease) may be retreated with a platinum-containing regimen, with the probability of response increasing with the duration of the disease-free interval. A number of regimens combining carboplatin or cisplatin with paclitaxel, docetaxel, liposomal doxorubicin, gemcitabine and other chemotherapeutics are used. For patients who have isolated recurrent platinum-sensitive disease, a secondary cytoreductive surgery may be beneficial provided that the recurrence can be completely resected. This option is particularly viable for patients who have relapsed after a recurrence-free period of at least 24 months. In patients with platinum-resistant disease (i.e., disease relapsing within the first 6 months after completing platinum-based chemotherapy), response may be obtained with several nonplatinum treatments, like docetaxel, topotecan, oral etoposide, liposomal doxorubicin or gemcitabine, usually administered as sequential single-agent therapies rather than combined regimens. Treatment with capecitabine or other alkylating agents (like cyclophosphamide or melphalan) may be useful in patients with platinum- and taxanes-resistant disease. Hormonal therapy with tamoxifen or aromatase inhibitors may be considered for patients intolerant to chemotherapy but the response rates are rather low.

Given poor prognosis and high recurrence rates, much hope is put in novel therapies with targeted molecular and biologic agents for treatment of advanced epithelial ovarian cancer, and in particular recurrent platinum-resistant disease. Adding bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), to the upfront carboplatin/paclitaxel chemotherapy followed by the maintenance bevacizumab has been shown to improve survival in a subset (but not all) of advanced ovarian cancer patients. Bevacizumab has been studied for use in combination with other chemotherapeutics or as a single-agent therapy. The experts disagree on the utility of this treatment, in particular for the maintenance therapy. So far, bevacizumab is approved by FDA for treatment of recurrent platinum-resistant disease, becoming the first antiangiogenic agent clinically available in the United States. A supplemental biologics license application for bevacizumab (Avastin, Genentech) for the first-line treatment of advanced ovarian cancer is currently being treated by FDA. Other antiangiogenic agents which may be beneficial in treatment of epithelial ovarian cancer, like pazopanib (an oral receptor tyrosine kinase inhibitor with an activity against VEGF receptor (VEGFR)), or suritinib and sorafenib (multiple tyrosine kinase inhibitors with anti-VEGFR activity), are currently being evaluated in clinical trials.

A very promising class of targeted molecular therapeutics are inhibitors of poly (ADP-ribose) polymerase (PARP). PARP inhibitors target DNA repair mechanisms and are particularly efficient in the treatment of *BRCA1*- and/or *BRCA2*-deficient tumors. The most widely used PARP inhibitor is olaparib (oral therapeutic) which was the first FDA-approved agent in this class. It is approved for treatment of advanced epithelial ovarian cancer in patients with germline *BRCA1/2* mutations who had been treated with three or more lines of chemotherapy. It is also recommended as a single-agent treatment for patients with recurrent platinum-resistant EOC. In addition to olaparib, two other PARP inhibitors: rucaparib and niraparib, are approved by FDA for treatment of EOC in a similar setting. The NCCN expert panel recommends rucaparib monotherapy in particular for recurrent platinum-resistant EOC, whereas niraparib for platinum-sensitive recurrent disease.

Inhibition of signaling pathways using MTOR and MAPK inhibitors has also been tested as a potential treatment modality of advanced EOC. However, early clinical trials (phase I) with these agents as monotherapies have shown no benefit and the trials have been stopped.

Multiagent regimens combining targeted therapies with known cytotoxic drugs or combinations of different classes of targeted therapeutics (like antiangiogenics with PARP inhibitors) seem to be the future of EOC treatment (in particular advanced disease), with a number of regimens being currently tested in clinical trials.

All EOC treatment regimens may be associated with serious complications and side effects. Therefore, providing supportive care throughout all stages of therapy is also very important. In particular, younger women will abruptly enter menopause following any non-fertility-sparing treatment and therefore require particular supportive care to this effect. Palliating symptoms, especially in patients with incurable advanced disease is also a serious concern. Palliative chemotherapy is the most common option. In patients who develop bowel obstruction and resistance to chemotherapy but have a reasonably good performance status, a palliative surgery may be considered. However, the mortality and complication rates are high and this decision should be thoroughly discussed with the patient, weighing the risks associated with the surgery against potential benefits in view of a short predicted life expectancy.

See also: Ovarian Cancer, Pathology and Genetics.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer.
Henderson, J.T., et al., 2018. Screening for ovarian Cancer: Updated evidence report and systematic review for the US preventive services task force. Journal of the American Medical Association 319 (6), 595–606.
Kurman, R.J., Carcangiu, M.L., Herrington, C.S., Young, R.J. (Eds.), 2014. WHO classification of tumors of female reproductive organs, 4th edn. International Agency for Research on Cancer, Lyon, France.
National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Ovarian Cancer. Version 2.2018 – March 9, 2018: https://www.nccn.org/professionals/physician_gls/default.aspx#site
Vetter, M.H., Hays, J.L., 2018. Use of targeted therapeutics in epithelial ovarian Cancer: A review of current literature and future directions. Clinical Therapeutics 40 (3), 361–371.

Relevant Websites

https://www.figo.org/—International Federation of Gynecology and Obstetrics (FIGO).
https://www.sgo.org/—Society of Gynecologic Oncology (SGO).

Ovarian Cancer: Pathology and Genetics

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Glossary

Gamma-H2AX Double stranded breaks (DSBs) resulting from DNA damage are followed by the phosphorylation of the H2AX histone. H2AX is a variant of the H2A protein family, a component of the histone octomer in nucleosomes. This newly phosphorylated protein is the first step in recruiting and localizing DNA repair proteins.

Homologous recombination repair (HRR) A DNA repair process that includes the invasion of an undamaged DNA molecule by a damaged molecule of identical or very similar sequence. Re-synthesis of the damaged region is accomplished using the undamaged molecule as a template.

P53 signature Small foci of strongly p53-immunoreactive cells in largely histologically normal fallopian tube epithelium. Like serous tubal intraepithelial carcinoma (STIC), p53 signatures are comprised exclusively of secretory cells (at least 12 consecutive immunoreactive cells).

Serous tubal intraepithelial carcinoma (STIC) Intraepithelial high-grade serous carcinoma in the distal fimbriated end of the fallopian tube as the probable precursor of some advanced high-grade serous carcinomas.

SWI–SNF-A complex A nucleosome remodeling complex found in both eukaryotes and prokaryotes. It is a group of proteins that associate to remodel the way DNA is packaged.

Introduction

About two-thirds of ovarian tumors occur in women of reproductive age and 80%–90% of them between the ages of 20–65 years; less than 5% develop in children. Approximately 80% are benign tumors, and 60% of them are diagnosed in women under the age of 40 years. Conversely, 90% of ovarian epithelial cancers, including borderline tumors, are diagnosed after the age of 40 years, and 30%–40% of them after the age of 65 years. The odds that an ovarian epithelial tumor is borderline or carcinoma in a patient under 40 years is approximately 1:10, but beyond that age it increases to 1:3.

Ovarian cancer is the 5th most common cancer in females in the United States, with an annual incidence of 25,000 new cases and over 14,000 deaths; it accounts for 4% of the total cancers in women (ranked behind neoplasms of the lung, breast, intestine, and uterus) and 25% of the malignancies of the female genital tract. It has been estimated that 1 woman in 70 (1.4%) will develop ovarian cancer at some point during her lifetime. Its low cure rate (less than 40%) results in 6% of the total cancer deaths in women in the United States.

As with tumors of other organs, the most logical classification and system of terminology for ovarian tumors would be based upon histogenesis. However, implementation is difficult because: (a) gonadal embryology is unsettled; (b) several of these tumors may have a diverse or even unknown origin; and (c) many tumors have overlapping histologic features. Nevertheless, for the purpose of facilitating communication on epidemiologic features, biologic behavior, and therapy, a uniform terminology is strongly recommended. The classification system formulated in 1999 by the World Health Organization (WHO), and the International Society of Gynecological Pathologists, also adhered to by the authors of the last edition (1998) of the fascicle published by the Armed Forces Institute of Pathology, is based, if at all possible, on cell types and patterns of growth. The 2003 and 2014 WHO *blue books* follow essentially the same classification system with only minor changes. Tumors of the ovary ultimately arise from one of the three ovarian components: (1) the surface epithelium and the underlying stroma which embryologically give rise to the müllerian ducts; (2) the specialized ovarian stroma which includes the sex cords, precursors of the endocrine cells of the postnatal ovary; and (3) the germ cells, which migrate to the ovary from the yolk sac and are totipotential. As usual, there is a group of tumors that defy classification, and finally there are secondary or metastatic tumors, given that the ovary is a common site of metastases from various other cancers.

Epithelial Ovarian Tumors

Epithelial ovarian tumors account for approximately two-thirds of all ovarian tumors and for about 90% of all ovarian cancers in the Western World. They comprise a group of heterogeneous neoplasms which are primarily classified according to cell type into serous, mucinous, endometrioid, clear cell, transitional, and squamous cell tumors. Paradoxically, benign counterparts of these cells are not found in the normal ovary and their development during neoplasia has long been attributed to mullerian "neometaplasia" of the ovarian surface epithelium (mesothelium) which derives from the coelomic epithelium. Specifically, since the coelomic

epithelium gives rise to the müllerian ducts, it was proposed that, as the surface epithelium becomes malignant, it would acquire the morphologic features of the müllerian duct epithelium; that is, serous (fallopian tube-like), endometrioid (endometrium-like), and mucinous (endocervical-like). This aberrant differentiation constitutes the basis for ovarian tumor classification. Currently, tumors are thought to develop from embryonic stem cells of the ovarian surface epithelium. However, there is now evidence that a number of tumors thought to be primary ovarian cancers actually originate in other pelvic organs and involve the ovary secondarily. It has been recognized that high-grade serous carcinomas (the most common type of ovarian cancer) may sometimes arise from precursor epithelial lesions in the fimbriated end of the fallopian tube, whereas endometrioid and clear cell carcinomas result from ovarian endometriosis. The relative importance of the fallopian tube mucosa compared with the ovarian surface epithelium in the genesis of high-grade serous ovarian cancer is still a subject of debate. On the other hand, it can be argued that tumors arising in endometriosis are ultimately of endometrial origin. Thus, in some cases, the term ovarian cancer may not be accurate and it has been suggested that it should be replaced with the terms "pelvic" or "peritoneal" cancer. However, given the confusion that might follow in the literature, we think best keeping the term ovarian cancer until the various possible origins of these diseases are known better.

Borderline Tumors

Epithelial ovarian tumors are subdivided into benign, borderline and malignant (carcinomas) and this subdivision is most important since it correlates with behavior. Borderline ovarian tumors show epithelial proliferation greater than that seen in their benign counterparts and variable nuclear atypia; however, in contrast to carcinomas, there is no destructive stromal invasion, and their prognosis is much better than that of carcinomas. In spite of the lack of stromal invasion, serous borderline tumors, particularly those with exophytic growth, can implant on peritoneal surfaces and, rarely (about 10% of peritoneal implants), progress to low-grade serous carcinoma (LGSC) and invade the underlying tissues.

Although favorable in the great majority of cases, the biologic behavior of the borderline tumors differs from that of the obviously benign tumors of the same cell type(s) and, rarely ($<1\%$–3%), progression to invasive carcinoma occurs justifying the term "borderline tumor." Alternative terms such as *atypical proliferative* are not recommended since they are nonspecific; that is, all nonbenign epithelial tumors (borderline and carcinomas) are, by definition, proliferative neoplasms which exhibit nuclear atypia; also, this term is misleading as it does not take into account the malignant potential of a small but significant number of these tumors and discourage follow-up. Although the term "borderline" may suggest uncertainty, it accurately describes the ambiguous histologic and biologic features of these neoplasms and remains the most appropriate term. Accordingly, it has been recommended by the World Health Organization (WHO) for the last four decades. The majority of these tumors are associated with a favorable prognosis and the term "tumors of low malignant potential" is no longer recommended.

The distinction between borderline tumors and carcinomas is one of the most common problems in ovarian tumor pathology, yet the literature on borderline tumors is confusing, particularly with regard to their diagnostic features and treatment. With the exception of squamous cell tumors, the borderline concept applies to all types of ovarian epithelial tumors listed before; however, most data in the literature refer to the serous and mucinous intestinal categories which are the two most common types and show significant clinicopathological differences.

Serous and Mucinous Borderline Tumors

Serous borderline tumors (SBTs) account for from 5% to 10% of ovarian serous tumors and occur at an average of 42 years. Approximately 70% are confined to one or both ovaries (stage I) at the time of diagnosis; the remaining tumors have spread within the pelvis (stage II) or upper abdomen (stage III). One-third of stage I tumors are bilateral. Macroscopically, SBTs have one or more cysts that are lined by polypoid excrescences and closely packed papillae (endophytic growth) (**Fig. 1**). In almost half of SBTs the papillary growth covers the outer surface of the ovary (exophyytic growth). Microscopically, SBTs show stromal polyps, glands, and papillae lined by stratified cuboidal to columnar epithelial cells and ciliated cells resembling those of the fallopian tube (**Fig. 2**). The ramifying papillae form increasingly smaller branches ending in epithelial cells clusters apparently detached from the stroma (hierarchical branching); the tumor cells show varying degree of nuclear atypia; and there is absence of "destructive" stromal invasion or solid sheets of tumor with a cribriform pattern. Nuclear atypia varies from mild to moderate.

Mucinous borderline tumors (MBTs) have been subclassified into two different clinicopathologic forms: the most common form (85% to 90%) is composed of gastrointestinal-type epithelium and is designated MBT of gastrointestinal type. A second and less common variant of MBT contains endocervical-type epithelium and has been named MBT endocervical-like (**Fig. 3**).

Endocervical-like MBTs also designated as müllerian MBTs, account for 10%–15% of MBTs. About 140 cases have been reported. These tumors differ in many respects from intestinal MBTs. The average age of the patients with endocervical MBTs is 40 years and association with endometriosis is frequent (35%–50%). At the time of diagnosis, most tumors are confined to the ovary and approximately 20% have spread to the peritoneum or regional lymph nodes. The prognosis of endocervical-type MBT is excellent and approximates that of SBTs. Recent studies report that foci of intraepithelial carcinoma (IEC) or microinvasion do not influence the prognosis. No deaths from these tumors have been reported.

MBTs of intestinal type account for 10%–15% of ovarian mucinous tumors and are more common in the first two decades than their serous counterparts. About 80%–90% are stage I and only 5% are bilateral. Of note, metastatic mucinous tumors in the ovary often mimic primary ovarian mucinous neoplasms, particularly adenocarcinomas of the pancreas and large intestine. Nearly all

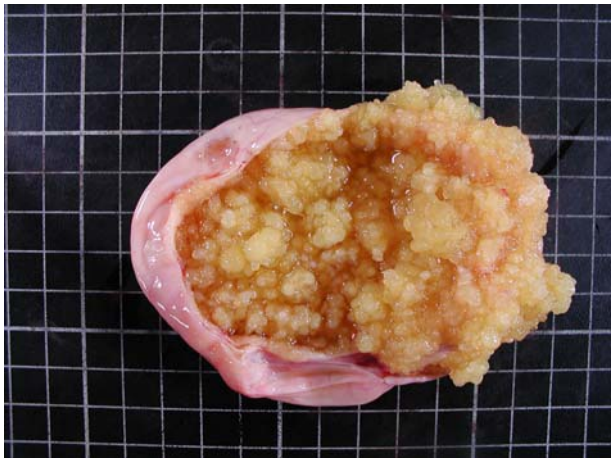

Fig. 1 Serous borderline tumor. Intracystic tumor with exhuberant papillary growth. The papillae are soft and edematous. Mutter, G. L. and Prat, J. eds. (2014). Pathology of the female reproductive tract. 3rd edn. Churchill Livingstone: Elsevier.

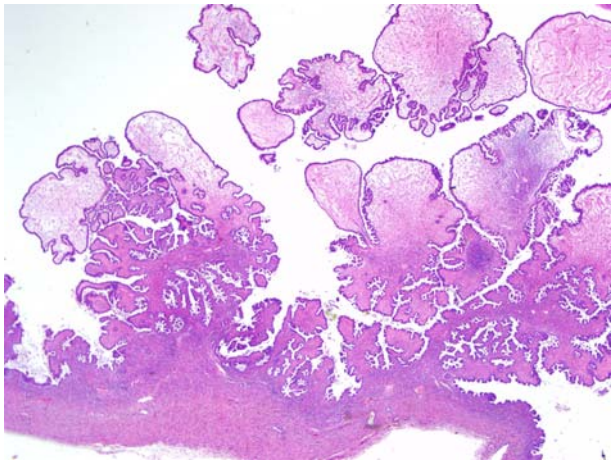

Fig. 2 Serous borderline tumor. The arborizing papillae form increasingly smaller branches ending in epithelial cells clusters apparently detached from the stroma (hierarchical branching).

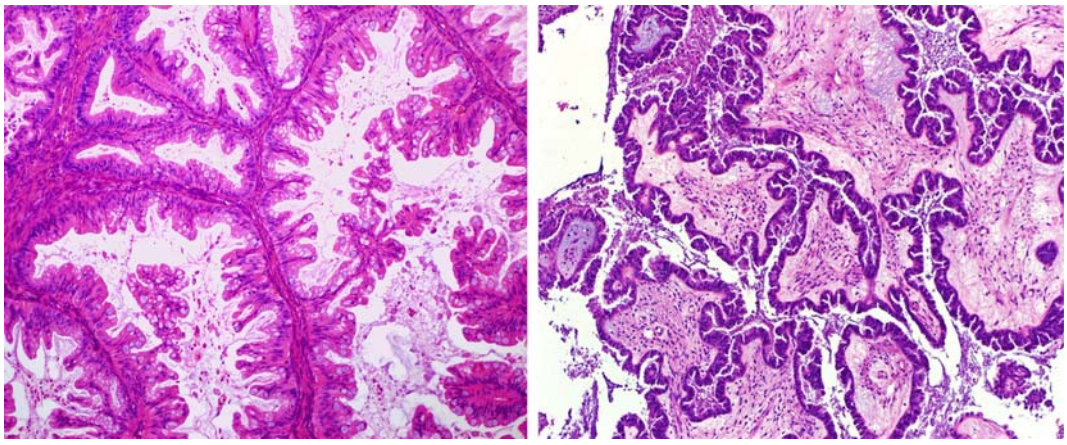

Fig. 3 Mucinous borderline tumors. *Left*: the intestinal-type tumor resembles a colonic polyp with globet cells. *Right*: the endocervical-like or mullerian tumor shows mucinous epithelial cells resembling endocervical epithelium.

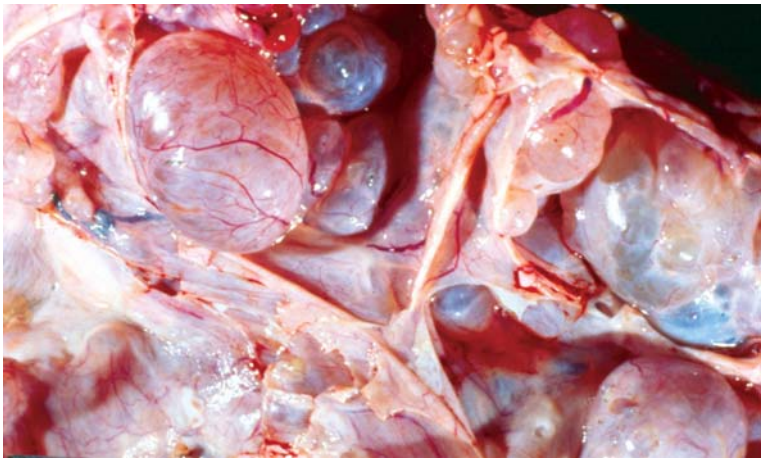

Fig. 4 Mucinous borderline tumor. Cystic and multilocular tumor with mucinous fluid. Lee, K. R., Tavassoli, F. A., Prat, J., et al. (2003). Surface epithelial-stromal tumours (Ch 2: tumours of the ovary and peritoneum). In: Tavassoli, F. A., Devilee, P. (eds.). *World Health Organization classification of tumours: pathology and genetics of tumours of the breast and female genital organs*, pp 117–145. Lyon: IARC Press.

stage II–III MBTs are associated with *pseudomyxoma peritonei* and, in these patients, the ovarian tumor is virtually always secondary (metastatic) from a primary low-grade mucinous appendiceal tumor.

On gross examination, MBTs of intestinal type average 19 cm in diameter, are usually cystic and multilocular, and contain mucinous fluid (Fig. 4). Microscopically, the tumor consists of cysts and glands lined by atypical epithelium of gastric pyloric-type. The cysts may contain papillae that are typically thin and branching The lining epithelium almost always contains goblet cells and may have argyrophil cells and occasional Paneth cells. The epithelial cells are usually stratified to two or three layers, nuclear atypia is mild to moderate, and mitotic figures vary from few to numerous. High grade nuclear features are absent and stromal invasion is not seen (Fig. 5).

MBTs of intestinal-type may exhibit areas of epithelial cell proliferation of four or more layers, scattered foci of cribriform or stroma-free papillary architecture, and moderate (grade 2) or severe atypical (grade 3) nuclei (Fig. 6). Numerous studies have shown that these tumors are almost always clinically benign, and we recommend classifying them as mucinous borderline tumors with intraepithelial carcinoma.

In contrast to serous tumors which are usually homogeneous, mucinous intestinal tumors often are heterogeneous. Benign-appearing, borderline, and invasive patterns may coexist within an individual neoplasm; this continuum suggests that progression occurs from cystadenoma and borderline tumor to noninvasive, microinvasive, and invasive carcinoma. This is supported by studies of *K-RAS* mutations, which represent an early event in mucinous ovarian tumorigenesis.

The overall outcome of SBTs is very favorable. The 5-year survival rates for patients with disease that is stages I to IIIb are between 88% and $>95\%$. For patients with stage I tumors, the risk of recurrence or the development of a second SBT has been estimated to be

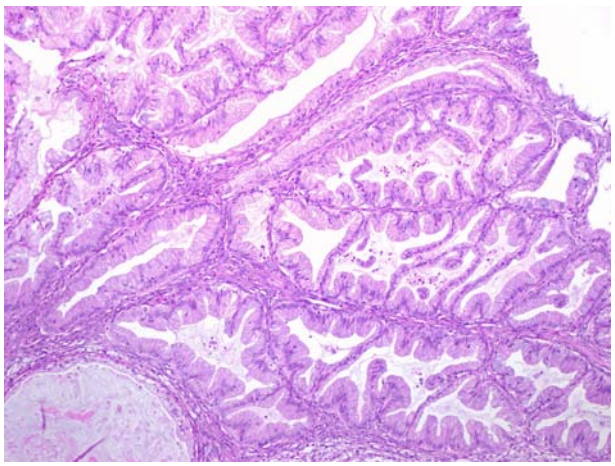

Fig. 5 Mucinous borderline tumor of intestinal-type. Packed intraglandular proliferation of mucinous epithelium with thin and branching papillae. There is no stromal invasion. Prat, J. (2017). Pathology of borderline and invasive cancers. *Best Practice & Research: Clinical Obstetrics and Gynaecology* ***41***, 15–30.

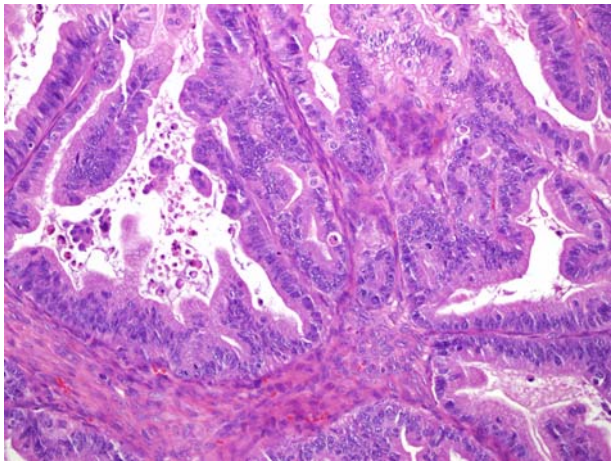

Fig. 6 Mucinous borderline tumor with intraepithelial carcinoma. Epithelial cell proliferation with foci of cribriform or stroma-free papillary architecture, grade 2–3 nuclei and mitotic figures.

only 5%–10%. Risk factors for recurrence include conservative treatment, particularly cystectomy, bilaterality (stage IB), and incomplete staging. In SBTs, the presence of tumor at the resection margin of the cystectomy specimen and multifocality with removal of more than one cyst, are strong predictors of failure of cystectomy to control the disease. Transformation of SBT into low-grade serous carcinoma (LGSC) occurs in 6%–7% of patients late in the course of the disease. Other than the adverse effect of invasive implants (Fig. 7), there is no agreement in the literature as to which prognostic factors are important. Subdivision of SBTs into benign and malignant, based on the presence of a micropapillary architecture (Fig. 8), is artificial since SBTs with or without micropapillary pattern may rarely be associated with invasive peritoneal implants and poor outcome. The term "micropapillary" is flawed as all SBTs have micropapillae descriptively and their extension may range from a few papillae to a huge number of them. Bilaterality, ovarian surface growth, and advanced stage (mainly noninvasive peritoneal implants) are more common features of extensively micropapillary SBTs than of typical SBTs, but a strong association of the former tumors with invasive implants and poor outcome has been inconsistent. According to most investigators, SBTs with micropapillary pattern or SBTs with microinvasion have a prognosis similar to that of tumors lacking these features. Likewise, focal lymph node involvement has not demonstrated any effect on survival. The 5-year survival rate for patients with stage I MBT nears 100%. In a recent report, 6 of 144 patients (4.2%) had tumor recurrence. Risk factors for recurrence included FIGO stage IC, microinvasive carcinoma, age less than 45 years, and intraepithelial carcinoma.

In young patients who undergo fertility-sparing surgery, the nature of the recurrent tumor varies according to the histological type. In these patients, MBTs of intestinal type recur less frequently than SBTs, but when they do it is more often as an invasive carcinoma. Most SBTs maintain their microscopic features in the recurrences and usually do not progress to frankly invasive carcinoma. In contrast, MBTs of intestinal type represent intermediate stages of mucinous tumorigenesis and, even if a MBT per se is essentially a benign neoplasm, it may be accompanied by or may progress to intraepithelial and frankly invasive carcinoma. However, in

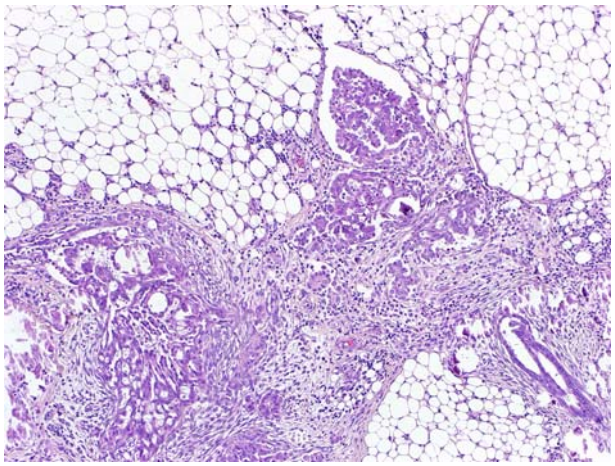

Fig. 7 Invasive implant of serous borderline tumor. The implant is composed predominantly of epithelial cells which disorderly infiltrate the adipose tissue.

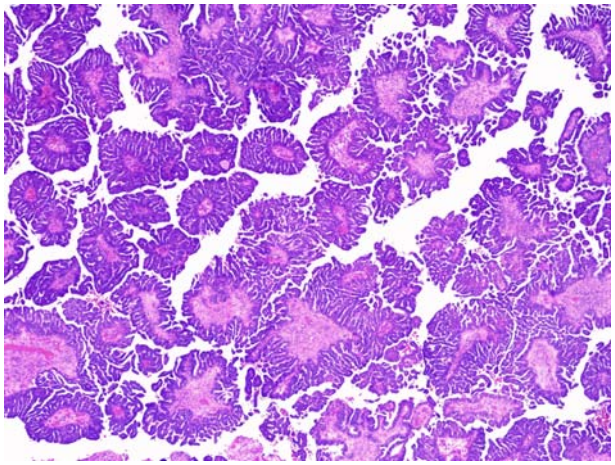

Fig. 8 Serous borderline tumor with micropapillary proliferation. Highly complex micropapillae growing in a nonhierarchical fashion from fibrovascular stalks ("Medusa head-like appearance").

patients with MBTs initially treated by unilateral salpingo-oophorectomy, recurrences that occur in the contralateral ovary most likely represent independent primary mucinous tumors, typically heterogeneous and containing benign-appearing, borderline, and carcinomatous elements. In other words, SBTs and MBTs of intestinal type are different diseases with different biologic behavior.

Endometrioid Borderline Tumors

Endometrioid borderline tumors are uncommon and constitute only 0.2% of all epithelial ovarian tumors and 2%–3% of borderline tumors. The average age of patients is 51 years. The majority of tumors are unilateral; bilateral disease is rare (4%). The average size is 9 cm. In one-half of cases an adenofibroma is present in the background. The glandular proliferation may consist of crowded, back-to-back endometrioid glands with mild or moderate cytological atypia, often with epithelial stratification. Squamous (morular) metaplasia is common (Fig. 9) and typically exhibits strong nuclear immunoreaction for beta-catenin indicating the occurrence of *CTNNB1* mutation. Prognosis is excellent.

Clear Cell Borderline Tumors

Clear cell borderline tumors comprise less than 1% of borderline tumors. Almost all the women are postmenopausal (mean age: 59–68 years). The majority of tumors are unilateral. Their size is variable with a mean of about 6 cm. These neoplasms are clear cell adenofibromatous tumors with atypia of the glandular epithelium without stromal invasion. The cyst and glands are lined by cuboidal, hobnail, or flattened cells with clear or eosinophilic cytoplasm (Fig. 10). All well documented cases have been benign.

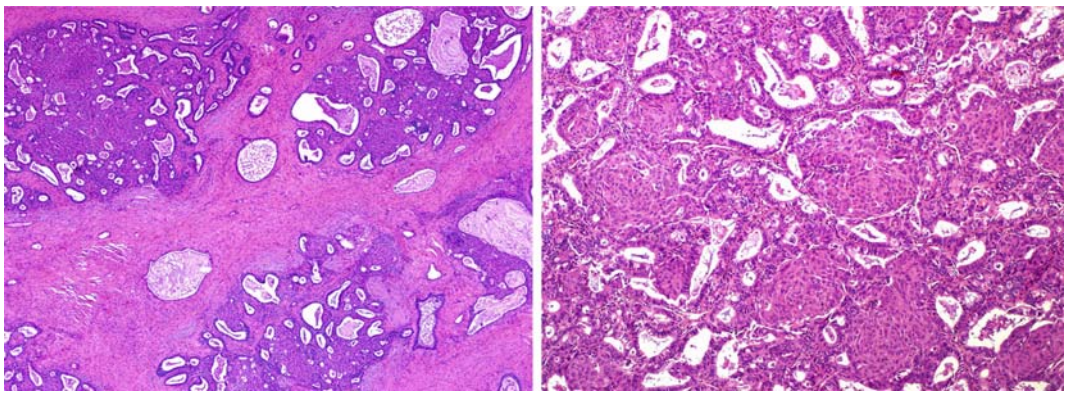

Fig. 9 Endometrioid borderline tumor. Left: endometrioid borderline adenofibroma. Right: endometrial glands are partly replaced by squamous morules.

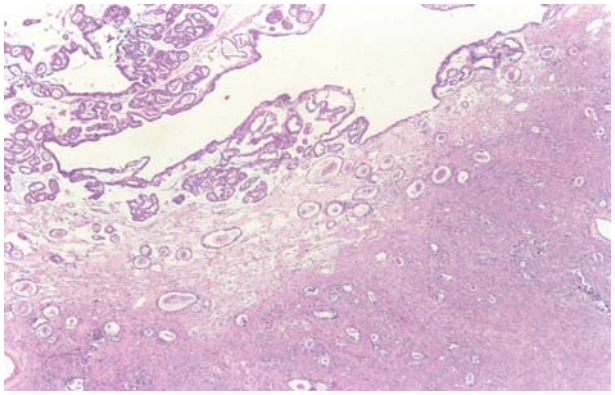

Fig. 10 Clear cell adenocarcinoma (upper left) with a borderline adenofibroma component (bottom right). Prat, J. (2004). Pathology of the ovary. Philadelphia: Saunders.

Borderline Brenner Tumors

Borderline Brenner tumors comprise less than 0.5% of borderline tumors. The mean patient age is 59 years. Tumors are unilateral. These are typically large, cystic tumors measuring, on average, 18 cm (range 10–28 cm) with papillary masses project into the cyst lumens. The papillary component closely resembles low-grade noninvasive papillary transitional cell (urothelial) tumors. Mucinous metaplasia is often present. A solid area of benign Brenner tumor is almost always present. The behavior is benign, although local recurrence may rarely occur (Fig. 11).

Ovarian Carcinomas

Ovarian carcinomas (malignant epithelial tumors) are the most common ovarian cancers and also the most lethal gynecological malignancies. Although traditionally referred to as a single entity, ovarian carcinoma is not a homogeneous disease but rather a group of diseases, each with different morphology and biologic behavior. Currently, based on histopathology, immunohistochemistry, and molecular genetic analysis, at least five main types of ovarian carcinomas are identified: high-grade serous carcinomas (70%), endometrioid carcinomas (10%), clear cell carcinomas (10%), mucinous carcinomas (3%), and low-grade serous carcinomas (<5%) (Table 1). These tumors account for over 95% of ovarian carcinomas, can be reproducibly diagnosed by light microscopy, and are inherently different diseases, as indicated by differences in epidemiological and genetic risk factors, precursor lesions, patterns of spread, molecular genetic alterations, response to chemotherapy, and prognosis. The fact that one tumor type (high-grade serous carcinomas) accounts for over two-thirds of cases, does not justify classifying ovarian carcinomas into only two

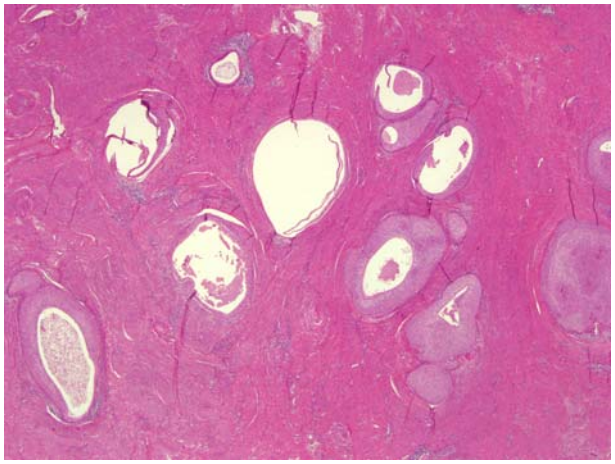

Fig. 11 Recurrent borderline Brenner tumor. Partly cavitated masses of transitional tumor cells invade the myometrium. Cuatrecasas, M., Catasus, L., Palacios, J. and Prat, J. (2009). Transitional cell tumors of the ovary: A comparative clinicopathologic, immunohistochemical, and molecular genetic analysis of Brenner tumors and transitional cell carcinomas. *The American Journal of Surgical Pathology* ***33,*** 556–567.

Table 1 Main types of ovarian carcinoma

	High-grade serous	*Low-grade serous*	*Mucinous*	*Endometrioid*	*Clear cell*
Usual stage at diagnosis	Advanced	Early or advanced	Early	Early	Early
Presumed tissue of origin/precursor lesion	Fallopian tube or tubal metaplasia in inclusions of ovarian surface epithelium	Serous borderline tumor	Adenoma–borderline–carcinoma sequence; teratoma	Endometriosis, adenofibroma	Endometriosis, adenofibroma
Genetic risk	*BRCA1/2*	?	?	HNPCC	?
Significant molecular abnormalities	p53 and pRb pathways	*B-RAF* or *K-RAS*	*HER2*	*PTEN*, β-catenin, *ARID1A*, and *PIK3CA* *K-RAS* MI	HNF-1β, *ARID1A*, and *PIK3CA*
Proliferation	High	Low	Intermediate	Low	Low
Response to primary chemotherapy	80%	26%–28%	15%	?	15%
Prognosis	Poor	Favorable	Favorable	Favorable	Intermediate

Prat, J. (2017). Pathology of borderline and invasive cancers. *Best Practice & Research: Clinical Obstetrics & Gynaecology*. ***41,*** 15–30.

pathogenetic types, lumping together the other four (endometrioid, clear cell, mucinous, and low-grade serous carcinomas) as "type I carcinomas." The latter tumors are clinically, morphologically, and molecularly distinct diseases that individually bear resemblance neither to high-grade serous carcinomas nor to each other.

Serous Carcinomas

Low-grade serous carcinoma (LGSC) and high-grade serous carcinoma (HGSC) are fundamentally different tumor types and, consequently, different diseases. LGSCs are associated, in most cases, with a serous borderline component, carry *K-RAS* and *B-RAF* mutations, and are unrelated to *TP53* mutations and *BRCA* abnormalities. In contrast, HGSCs are not associated with SBTs and typically exhibit *TP53* mutations and *BRCA* abnormalities.

High-grade serous carcinoma (HGSC)

HGSCs are the most common ovarian carcinomas and most patients present with advanced stage disease (approximately 80%); tumors limited to the ovary at diagnosis are distinctly uncommon (< 10%) (Table 2).

Microscopically, HGSCs show papillary and solid growth with slit-like glandular lumens. The tumor cells are typically of intermediate size, with scattered bizarre mononuclear giant cells exhibiting prominent nucleoli (Fig. 12). In contrast to LGSCs, these tumors show more than threefold variation in nuclear size. In cases with equivocal degrees of nuclear pleomorphism, mitotic activity greater than 12/10 high-power microscopic fields (HPF) favors a diagnosis of HGSC. High grade and predominantly solid carcinomas showing serous differentiation, even in a minority of the tumor, should be classified as HGSC.

Most HGSC immunoreact for p53, BRCA1, WT1, and p16. They also exhibit a high proliferation index as indicated by an increased nuclear expression of Ki-67. Only strong and diffuse p53 and p16 reactions should be considered abnormal. Nuclear WT1 reaction occurs in approximately 80% of cases of HGSC and LGSC but in less than 5% of ovarian carcinomas of other types. Estrogen receptor (ER) is expressed in approximately two-thirds of cases of HGSC and is also expressed in LGSCs and ECs but is negative in almost all CCCs and MCs.

Women with germline mutations in *BRCA1* or *BRCA2* have a 30%–70% lifetime risk of developing ovarian cancer mainly HGSC. Carcinomas arising in patients with germline *BRCA1* or *BRCA2* mutations are almost invariably of high-grade serous type. Like *TP53* mutations, *BRCA* inactivation seems to be a consistent genetic alteration of HGSC. Besides germline mutation, inactivation of the BRCA pathway may result from somatic mutation in either *BRCA1* or *BRCA2*, or promoter hypermethylation in *BRCA1*.

The finding of serous tubal intraepithelial carcinoma (STIC) in risk-reducing salpingo-oophorectomy (RRSO) specimens from women with known *BRCA* mutations and/or a strong family history of ovarian cancer resulted in extensive research into the role of the fallopian tube in pelvic serous carcinogenesis. Early studies showed small foci of strongly p53-immunoreactive cells in histologically normal fallopian tube epithelium. These foci, which predominate in the distal portion of the fallopian tube, have been designated "p53 signatures." Like STIC, p53 signatures are comprised exclusively of secretory cells (at least 12 consecutive immunoreactive cells), and the majority exhibit evidence of DNA damage by immunoreaction for gamma-H2AX. They are more frequent and multifocal in tubes with STIC and, in some cases, can be identified in direct continuity with STIC. About 57% of p53 signatures contain *TP53* mutations; however, Ki-67 proliferation index is low (mean, 3%). p53 signatures probably represent early clonal expansion insufficient for neoplastic proliferation and, surprisingly, are found in both women with and without *BRCA1* or *BRCA2* mutations at the same frequency (10%–38% vs. 17%–33%, respectively). *TP53* mutation is an early event in the genesis of HGSC, occurring in p53 signature foci and leading to STIC in the distal fallopian tube. *BRCA1* mutation also occurs early in the development of STIC but after *TP53* mutation.

Table 2 Clinical staging of cancer of the ovary, fallopian tube, and peritoneum (International Federation of Gynecology and Obstetrics, FIGO, 2014)

I	Tumor confined to ovaries or fallopian tube(s)			
	IA	Tumor limited to one ovary (capsule intact) or fallopian tube, no tumor on ovarian or fallopian tube surface. No malignant cells in the ascites or peritoneal washings		
	IB	Tumor limited to both ovaries (capsules intact) or fallopian tubes. No tumor on ovarian or fallopian tube surface. No malignant cells in the ascites or peritoneal washings		
	IC	Tumor limited to one or both ovaries or fallopian tubes, with any of the following:		
		IC1	Surgical spill intraoperatively	
		IC2	Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface	
		IC3	Malignant cells in the ascites or peritoneal washings	
II	Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim)			
	IIA	Extension and/or implants on the uterus and/or fallopian tubes/and/or ovaries		
	IIB	Extension to other pelvic intraperitoneal tissues		
III	Tumor involves one or both ovaries, fallopian tubes, or primary peritoneal cancer, with microscopically confirmed spread to the peritoneum outside the pelvis or metastasis to the retroperitoneal lymph nodes			
	IIIA	Metastasis to the retroperitoneal lymph nodes with or without microscopic peritoneal involvement beyond the pelvis		
		IIIA1	Positive retroperitoneal lymph nodes only. Microscopically proven	
			IIIA1 (i)	Metastasis $\leq$10 mm in greatest dimension
			IIIA1 (ii)	Metastasis $>$10 mm in greatest dimension
		IIIA2	Microscopic extrapelvic (above pelvic brim) peritoneal involvement with or without positive peritoneal lymph nodes	
	IIIB	Macroscopic peritoneal metastasis beyond the pelvic brim 2 cm or less in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes		
	IIIC	Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (Note 1)		
IV	Distant metastasis excluding peritoneal metastases			
	IVA	Pleural effusion with positive cytology		
	IVB	Metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside the abdominal cavity)		

Note 1: Includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ; *Note* 2: Parenchymal metastases are stage IVB.
OV: Primary tumor, ovary, Tov; FT: Primary tumor, fallopian tube, Tft; P: Primary tumor, peritoneum, Tp; X: Primary tumor cannot be assessed, Tx.
Modified from Prat, J. for the FIGO committee on gynecologic oncology: FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: Abridged republication. *Journal of Gynecologic Oncology* ***26*** (2), 87–89.

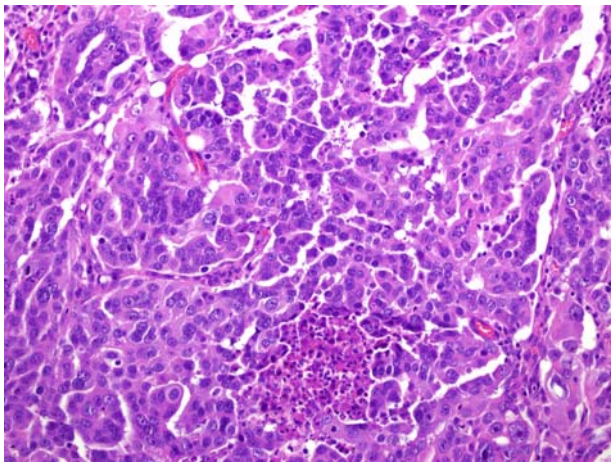

Fig. 12 High-grade serous carcinoma. Solid masses of cells with slit-like glandular lumens, high grade nuclear atypia, mitotic figures, and necrosis.

The sequence of epithelial changes taking place in the distal fallopian tube have been described as follows: normal fallopian tube epithelium, overexpression of p53, serous tubal intraepithelial carcinoma (STIC), and finally, invasive serous carcinoma. The secretory cells of the tubal epithelium have a limited ability to repair DNA DSB, as shown by the persistence of gamma-H2AX immunoreactive foci after DNA damage, which might explain why this tissue seems to be especially sensitive to inactivating *BRCA* mutations.

A pathogenetic model that includes the stages of initiation and progression of HGSC have been described (Fig. 13). It proposes as primary events early p53 loss followed by BRCA loss, leading to deficiency in homologous recombination repair (HRR) of DSB, which triggers chromosomal instability (genetic chaos) and widespread copy number changes. Once chromosomal instability is set up by mutation in *TP53* and *BRCA* inactivation, gene copy number is the major determinant of progression of HGSC.

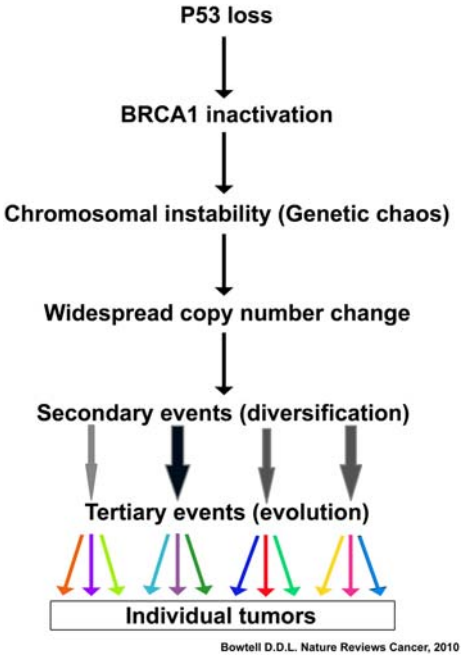

Fig. 13 Pathogenetic model that includes the stages of initiation and progression of HGSC. Modified from Bowtell, D. D. (2010). The genesis and evolution of high-grade serous ovarian cancer. *Nature Reviews Cancer* ***10,*** 803–808.

Approximately 5%–10% of *BRCA*-positive asymptomatic women have early HGSC, and 80% of them are associated with STIC. As a result, speculation that "all" HGSCs originated in the fallopian tube followed. However, the pathogenesis of HGSC appears to be more complex as indicated by the following: (a) only 40% of advanced HGSCs show STIC; (b) only 8% of HGSCs with SET (solid, pseudoendometrioid, transitional) morphology show STIC; and (c) high-grade serous carcinomas with SET morphology tend to be *BRCA*-positive, patients are younger, and outcome is better. Thus, it seems that HGSC (*BRCA* positive and *BRCA* negative) is not a homogeneous disease. Several variables, including age, histotype, STIC ±, and patient outcome, allow segregation of two tumor groups: (a) younger *BRCA*-positive patients, without STIC and with favorable outcome; and (b) older *BRCA*-negative patients, with STIC, and with unfavorable prognosis. Therefore, recent studies suggest that HGSCs may have at least two different origins; that is, ovarian surface epithelium and tubal epithelium.

Low-grade serous carcinoma (LGSC)

Low-grade serous carcinoma are uncommon and account for less than 5% of all cases of ovarian carcinoma. They frequently have a noninvasive SBT component (with or without micropapillary pattern) and most likely represent progression of SBTs. The presence of small foci of LGSC in an ovarian borderline tumor is associated with an excellent prognosis; however, patients with advanced stage disease fare less favorably even if the disease usually follows a relatively indolent course.

Microscopically, LGSCs show small papillae of tumor cells exhibiting uniform small nuclei within variable amounts of hyalinized stroma, which often contains psammoma bodies (Fig. 14). Uniformity of the nuclei is the principal criterion for distinguishing between LGSC and HGSC, with less than threefold variability. LGSC rarely progress to high-grade tumors.

The biomarker expression profile of LGSC is similar to that of their high-grade counterparts, but the median Ki-67 labelling index is of 2.5% in LGSC compared to 22.4% in HGSC. *BRAF* or *KRAS* mutations are present in LGSCs (30% and 35%, respectively, in one study and 5% and 40%, respectively, in another). LGSCs do not show chromosomal instability and lack the complex genetic abnormalities seen in HGSCs. LGSCs are not associated with *BRCA* germline mutations.

With regard to the distinction between LGSC and serous borderline tumor, micropapillarity, by itself, is not sufficient to warrant a diagnosis of carcinoma in the absence of invasion. If there are invasive foci measuring less than 10 mm^2, the tumor is considered to be borderline with microinvasion. Tumors with larger invasive components are classified as LGSC. Histopathologically, invasive peritoneal implants and LGSC are identical lesions which are only distinguished by the timing of the disease and the volume of the tumor. Whereas invasive implants are early superficial lesions of microscopic or small macroscopic size ($\leq$1–2 cm), LGSC frequently presents as bulky disease. Although the independent origin of the invasive peritoneal implants associated with ovarian SBT cannot be completely excluded, we have demonstrated identical *B-RAF* y *K-RAS* mutations as well as identical LOH in a series of ovarian SBT and peritoneal implants. Such findings support a monoclonal origin of these tumors and the secondary nature of the implants.

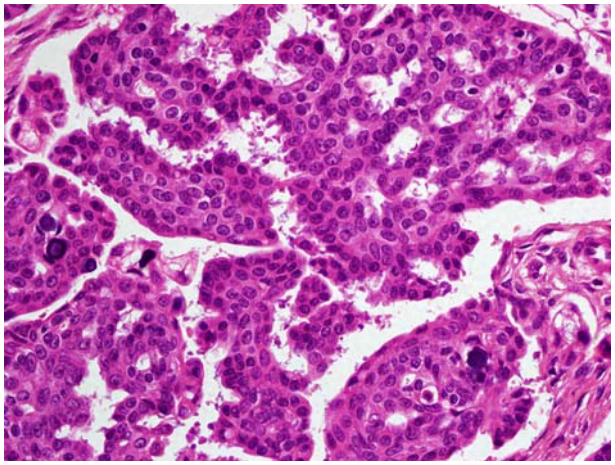

Fig. 14 Low-grade serous carcinoma. The papillary tumor shows epithelial cells with small and uniform nuclei (grade 1). The stroma contains psammoma bodies. Prat, J. (2017). Pathology of borderline and invasive cancers. *Best Practice and Research: Clinical Obstetrics and Gynaecology* ***41,*** 15–30.

Mucinous Carcinomas (MC)

Mucinous tumors account for 10%–15% of all primary ovarian tumors; however, approximately 80% are benign and most of the remainder are borderline tumors. If metastases to the ovary, particularly from the gastrointestinal tract, are carefully excluded, only 3%–4% of ovarian carcinomas are of mucinous type. The cells of MCs may resemble those of the gastric pylorus, intestine, or endocervix; nevertheless, the vast majority of these tumors show gastrointestinal differentiation. The origin of these tumors is unknown. Large size (>13 cm) and unilaterality are features suggestive of a primary MC, while metastases are typically smaller and bilateral. Primary MCs of the ovary are usually confined to the ovary, without ovarian surface involvement or pseudomyxoma peritonei.

As indicated before, malignant mucinous ovarian tumors are often heterogeneous. Benign-appearing, borderline, noninvasive carcinoma, and invasive components may coexist within an individual tumor and suggest tumor progression from benign to borderline and from borderline to carcinoma. Therefore, extensive sampling for histological examination is necessary. The category of MBT with intraepithelial carcinoma is used for those tumors that lack obvious stromal invasion but show areas, less than 10 mm^2, where the cytological features of the tumor cells are unequivocally malignant. MBTs with intraepithelial carcinoma have a very low risk of recurrence of less than 5%.

MCs have been divided into two categories: (a) an expansile type without obvious stromal invasion, but exhibiting back-to-back or complex malignant glands with minimal or no intervening stroma, and exceeding 10 mm^2 in area (>3 mm in each of two linear dimensions) (Fig. 15); and (b) an infiltrative type, showing evident stromal invasion in the form of glands, cell clusters, or individual cells, disorderly infiltrating the stroma and frequently associated with a desmoplastic stromal reaction. The expansile pattern of growth has also been referred to as the "noninvasive" pattern and is associated with a more favorable prognosis than the infiltrative pattern. A histopathological feature unique to mucinous tumors is the occasional finding of mural nodules of anaplastic carcinoma or high-grade sarcoma (Fig. 16). When such nodules are localized in the wall of an unruptured cyst, the prognosis may be favorable, but such tumors may recur and do so as the anaplastic component.

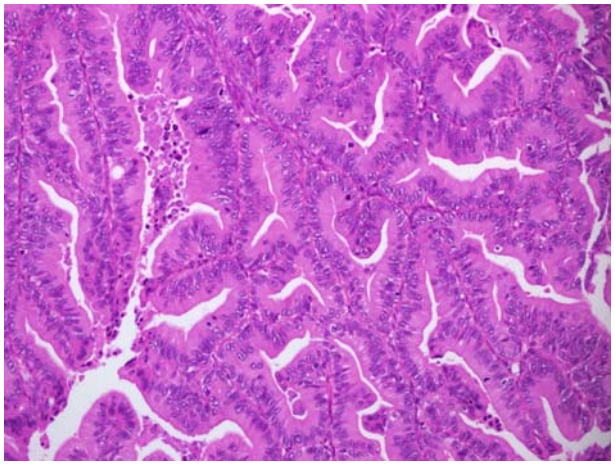

Fig. 15 Mucinous carcinoma with expansile invasion. Closely packed (back-to-back) malignant glands with no stromal response. Stromal invasion is not obvious.

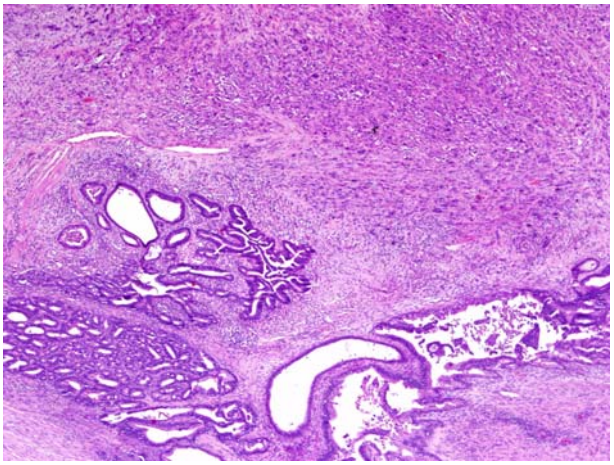

Fig. 16 Anaplastic carcinoma in a mucinous tumor. The epithelial lining appears to be of borderline malignancy (*bottom*). The undifferentiated component is composed of cells with pleomorphic (grade 3) nuclei (*top*). Longacre, T. A., Bell D. A., Malpica, A., et al. (2014). Mucinous tumours. In Kurman, R. J., Carcangiu, M. L., Herrington, C. S. and Young, R. H. eds. *World Health Organization Classification of tumours of female reproductive organs*. Lyon: IARC Press, pp. 25–28.

The gene expression profile of MCs differs from those of serous, endometrioid, and clear cell carcinomas. *KRAS* mutations, which are an early event in mucinous tumorigenesis, occur in 43.6% of MCs and 78.8% of MBTs. On the other hand, overexpression/amplification of *HER2* has been found in 18.8% of MCs and 6.2% of MBTs. Interestingly, *KRAS* mutations are near mutually exclusive of *HER2* amplification. Approximately 34% of MCs have neither *HER2* amplification nor *KRAS* mutation and these cases are associated with increased likelihood of disease recurrence or death when compared to tumors with either genetic alteration. Using highly-sensitive next-generation sequencing, *KRAS* mutations remain the most frequent alteration among MCs and MBTs (92.3%). *TP53* mutation occurred more frequently in carcinomas (68%) than borderline tumors (20%).

Primary ovarian mucinous tumors are almost always (up to 80%) immunoreactive for cytokeratin 7 (CK7) whereas metastatic colorectal adenocarcinomas are usually CK7 negative. Ovarian MCs are immunoreactive for CK20 in 65% of cases, but the reaction is typically weak and focal; staining for CDX-2 (nuclear immunoreaction) is similar. In contrast, colorectal adenocarcinomas are diffusely and strongly reactive for CK20 and CDX-2. Human papilloma virus (HPV) DNA assessment may be helpful for distinguishing mucinous adenocarcinoma of the cervix metastatic to the ovary from a primary ovarian MC. p16 expression is also a reliable surrogate marker for HPV. MCs are uniformly negative for estrogen receptor (ER) and WT1, in contrast to endometrioid (ER+) and serous (ER+ and WT1+) carcinomas.

FIGO stage is the single most important prognostic factor, and stage I MCs have an excellent prognosis. However, the prognosis in cases with extraovarian spread is poor.

Endometrioid Carcinomas (EC)

Endometrioid tumors of the ovary closely mimic their uterine counterparts. ECs account for 10% of all ovarian carcinomas, occur most frequently in women of perimenopausal age, and most are found at an early stage. The tumors are bilateral in 28% of cases and are associated in 15%–20% of cases with carcinoma of the endometrium. Most ECs are low-grade adenocarcinomas (**Fig. 17**) and seem to arise from endometriotic cysts. Up to 42% of cases have evidence of ipsilateral ovarian or pelvic endometriosis. Squamous differentiation occurs in 50% of cases. In contrast, high-grade ECs are morphologically indistinguishable from HGSCs (**Fig. 18**) and often express WT1. Gene expression profiling is also similar, suggesting that high-grade EC is not a distinct tumor type.

Atypical endometriosis is the precursor lesion of endometrioid and clear cell carcinomas of the ovary and a direct transition from ovarian atypical endometriosis to endometrioid or clear cell carcinomas has been described in 15%–32% of cases (**Fig. 19**). In these cases, common genetic alterations have been encountered in the adjacent endometriosis, atypical endometriosis, and adenocarcinoma. In mice harboring *K-RAS* mutations that result in the development of benign lesions reminiscent of endometriosis, deletion of *PTEN* leads to the induction of invasive EC. These results indicate that inactivation of tumor suppressor genes such as *PTEN* may represent early events in the malignant transformation of endometriosis.

ARID1A (AT-rich interactive domain 1A gene) mutations occur in both endometrioid (30%) and clear cell carcinomas (50%) and their finding in adjacent endometriosis has renewed interest in the molecular events that occur in precursor lesions. *ARID1A* is a component of the SWI–SNF-A complex, a large, multiprotein chromatin remodeling complex which is known to both enhance and repress transcription. It behaves as a tumor suppressor gene. BAF250 protein, encoded by *ARID1A*, plays a crucial role in chromatin remodeling. The question has been raised whether patients with endometriotic lesions that show loss of expression of BAF250 should be viewed as being at high risk for the development of CCC or endometrioid ovarian cancers.

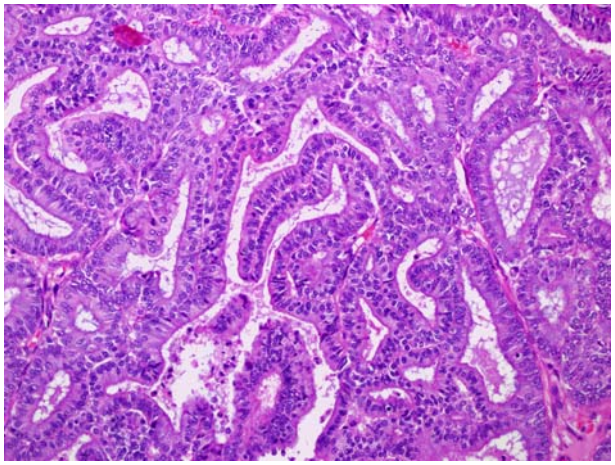

Fig. 17 Well-differentiated endometrioid adenocarcinoma. Confluent growth of glands which replace the stroma. Zaino, R. J., Carinelli, S. G. and Ellenson, L. H. et al. (2014). Tumours of the uterine corpus-epithelial tumours and precursors, In Kurman, R. J., Carcangiu, M. L., Herrington, C. S. and Young, R. H. eds. *World Health Organization Classification of tumours of female reproductive organs*. Lyon: IARC Press, pp. 125–133.

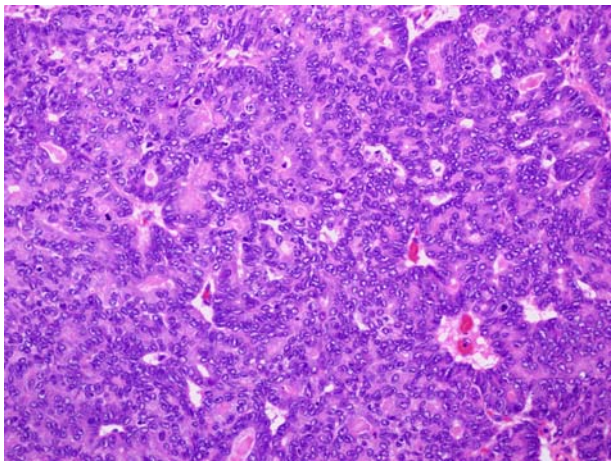

Fig. 18 Grade 3 endometrioid adenocarcinoma with a solid tumor growth

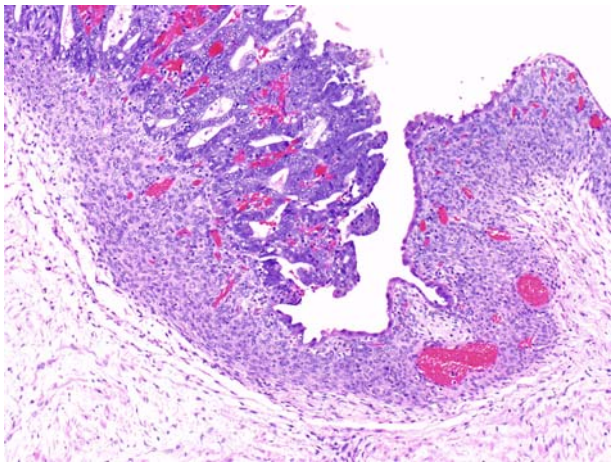

Fig. 19 Endometrioid adenocarcinoma arising from endometriosis. Mutter, G. L. and Prat J. eds. (2014). Pathology of the female reproductive tract. 3rd edn. Churchill Livingstone: Elsevier.

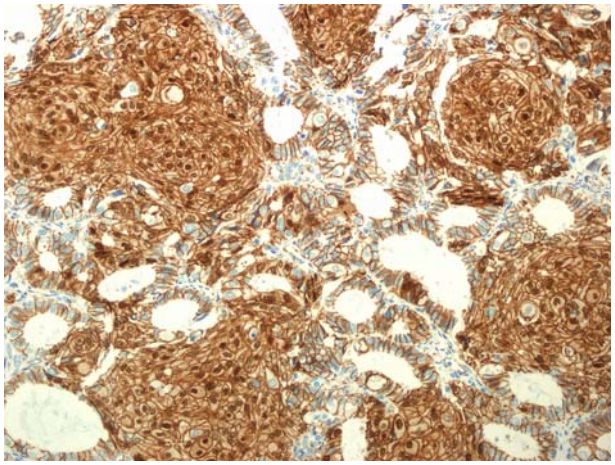

Fig. 20 Endometrioid borderline tumor. Nuclear immunoreactivity for beta-catenin in the squamous morules.

Somatic mutations of the beta-catenin (*CTNNB1*) and *PTEN* genes are the most common genetic abnormalities identified in ovarian ECs. Compared with uterine ECs, ovarian ECs have a similar frequency of beta-catenin abnormalities but lower rate of microsatellite instability (MI) and *PTEN* alterations. *CTNNB1* mutations, which occur in 38%–50% of cases, are associated with squamous differentiation, low tumor grade, and favorable outcome. Beta-catenin is immunohistochemically detectable in carcinoma cells in more than 80% of the cases (Fig. 20).

PTEN inactivation results in activation of the PI3K-AKT signaling pathway that inhibits apoptosis. *PTEN* is mutated in approximately 20% of ovarian ECs and in 46% of those with 10q23 LOH. An alternative mechanism for activation of the PI3K signaling in ECs is through activating mutations of *PIK3CA*, which encodes the p110 catalytic subunit of PI3K. *PIK3CA* mutations in exons 9 and 20 have been identified in 20% of ovarian ECs and CCCs but in only 2% of HGSC. *PIK3CA* mutations are associated with adverse prognostic parameters.

ECs are the types most commonly encountered in patients with hereditary nonpolyposis colon cancer syndrome. The reported frequency of MI in ovarian ECs ranges from 12.5% to 19%. Like endometrial carcinomas, ovarian ECs with MI follow the same process of *MLH-1* promoter methylation and frameshift mutations at coding mononucleotide repeat microsatellites. ECs are immunoreactive for vimentin, cytokeratins (CK7, 97%; CK20, 13%), epithelial membrane antigen (EMA), and estrogen and progesterone receptors. Immunoreaction for alpha-inhibin, WT-1, and calretinin are negative in most ECs. The median Ki-67 labelling index for endometrioid is 8.2%.

Simultaneous carcinomas of the uterine corpus and ovary, occur in 15%–20% of ovarian tumors and in approximately 5% of uterine tumors. Both tumors are of endometrioid type in the majority of cases. Besides prognostic implications, accurate diagnosis as independent primaries or metastases is necessary for appropriate staging and treatment. Assessment of conventional pathological features including tumor size, histological type and grade, pattern of tumor growth, vascular invasion, and coexisting atypical hyperplasia or endometriosis allows the distinction in most cases. Occasionally, however, the differential diagnosis can be difficult or impossible as the tumors may show overlapping features. In such cases, patient follow-up is the single most conclusive factor, but ancillary techniques may help to establish the correct diagnosis.

Clonality analysis using LOH and gene mutation have been used for distinguishing independent primary carcinomas from metastatic carcinomas. The frequency of molecular alterations in both independent and metastatic tumors, including MI and *PTEN* mutations, is higher than that observed in single sporadic tumors. Nuclear immunoreactivities for beta-catenin and *CTNNB1* mutations were restricted to independent uterine and ovarian tumors and were absent in metastatic tumors. These findings correlated with the clinical outcome. Recently, however, whole-exome massively parallel sequencing done in five patients with synchronous endometrioid endometrial and ovarian carcinomas revealed that the tumors of each case displayed strikingly similar repertoires of somatic mutations and gene copy number alterations. These results favor that sporadic synchronous EC of the endometrium and ovary are clonally related, and likely constitute dissemination from one site to the other. Nevertheless, the "independent" origin of the ovarian EC arising from endometrial stem cells that reached the ovary through menstrual reflux cannot be excluded.

EC is the type of ovarian carcinoma with the most favorable prognosis and this may be due to its lower grade and lower stage in most cases (Table 2).

Clear Cell Carcinomas (CCC)

Clear cell carcinomas account for approximately 10% of ovarian carcinomas and patients typically present with stage 1 or 2 disease (Table 2). Tumors are rarely bilateral. CCCs are associated with an unfavorable prognosis when they present at advanced stage. As with EC, there is a strong association with endometriosis, and CCCs associated with endometriosis have a favorable prognosis.

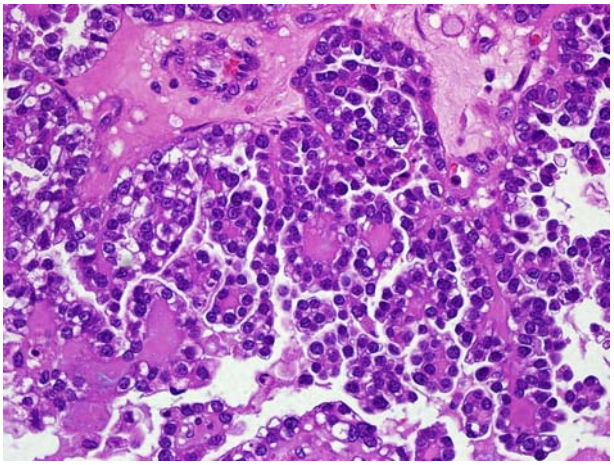

Fig. 21 Clear cell adenocarcinoma. The papillae are lined by clear cells and have hyalinized cores. Prat, J. (2017). Pathology of borderline and invasive cancers. *Best Practice & Research: Clinical Obstetrics & Gynaecology* ***41,*** 15–30.

The presence of clear cells alone is not sufficient for a diagnosis of CCC, as cells with clear cytoplasm can be seen in HGSC and EC. Besides the characteristic clear or hobnail cells with eccentric, rounded, and bulbous nuclei, the diagnosis is based on the following histological findings: (a) multiple complex papillae; (b) densely hyaline basement membrane material expanding the cores of the papillae (Fig. 21); and (c) hyaline bodies, which are present in approximately 25% of cases. Mitoses are less frequent than in other types of ovarian carcinomas (usually less than 5/10 HPFs).

Clear cell carcinomas lack the *BRCA* abnormalities, chromosomal instability, or complex karyotypes of HGSC. Also, mutations in *TP53*, which are common in HGSCs, are usually absent in CCCs. This suggests that other antiapoptotic mechanisms are likely to be involved in the development of CCC. Loss of PTEN expression has been found in 40% of early stage CCCs and inactivating mutations in 8% of cases. On the other hand, activating mutations in *PIK3CA* occur in 33% of cases. Recently, it has been found that nearly half the CCCs (46%–57%) carry *ARID1A* mutations and lack BAF250 protein. *ARID1A* mutations and loss of BAF250a expression have been found in the tumor and adjacent endometriosis, which suggests that *ARID1A* inactivation occurs early during malignant transformation of endometriosis. CCCs are usually positive for HNF1-beta (>90%) and are negative for ER and WT1 in more than 95% of cases. Mutations in *KRAS* are present in some CCCs but their frequency is very low. Microsatellite instability may be encountered in up to 15% of cases.

The genetic profiles of CCC are similar to those of renal and endometrial CCCs, which suggest that some molecular genetic alterations may be common to CCCs regardless of the organ of origin.

Hepatocyte nuclear factor-1beta (HNF-1beta) is upregulated in ovarian clear cell tumors, including benign, borderline tumors, and carcinomas (Fig. 22). This transcription factor facilitates glycogen synthesis and is expressed in mid-to-late secretory and gestational endometrium (Arias–Stella reaction), atypical and inflammatory endometriosis, and clear cell carcinoma. HNF-1beta regulates several specific genes of clear cell carcinoma, including dipeptidyl peptidase IV (glycogen synthesis), osteopontin (progesterone-regulated endometrial secretory protein), angiotensin converting enzyme 2 (ferritin induction, iron deposition,

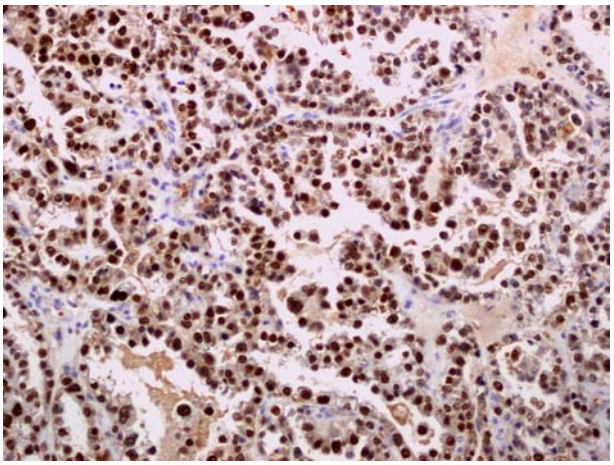

Fig. 22 Clear cell adenocarcinoma. HNF-1beta immunostaining.

antiapoptosis), annexin 4 (paclitaxel resistance), and *UGT1A1* (detoxification). Thus, HNF-1beta appears to play an important role in the pathogenesis and behavior of clear cell carcinoma.

Sex Cord-Stromal Tumors

Sex cord-stromal tumors account for only 8% of ovarian tumors and are the most common functioning ovarian neoplasms associated with endocrine symptoms. They may differentiate toward female (granulosa and theca cells) or male (Sertoli and Leydig cells) structures. Most of these tumors are benign and less frequently of low-grade malignancy. Rarely, malignant sarcomatoid forms occur.

Malignant Sex Cord-Stromal Tumors

Granulosa cell tumor

Granulosa cell tumors account for 12% of all sex cord-stromal tumors. Most granulosa cell tumors (95%) occur after the menopause (adult form) and are unusual before puberty. A juvenile form that develop in children and young women has distinct clinical and pathologic features (hyperestrinism and precocious puberty). Development of granulosa cell tumors is linked to loss of oocytes. Oocytes appear to regulate granulosa cells, and tumorigenesis occurs when follicles are disorganized or atretic. Adult-type granulosa cell tumors, are clinically palpable and, like most ovarian tumors, focally cystic and solid. The cut surface shows yellow areas, owing to lipid-rich luteinized granulosa cells, white zones of stroma, and focal hemorrhages (**Fig. 23**). Granulosa cell tumors show diverse growth patterns: (1) diffuse (sarcomatoid), (2) insular (islands of cells) or (3) trabecular (anastomotic bands of granulosa cells). Random nuclear arrangement about a central degenerative space (Call-Exner bodies) gives a characteristic follicular pattern (**Fig. 24**). Tumor cells are typically spindle shaped and have a cleaved, elongated nucleus (coffee bean appearance). They secrete inhibin, a protein that suppresses pituitary release of follicle-stimulating hormone (FSH). These tumors can also express calretinin, a primarily neuronal protein, which suggests possible neural differentiation or derivation for these neoplasms. Three fourths of granulosa cell tumors secrete estrogens; thus, endometrial hyperplasia is a common presenting sign. Endometrial hyperplasia or low-grade endometrioid carcinoma may develop if a functioning granulosa cell tumor remains undetected. At diagnosis, 90% of granulosa cell tumors are within the ovary (stage I). Over 90% of these patients survive 10 years. Tumors that have extended into the pelvis and lower abdomen have a poorer prognosis. Late recurrence, 5–10 years after surgical removal, is not uncommon and is usually fatal. The most common chromosomal abnormalities are trisomy 12, trisomy 14, monosomy 16, deletion of 16q, and monosomy 22. Missense somatic point mutations in the *FOXL2* gene (402 C to G) are found in over 90% of adult granulosa cell tumors.

Setoli–Leydig cell tumor

Sertoli–Leydig cell tumors (SLCT) are rare androgen-secreting mesenchymal neoplasms that resemble embryonic testis. They account for 0.5% of all sex-cord stromal tumors. Tumor cells typically secrete weak androgens (dehydroepiandrosterone), so tumors are usually quite large before patients complain of masculinization. Sertoli–Leydig cell tumors occur at all ages but are most common in young women of childbearing age. Sertoli–Leydig cell tumors are unilateral, usually 5–15 cm, and tend to be lobulated, solid, and brown to yellow. They vary from well to poorly differentiated and some contain heterologous elements (e.g., mucinous

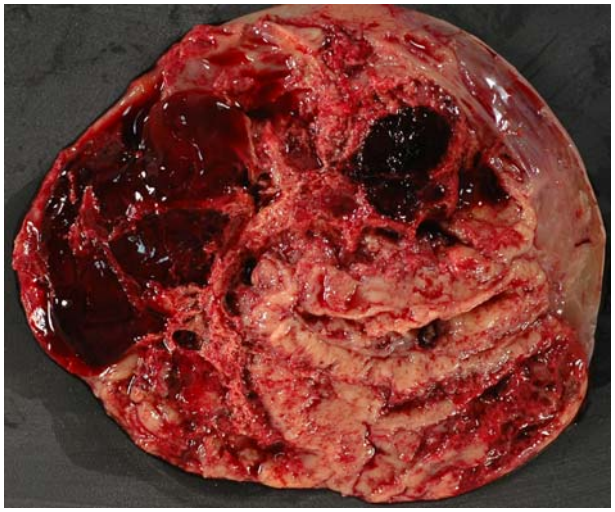

Fig. 23 Adult granulosa cell tumor. Cystic and solid tumor. The solid component is *yellow* and the cysts contain clotted blood.

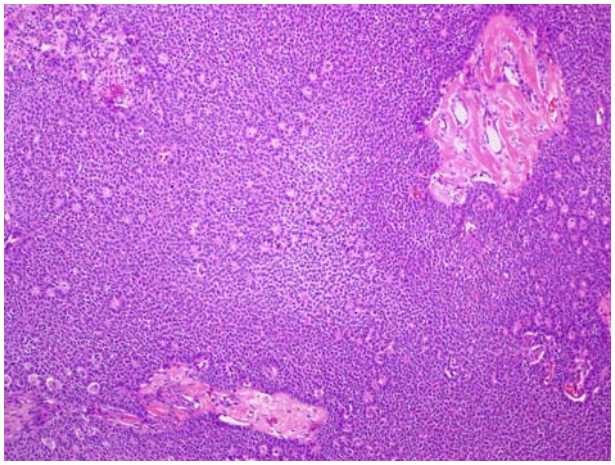

Fig. 24 Adult granulosa cell tumor. Microfollicular pattern (Call-Exner bodies).

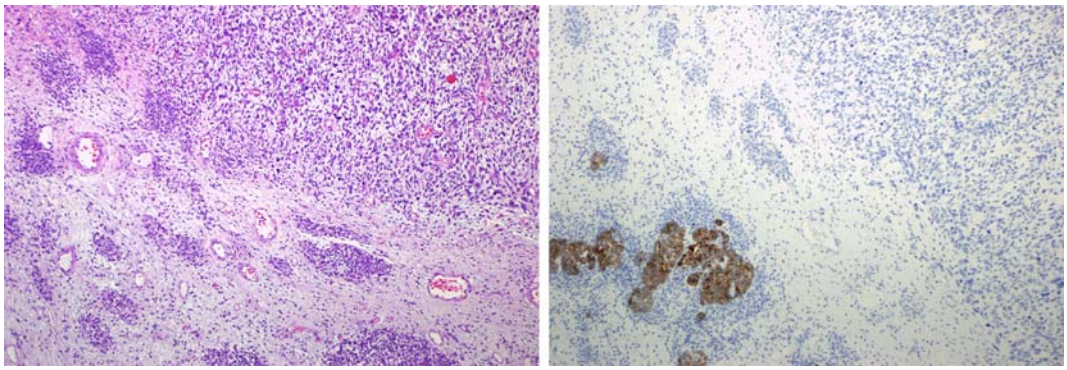

Fig. 25 Sertoli–Leydig cell tumor poorly differentiated with heterologous mesenchymal element (rhabdomyosarcoma) (*upper half*). Positive inhibin immunoreaction i-n the Sertoli-Leydig cell component (*right*).

glands and, rarely, even cartilage). Large Leydig cells have abundant eosinophilic cytoplasm and a central round to oval nucleus with a prominent nucleolus. The stroma in some areas often differentiates into immature solid tubules of embryonic Sertoli cells (Fig. 25). Nearly half of all patients with Sertoli–Leydig cell tumors exhibit signs of virilization: hirsutism, male escutcheon, enlarged clitoris, and deepened voice. Initial signs are often defeminization, manifested as breast atrophy, amenorrhea and loss of hip fat. Once the tumor is removed, these signs disappear or lessen. Well-differentiated tumors are benign and virtually always cured by surgical resection, but the rare cases of poorly differentiated (or sarcomatoid) SLCT may metastasize. Mutations in *DICER1*, a gene encoding an RNase III endoribonuclease, are found in 60% of Sertoli–Leydig cell tumors. Germline mutations in this gene are seen in familiar multinodular goiter with Sertoli–Leydig tumor, and tumor susceptibility includes pleuropulmonary blastoma in childhood. Sertoli–Leydig cell tumor has been reported in association with cervical embryonal rhabdomyosarcoma in four patients.

Malignant Germ Cell Tumors

Dysgerminoma

Dysgerminoma, the ovarian counterpart of testicular seminoma, is composed of primordial germ cells. It accounts for less than 2% of ovarian cancers but 10% of those occurring in women younger than 20 years. Dysgerminoma represents 45% of malignant germ cell tumors, Most patients are between 10 and 30. The tumors are bilateral in about 15% of cases, are often large and firm, and have a bosselated external surface. The cut surface is soft and fleshy (Fig. 26). They contain large nests of monotonously uniform tumor cells that have clear glycogen-filled cytoplasm and irregularly flattened central nuclei. Fibrous septa containing lymphocytes traverse the tumor (Fig. 27). The tumor cells show diffuse nuclear expression for the stem cell/primitive germ cell nuclear transcription factors OCT3/4, NANOG, and SALL4. Dysgerminomas are treated surgically; 5-year survival for patients with stage I tumor approaches 100%. Because the tumor is highly radiosensitive and also responsive to chemotherapy, even higher-stage tumors have 5-year survival rates exceeding 80%. The majority of dysgerminomas show isochromosome 12p. *c-Kit* mutations are seen in 25%–50% of tumors, most commonly in exon 17, not in the exon 11 location that confers susceptibility to imatinib therapy.

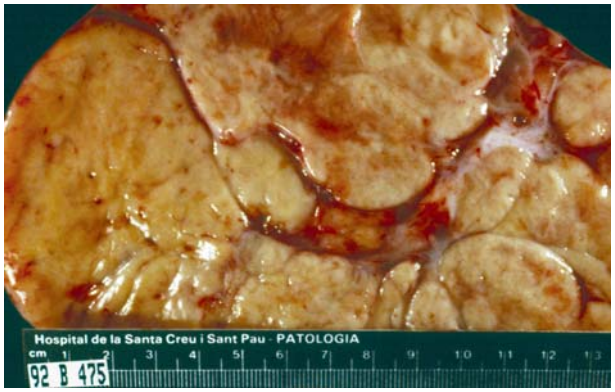

Fig. 26 Dysgerminoma. Solid, *yellowish–white*, and lobulated tumor.

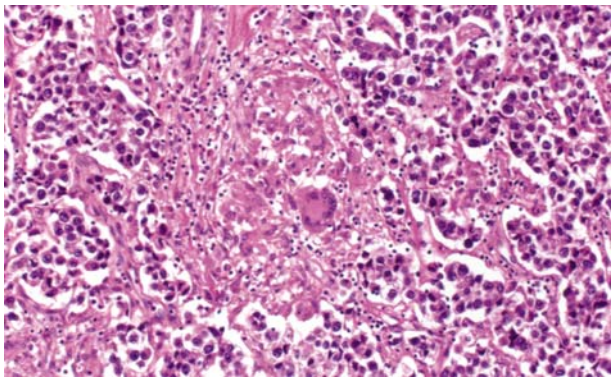

Fig. 27 Dysgerminoma. Nest of tumor cells separated by delicate fibrous septa. The stroma contains numerous lymphocytes and a sarcoid-like granuloma. Prat, J. (2004). Pathology of the ovary. Philadelphia: Saunders.

Yolk Sac Tumor

Yolk sac tumors are highly malignant tumors of women under the age of 30 that histologically resemble mesenchyme of the primitive yolk sac. They are the second most common malignant germ cell tumors (~20%) and are almost always unilateral. Yolk sac tumors are large, with extensive necrosis and hemorrhage. Several patterns are seen. The most common is a reticular, honeycombed structure of communicating spaces lined by primitive epithelial cells with glycogen-rich, clear cytoplasm and large hyperchromatic nuclei (primitive endoderm). Glomerular or Schiller–Duval bodies (Fig. 28) are found sparingly in a few tumors but are characteristic. They consist of papillae that protrude into a space lined by tumor cells, resembling the glomerular Bowman space. The papillae

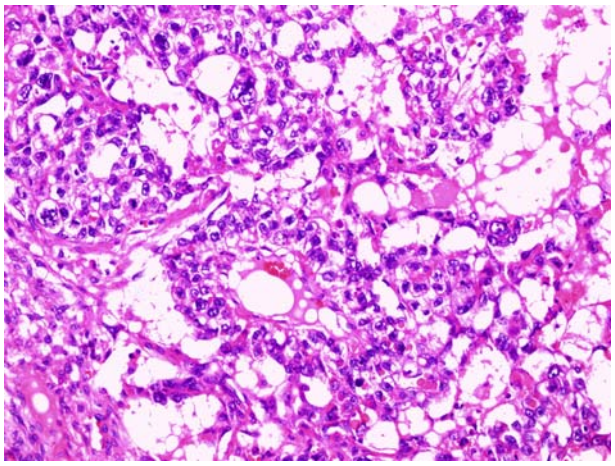

Fig. 28 Yolk sac tumor. Glomerular or Schiller–Duval body.

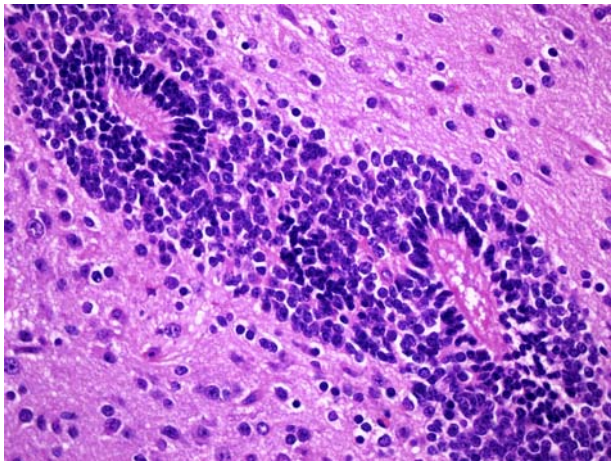

Fig. 29 Immature teratoma (grade 3). Immature neuroectodermal tissue with rosettes. Mutter, G.L. and Prat, J. eds. (2014). Pathology of the female reproductive tract. 3rd edn. Churchill Livingstone; Elsevier.

are covered by a mantle of embryonal cells and contain a fibrovascular core and a central blood vessel. Yolk sac tumor should not be confused with embryonal cell carcinoma, which is far more common in the testis. The former secretes α-fetoprotein, which can be demonstrated by immunohistochemistry. Detection of α-fetoprotein in the blood is useful for diagnosis and for monitoring the effectiveness of therapy. Although once uniformly fatal, 5-year survival with chemotherapy for stage I yolk sac tumors now exceeds 80%.

Immature Teratoma

Immature teratomas of the ovary contain elements derived from the three germ layers. However, unlike mature cystic teratomas, immature teratomas contain embryonal tissues. These tumors account for over 20% of malignant germ cell tumors. They occur predominantly in women under the age of 20 and become progressively less common in older women. Immature teratomas are predominantly solid and lobulated, with numerous small cysts. Solid areas may contain grossly recognizable immature bone and cartilage. Multiple tumor components are often seen, including those differentiating toward nerve (neuroepithelial rosettes and immature glia), glands and other structures found in mature cystic teratomas (Fig. 29). Grading is based on the amount of immature neural tissue present. Survival reflects tumor grade. Well-differentiated immature teratomas have a good prognosis, but high-grade tumors (mainly embryonal tissue) are often lethal.

Summary

Epithelial ovarian tumors are heterogeneous neoplasms primarily classified according to cell type and further subdivided into benign, borderline, and malignant (carcinoma). Borderline tumors show epithelial proliferation and nuclear atypia but, contrary to carcinomas, show no destructive stromal invasion and their prognosis is usually favorable. The term "borderline" accurately describes the ambiguous histologic and biologic features of these neoplasms. The five main types of ovarian carcinoma (HGSC, LGSC, CCC, EC, and MC) account for over 95% of ovarian carcinomas, can be reproducibly diagnosed, and are inherently different diseases. Correct histopathological diagnosis is critical for treating them successfully.

An undetermined number of cases of HGSCs originate in the tubal fimbria; however, an origin from embryonic stem cells of the ovarian surface epithelium cannot be excluded. HGSC is not a homogeneous disease and several variables, including age, histotype and association with STIC allow segregation of two tumor groups: (a) younger *BRCA*-positive patients, without STIC and with favorable outcome; and (b) older *BRCA*-negative patients, with STIC, and with unfavorable prognosis.

Endometrioid and clear cell carcinomas originate from ovarian endometriosis. Although endometriosis is essentially a benign disease, it exhibits some features reminiscent of malignancy such as metastatic potential and monoclonality. The incidence of malignant tumors arising from ovarian endometriosis is minimal, but the frequency of endometriosis in women with certain types of ovarian cancer (i.e., endometrioid and clear cell carcinomas) is more significant. Atypical endometriosis is the precursor lesion of EC and CCC of the ovary and a direct transition from ovarian atypical endometriosis to EC or CCC has been found.

Much less common are malignant germ cell tumors (dysgerminomas, yolk sac tumors, and immature teratomas) (3% of ovarian cancers) and potentially malignant sex cord-stromal tumors (1%–2%) (granulosa cell tumors).

See also: Ovarian Cancer: Diagnosis and Treatment.

Further Reading

Gilks, C.B., Prat, J., 2009. Ovarian carcinoma pathology and genetics: Recent advances. Human Pathology 40 (9), 1213–1223.
Kurman, R.J., Carcangiu, M.L., Herrington, C.S., Young, R.H. (Eds.), 2014. World Health Organization classification of tumours of female reproductive organs. IARC Press, Lyon.
Mutter, G.L., Prat, J. (Eds.), 2014. Pathology of the female reproductive tract, 3rd edn. Elsevier, Churchill Livingstone.
Prat, J., 2017. Pathology of borderline and invasive cancers. Best Practice & Research. Clinical Obstetrics & Gynaecology 41, 15–30.
Prat, J., 2012. Ovarian carcinomas: Five distinct diseases with different origins, genetic alterations, and clinicopathological features. Virchows Archiv 460, 237–249.
Prat, J., 2012. New insights into ovarian cancer pathology. Annals of Oncology 23 (Suppl 10), x111–x117.
Prat, J., 2004. Pathology of the ovary. Saunders, Philadelphia.
Strayer, D.S., Rubin, R., 2016. Rubin's pathology: Clinicopathologic foundations of medicine, 7th edn. Lippincott Williams & Wilkins, Philadelphia. Chapter 18.

Relevant Websites

http://library.med.utah.edu/WebPath/webpath.html - The Internet Pathology Laboratory for Medical Education Hosted By The University of Utah Eccles Health Sciences Library.
http://www.brown.edu/Courses/Digital_Path/ - Digital Pathology Brown Medical School.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4397237 - Prat J, FIGO Committee on Gynecologic Oncology. FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: abridged republication. Journal of Gynecologic Oncology. 2015;26(2):87-89. https://doi.org/10.3802/jgo.2015.26.2.87.
https://www.cancer.org/cancer/ovarian-cancer/ - American Cancer Society. Ovarian Cancer.

Pancreatic Cancer: Diagnosis and Treatment

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Abbreviations

5-FU 5-Fluorouracil
CA 19-9 Cancer antigen 19-9
CT Computed tomography
CTLA4 Cytotoxic T-lymphocyte-associated protein 4
ERCP Endoscopic retrograde cholangiopancreatography
EUS Endoscopic ultrasonography
FNA Fine-needle aspiration/aspirate
FOLFIRINOX Leucovorin/5-FU/irinotecan/oxaliplatin
ICD-O International Classification of Disease for Oncology
MDCT Multidetector CT
MRCP Magnetic resonance cholangiopancreatography
MRI Magnetic resonance imaging
NCCN US National Comprehensive Cancer Network
PDAC Pancreatic ductal adenocarcinoma
PET Positron emission tomography
SBRT Stereotactic body radiation therapy

Definition

The most common cancer of the pancreas is pancreatic ductal adenocarcinoma (PDAC; ICD-O code: 8500/3) which accounts for over 90% of all pancreatic malignancies. This entry is entirely devoted to this cancer type and does not cover the rare neuroendocrine and mesenchymal pancreatic cancers which are very different and require different clinical approaches.

Presentation and Diagnosis

Presentation

Early pancreatic cancer is usually asymptomatic and effective screening strategies do not exist. The initial presentations manifest with a range of nonspecific symptoms, which further delays the diagnosis. Therefore, most patients present with advanced symptomatic disease. Patients typically report a gradual onset of nonspecific symptoms, such as anorexia, malaise, nausea, fatigue or weakness, and mid-epigastric or back pain.

The most common presenting symptom is abdominal (mid-epigastric) pain (present in about 80% of patients), often unrelenting and/or during the night. The pain may be worse when the patient is lying flat. Some patients may experience increased discomfort after eating. Radiation of the pain to the back may occur due to retroperitoneal invasion of the splanchnic nerve plexus by the tumor. All patients experience pain at some point during the clinical course of the disease.

Significant unexplained weight loss occurs in about 60%–65% of patients as a result of cancer-associated anorexia and/or subclinical fat malabsorption due to pancreatic exocrine insufficiency caused by pancreatic duct obstruction by the tumor. Patients with malabsorption usually complain about diarrhea and malodorous, greasy stools. Nausea and early satiety may also occur and contribute to weight loss.

Diabetes mellitus is present in almost three quarters of pancreatic cancer patients and a new-onset diabetes may be the first PDAC symptom. Therefore, pancreatic cancer should be considered in patients with a sudden-onset diabetes, in particular if it occurs at older age and/or has uncommon manifestations, such as abdominal symptoms and continuous weight loss.

Depression which is disproportionate to the severity of the disease occurs in almost half of the patients and may be the most prominent presenting symptom. Sleeping problems may also occur.

The most characteristic sign of carcinoma of the pancreatic head is painless obstructive jaundice. Patients usually notice a darkening of their urine and lightening of their stools before noting the change in skin pigmentation. Pruritus may accompany or (often) precede clinical obstructive jaundice, and actually be the patient's most distressing symptom. Patients presenting with clinical jaundice may also have a palpable gallbladder at physical examination as well as skin excoriations from unrelenting pruritus.

Other, less common, signs and symptoms include xerostomia, dyspepsia, hoarseness, taste change, bloating or belching, hiccup, dysphagia as well as dyspnea, dizziness, or edema. Occasionally, patients may present with migratory thrombophlebitis or venous thrombosis, acute pancreatitis, hypoglycemia, and/or hypercalcemia. Marantic endocarditis may also develop in pancreatic cancer, occasionally being confused with subacute bacterial endocarditis.

In general, tumors of the pancreas body and tail cause symptoms later in the tumor development than those of the pancreatic head.

Workup and Diagnosis

The initial workup and staging of the disease are based on imaging techniques, and the definitive diagnosis is made upon a pathologic evaluation of a tissue sample. Noteworthy, tissue-based confirmation of pancreatic cancer diagnosis before surgery is not necessarily required. As surgery remains the only cure for PDAC, the priority is to resect all resectable disease rather than delay surgical procedures waiting for definitive diagnosis, and high-quality multiphase imaging can be sufficient to distinguish between patients eligible for a curative tumor resection and those with unresectable tumors. There is much debate on sensitivity and specificity of different imaging modalities for the diagnosis and staging of PDAC. No single modality is considered sufficiently sensitive and accurate to be used on its own during the initial workup. However, a combination of different imaging techniques performed and evaluated by experienced clinicians may achieve the accuracy exceeding 95%.

The mainstay of initial diagnostic modalities in case of a suspected pancreatic cancer or evidence of a dilated duct (stricture) is computed tomography (CT) scanning of the abdomen. The expert panel of the US National Comprehensive Cancer Network (NCCN) recommends multidetector CT (MDCT) angiography performed by acquiring thin (preferably submillimeter) axial sections using a dual-phase pancreatic protocol, with images obtained in the pancreatic and portal venous phase of contrast enhancement. The scan coverage may be extended to cover the chest and pelvis for complete staging. In up to 92% of patients, pancreatic adenocarcinomas appear as hypodense masses on CT scans. Early findings include abrupt cut-off and dilatation of the pancreatic duct. Dilatation of both biliary and pancreatic ducts indicates a cancer arising in the head of the pancreas where up to 70% of PDACs originate. Multiplanar reconstruction allows to precisely visualize the relationship of the primary tumor to the mesenteric vasculature and predict involvement of the superior mesenteric artery as well as to detect subcentimeter metastatic deposits. However, CT sensitivity for small hepatic and peritoneal metastases is limited. Moreover, small pancreatic tumors (below 2 cm) can be missed even with the most advanced CT-scanning techniques.

Endoscopic ultrasonography (EUS) may be complementary to CT, providing additional staging information in particular in patients with no lesions visible on initial scans and/or when vascular or lymph node involvement is questionable. Because of the proximity of the pancreas to the EUS transducer, high-frequency ultrasounds can be used to produce very high-resolution (submillimeter) images and detect pancreatic tumors smaller than 2 cm. An additional diagnostic advantage of EUS is the possibility to guide fine-needle aspiration (FNA), which allows for simultaneous cytological confirmation of PDAC diagnosis. However, introducing newer CT scanning techniques has significantly improved the sensitivity and specificity of abdominal CT-scan findings in patients with suspected pancreatic carcinoma and, in patients with visible lesions, also CT can be used to direct FNA. Expert opinions so as to which of these two techniques is superior in the initial workup of suspected PDAC vary and many guidelines actually consider them complementary. Overall, EUS appears to be equivalent to dual-phase spiral CT scanning for assessing tumor resectability. It is probably superior to CT scanning as a means of assessing the T stage of the tumor, especially when the clinician is looking for portal vein involvement in pancreatic head lesions, but it is probably inferior to CT scanning in assessing arterial involvement and distant metastases. Both EUS and CT scanning are poor at detecting occult nodal involvement.

Positron emission tomography (PET) combined with CT may be considered in selected high-risk patients following a CT scan. However, PET/CT does not substitute for high-quality contrast-enhanced CT.

Pancreas protocol magnetic resonance imaging (MRI) may have added value in the initial diagnostic workup and staging of PDAC, in particular when suspected pancreatic lesions are not visible on CT scans or if the patient is allergic to the contrast medium. It may also help characterize indeterminate liver lesions.

Endoscopic retrograde cholangiopancreatography (ERCP), which combines endoscopic and fluoroscopic procedures, is highly sensitive in detecting pancreatic and/or biliary ductal abnormalities. However, the findings are not specific to pancreatic cancer (it is particularly difficult to differentiate them from changes observed in patients with chronic pancreatitis). Moreover, ERCP is more invasive than the other available diagnostic imaging modalities and so associated with a higher risk of complications. Therefore, it is mostly used for specific therapeutic interventions, especially to relieve biliary obstruction by placing a stent. This may also be achieved using magnetic resonance cholangiopancreatography (MRCP) which is considered equivalent to ERCP in terms of sensitivity. Both modalities allow to obtain brushings for diagnostic cytological evaluation.

Laparoscopy can detect extrapancreatic involvement in approximately 40% of patients without visible lesions on CT. NCCN panel recommends considering laparoscopy for patients with resectable pancreatic cancer who are at increased risk for disseminated disease.

Laboratory findings in PDAC patients are usually nonspecific. They may include a mild normochromic anemia and thrombocytosis, as well as findings related to obstructive jaundice (elevated bilirubin, alkaline phosphatase, gamma-glutamyl transpeptidase, and—to a lesser extent—aspartate aminotransferase and alanine aminotransferase). Serum amylase and/or lipase levels are elevated in less than half of the patients with resectable pancreatic cancers and in only one quarter of those with unresectable tumors. However, about 5% of patients initially present with acute pancreatitis, in which case amylase and lipase would be

uniformly elevated. Liver metastases may result in relatively low-grade elevations of serum alkaline phosphatase and transaminase levels. Patients with advanced disease and weight loss may also have general laboratory evidence of malnutrition (e.g., low serum albumin or cholesterol levels).

No reliable and specific blood (serum) biomarker for PDAC screening and/or diagnosis has so far been identified. The most established of the existing biomarkers is cancer antigen 19-9 (CA 19-9), a sialylated oligosaccharide Lewis A blood antigen commonly found on circulating mucins in cancer patients. CA 19-9 levels are often increased in PDAC patients. However, this antigen is commonly expressed and shed in various pancreatic and hepatobiliary diseases as well as in many different malignancies, and so it is not specific to PDAC diagnosis. Moreover, up to 10% of PDAC patients do not produce this antigen because they lack the necessary enzyme. Still, in most patients, preoperative CA 19-9 levels correlate with the tumor stage and resectability, making it a useful prognostic marker. Moreover, variations in CA 19-9 levels may be of some diagnostic value helping to differentiate PDAC from pancreatitis. However, CA 19-9 is not an independent diagnostic PDAC marker.

Since a pathologic confirmation of PDAC diagnosis is not required before surgery, in patients with resectable tumors, the pathologic evaluation is made on surgical specimens. In patients who are not candidates for surgery, EUS- or CT-guided FNA biopsies are obtained. EUS-guided procedure is safer than CT-guided percutaneous approach because it limits the risk of internal bleeding and infections, and—more importantly—that of peritoneal seeding from the tumor. The NCCN panel recommends EUS-guided FNA as the preferred technique to obtain biopsy material in nonsurgical patients with a suspicion of PDAC. If performing neither EUS-, nor CT-guided FNA is possible, biopsy specimens can be obtained via endoscopic cholangioscopy (intraductal biopsies) or laparoscopy. Pancreatic ductal brushings or biopsies obtained at ERCP also often reveal cytological findings consistent with PDAC diagnosis. One negative biopsy does not exclude PDAC diagnosis and at least one repeat biopsy should be performed. It must be emphasized again, however, that the absence of histologically confirmed diagnosis (negative biopsy) should not delay surgery in patients with resectable disease in whom the clinical suspicion of a pancreatic malignancy is high.

Management and Therapy

The management of PDAC remains a considerable clinical challenge. This cancer type has a particularly bad prognosis and the existing therapeutic modalities, except for complete negative-margin surgical resection of localized tumors, provide only limited clinical benefits. The reported overall 5-year survival rates for treated PDAC patients range from 5% to 25%, while the life expectancy of untreated PDAC patients is no more than several months.

The fist-choice treatment of PDAC is surgery and surgical resection of the tumor remains the only potentially curative procedure. As patients usually present with advanced disease, defining resectability criteria is crucial to guide management decisions. These criteria may differ between centers. In general, extrapancreatic disease precludes surgery with a curative intent, while obtaining negative surgical margins is the most significant positive prognostic factor in patients with nonmetastatic disease. The NCCN panel recommends that patients be selected for surgery based on the probability of cure defined as the probability of obtaining negative resection margins ("R0 resection") as the only criterion, emphasizing that assessing this probability should involve a multidisciplinary expert team at a high-volume center with extensive expertise in managing PDAC.

Management of Resectable Disease

Curative resection options include pancreaticoduodenectomy, distal pancreatectomy, and total pancreatectomy.

Pancreaticoduodenectomy (Whipple procedure) involves removal of the pancreatic head, duodenum, gallbladder, and the antrum of the stomach, with surgical drainage of the distal pancreatic duct and biliary system, usually through an anastomosis to the jejunum. This procedure is most likely to be beneficial to patients who have a tumor located in the head of the pancreas or in the periampullary region. A pylorus-sparing variant of the standard Whipple procedure may also be used as an alternative to classic pancreaticoduodenectomy with antrectomy. Portal vein resection, even though practiced in some centers, as well as the extent of lymphadenectomy, remain controversial.

Distal (left-sided) pancreatectomy involves isolation and resection of the distal portion of the pancreas containing the tumor, with oversewing of the distal pancreatic duct. Preservation of the spleen is not recommended. This procedure may be effective for tumors located in the body and tail of the pancreas. However, as tumors at these locations give later symptoms, patients with these tumors have higher unresectability rates. Distal pancreatectomy may also be performed with a laparoscopic approach which gains increasing popularity over an open surgery approach due to lower complication rates.

Total pancreatectomy may be an option when the tumor involves the neck of the pancreas. However, this procedure is associated with the highest mortality rate and the most severe comorbidities (like exocrine insufficiency and diabetes mellitus), and is therefore the least commonly performed.

Preoperative biliary drainage may be considered before any of these procedures in order to alleviate symptoms of pruritus and cholangitis, and potentially decrease the probability of surgery-related morbidities by improving liver function. Biliary decompression is necessary in patients with jaundice who receive neoadjuvant induction therapy prior to pancreatic resection.

Postsurgical surveillance includes regular physical examinations (symptom assessment) and CT scans as well as serial measurements of serum CA 19-9 levels. Increasing CA 19-9 levels indicate disease progression or recurrence. However, some patients progress without an increase in this marker levels.

Postsurgical recurrence rates are generally high, even after R0 resection (complete tumor resection, with clear margins), with some patients relapsing as early as a few weeks after surgery. Therefore, all patients receive adjuvant therapy following surgery. Chemotherapy with gemcitabine is a widely accepted standard in this setting, either as monotherapy or with capecitabine. Another option is 5-fluorouracil (5-FU) with leucovirin. NCCN guidelines list all these options as equivalent in view of the current knowledge (**Table 1**). The usefulness of adjuvant FOLFIRINOX (leucovorin/5-FU/irinotecan/oxaliplatin) is currently being tested in clinical trials. Chemoradiation may also be administered in the adjuvant setting, either after or before chemotherapy. Chemotherapy with S-1, an oral antimetabolite manufactured by Taiho Pharmaceuticals, is used for postsurgical adjuvant therapy in some Asian countries but as of now it is not approved for treatment of PDAC in Western countries.

Management of Borderline Resectable and Nonresectable Disease

While immediate resection and postoperative adjuvant therapy is considered standard treatment for patients with resectable tumors, neoadjuvant chemotherapy may be beneficial to those with borderline resectable tumors, potentially downsizing the tumor and thus increasing the probability of a subsequent R0 resection. Neoadjuvant therapy is also sometimes used in high-risk resectable patients, however the benefits remain controversial. The most established neoadjuvant regimens include FOLFIRINOX, gemcitabine with albumin-bound paclitaxel, and gemcitabine with cisplatin (for patients with mutations in DNA repair genes, including *BRCA1* and *BRCA2*). Some studies suggest that neoadjuvant chemoradiation, followed by chemotherapy or without it, may be even more beneficial. Chemotherapy followed by stereotactic body radiation therapy (SBRT) may also be a safe and feasible neoadjuvant option. However, these remain to be validated. Of note, histological confirmation of PDAC diagnosis is necessary before administering neoadjuvant therapy in all cases.

The reported resectability rates vary between centers, yet they are consistently low. Overall, approximately 80%–95% of patients present with unresectable disease. A palliative surgery may be considered in these patients but the mainstay of the frontline treatment is chemotherapy and/or chemoradiation. In patients with metastatic or locally advanced disease and good performance status, FOLFIRINOX is currently the preferred first-line treatment. Before introduction of FOLFIRINOX, gemcitabine monotherapy had been the most widely used regimen in unresectable patients. As it has an advantage of relieving symptoms, it may be used regardless of the patient's performance status and may therefore still be the first choice for patients with metastatic disease and poor performance status. A number of gemcitabine-containing combination regimens have also been studied. Of these, only gemcitabine with albumin-bound paclitaxel and gemcitabine with erlotinib (an inhibitor of epidermal growth factor receptor) have been shown to significantly improve survival when used as frontline treatment in patients with unresectable disease. However, combining gemcitabine with some other targeted therapeutics have been shown to improve outcomes when used as maintenance therapy. Patients with mutations in DNA repair genes may benefit from a frontline combination of gemcitabine with cisplatin. Upfront SBRT may be used in patients who are not candidates for systemic treatment. Patients who maintain good performance status after the primary treatment may undergo second-line therapy. This may involve chemotherapy using a different regimen than that administered as a frontline treatment, or chemoradiation.

Different currently available PDAC treatment options and their therapeutic indications as recommended in the most recent NCCN guidelines are summarized in **Table 1**.

Other Considerations

Given poor outcomes of PDAC patients, best supportive care and palliation of symptoms are extremely important. Specific measures are needed in particular to relieve symptoms of biliary and/or gastric outlet obstruction, severe abdominal pain, thromboembolic disease, depression, and malnutrition. These may include surgical, radiotherapeutic and/or chemotherapeutic palliation. Local ablative techniques, such as radiofrequency ablation or irreversible electroporation may also be feasible options.

Prospective Vision

The detection and management of pancreatic cancer remains one of the biggest challenges of clinical oncology. While a multitude of putative or validated biomarkers combined with novel therapeutic approaches have significantly improved the outcomes of patients with many other cancer types, pancreatic cancer is usually diagnosed late and remains to be fatal in most cases. Therefore, identifying reliable early detection biomarkers as well as effective therapies is an extremely urgent need.

A large variety of candidate biomarkers for early detection of PDAC have been studied, including among others thrombospondin-2 (THBS-2), mucin-5 AC (MUC5AC), a panel of methylated gene markers to predict the grade of dysplasia in pancreatic cysts, exosome-derived DNA and RNA, genetic alterations found in cell-free DNA, and miRNA markers. Biomarkers measurable in pancreatic juice and cystic fluid seem to be the closest to large-scale validation studies. However, it becomes clear that no single biomarker will be sufficiently specific for PDAC diagnosis and that research should focus on identifying panels of molecular markers combined with modern imaging markers as a strategy for early detection of this cancer.

A search for novel therapeutic solutions for PDAC has also been the focus of extensive research. In particular, numerous potential molecular targets have been identified. Unfortunately, most of the tested targeted therapeutics, many of which have been successfully incorporated into treatment regimens for other cancer types, have failed to provide clinical benefit to PDAC patients. The

Table 1 Various therapy regimens for the treatment of pancreatic ductal adenocarcinoma and their clinical indications as recommended by the US National Comprehensive Cancer Network (NCCN).

Regimen	*Resectable (adjuvant)*	*Borderline Resectable/ resectable (neoadjuvant)*	*Locally advanced (category recommendations for good performance status only unless otherwise noted)*	*Metastatic (category recommendations for good performance status only unless otherwise noted)*	*Second-line therapy (good performance status only)*
Gemcitabine	√ (category 1)		√ (category 1 for poor performance status)	√ (category 1 for good and poor performance status)	√ (if previously treated with fluoropyrimidine-based therapy)
Gemcitabine/albumin-bound paclitaxel			√	√ (category 1; preferred)	√ (if previously treated with fluoropyrimidine-based therapy)
Gemcitabine/eriotinib			√	√ (category 1)	√ (if previously treated with fluoropyrimidine-based therapy)
Gemcitabine/cisplatin			√ (especially for patients with *BRCA1/2* or other DNA repair mutations)	√ (especially for patients with *BRCA1/2* or other DNA repair mutations)	√ (if previously treated with fluoropyrimidine-based therapy)
Gemcitabine/capecitabine	√ (category 1)		√	√	√ (if previously treated with fluoropyrimidine-based therapy)
Fixed-dose-rate gemcitabine			√ (poor performance status only; category 2B)	√ (poor performance status only; category 2B)	√ (if previously treated with fluoropyrimidine-based therapy)
GTX [fixed-dose-rate gemcitabine/docetaxel/ capecitabine]			√ (category 2B)	√ (category 2B)	√ (if previously treated with fluoropyrimidine-based therapy)
5-FU/leucovorin	√ (category 1)				√ (if previously treated with gemcitabine-based therapy)
5-FU/leucovorin/liposomal irinotecan					√ (category 1 if previously treated with gemcitabine-based therapy and metastatic disease)
FOLFIRINOX		√	√	√ (category 1; preferred)	√ (if previously treated with gemcitabine-based therapy)
Capecitabine	√ (category 2B)		√ (category 2B)	√ (poor performance status only; category 2B)	√ (if previously treated with gemcitabine-based therapy)
Continuous infusion 5-FU	√		√ (category 2B)	√ (poor performance status only; category 2B)	√ (if previously treated with gemcitabine-based therapy)
Fluoropyrimidine/oxaliplatin (eg, FOLFOX, CapeOx)			√ (category 2B)	√ (category 2B)	√ (if previously treated with gemcitabine-based therapy)
Chemoradiation	√ (following induction chemotherapy, with or without subsequent chemotherapy)	√ (subsequent chemoradiation is sometimes included)	√ (in select patients who are not candidates for combination therapy, and following induction chemotherapy in select patients without systemic metastases)		√ (if locally advanced disease; if not previously given; and if primary site is the sole size of progression)

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biggest hopes for now seem to be raised by immunotherapeutics, such as cancer vaccines and immune checkpoint inhibitors. Out of the latter category, tremelimumab, a monoclonal antibody against the human cytotoxic T-lymphocyte-associated protein 4 (CTLA4), is currently under clinical trials.

See also: Pancreatic Cancer: Pathology and Genetics.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.

International Agency for Research on Cancer, 2010. In: Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D. (Eds.), WHO Classification of Tumors of the Digestive System, 4th edn.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Pancreatic Adenocarcinoma. Version 3.2017 – September 11, 2017: https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Roth, M.T., Berlin, J.D., 2018. Current concepts in the treatment of Resectable pancreatic Cancer. Current Oncology Reports 20, 39.

Young, M.R., et al., 2018. Validation of biomarkers for early detection of pancreatic Cancer: Summary of the alliance of pancreatic Cancer consortia for biomarkers for early detection workshop. Pancreas 47 (2), 135–141.

Relevant Website

https://clinicalTrials.gov/—Clinical trial information at the US National Library of Medicine, National Health Institutes.

Pancreatic Cancer: Pathology and Genetics

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Glossary

Chromothripsis Single event in localized and confined genomic regions where up to thousands of chromosomal rearrangements occur.

Desmoplastic stroma Growth of dense connective tissue around a tumor.

Metaplasia Form of cell adaptation to an abnormal stimulus consisting of reversible transformation of a differentiated cell type into another differentiated cell type.

Precursor lesion (Usually local) change of a tissue occurring based on genetic aberrations and identifiable on a morphological level as a forerunner of a malignant tumor.

R1-resection Surgical resection with cancer cells present microscopically at the resection margin.

TNM-Classification A classification system to describe cancer stage. T: size of the primary tumor; N: involved lymph nodes; M: distant metastasis.

Introduction

Pancreatic cancers are traditionally classified according to their morphological and marker expression profile, which supposedly reflect their cell/tissue of origin. Accordingly, exocrine and endocrine cancers represent the main malignant epithelial pancreatic neoplasms. Exocrine cancers are further divided into ductal and acinar cell cancers. Pancreatic ductal adenocarcinoma (PDAC) represents the most common form of pancreatic cancer; therefore, the term pancreatic cancer is often used as synonym of PDAC in the scientific literature and in the social media as well.

Genetically engineered mouse models (GEMM) for PDAC have recently challenged the above outlined model of cancer origin, suggesting that a process of metaplasia of the acinar cell is involved in the origin of PDAC. Likewise, different cells of origin have been suggested for neuroendocrine neoplasms with different biological behavior. In addition, evidence coming from studies based on large-scale genomic and transcriptomic analyses has dramatically affected standard histology-based tumor classifications and is most likely going to affect pancreatic cancer classification as well.

In this article, the main morphological and genetic characteristics of pancreatic cancers are reviewed focusing on exocrine neoplasms; special attention will be set on new concepts concerning the origin and the molecular subtyping of PDAC.

Classification of Pancreatic Cancers and Their Precursor Lesions

Pancreatic neoplasms are currently classified according to standard criteria of tissue and supposed cell of origin and of biological behavior. Accordingly, epithelial and mesenchymal as well as benign and malignant tumors of the pancreas are recognized.

A classification of pancreatic epithelial neoplasms based on the current WHO systems for tumors of the exocrine and endocrine pancreas and incorporating the recent classification of precursor lesions of PDAC is shown in **Table 1**.

Pathology of Pancreatic Cancers

Exocrine Pancreas

Pancreatic ductal adenocarcinoma

Gross morphology

PDAC is most frequently (60%–70%) localized in the head of the organ, where it often causes obstruction of the main pancreatic duct and/or of the intrapancreatic bile duct. At diagnosis, most tumors have already grown beyond the organ's anatomical boundaries with infiltration of the peripancreatic fat tissue or of the duodenum and usually reach a size of about 3 cm (*pT2 UICC stage*). PDAC of the body and tail of the organ are usually larger and might infiltrate surrounding structures, such as the spleen, the mesocolon and the bowel wall, the stomach or the left adrenal gland. Tumors < 2 cm are rare and mostly associated with cystic precursors, such as intraductal pancreatic mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) (see below). Finally, an origin of PDAC in ectopic pancreatic tissue, for example in the stomach or in the small bowel, has been rarely reported. Grossly, PDAC appears as a mostly solid, whitish mass with firm consistency and irregular contours (**Fig. 1**A). Cystic changes as well as necrosis and hemorrhage rarely occur. The surrounding tissue often shows obstructive changes with fibrosis and acinar cell atrophy.

Table 1 Classification of epithelial pancreatic neoplasms

Exocrine neoplasms		
	Entity	*Subtype*
Benign	Acinar cell cystadenoma	
	Serous cystadenoma	
	Pyloric gland adenoma	
Premalignant	Pancreatic intraepithelial neoplasia, grade 3 (PanIN-3)	
	Intraductal papillary mucinous neoplasm	With low-grade dysplasia
		With high-grade dysplasia
	Intraductal tubulopapillary neoplasm	
	Mucinous cystic neoplasm	With low-grade dysplasia
		With high-grade dysplasia
Malignant	Acinar cell carcinoma	
	Acinar cell cystadenocarcinoma	
	Ductal adenocarcinoma	Adenosquamous carcinoma
		Colloid carcinoma
		Hepatoid carcinoma
		Medullary carcinoma
		Signet ring cell carcinoma
		Undifferentiated carcinoma
		Undifferentiated carcinoma with osteoclast-like giant cells
	Intraductal papillary mucinous neoplasm with associated invasive carcinoma	
	Intraductal tubulo-papillary neoplasm with associated invasive carcinoma	
	Mixed acinar/ductal/neuroendocrine carcinoma	
	Pancreatoblastoma	
	Serous cystadenocarcioma	
	Solid-pseudopapillary neoplasms	
Neuroendocrine neoplasms	Pancreatic neuroendocrine microadenoma	
	Neuroendocrine tumor	Nonfunctional pancreatic NET
		NET G1
		NET G2
		NET G3
	Neuroendocrine carcinoma (NEC)	Small cell NEC
		Large cell NEC
	EC-cell, serotonin producing NET (carcinoid)	
	Gastrinoma	
	Glucagonoma	
	Insulinoma	
	Somatostatinoma	
	VIPoma	

Regional invasion—Impact on resection status

Due to its retroperitoneal localization, the absence of a true capsule and its aggressive biology, infiltration of the retroperitoneal space accompanied by perineural and lymphovascular invasion and frequently by direct infiltration or metastasis in regional lymph nodes is commonly observed. All these aspects contribute to explain the high frequency of microscopically incomplete resections (*R1-resections*) that are encountered also in high-volume centers if pancreatic resection specimens are carefully analyzed according to standardized procedures. Particularly relevant is the microscopic analysis of the circumferential resection margins (CRM), which include the dorsal, the ventral and—in cases of a pancreatic head resection- the medial surface (vascular groove, superior mesenteric artery/superior mesenteric vein margin, uncinated margin) of the organ (**Fig. 1**A). According to the distance of the tumor cells from a resection margin observed microscopically, three different situations are encountered: R1, if tumor cells are present in the margin (**Fig. 1**B); R0 narrow, if tumor cells are encountered ≤ 1 mm from the margin (**Fig. 1**C); R0 wide, if tumor cells are encountered >1 mm from a given margin. This classification has been shown to be relevant for patient survival as well.

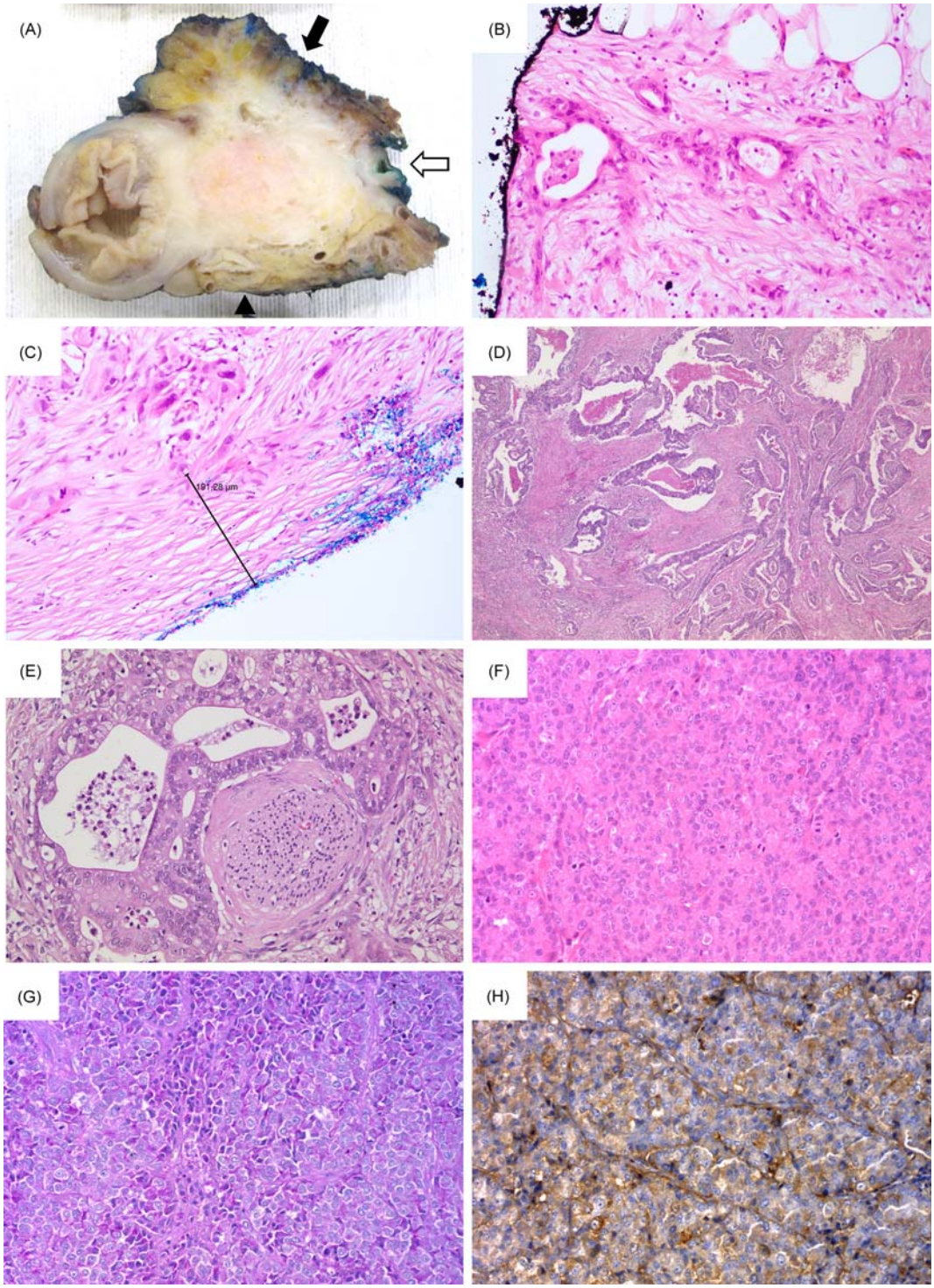

Fig. 1 *Morphology of exocrine pancreatic cancers*. (A) Gross morphology of pancreatic ductal adenocarcinoma: whitish mass with irregular contours infiltrating the duodenal wall. Margins are inked according to a defined color code. *Arrow*: ventral margin; *dotted arrow*: medial margin; *arrowhead*: dorsal margin. (B) R1-situation with tumor cells detected at the *black-inked* margin (HE, 20×). (C) R0 "narrow" situation with tumor cells within 1 mm from the margin (HE, 20×). (D) Histopathology of pancreatic ductal adenocarcinoma: neoplastic glands are surrounded by a desmoplastic stroma (HE, 4×). (E) Detail of a tumor gland infiltration a perineural space (HE, 20×). (F) Histopathology of acinar cell carcinoma displaying a typical acinar growth pattern and consisting of cylindrical cells with round nuclei and prominent nucleoli (HE, 20×). (G) PAS-positive zymogen granules in the cytoplasm of acinar cell carcinoma cells (PAS, 20×). (H) Trypsin expression in acinar cell carcinoma (20×).

Distant metastases and recurrence

Direct infiltration/metastasis of regional lymph nodes is observed in 75%–85% of the cases at diagnosis. The anterior and posterior pancreaticoduodenal nodes and the lymph nodes of the hepatoduodenal ligament are most commonly involved in cases of PDAC of the pancreatic head; lymph nodes on the upper pancreatic contour are most frequently positive in cases of PDAC of the body-tail. Depending on the number of affected lymph nodes a pN1 (1–3) or a pN2 (>3)-stage are distinguished according to the latest TNM classification (Brierley et al., 2016). This is based on studies showing a significant impact on prognosis of the number of affected lymph nodes. In addition to the presence of lymph node metastases and their number, the ratio between affected lymph nodes to the total number of retrieved lymph nodes (so-called lymph node ratio) has been shown to significantly impact on prognosis as well.

Distant metastases are commonly found in the liver (65%), lungs, and more rarely in bones or adrenals. About 50% of patients already have distant metastases at diagnosis.

Local tumor recurrences and/or distant metastases occur in the first 2 years after resection in about 70% of patients.

Histopathology

Classical PDAC consists of tubular structures reminding of pancreatic ducts and in most cases display a moderate degree of differentation (**Fig. 1**D–E). However, about half of the cases show heterogeneous differentiation with additional growth components (e.g., clear cell, micropapillary, cribriform). Well or poorly differentiated (G1 or G3) tumors are reported in 10%–30% of the cases. Tumor grading is performed according to the WHO criteria, which take into consideration the percentage of tubular structures (vs. solid areas), mucin production, nuclear abnormalities and the mitotic rate (**Table 2**). Well- and moderately differentiated PDAC display a characteristic *desmoplastic* stroma (**Fig. 1**D), consisting of whorls of connective tissue fibers with mesenchymal cells, including pancreatic stellate cells (PSC) and inflammatory cells. Histopathologic grade is a relevant prognostic factor for PDAC.

PDAC variants

Homogenous variants of PDAC, defined as a growth component making up at least 50% of the tumor mass, are found in about 10% of the patients. These include the *adenosquamous* carcinoma, characterized by solid-squamous differentiation, the *mucinous* (colloid) carcinoma, characterized by the presence of abundant extracellular mucin, the rare *signet-ring* cell carcinoma, the *large-duct* type adenocarcinoma, characterized by the presence of well-differentiated, large ductal structures, the *medullary* carcinoma, the *hepatoid* carcinoma, the *undifferentiated* (anaplastic) carcinoma and the undifferentiated carcinoma with osteoclastic-like giant cells. Some of these variants (e.g., mucinous, medullary, hepatoid) develop according to molecular pathways that differ from those of conventional PDAC (see below). An impact on survival depending on variant type has been described, with the adenosquamous and undifferentiated variants bearing the worst prognosis.

Immunohistochemistry

Usually immunohistochemistry is not necessary to make a diagnosis of PDAC. Classical PDAC express pancreatic ductal cyto keratins (CK), such as CK7, 8, 18, 19 and sometimes 20. Moreover, they show apical and cytoplasmic expression of MUC1 as well as a cytoplasmic expression of MUC4 and MUC5AC, whereas they are negative for MUC2. CEA also displays an apical and cytoplasmic pattern of expression; CA19–9 and CA125 are frequently expressed as well. Immunostains may be useful to distinguish PDAC from other primary cancers in case of metastasis and to distinguish well-differentiated tumor glands from reactive tubular structures, for example, when examining a pancreatic resection margin. Reactive tubules show no or only apical expression of CEA and MUC1; moreover, they usually do not display any stained cells when detecting proliferation-associated antigens, such as Ki-67.

Acinar cell carcinoma

Gross morphology

Acinar cell carcinoma (ACC) can occur anywhere in the pancreas and typically presents as a large, well-circumscribed, solid mass. Multinodular growth, as well as the presence of areas of necrosis and hemorrhage, have been described. A cystic variant, which may reach a very large size (>20 cm), and an intraductal variant with papillary growth have been reported.

Table 2 Grading of PDAC

Features	*G1*	*G2*	*G3*
Architecture	Tubular, middle-sized duct-like structures, papillary projections	Middle and small-sized duct-like structures, cribriform structures	Solid areas, budding, single cell infiltration
Cells	Cylindrical, retained mucin	Cubic, partial loss of mucin	Polygonal, pleomorphic, spindle Loss of mucin production
Nuclei	Slightly polymorphous	Moderately polymorphous	Very polymorphous
Mitoses	1–5/10 HPF	6–10/10 HP	>10/10 HPF

HPF = high-power field.

Adapted from Bosman, F. T., Carneiro, F., Hruban, R. H., Theise, N.D. (Eds.) (2010): WHO/IARC classification of tumours. WHO classification of tumours of the digestive system. IARC Press: Lyon.

Spread and metastases

ACC can invade adjacent structures (duodenum, spleen, large vessels) and spread to regional lymph nodes and distant sites, mostly to the liver.

Histopathology and immunohistochemistry

ACC display different growth patterns, including a classical acinar pattern, composed of acinar complexes with small lumina, a glandular pattern, composed of tubular structures with larger lumina, and a solid pattern. The tumor cells have round to oval nuclei, in some cases with a prominent central nucleolus (**Fig. 1**F). The cytoplasm is slightly eosinophilic, due to the presence of zymogen granules, which might be slightly PAS-positive (**Fig. 1**G). Immunostains for detection of pancreatic digestive enzymes (trypsin, lipase, amylase) are very useful to establish a diagnosis of ACC (**Fig. 1**H). A prominent stromal reaction is not a typical feature of ACC.

Others

Tubulo-papillary carcinomas

This is a rare form of pancreatic cancer, which has been described recently and occurs in association with an intraductal lesion known as intraductal tubulo-papillary neoplasm (ITPN), described below. Grossly, tubulo-papillary carcinomas form a solid whitish mass with pushing borders. Invasion of adjacent structures, as for example the duodenum, has been reported. Histologically, they consist of back-to-back tubules or glands as well as papillary structures without overt mucin production. Nuclear grade is usually high. Tubulo-papillary carcinomas are reminiscent of acinar cell or even neuroendocrine neoplasms, and immunohistochemistry may be useful to make the diagnosis.

Carcinomas with mixed differentiation

Carcinomas with mixed acinar-ductal, ductal-neuroendocrine, acinar-neuroendocrine and even acinar-ductal-neuroendocrine features have been reported. Each component should be detected in at least one-third of the tumor mass to warrant a diagnosis of mixed neoplasm.

Endocrine Pancreas

Neuroendocrine neoplasms (NEN) represent only 2%–5% of all pancreatic neoplasms. According to the latest WHO classification, NEN are classified into three groups: the well-differentiated neuroendocrine tumors (NET), further split into functioning and nonfunctioning tumors, the poorly-differentiated neuroendocrine carcinomas (NEC) and the mixed neuroendocrine-non-neuroendocrine neoplasms (MiNEN) (**Table 1**). These subtypes are distinguished on the basis of their growth pattern and proliferative activity, assessed by mitotic count or preferably by the Ki-67 index. The different morphology and biological behavior of PanNET and PanNEC argue against a direct evolutionary connection between these two entities and suggest that they might arise in different cells of origin. Conceivably, PanNET may originate in already differentiated neuroendocrine cells, which are capable of hormone synthesis and release and closely resemble normal neurendocrine cells of the islets of Langerhans. On the other hand, PanNEC, which have a less differentiated appearance and marker profile may originate from stem cells that are neuroendocrine committed, but not yet differentiated. The different key genetic events involved in these two entities support this concept.

PanNET

Nonfunctioning pancreatic NET (about 40%) most often (about two-thirds of cases) occur in the head of the pancreas and range in size 2–5 cm. PanNET smaller than 0.5 cm are called microadenomas; they are biologically benign and, if multiple, are often associated with Multiple Endocrine Neoplasia (MEN) type 1. PanNET are usually well-demarcated yellowish masses of soft consistency; a capsule may be present. Multinodular growth and a prominent stromal component may occur. Necrosis and hemorrhage are rare; a cystic variant has been described. Malignant PanNET may infiltrate adjacent organs and metastasize to regional lymph nodes. Distant metastases occur late and may affect different organs, such as liver, lung and bone. Histologically, several growth patterns (trabecular, nesting, gyriform, glandular, solid) are seen (**Fig. 2**A). The cells are monotonous and often display granular cytoplasm and granular chromatin, known as "salt and pepper" chromatin. Functioning PanNET, including insulinomas, glucagonomas, somatostatinomas, gastrinomas, VIPomas and others may become apparent due to clinical syndromes caused by hormone hypersecretion. PanNET are classified into three groups (G1–3) according to their Ki-67 or mitotic index (**Table 3**). Stage and grade have emerged as the most potent predictive factors in most studies. After surgical resection, the prognosis of PanNET is usually better than that of PDAC.

PanNEC

Pancreatic neuroendocrine carcinomas account for 2%–3% of PanNEN and are more frequently localized in the pancreatic head. They are usually large, whitish masses with ill-defined borders and areas of necrosis and hemorrhage. In the majority of cases distant metastases are present at diagnosis. Histologically, a small cell and a large cell variant of PanNEC exist. NEC are by definition G3 with a Ki-67 index of usually $>50\%$. Survival ranges from 1 month to a year, despite occasional initial favorable response to chemotherapy.

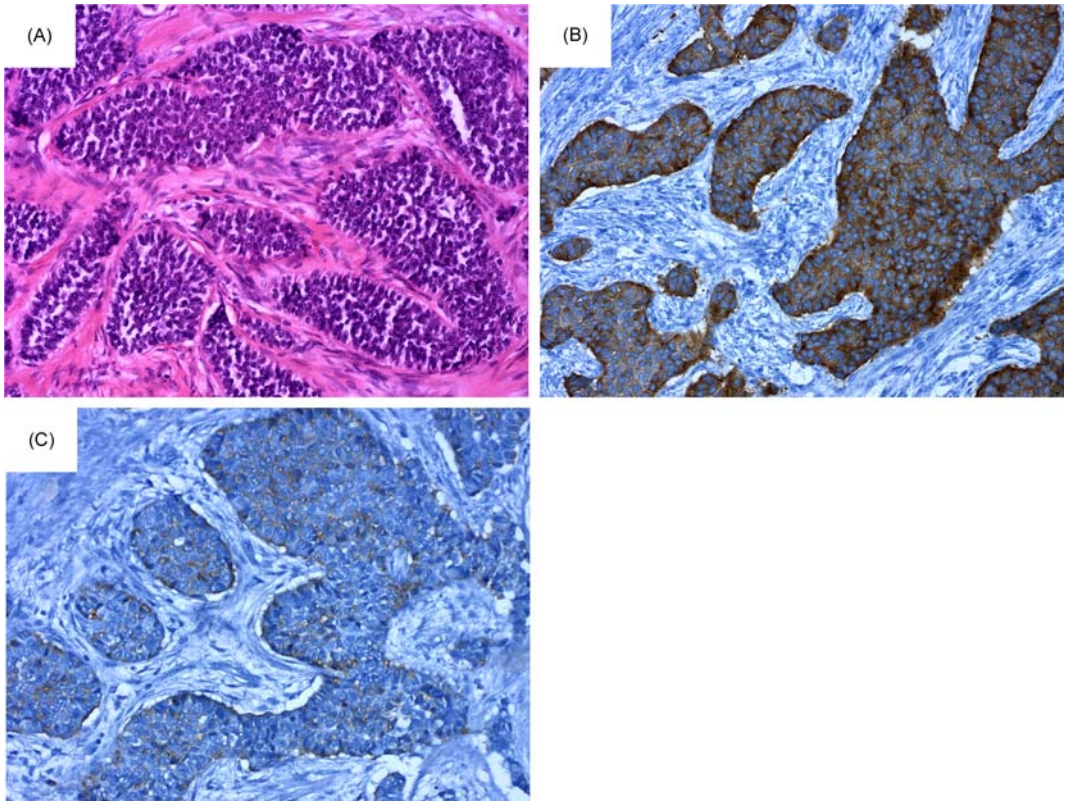

Fig. 2 *Morphology of pancreatic neuroendocrine neoplasms.* (A) Well-differentiated NET, G2, displaying an insular growth pattern and consisting of a monotonous cell population. Note the absence of nucleoli in the tumor cells (HE, 10×). (B) Immunostaining for synaptophysin (10×). (C) Immunostaining for chromogranin A (10×).

Table 3 Grading of PanNEN

	G1		*G2*		*G3*	
Entity	Ki67	Mitotic count	Ki67	Mitotic count	K67	Mitotic count
NET	<3%	0–2/10 HPF	3–20%	3–20/HPF	>20%[a]	>20/10 HPF
NEC	–		–		>20%[b]	>20/10 HPF

HPF = high power field.
[a]Usually max. 35%
[b]Usually >50%.
Adapted from Lloyd, R. V., Osamura, R. Y., Klöppel, G., Rosai, J. (Eds.) (2017). WHO/IARC classification of tumours. WHO classification of tumours of endocrine organs. IARC Press: Lyon.

MiNEN

As stated above, mixed neoplasms composed of a ductal or acinar cell adenocarcinoma and a neuroendocrine neoplasm are defined by the presence of each component accounting for at least 30% of the tumors. Each component is graded separately.

Immunohistochemistry

Immunohistochemistry is essential for the diagnosis and further classification of PanNEN. Immunostains for chromogranin A and synaptophysin (Fig. 2B–C) define the neuroendocrine subtype; Ki-67 establishes the grade. Expression of chromogranin A is usually lost in NEC. Immunohistochemistry using antibodies specific for the different hormones assists in subtyping PanNET. The use of an epithelial marker (cytokeratin) is necessary to distinguish PanNET from other pancreatic tumors with similar morphology and at least partial synaptophysin expression (solid-pseudopapillary neoplasms, paragangliomas) and is very useful in poorly differentiated neoplasms. Immunostains are essential for the diagnosis of MiNEN as well. The transcription factor islet-1 can be used in the differential diagnosis of metastatic NEN, but it cannot usually be detected in PanNEC. PanNET usually expresses somatostatin receptors (SSTR2A and SSTR5), whereas this expression is lost in NEC.

Genetics of Pancreatic Cancers

Genetics of PDAC

During recent years, next generation sequencing (NGS), which enables large-scale whole-genome analysis, has become a standard tool also in pancreatic cancer research. Multiple genetic aberrations that occur during the development and progression of PDAC have been found in various whole genome screens. In 2008, one of the first global genomic screens in pancreatic cancer revealed an average of 63 genetic alterations (mostly point mutations), which result in an activation of multiple core pathways (e.g., KRAS-, TGF-β-, Wnt-signaling). However, alterations in four genes are found in most cases of PDAC: *KRAS*, *CDKN2A*, *TP53* and *SMAD4* (Table 4).

KRAS is part of the canonical RAS family (KRAS, NRAS and HRAS), which consists of small GTPases switching from an active state (GTP-bound) to an inactive state (GDP-bound) via guanine nucleotide exchange factors (GDP -> GTP exchange) and GTPase-activating proteins (GTP-hydrolysis). In PDAC, the frequency of *KRAS* mutation is about 95%, whereas *NRAS* and *HRAS* mutations are almost nonexistent. Most common *KRAS* mutations are located at codon 12 of exon 2 (e.g., $KRAS^{G12D}$, $KRAS^{G12V}$, $KRAS^{G12R}$) and result in constitutive activation of the protein. Via multiple signal transduction cascades, persistently active KRAS promotes activation of transcriptional programs (e.g., proliferation, metabolism, immune evasion, migration), which facilitate cancer development and progression. Evolution of PDAC may occur via gradual disease progression with stepwise accumulation of additional mutations of *CDKN2A*, *TP53* and *SMAD4* or by a sudden event via genomic instability/chromothripsis. CDKN2A/p16INK4 negatively regulates proliferation of normal cells through interaction with the cyclin dependent kinases CDK4 and CDK6. Epigenetic silencing or loss of *CDKN2A* is found in most PDAC patients. The "guardian of the genome" *TP53* initiates death of DNA damaged cells before they become cancerous. Loss of the tumor suppressor *SMAD4/DPC4* (deleted in pancreatic cancer, locus 4) results in disruption of TGF-β signaling, which generally suppresses growth of epithelial cells.

Molecular Subtyping of PDAC

In recent years, single marker-based subtyping of PDAC has been replaced by large scale genomic- and transcriptomic data-based studies and is now widely accepted for pancreatic cancer stratification. Four main molecular subtypes have been described, based on gene expression profiles or on the distribution of structural chromosomal rearrangements. One of the first studies in 2011 by Collisson et al., performed on microdissected specimens and human and murine cell lines, described three subtypes of pancreatic cancer: the classical, the quasi-mesenchymal and the exocrine-like subtype. This classification was based on differences in subtype-specific gene expression profiles. The classical subtype was characterized by the expression of adhesion-associated and epithelial genes; the quasi-mesenchymal subtypes was characterized by mesenchyme-associated genes, whereas digestive enzyme genes were strongly expressed in the exocrine-like subtype. Recently, Bailey et al. identified four subtypes of PDAC using whole exome sequencing and copy number variation (CNV) analysis. They identified a squamous subtype similar to the quasi-mesenchymal subtype of Collisson et al., and an aberrantly differentiated endocrine exocrine (ADEX) subtype, which recapitulates characteristics of the exocrine subtype of Collisson et al. In addition, Bailey et al. identified a pancreatic progenitor and an immunogenic subtype of PDAC. Genes like *PDX1*, which are involved in early pancreatic development, were characteristic for pancreatic progenitor PDAC subtype. In the immunogenic subtype, immune networks, such as acquired immune suppression pathways were found to be upregulated. Besides transcriptome profiling and gene set expression analysis, subtyping of PDAC has been performed also based only on copy number variations. Using this approach, Waddel et al. identified four subtypes of PDAC: stable (≤ 50 structural variation events), locally rearranged, scattered and unstable (>200 structural variation events). Inactivation of DNA maintenance genes (e.g., *BRCA1*, *BRCA2*, *PALB2*) and a high grade of genomic instability with defects in DNA damage repair were observed in the unstable subtype. Evaluation of these subtypes revealed possible targets for new therapeutic interventions. As an example, the unstable subtype described by Waddel et al. may be sensitive to DNA damaging agents such as PARP-inhibitors. For the classical and

Table 4 Top 10 mutated genes in PDAC

COSMIC (n = *5408*)		Bailey et al. (2016) (n = *384*)		*TCGA* (n = *150*)	
Gene ID	*(%)*	*Gene ID*	*(%)*	*Gene ID*	*(%)*
KRAS	70.0	*KRAS*	89.8	*KRAS*	90.7
TP53	44.0	*TP53*	66.1	*TP53*	69.3
SMAD4	15.0	*SMAD4*	22.5	*SMAD4*	24.7
CDKN2A	13.0	*CDKN2A*	18.5	*CDKN2A*	14.7
GNAS	6.0	*ARID1A*	7.6	*OBSCN*	7.3
LRP1B	5.0	*SYNE1*	7.1	*FLG*	6.7
ARID1A	4.0	*LRP1B*	5.7	*GNAS*	6.7
RNF43	4.0	*RNF43*	5.5	*ADAMTS16*	6.0
KMT2C	4.0	*KMT2C*	5.5	*LRP1B*	6.0
TGFBR2	3.0	*FLG*	5.0	*FAT3*	6.0

the quasi-mesenchymal subtype of PDAC, patients drug resistance markers, such as cytochrome P450 3A5 (CYP3A5), may be predictive for therapeutic efficacy.

Genetics of Special PDAC Subtypes

Hepatoid carcinoma

Hepatoid carcinoma is a rare subtype of PDAC, which is not associated with *KRAS* mutation. Amplicon sequencing of 600 cancer associated genes revealed mutations in *NOTCH1* and *BAP1*, a BRCA1-associated protein involved in DNA damage repair. In a pancreatic insertional mutagenesis screen in mice, based on a conditional *piggyBac* transposition system (*Pdx1-Cre; LSL-Kras^{G12D}; Rosa26^{LSL-PB}; ATP1*), the hepatoid variant of PDAC was significantly associated with *FIGN* insertions, which lead to its overexpression. *FIGN* insertions were observed in 11 of 15 hepatoid and 3 of 34 nonhepatoid tumors. Fign is part of the superfamily of AAA-ATPases, which are involved in various cellular processes, such as vesicle-mediated transport, microtubule regulation and proteasome function. These findings may help to identify this rare subtype of PDAC.

Medullary carcinoma

Medullary carcinoma has a low prevalence of somatic mutations. Most often observed aberrations are microsatellite instability and a loss of the expression of one of the DNA mismatch repair proteins, Mlh1 and Msh2. Alterations of the *KRAS* gene have been found in 31% of the analyzed cases. In principle, patients with medullary carcinoma are more likely to have a family history of cancer with deficiencies of DNA repair.

Hereditary Pancreatic Cancer

Through unbiased whole-exome sequencing of familial pancreatic cancer (FPC) patients ($n = 96$), *BRCA2* was found to be the most commonly and its binding partner *PALB2* the second most commonly mutated gene in hereditary pancreatic cancer. In addition to *BRCA2* and *PALB2*, both involved in double-strand break repair and homologous recombination, *ATM* gene mutations predispose some families to pancreatic cancer. ATM (serine/threonine kinase ataxia telangiectasia mutated) coordinates DNA double-strand break repair and is associated with an increased risk of cancer, particularly lymphoma and leukemia. Other known predisposition genes, such as *CDKN2A*, *TP53*, *STK11*, *MLH1*, *MSH2*, *MSH6 and PMS2* are also involved in DNA double strand or DNA mismatch repair.

Genetics of Other Cancers of the Exocrine Pancreas

Acinar cell carcinoma (ACC)

The most common gene alteration in ACC is found in the *APC* gene. *APC* is one of the most frequently mutated genes in colorectal cancer. By promoting degradation of the Wnt-key component β-catenin, APC negatively regulates the Wnt-signaling pathway. The most frequent alterations are gene loss (48%) or gene/promoter hyper-methylation (56%) while *APC* mutations are relatively uncommon (7%). In about 23% of ACC, rearrangements of *BRAF* and *RAF1* have been described, with, *SND1-BRAF* as most prevalent fusion, which results in activation of the MAPK pathway. Although contradictory results have been published, recent sequencing analysis revealed in 13% of 44 patients a mutation in the *TP53* gene. Whereas mutations of *KRAS*, *CDKN2A* are almost not present in this rare tumor type, *SMAD4/DPC4* mutations are found in ACC cases. In a recent study, whole exome sequencing of 73 ACC and 34 normal pancreatic tissue samples revealed that the four tumor suppressor genes *ID3*, *ARID1A*, *APC*, and *CDKN2A* are altered or downregulated in the vast majority of the analyzed ACC specimens. Alterations of the genes *CDKN2A*, *APC* and *ID3* are involved in cell cycle progression, for instance by interference with CDK4/6. Also, mutational signatures associated with defective DNA repair have been identified.

Pathology and Genetics of PDAC Precursors

Pancreatic Intraepithelial Neoplasms (PanIN)

Pancreatic intraepithelial neoplasms (PanIN), microscopic (<0.5 cm) ductal lesions, are probably the best-known precursors of PDAC. Natural history and step-wise progression to invasive carcinoma of PanIN, as well as underlying molecular alterations, have been studied extensively in transgenic mouse models. Like PDAC, PanIN lesions are more often located in the head than in the body-tail of the organ. While they are common findings in pancreas resection specimens both with and without invasive PDAC, the frequency of PanIN in the normal pancreas is lower than that in pancreas specimens with chronic pancreatitis and PDAC (16% vs. 60% vs. 82%, respectively). For high-grade PanIN, these numbers are 0%, 4% and 40%. PanIN lesions are especially common in patients with a strong family history of PDAC and then are often multifocal.

PanIN are characterized by a flat mucinous columnar or cuboidal epithelium in the earliest stage, but display an increasingly papillary architecture during the progression from low-grade to high-grade dysplasia (Fig. 3A–B). A two-tiered system in low- and high-grade PanIN (Table 1) has recently replaced the traditional three-tiered classification (PanIN 1–3). This new classification system is more in line with the clinical consequences: while the significance of PanIN with low-grade dysplasia for the development

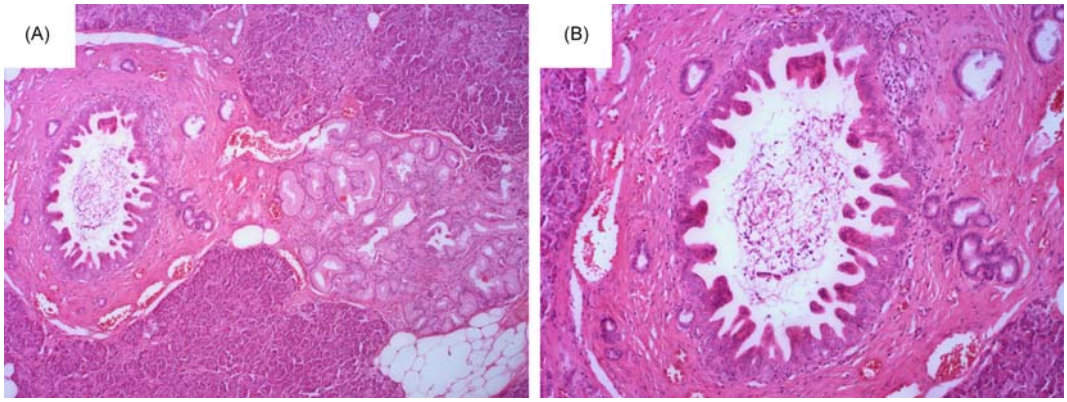

Fig. 3 *Morphology of PanIN.* (A) PanIN. Low (right) and high-grade (left) PanIN (HE, 10×). (B) Detail of high-grade PanIN with micropapillary architecture and nuclear atypia (HE 20×).

of PDAC is still controversial, PanIN with high-grade dysplasia is a direct precursor of PDAC and therefore warrants resection. Immunohistochemically, MUC5A is regularly expressed in both low-grade and high-grade PanIN lesions, while MUC1 is usually only expressed in high-grade PanIN.

A single *KRAS* mutation ($KRAS^{G12D}$) is sufficient for the development of PDAC via a precursor lesion in genetically engineered mice, which provides an argument in favor of the notion that mutant *KRAS* alone is sufficient to drive PDAC also in humans. Indeed, in more than 84% of microdissected human low-grade PanIN lesions, only a *KRAS*- mutation was identified. Sequencing data of 66 PanIN lesions in the COSMIC (catalogue of somatic mutations in cancer) database showed *KRAS* as the most frequently mutated gene, with most cases bearing only a *KRAS* mutation. In addition to *KRAS* mutations, alterations in *CDKN2A*, *GNAS* and *BRAF* have been observed in low grade PanIN. Alterations of *TP53* or *Smad4* are extremely rare in PanIN.

Intraductal Papillary Mucinous Neoplasm (IPMN)

Intraductal papillary mucinous neoplasms (IPMN) comprise a heterogeneous group of mucin-producing epithelial tumors with papillary architecture in the pancreatic duct system, which lead to cystic dilation of the involved duct segments (Fig. 4A). IPMN are most commonly located in the pancreatic head and are macroscopic lesions, by definition with a diameter of at least 1 cm. According to their site of origin, IPMN are distinguished in main duct-type IPMN, branch duct-type IPMN and combined-type (or mixed-type) IPMN.

In addition to these macroscopic subtypes, IPMN are classified by histomorphology and immunohistochemistry into four distinct histologic subtypes: gastric-type IPMN, intestinal-type IPMN, oncocytic-type IPMN and pancreatobiliary-type IPMN (Table 5). Main duct-type IPMN are usually intestinal-type or, less frequently, oncocytic-, pancreatobiliary- or gastric-type IPMN, while branch-duct type IPMN are most commonly gastric-type.

Gastric-type IPMN mimic gastric foveolar epithelium: they form short, wide papillae, which consist of columnar epithelial cells containing large amounts of mucin (Fig. 4B–D). By immunohistochemistry, gastric-type IPMN are characterized by expression of MUC5A; but not of MUC2 and MUC1 (Fig. 4C). Gastric-type IPMN have a similar, if not identical, phenotype as PanIN lesions, which complicates the differentiation between small gastric-type IPMN and large PanIN for lesions ranging between 0.5 and 1 cm. These lesions are reported descriptively, although the term "incipient IPMN" is used if molecular or morphologic features typical of IPMN but absent in PanIN are present, (e.g., *GNAS* mutation or villous, pancreatobiliary or oncocytic differentiation of the epithelium).

In contrast, intestinal-type IPMN are characterized by the formation of papillae similar to those of villous adenoma of the colon. The columnar epithelial cells have elongated basal nuclei and apical mucin and form villous structures protruding into the duct lumen (Fig. 4E). Intestinal-type IPMN express MUC5A, MUC2 and CDX2 (Fig. 4F–G), but show no expression of MUC1.

The papillae of pancreatobiliary-type IPMN are complex branch-like structures, consisting of cuboid cells with round nuclei, often displaying prominent nucleoli, and with considerably less mucin than gastric-type or intestinal-type IPMN epithelium (Fig. 4H). The pancreatobiliary-type IPMN epithelium stains positive for MUC5A and MUC1 (Fig. 4I), but is negative for MUC2 (Fig. 4G).

Oncocytic-type IPMN are similar to pancreatobiliary-type IPMN in morphology and marker profile. In analogy to pancreatobiliary-type IPMN, they consist of cuboid epithelial cells forming complex branched papillae. However, these cells show marked cytoplasmic eosinophilia and the round nuclei often have one single prominent eccentric nucleolus. Oncocytic-type IPMN focally express MUC1 and may show MUC2 expression in goblet cells as well as a diffuse expression of MUC5A and MUC6.

The degree of dysplasia in IPMN varies greatly, which calls for extensive sampling of these lesions. They are classified as IPMN with low-grade or high-grade dysplasia depending on the highest grade of dysplasia that can be found in the lesion. While all types

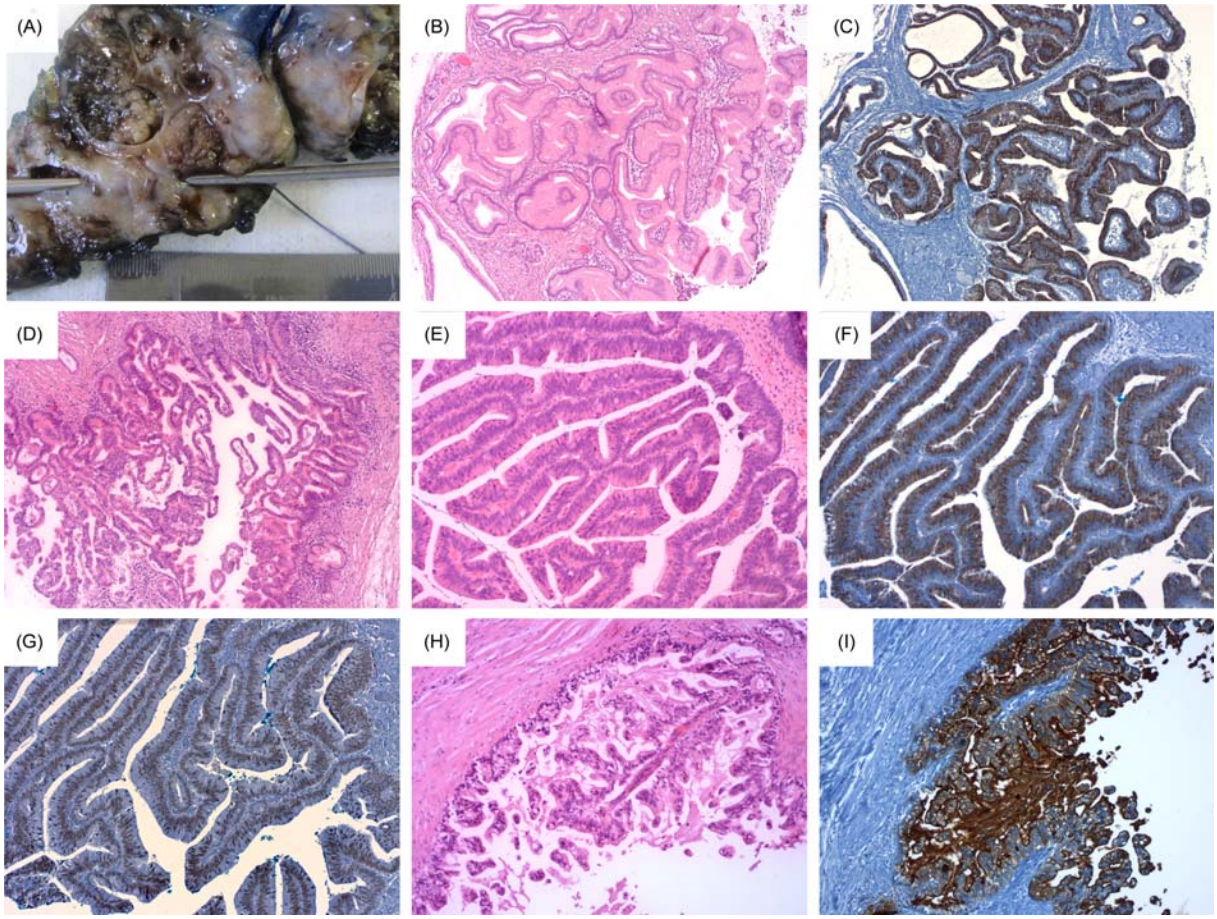

Fig. 4 *Morphology of IPMN.* (A) Gross aspect of an IPMN displaying cystic dilation of pancreatic ducts filled with papillary structures and mucus. (B) Gastric-type IPMN, low-grade, displaying a gastric-foveolar phenotype and basally located nuclei (HE, 4×). (C) MUC 5AC expression in gastric-type IPMN, low-grade (4×). (D) Gastric-type IPMN, high-grade, with irregular architecture and hyperchromatic nuclei (HE, 4×). (E) Intestinal-type IPMN, with moderate dysplasia (low-grade) with villous-like papillae (HE 10×). (F) MUC 2 expression in intestinal IPMN (10×). (G) Nuclear CDX-2 expression in intestinal IPMN (10×). (H) Pancreatobiliary IPMN, high-grade, with complex papillary and cribriform architecture (HE 10×). (I) MUC 1 expression in pancreatobiliary IPMN (10×).

Table 5 Morphology and immunohistochemical profile of intraductal neoplasms of the pancreas

Intraductal neoplasm	*Morphology*	*Dysplasia*	*MUC1*	*MUC2*	*MUC5A*	*MUC6*	*Others*
IPMN gastric	Predominantly papillary, mucinous	Low- to high-grade	−	−	+	−	−
IPMN intestinal	Predominantly papillary, mucinous	Low- to high-grade	−	+	+	−	CDX2+
IPMN pancreatobilliary	Predominantly papillary, mucinous	Low- to high-grade	+	−	+	(+)	−
IPMN oncocytic	Predominantly papillary, mucinous	Low- to high-grade	Focal	Focal (goblet cells)	+ (diffuse)	+ (diffuse)	−
ITPN	Predominantly tubular, nonmucinous	High-grade	+	−	−	+	CK7+ CK19+

of IPMN can progress to invasive PDAC, they differ both in their risk of progression and in the resulting type of carcinoma. It has been shown that gastric-type IPMN have the least malignant potential (invasive carcinoma in about 6% of cases), while oncocytic-type and intestinal-type IPMN have a moderate malignant potential (invasive carcinoma in about 25% or 34% of cases, respectively), and pancreatobiliary-type IPMN harbor the highest malignant potential with an invasive component in about 58% of cases. The subtype of the associated invasive carcinoma is dependent on the IPMN subtype. Thus, intestinal-type IPMN usually result in

colloid (mucinous) carcinomas, whereas pancreato-biliary and gastric type IPMN usually progress to classical tubular PDAC. Rare oncocytic carcinomas may arise from oncocytic IPMN.

These profound differences in morphology and immunohistochemical marker profile, together with the different molecular pathways involved into the pathogenesis of different IPMN, suggest that the four IPMN types represent different tumor entities more than a mere morphological spectrum. *GNAS* (Guanine nucleotide-binding protein G(s) subunit alpha isoforms short) mutations are found in PanIN lesions, but are more frequently present in IPMN. In approximately two thirds of IPMN cases, the hotspot mutation in codon 201 (mostly $GNAS^{R201C}$ or $GNAS^{R201H}$) has been identified. This mutation leads to constitutive activation of the *GNAS* encoded protein GS-alpha (adenylate cyclase stimulator), which induces cAMP (cyclic adenosine monophosphate) signaling to activate PKA (protein kinase A) and RAS stimulation via RAPGEF2 (Rap guanine nucleotide exchange factor 2). In pancreatic- and biliary-intraductal papillary neoplasms mutant *GNAS* and mutations in *RNF43* (ring finger protein 43), which are correlated with the downregulation of RNF43, occur together. The tumor suppressive ubiquitin E3 ligase RNF43 induces Frizzled ubiquitination, which leads to suppression of Wnt signaling. Hence, loss of RNF43 function promotes Wnt signaling, enhances cellular proliferation, and results in neoplastic transformation.

Accumulation of additional mutations in *TP53* and *SMAD4* (and *CDKN2A*), which can be found in high grade IPMNs, are responsible for progression to highly invasive carcinoma with a high metastatic propensity.

Gene alterations of the tumor suppressors *ARID1A*, *ARID1B*, *BRG1*, *PBRM1* and *SMARCA2*, which are all part of the SWI/SNF chromatin remodeling complex, have been identified as central players in PDAC. Especially loss of *BRG1* function seems to be associated with IPMN pathogenesis. A high incidence of IPMN has also been observed in patients with a somatic mutation of the *STK11* gene (responsible for Peutz-Jeghers syndrome). The Serine/threonine-protein kinase STK11 mediates *TP53* dependent cell death and controls the activity of AMPK (AMP-activated protein kinase). Certain mutations are associated with a particular IPMN subtype: *GNAS*-only mutations have a higher frequency in the intestinal type of IPMNs, whereas *KRAS*-only mutations are more frequent in the gastric and the pancreatobiliary IPMN subtypes. Of *GNAS* wild-type gastric-type IPMN, 43% were associated with PDAC, whereas in only 9% of cases with nongastric-type IPMN PDAC was found. Very rare *BRAF* mutations have been found in the oncocytic type IPMN.

Intraductal Tubular-Papillary Neoplasms (ITPN)

Similar to IPMN, intraductal tubular-papillary neoplasms (ITPN) are macroscopic (≥ 1 cm in diameter) epithelial neoplasms of the pancreatic duct system (Fig. 5A). However, in contrast to IPMN, their epithelium is not or only minimally mucin-producing, and as a result ITPN present much less commonly as a cystic lesion.

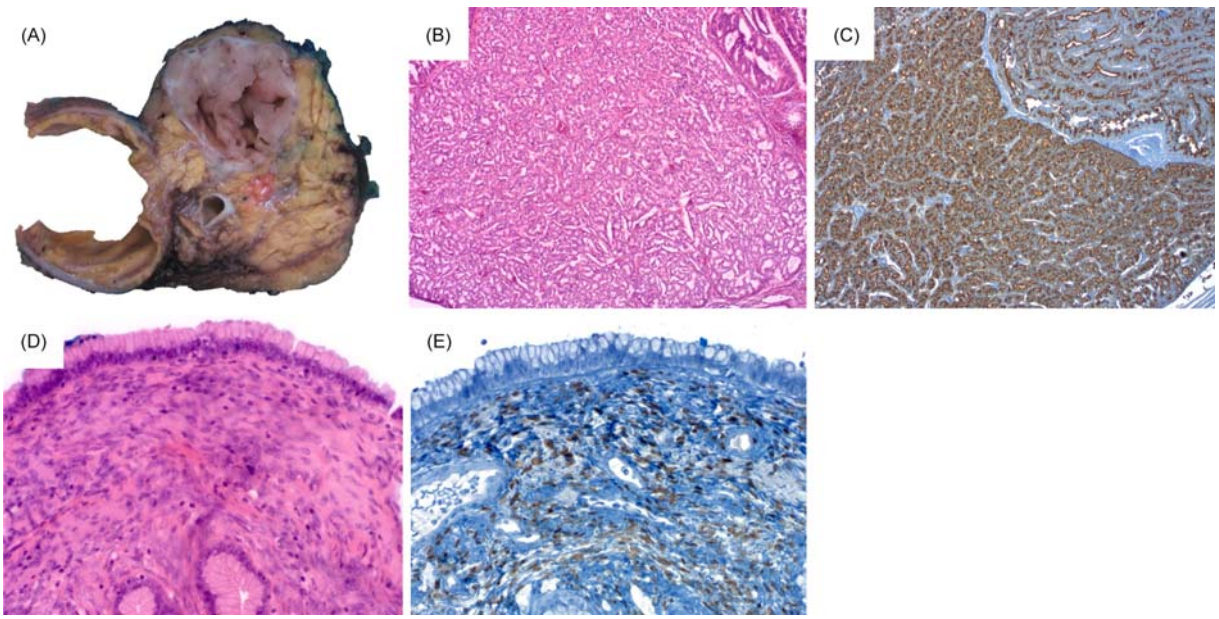

Fig. 5 Morphology ITPN and MCN. (A) Macroscopic aspect of ITPN without associated carcinoma. ITPN forms an intraductal soft, whitish and well-circumscribed mass. (B) Histology of ITPN showing back-to-back tubular structures and papillae (right) (HE, 4×). (C) Expression of MUC1 in ITPN with typical luminal pattern (4×). (D) Histopathology of MCN showing a cylindrical mucinous epithelium lying over a cellular stroma (HE, 20×). (E) Expression of the estrogen receptor in the stromal cells (20×).

Histomorphologically, the growth pattern of ITPN is predominantly tubular, although papillary components are also present. They form densely packed tubules, which can display a cribriform architecture and consist of cuboidal cells with round to oval nuclei, which usually show high-grade dysplasia and can contain comedo-like necrotic foci (Fig. 5B). By immunohistochemistry, ITPN express MUC1 and MUC6, similar to IPMN of the oncocytic subtype, but also show expression of CK7 and CK19 as well as absence of expression of MUC5A and MUC2 (Table 5). ITPN and IPMN are also different at the molecular level, for example, *KRAS* and *GNAS* mutations are distinctively less frequent to absent in ITPN compared to IPMN, which provides further support for the notion that ITPN and IPMN are indeed different entities.

More than half of the ITPN cases have been associated with an invasive carcinoma, larger tumor size and high Ki67 proliferation index being linked to invasiveness.

Mucinous Cystic Neoplasms (MCN)

Mucinous cystic neoplasms (MCN) are usually large uni- or multilocular thick-walled cysts, which are most often filled with mucinous fluid, but may also have a hemorrhagic or serous content. In high-grade MCN, the cysts may contain solid areas, mural nodules or papillary projections. MCN almost exclusively occur in the body or tail of the pancreas of women between 40 and 60 years, although rare cases in male patients have been reported.

The defining histomorphologic characteristics of MCN are mucinous columnar epithelium with an underlying dense *ovarian-like* stroma (Fig. 5D). The epithelial cells of MCN display various grades of dysplasia, even in one single lesion, warranting extensive sampling. Similar to the above-mentioned precursor lesions, MCN should be classified as MCN with low-grade or high-grade dysplasia according to the highest grade of dysplasia found in the lesion. As papillary epithelium may be present in MCN with high-grade dysplasia, the presence of *ovarian-like* stroma must be used to distinguish those lesions from IPMN. While the MCN epithelium expresses MUC5A and CEA, the spindle cells of the *ovarian-like* stroma express estrogen and progesterone receptors (Fig. 5E), smooth muscle markers like α-smooth muscle actin, desmin and vimentin, and can also undergo luteinization and show expression of α-inhibin, tyrosine hydroxylase and calretinin.

MCN can progress to invasive pancreatic cancer, usually invasive pancreatic adenocarcinoma of the classical (ductal) type. In a recent multicenter study, 12.6% of MCN cases were associated with invasive adenocarcinoma.

The most frequent alteration in mucinous cystic tumors is a mutation in *KRAS*. By whole-exome sequencing, mutations in the E3 ligase *RNF43* have been identified in mucinous cystic neoplasms. Hypermethylation of *CDKN2A*, which is commonly altered in PDAC, has been found in MCNs as well. *PIK3CA* mutations have been described in MCN lesions with very low frequency.

New Concepts Emerging From Genetically Engineered Mouse Models

PDAC has long been assumed to be of ductal origin due to its ductal phenotype and due to ductal differentiation of its precursor lesions. However, genetically engineered mouse models have challenged the ductal carcinogenesis model of pancreatic cancer. In these mouse models, activation of the *KRAS* oncogene induced PanIN lesions, faithfully resembling those in humans. While PanIN could be induced in adult acinar and other pancreatic cell types, they did not arise in ductal epithelial cells. Ductal epithelium therefore seems rather refractory to the induction of PanIN lesions, with the exception of early-stage PanIN-like lesions. Such findings have led to the emerging concept of pancreatic carcinogenesis through an acinar-ductal metaplasia-dysplasia sequence. According to this concept, cells of the centroacinar/acinar compartment undergo ductal metaplasia and then form PanIN, which then may progress to invasive pancreatic cancer. Newer data suggest that PDAC can even arise directly from centroacinar/acinar cells, without preceding PanIN lesions: in a $Kras^{G12D/+}$; $Ptf1a\text{-}Cre^{ex1/+}$ mouse model, atypical flat lesions (AFL) were identified as alternative precursor lesions of PDAC. Although they have a ductal phenotype, these lesions are found in the centroacinar/acinar compartment, which suggests that they are a result of acinar-ductal metaplasia. Adult acinar cells have recently been confirmed as cells of origin of AFL. Histomorphologically, AFL are small tubular lesions, which consist of flat to cuboidal cells with cytologic atypia, expressing CK19 and MUC1 by immunohistochemistry, and surrounded by reactive stroma (Fig. 6). Lesions resembling murine AFL have been reported in patients with familial pancreatic cancer, which is an umbrella term for patients with increased life-time risk for pancreatic cancer associated with various genetic syndromes. This confirms the clinical relevance of the ADM/AFL carcinogenesis model of pancreatic cancer.

Prospective Vision

Conventional morphological methods are essential for the diagnosis and classification of pancreatic cancers and deliver fundamental information for patient management. New knowledge coming from genetically engineered mouse models has revealed possible alternative pathways for pancreatic cancer development, which might be exploited for the identification of better biomarkers and, possibly, screening strategies. Additionally, high-throughput molecular methods have contributed significantly to the discovery of molecular signatures that define PDAC subtypes, which may in turn be associated with different therapeutic approaches. The next big challenge will be to integrate this large amount of information in feasible clinical, diagnostic and therapeutic algorithms for better patient care.

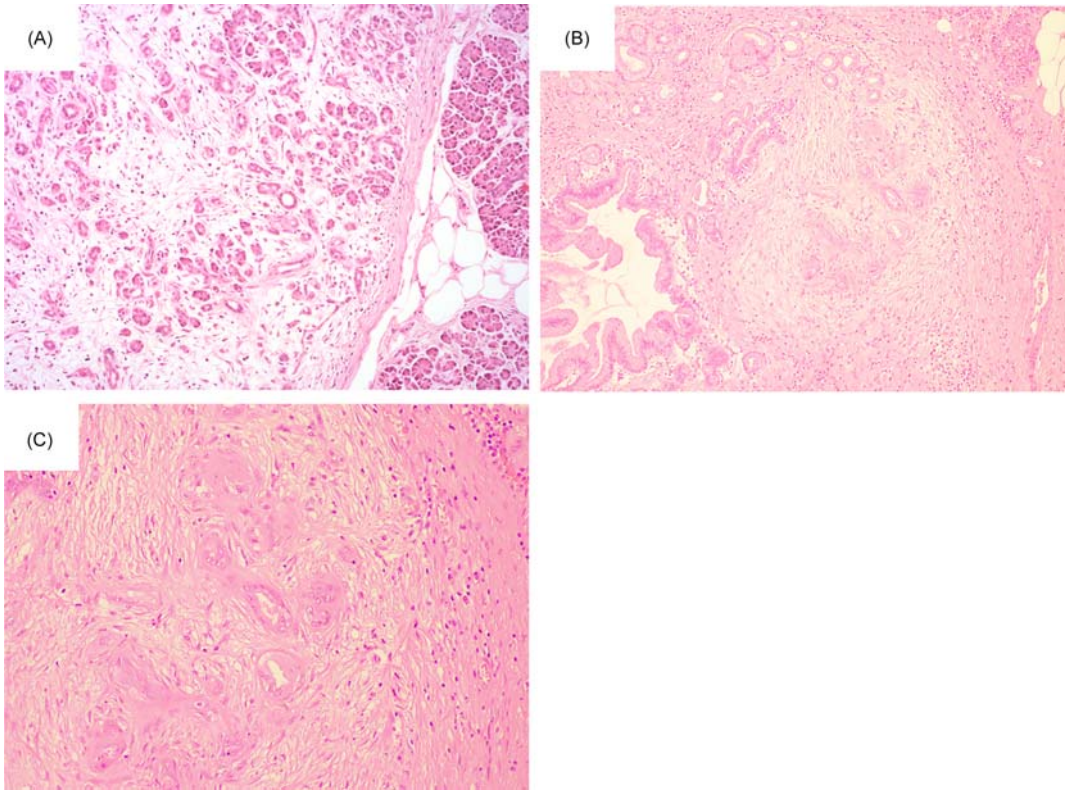

Fig. 6 *Morphology of ADM and AFL*. (A) Area of acinar-ductal metaplasia (ADM) characterized by ductuli and residual acini without cytologic atypia surrounded by loose stroma (HE, 10×). (B) Atypical flat lesion (AFL) characterized by irregularly formed ductular structures surrounded by myxoid stroma (HE, 10×). (C) Detail of cytologic atypia of AFL (HE, 20×).

See also: Pancreatic Cancer: Diagnosis and Treatment.

References

Bailey, P., Chang, D.K., Nones, K., et al., 2016. Genomic analyses identify molecular subtypes of pancreatic cancer. Nature 531 (7592), 47–52.
Brierley, J.D., Gospodarowicz, M.K., Wittekind, C. (Eds.), 2016. TNM classification of malignant tumours, 8th edn. Wiley Blackwell.

Further Reading

Basturk, O., Hong, S.M., Wood, L.D., et al., 2015. Baltimore consensus meeting. A revised classification system and recommendations from the Baltimore consensus meeting for neoplastic precursor lesions in the pancreas. The American Journal of Surgical Pathology 39, 1730–1741.
Bijlsma, M.F., Sadanandam, A., Tan, P., et al., 2017. Molecular subtypes in cancers of the gastrointestinal tract. Nature Reviews. Gastroenterology & Hepatology 14, 333–342.
Cancer Genome Atlas Research Network, 2017. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. Cancer Cell 32, 185–203.e13.
Esposito, I., Segler, A., Steiger, K., et al., 2015. Pathology, genetics and precursors of human and experimental pancreatic neoplasms: An update. Pancreatology 15, 598–610.
Giroux, V., Rustgi, A.K., 2017. Metaplasia: Tissue injury adaptation and a precursor to the dysplasia-cancer sequence. Nature Reviews. Cancer 17, 594–604.
Hwang, C.I., Boj, S.F., Clevers, H., et al., 2016. Preclinical models of pancreatic ductal adenocarcinoma. The Journal of Pathology 238, 197–204.
Kidd, M., Modlin, I., Öberg, K., 2016. Towards a new classification of gastroenteropancreatic neuroendocrine neoplasms. Nature Reviews. Clinical Oncology 13, 691–705.
Kleeff, J., Korc, M., Apte, M., et al., 2016. Pancreatic cancer. Nature Reviews. Disease Primers 2, 16022.
Klimstra, D.S., Adsay, V., 2016. Acinar neoplasms of the pancreas-A summary of 25 years of research. Seminars in Diagnostic Pathology 33, 307–318.
Klöppel, G., Basturk, O., Schlitter, A.M., et al., 2014. Intraductal neoplasms of the pancreas. Seminars in Diagnostic Pathology 31, 452–466.
Pittman, M.E., Rao, R., Hruban, R.H., 2017. Classification, morphology, molecular pathogenesis, and outcome of premalignant lesions of the pancreas. Archives of Pathology & Laboratory Medicine 141, 1606–1614.
Singhi, A.D., Klimstra, D.S., 2018. Well-differentiated pancreatic neuroendocrine tumors (PanNETs) and poorly differentiated pancreatic neuroendocrine carcinomas (PanNECs): Concepts, issues and a practical diagnostic approach to high-grade (G3) cases. Histopathology 72, 168–177.
Storz, P., 2017. Acinar cell plasticity and development of pancreatic ductal adenocarcinoma. Nature Reviews. Gastroenterology & Hepatology 14, 296–304.
Welinsky, S., Lucas, A.L., 2017. Familial pancreatic cancer and the future of directed screening. Gut and Liver 11, 761–770.

Papillomaviruses☆

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Glossary

Epigenetic modifications Posttranslational modifications of the DNA or DNA bound proteins, referred to as histones, that determine transcriptional competence of chromosomal loci.

Episome An extrachromosomal, autonomously replicating genetic element.

Pap-smear The Papanicolaou-smear is a procedure where cells derived from a cervical smear are microscopically evaluated for cytological abnormalities. This procedure has dramatically decreased the incidence and mortality of cervical cancer.

Posttranslational modifications In many proteins, certain amino acids are subject to modification by a number of chemical reactions including phosphorylation, acetylation, and ubiquitination. These reversible enzymatic reactions alter the biochemical and functional activities of a protein.

Papillomaviruses

Papillomaviruses are small nonenveloped viruses with circular double-stranded DNA genomes of approximately 8000 base pairs in size. They preferentially infect squamous epithelial cells and have been isolated from a wide range of animal species, from fish to birds to humans. Papillomaviruses are species-specific and there is little evidence for transmission from one species to another. A schematic outline of the HPV16 genome is represented in Fig. 1A. All the major open reading frames (ORFs) are located on one of the two DNA strands. They are overlapping and encoded in each of the three possible reading frames. Papillomaviruses contain less than 10 ORFs; early and late ORFs are identified by the letters, E and L, respectively, followed by a number. The lowest number designates the longest ORF. Early ORFs encode nonstructural, regulatory proteins, whereas the two late ORFs, L1 and L2, encode the major and minor viral capsid proteins, respectively. Viral gene expression involves extensive splicing, and some early viral mRNAs contain coding information derived from more than one ORF. In addition to the early and late coding sequence, there is an approximately 1000 bp portion of the genome that does not specify major ORFs. This region is referred to as the long control region (LCR) and contains regulatory DNA elements, including the origin of viral genome replication and binding sites for viral E2 transcription factors as well as cellular transcription factors (Fig. 1A).

Regulation of Papillomavirus Replication and Gene Expression

Like other viruses, papillomaviruses are obligate intracellular parasites that heavily rely on host cellular replication factors for synthesizing their genomes. Importantly, however, the papillomavirus viral life cycle is tightly coupled to the process of epithelial cell differentiation. Papillomaviruses initially infect undifferentiated, proliferative basal epithelial cells, which are located in the deepest, basal layer of the skin. Papillomaviruses gain access to these cells through microscopic abrasions or at squamo-columnar transformations zones, where basal-like epithelial cells are exposed and readily accessible for infection. The viral entry receptor remains unknown but upon reaching the nucleus, viral genomes replicate to a low copy number. Basal epithelial cells can remain persistently infected for years or decades. High-level synthesis of viral genomes, expression of viral capsid proteins, and formation of progeny virus, however, are confined to cells within the uppermost, differentiated layers of the skin. Infectious viral particles are shed together with the dead cells in the uppermost layers of the skin.

Differentiated epithelial cells no longer undergo DNA synthesis, and thus they do not express the cellular enzymes that are necessary to support viral genome replication. To complete their infectious life cycles, papillomaviruses have evolved to prevent cell cycle withdrawal during epithelial cell differentiation. The strategies that some papillomaviruses have evolved to accomplish this, form the basis for their oncogenic activities.

The E1 and E2 proteins are key to viral replication and genome maintenance. E1 is an origin binding protein with ATPase and DNA helicase activity. Papillomavirus origins of replication are flanked by E2 binding sites and E2 associates with E1 to allow for efficient E1 binding to the origin. E2 proteins are also major viral transcriptional regulators. E2 proteins bind to $ACCN_6GGT$ palindromic sequences in the viral genome to modulate viral gene expression. Like many transcription factors, E2 proteins are functional dimers. Each monomer consists of an amino terminal transcriptional activation domain, followed by a flexible hinge and carboxyl

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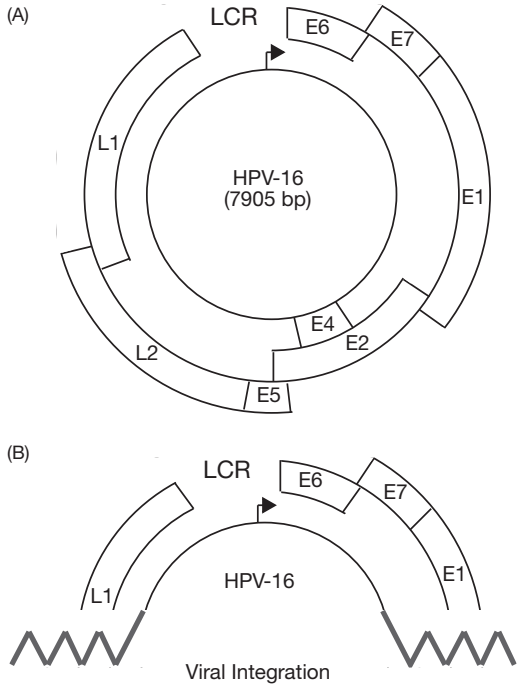

Fig. 1 (A) Schematic map of the genome of the high-risk alpha genus HPV16. LCR denotes the long control region, which harbors sequence elements important for the regulation of viral replication and transcription. Only one strand of the double stranded circular DNA genome is transcribed, open reading frames are encoded in all three different open reading frames. The early (E) genes each have functions relevant to the replication of the virus, the two late (L) genes encode the viral capsid proteins. The major early promoter that drives expression of early genes including E6/E7 is indicated by an *arrow*. (B) Integration of the viral genome into the host chromosome is a frequent hallmark of malignant progression. Integration retains expression of E6 and E7 and frequently disrupts expression of the E2 gene. Since E2 encodes a transcriptional repressor of E6/E7 expression, disruption of E2 as a consequence of viral genome integration causes dysregulated E6/E7 expression, which drives malignant progression.

terminal dimerization/DNA binding domains. E2 dimers associate with mitotic chromatin through amino terminal sequences, thereby enabling viral genome partitioning during cell division. Many papillomaviruses encode multiple E2 species with different transcriptional activities. The bovine papillomavirus type 1, BPV1, for example, encodes a full length E2 protein that can act as a transcriptional activator, as well as two smaller proteins. One is generated by initiation at an internal methionine residue and encodes a small part of the transactivation domain, the hinge, and the carboxyl terminal dimerization/DNA binding domains. The second shorter E2 protein is generated by a splicing event where a small segment of the E8 ORF is fused to the E2 hinge and dimerization/DNA binding domains. This protein functions as a potent transcriptional repressor. Other papillomaviruses also encode multiple, functionally distinct E2 transcription factors. In addition, viral transcription is also modulated by cellular factors. Of particular importance are differentiation specific proteins that may trigger the early to late switch of viral gene expression. In addition to transcription the early to late switch of the viral life cycle is also regulated at the level of RNA splicing and/or stability.

Cancer-Associated Human Papillomaviruses (HPVs)

More than 300 human papillomaviruses (HPVs) have been identified and new types are regularly added to this list. HPVs are classified as genotypes and can be phylogenetically classified into genera. The best studied HPVs fall within the alpha and beta genera. Most alpha HPVs infect mucosal epithelia, whereas beta HPVs are best known to infect cutaneous epithelia. HPV infections are very common. The majority of HPVs cause benign hyperplastic epithelial lesions or warts that have a negligible propensity for malignant progression, and these HPVs are referred to as "low-risk" HPVs. In contrast, "high-risk" HPVs cause lesions that have a certain potential for carcinogenic progression. High-risk alpha HPVs include HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66, with HPV16 being the most abundant high-risk HPV. Most high-risk HPV associated lesions spontaneously regress and malignant progression is quite rare and often occurs years or decades after the initial infection. Nonetheless, a high-risk HPV infection represents a greater risk factor for cervical cancer development than cigarette smoking for lung cancer formation. Overall, approximately 5% of all human cancer are caused by high-risk alpha HPV infections.

HPV-associated cervical cancers generally represent nonproductive HPV infections. This means that while HPV proteins are expressed, no infectious virus is produced. The best-studied mechanism that generates a nonproductive infection is integration of viral genomic sequences into a host cellular chromosome (Fig. 1B) although other mechanisms, including DNA methylation

of the viral genome, can affect viral gene transcription and also cause nonproductive infections. Hence, while cancer formation is not part of the normal life cycle of high-risk HPVs, it is caused by dysregulated expression of viral proteins that play important roles for the viral life cycle.

Alpha HPV Associated Anogenital Tract Lesions and Cancers

Low-risk alpha HPVs cause genital warts (condylomata acuminata) and more than 90% are low-risk HPV positive. Genital warts very rarely progress to malignancy although on occasion they can become locally invasive and form the giant condylomata described by Buschke and Löwenstein. The most abundant low-risk HPVs are HPV6 and HPV11.

In contrast, high-risk HPVs cause squamous intraepithelial lesions (SILs), which are at risk for malignant progression. The most prominent human cancer type associated with high-risk HPV infection is cervical carcinoma. More than 99% of cervical cancers are high-risk HPV positive, and cervical cancer is a leading cause of cancer death in women. Worldwide, there were an estimated 528,000 new cases of cervical cancer worldwide with approximately 266,000 deaths in 2012, and in United States there were 12,820 cases/4210 deaths estimated in 2017. The most abundant high-risk HPVs in the United States are HPV16 and HPV18; they are detected in approximately 70% of all cervical carcinomas. Cervical carcinoma is a venereal disease and early onset of sexual activity and multiple sexual partners have now been firmly established as the most prominent major risk factors for the development of SIL and cervical cancer.

High-risk HPV infections in males can cause penile intraepithelial neoplasia (PIN), which can progress to penile carcinoma. Although penile carcinoma is more rare than cervical cancer (2120 cases/360 deaths estimated in the United States in 2017), the incidence of cervical and penile carcinoma correlates in many parts of the world. In addition, a large percentage of other anogenital tract carcinomas, including vulvar, vaginal, and anal cancers are also caused by high-risk HPV infections. Anal cancer rates have increased in recent years. The incidence of high-risk HPV-associated cancers is higher in HIV positive patients; even in those patients with restored immune function due to antiretroviral therapy.

Most high-risk HPV infections resolve spontaneously and malignant progression often occurs years or decades after the initial infection. This extended period between HPV infection and cancer formation provides a window of opportunity to detect these potentially premalignant lesions before they progress to invasive carcinoma. The most commonly used technique is a cytological examination of cervical cells, the Papanicolaou-smear ("Pap-smear"), although this technology is gradually replaced by, or at least combined with HPV typing. Widespread implementation of the Pap-smear procedure has dramatically decreased the incidence rate of cervical cancer in many countries, and it is possibly the most impressive illustration of the value of early detection in cancer prevention. In countries where Pap-smears are not routinely performed, cervical cancer remains a leading cause of cancer death in women. An anal Pap-smear has been developed and is used in populations that are high risk for anal cancer development.

Alpha HPV-Associated Oral Lesions and Cancers

The oral mucosa is also readily infected with alpha HPVs. Oral infections with low-risk HPVs can give rise to recurrent respiratory papillomatosis (RRP), a rare disease with juvenile or adult onset. Juvenile onset RRP may be caused by vertical transmission, that is, infection during delivery, whereas adult onset RRP may be a venereal disease. HPV associated lesions in RRP patients grow very rapidly and frequent surgeries are required to keep the airways clear. On rare occasions, RRP lesions undergo malignant progression and can spread to the lungs. This is often associated with mutations and rearrangements in the viral genome particularly sequences in the viral LCR. Benign oral papillomas and oral focal epithelial hyperplasias have also been connected to with low-risk alpha HPV6 and HPV11 infections. Moreover, there is evidence for oral infections with beta and gamma genus HPVs, although it is unclear whether such infections are associated with any disease.

Approximately 20% of head and neck squamous cell carcinomas (HNSCCs) are HPV-positive. Of these, oropharyngeal squamous cell carcinomas (OPSCCs) are particularly highly associated with HPVs; approximately 65% are HPV positive. HPV16 is detected in more than 90% of the HPV associated OPSCCs. HPV positive OPSCCs frequently occur in patients that are younger and lack classical oral cancer risk factors such as poor oral hygiene and tobacco and alcohol abuse and are more common in males than in females. Transmission may be by oral sex practices and deep kissing. The incidence of HPV positive OPSCCs is rapidly increasing in many industrialized countries. There is no procedure for early detection of these cancers. Even though HPV positive OPSCCs appear more aggressive, they more readily respond to radiation and chemotherapy than HPV negative OPSCCs.

Beta HPV-Associated Nonmelanoma Skin Cancers

Studies with patients afflicted by a rare skin disease, epidermodysplasia verruciformis (EV), provided the first evidence that HPVs can contribute to human carcinogenesis. EV is a rare disease that is frequently linked to mutations in one of two genes on chromosome 17, *EVER1* (*TMC6*), and *EVER2* (*TMC8*). EV patients are not able to efficiently clear cutaneous, beta HPV infections, and they develop wart-like lesions that cover large parts of their bodies. Later in life, and particularly at sun-exposed areas of the body, these warts progress to malignant squamous cell carcinomas (SCCs), which contain beta HPVs, most prominently HPV5 and HPV8. These

results clearly implicate UV exposure as an important environmental cofactor for beta HPV associated skin cancer development in EV patients.

The HPV8 E6, E7, and E2 each are intrinsically oncogenic in that they can each contribute to skin cancer formation in transgenic mouse models. The molecular targets of the beta HPV oncoproteins are quite distinct from those of the corresponding high-risk alpha HPV proteins. Beta HPV E6 proteins inhibit NOTCH and TGF beta signaling and can inhibit apoptosis in response to UV irradiation potentially by inhibiting the proapoptotic BCL2 family member BAK1, and/or the ATR kinase and the acetyl transferases EP300/CREBBP that are involved in UV-induced DNA damage repair.

Beta HPV infections also contribute to cutaneous SCCs that frequently arise in organ transplant patients as a consequence of long term systemic immune suppression, but it has been more challenging to prove an etiologic role of these viruses in skin cancer formation in the general patient population because not every tumor cell is HPV positive and, more generally, beta as well as the rapidly expanding gamma genus HPVs appear to be part of the normal viral flora of the skin. This suggests that beta HPVs may contribute to skin cancer initiation, but that continuous HPV gene expression is not necessary for tumor maintenance and/or that noncell-autonomous mechanisms may be involved. Deep sequencing studies of skin cancer tissues have also detected some gamma HPVs in these cancers. Clearly, more work is required to clarify the roles that beta and, potentially gamma, HPVs may play in the genesis of human skin cancers in immunocompetent patients.

High-Risk Alpha HPV Oncogenes and Cancer Initiation

The transforming activities of the high-risk alpha HPV E6/E7 proteins are a consequence of the viral replication strategy that is to ensure long term persistent infection of rapidly dividing basal epithelial cells and viral genome replication and progeny synthesis in growth arrested, terminally differentiated epithelial cells. To accomplish this, HPVs encode proteins that subvert the normal programs of cell division, differentiation, senescence, and death (Fig. 2). During the productive viral life cycle, expression of these viral proteins is controlled by a complex interplay of viral and cellular regulatory factors. This causes only subtle disruptions of cellular functions, which can become clinically apparent as hyperplastic lesions. When the normal regulatory balance of viral gene expression is distorted, for example as a consequence of a nonproductive infection, the replication strategy of a high-risk HPV becomes a serious predicament for the cell that can lead to cancer and the eventual demise of the host.

Dysregulated HPV protein expression and a nonproductive infection can arise as a consequence of the accidental integration of parts of the HPV genome into a host cell chromosome, which terminates the viral life cycle (Fig. 1B). Viral genome integration is a frequent albeit not universal event during malignant progression, and HPV associated cancers frequently (also) contain extrachromosomal HPV genomes. HPV genome integration often involves common fragile sites in the human genome. There is a remarkable conservation of the pattern of integration with respect to the viral genome. The LCR as well as the E6 and E7 ORFs are consistently retained, while in contrast, expression of the E2 transcriptional repressor is frequently abrogated as a result of integration. Dysregulated E6/E7 expression caused by loss of E2 expression is a key step that drives malignant progression. Epigenetic alterations in HPV genomes may also contribute to abnormal E6/E7 expression and drive malignant progression even in cases where there is no viral genome integration.

Expression of high-risk alpha HPV E6 and E7 proteins is necessary for the induction and maintenance of the transformed state. High-risk but not low-risk HPV derived E6 and E7 proteins each have potent oncogenic activities. Expression of E6 and E7 in primary human genital epithelial cells causes cellular immortalization. While the resulting cell lines are initially nontumorigenic, they exhibit histopathological abnormalities that are reminiscent of high-grade precancerous SILs. Long-term continuous passaging of high-risk alpha HPV immortalized cell lines eventually yields fully transformed, tumorigenic lines. Transgenic mice with HPV16

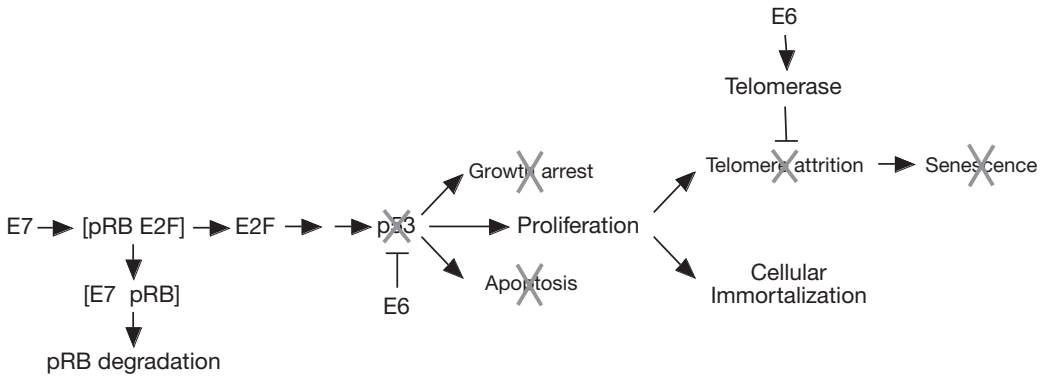

Fig. 2 Cellular targets of high-risk alpha HPV E6 and E7 proteins. Interfering with their activities is necessary for the ability of the high-risk HPVs to replicate their genomes in cells that would not normally support DNA synthesis, and for prolonged survival of HPVs within their infected host cells. In the context of nonproductive infection, when E6/E7 expression is dysregulated, inactivation of these cellular targets contributes to cellular transformation. See text for details.

E6/E7 expression targeted to basal epithelial cells develop cervical carcinomas when treated with low doses of estrogen. Similarly, oral and anal carcinoma formation can also be modeled in HPV16 E6/E7 expressing transgenic mice.

Persistent expression of the high-risk HPV E6/E7 is necessary for the maintenance of the transformed state. When endogenous high-risk HPV E6/E7 expression is inhibited by antisense, ribozyme, gene editing, or RNAi technologies or by re-expression of the E2 transcriptional repressor, cervical carcinoma cells undergo cell cycle arrest, replicative senescence or apoptosis. This demonstrates that cervical carcinoma cells remain "addicted" to HPV oncoprotein expression.

Even though HPV E5 is not consistently expressed in cervical cancers, it exhibits transforming activities in transgenic mice. Hence it is possible that E5 may contribute to tumor initiation, but given that it is not always expressed in cancer, it is likely dispensable for tumor maintenance.

Cellular Targets of High-Risk Alpha HPV E6 and E7 Proteins

E6 and E7 are small proteins of approximately 150 and 100 amino acid residues, respectively. There are no closely related cellular homologs, and E6 and E7 each contain related cysteine-rich zinc binding motives. The three-dimensional structures of E6 and E7 have been determined. E6 and E7 do not directly bind to specific DNA sequences, and they have no known intrinsic enzymatic activities, but function by binding to and functionally reprogramming host cellular protein/protein complexes. A large number of putative cellular partners of E6 and E7 have been reported and for the sake of simplicity only some of the main factors will be discussed here.

HPV E7 proteins share sequence similarity to oncoproteins encoded by other small DNA tumor viruses, including large tumor antigens (T Ag) of polyomaviruses and the adenovirus E1A proteins. A portion of the conserved sequence represents the interaction domain with the retinoblastoma tumor suppressor protein, RB1. Tumor suppressors are functional counterparts to cellular proto-oncogenes and drive cancer formation when they are inactivated. Mutational inactivation of RB1 is the rate limiting mutation that causes retinoblastoma, a rare childhood eye tumor. High-risk alpha HPV E7 proteins not only bind RB1, but target RB1 for degradation through the ubiquitin/proteasome system. Consequently, RB1-regulated cellular regulatory circuits, which include cell division, differentiation, senescence, apoptosis, and genome stability, are functionally compromised in high-risk HPV infected cells. The most studied biological activity of RB1 is the control of cell division. When a cell is in a resting state, referred to as G1, the intracellular RB1 pool is hypophosphorylated and tightly associated with E2F transcription factors. The RB1 protein contains a transcriptional repressor domain, and RB1/E2F complexes function as transcriptional repressors. When a cell receives a signal to undergo a round of cell division, a family of protein kinases, the cyclin dependent kinases (CDKs), are activated and drive progression through the cell division cycle. The very first CDK complex that is activated as a prelude to cell cycle entry (CDK4 or CDK6 in complex with a D-type cyclin) phosphorylates RB1. Phosphorylated RB1 species lose their transcriptional repressor activity and E2F family members activate expression of their target genes, thereby driving DNA synthesis and cell division. Since high-risk HPV E7 proteins bind to and destabilize growth suppressive, hypophosphorylated RB1, cells will be able to undergo aberrant DNA synthesis that is independent of CDK activity (Fig. 2).

However, a cellular failsafe mechanism exists that causes elimination of such aberrantly proliferating cells. In response to abnormal cell division in the absence of extrinsic growth factor availability, the TP53 tumor suppressor undergoes posttranslational modifications, is rendered more stable and accumulates in the nucleus. The TP53 protein functions as a DNA binding transcription factor and induces the expression of cellular target genes that signal either growth arrest or apoptosis (Fig. 2).

To counter elimination of HPV infected, E7 expressing cells by this mechanism, the high-risk HPV E6 proteins target the TP53 tumor suppressor for ubiquitination and proteasome-mediated degradation by forming a complex with the cellular UBE3A (E6AP) ubiquitin ligase (Fig. 2). Hence, high-risk HPV E6 proteins deflect the normal, abortive cellular response to RB1 inactivation. In combination, functional RB1 and TP53 tumor suppressor inactivation by high-risk alpha HPV E7 and E6 oncoproteins result in sustained, aberrant proliferation of infected cells.

Since the chromosomal termini, the telomeres, progressively shorten with every round of cell division, most normal cells can proliferate only for a limited time. Once telomeres have critically eroded, cells undergo replicative senescence, an irreversible withdrawal from proliferation. Some cell types, including stem cells maintain telomere length by expressing the telomerase enzyme.

To overcome the limitation of replicative senescence, high-risk HPV E6 proteins have evolved to enhance telomerase expression and activity (Fig. 2). Therefore, high-risk HPV E6/E7 expressing cells can divide for extended periods of time by inactivating the RB1 and TP53 tumor suppressors and overcome the normal limitation of replicative senescence by activating telomerase activity. Consistent with this model, ectopic expression of high-risk HPV E6/E7 extends the life span and facilitates immortalization of primary human epithelial cells (Fig. 2).

It is interesting to note that these cellular pathways targeted by the E6 and E7 oncoproteins are rendered dysfunctional by mutation in the majority of human solid tumors.

High-Risk Alpha HPV E6 and E7 and Malignant Progression

High-risk HPV immortalized cells are nontumorigenic, but tumorigenic clones will arise after long-term passaging in cell culture. This is reminiscent of high-risk HPV-induced lesions in patients that undergo carcinogenic progression relatively rarely and often

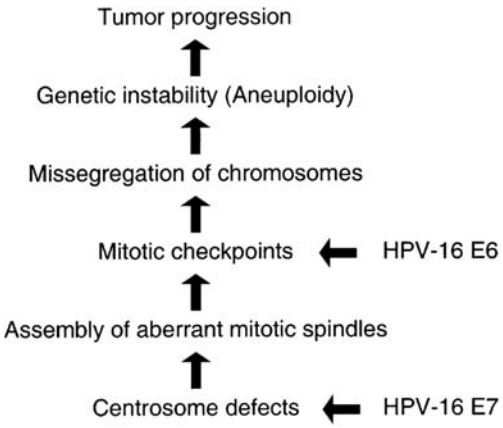

Fig. 3 High risk HPV E7 and E6 oncoproteins can induce and perpetuate genomic instability, respectively. See text for details.

only years or decades after the initial infection. This strongly suggests that additional cellular mutations are necessary to cause malignant progression. Consistent with this model, recurrent mutations affecting ERBB2, ERBB3, the phosphoinositide 3-kinase pathway, SHKBP1, CASP8, HLA-A and TGFBR2, and others have been identified in cervical cancers.

Normal cells effectively maintain the integrity of their genomes and accumulate mutations at an exceedingly low rate. Expression of E6 and E7 directly contributes to the loss of genomic integrity (Fig. 3). High-risk HPV E7 proteins increase the incidence of mitotic aberrations through several mechanisms. First, E7 expression triggers aberrant synthesis of centrosomes, which form the mitotic spindle poles (Fig. 3). In normal cells, centrosome synthesis is tightly coupled to cell division. It occurs once and only once per cell division to ensure the formation of two mitotic spindle poles and the symmetrical distribution of chromosomes during cell division. As a consequence of aberrant centrosome synthesis, the incidence of abnormal, asymmetrical mitoses generated by the presence of more than two mitotic spindle poles, is markedly increased in HPV16 E7 expressing. Such abnormalities have long been recognized as a hallmark of high-risk HPV-associated lesions and cancers. Daughter cells that arise after a cell undergoes aberrant, multipolar cell division contain abnormal chromosome numbers. Numerical chromosome abnormalities, referred to as aneuploidy, are the most frequent manifestation of genomic instability in solid tumors, and cervical cancers have been shown to be aneuploid. In addition, HPV E7 also increases the incidence of unrepaired double strand DNA breaks in cells, which can lead to chromosomal fusions and translocations. Other mitotic abnormalities, including defects in chromosome alignment during metaphase, have also been observed in E7 expressing cells. Lastly, HPV oncoprotein expression triggers activation of members of the APBEC3 family of cytidine deaminases, which cause G to A/C to T mutations and HPV associated cervical and oral cancer show APOBEC3 mutational signatures. APOBEC3 activation also causes extensive mutagenesis of the HPV genomes. Subversion of TP53 tumor suppressor activity by E6 compromises a critical surveillance mechanism that normally removes abnormal cells from the proliferative pool, which increases the possibility that abnormal cells with chromosomal abnormalities accumulate and malignant progression can ensue. Hence high-risk HPV E6 and E7 contribute directly and indirectly to accumulation of cellular mutations that drive malignant progression.

Clinical Intervention

Despite impressive advances in deciphering the molecular mechanisms of HPV-induced carcinogenesis, no HPV specific antivirals have been discovered. HPVs only encode a single enzyme, the E1 ATPase/DNA helicase, which would be an obvious target for the discovery of specific antiviral compounds. The oncogenic activities of E6 and E7 are mediated through cellular enzymes, and although these may be targeted for therapy, their functional inhibition may also have direct consequences for uninfected cells. Another possibility is to develop compounds that inhibit association of E6 and/or E7 with their cellular targets, but blocking protein–protein interactions with small molecule inhibitors remains challenging, despite the fact that three-dimensional structures of E6 and E7 are now available. Other approaches have focused on identifying cellular vulnerabilities that are caused by HPV E6 and/or E7 mediated rewiring of cellular control circuits. Such approaches have become feasible through RNAi based loss of function screens and a number of enzymes have been identified that are essential for proliferation/survival of HPV E6/E7 expressing cells but are largely dispensable for proliferation of normal cells. Last but not least, it may be possible to directly inactivate E6 and/or E7 by RNAi, gene editing or other approaches. Given the consistent "addiction" of HPV tumors to E6/E7 oncogene expression, such approaches, if technically feasible, appear particularly appealing.

A major breakthrough has been the commercial availability of extraordinarily efficacious prophylactic HPV vaccines, which essentially prevent infections with the most common high-risk HPVs as well as the low-risk HPV6 and HPV11. These vaccines consist of empty viral shells, "virus like particles," formed by the L1 major capsid protein that are produced in yeast or insect cells. Because these prophylactic vaccines only prevent new infections, they have to be administered, prior to the onset of sexual activity.

The incidence of premalignant cervical lesions has dramatically decreased in countries with national vaccination programs, however, in some countries including the United States, vaccination rates have been disappointingly low and in other countries these vaccines are not widely available due to high production costs and limited shelf life. In combination with the long latency period between the initial infection and cancer formation it will be years to decades before vaccination will have a measurable impact on the incidence and mortality of HPV associated lesions and cancer, worldwide.

Other strategies that are currently evaluated are immunological checkpoint inhibitors and postexposure, therapeutic vaccines. Such vaccines are directed against viral proteins expressed in tumors and they will have to be matched to the HPV type causing the lesion.

Large-scale deployment of the Pap-smear as a relatively inexpensive screening modality has decreased incidence and mortality of cervical cancer by approximately 70%. More sophisticated implementations of the basic Pap-smear technology that allow for more consistent sampling of the cells and automated processing of specimens are now widely used. Pap-smears, however, are increasingly complemented, and in many cases replaced by DNA typing and these innovations will further improve the vigilance of early detection to allow ablation of HPV associated lesions before they undergo malignant progression.

See also: Cervical Cancer: Screening, Vaccination, and Preventive Strategies. Uterine Cervix Cancer: Diagnosis and Treatment. Uterine Cervix Cancer: Pathology and Genetics.

Further Reading

Adey, A., Burton, J.N., Kitzman, J.O., Hiatt, J.B., Lewis, A.P., Martin, B.K., Qiu, R., Lee, C., Shendure, J., 2013. The haplotype-resolved genome and epigenome of the aneuploid HeLa cancer cell line. Nature 500, 207–211.
Bergvall, M., Melendy, T., Archambault, J., 2013. The E1 proteins. Virology 445, 35–56.
Cubie, H.A., 2013. Diseases associated with human papillomavirus infection. Virology 445, 21–34.
DiMaio, D., Petti, L.M., 2013. The E5 proteins. Virology 445, 99–114.
Gillison, M.L., Alemany, L., Snijders, P.J., Chaturvedi, A., Steinberg, B.M., Schwartz, S., Castellsagué, X., 2012. Human papillomavirus and diseases of the upper airway: Head and neck cancer and respiratory papillomatosis. Vaccine 30 (Suppl 5), F34–F54.
McBride, A.A., 2013. The papillomavirus E2 proteins. Virology 445, 57–79.
McLaughlin-Drubin, M.E., Meyers, J., Munger, K., 2012. Cancer associated human papillomaviruses. Current Opinion in Virology 2, 459–466.
Moody, C.A., Laimins, L.A., 2010. Human papillomavirus oncoproteins: Pathways to transformation. Nature Reviews Cancer 10, 550–560.
Moscicki, A.B., Schiffman, M., Burchell, A., Albero, G., Giuliano, A.R., Goodman, M.T., Kjaer, S.K., Palefsky, J., 2012. Updating the natural history of human papillomavirus and anogenital cancers. Vaccine 30 (Suppl 5), F24–F33.
Ojesina, A.I., Lichtenstein, L., Freeman, S.S., Pedamallu, C.S., Imaz-Rosshandler, I., Pugh, T.J., Cherniack, A.D., Ambrogio, L., Cibulskis, K., Bertelsen, B., Romero-Cordoba, S., Treviño, V., Vazquez-Santillan, K., Guadarrama, A.S., Wright, A.A., Rosenberg, M.W., Duke, F., Kaplan, B., Wang, R., Nickerson, E., Walline, H.M., Lawrence, M.S., Stewart, C., Carter, S.L., McKenna, A., Rodriguez-Sanchez, I.P., Espinosa-Castilla, M., Woie, K., Bjorge, L., Wik, E., Halle, M.K., Hoivik, E.A., Krakstad, C., Gabiño, N.B., Gómez-Macías, G.S., Valdez-Chapa, L.D., Garza-Rodríguez, M.L., Maytorena, G., Vazquez, J., Rodea, C., Cravioto, A., Cortes, M.L., Greulich, H., Crum, C.P., Neuberg, D.S., Hidalgo-Miranda, A., Escareno, C.R., Akslen, L.A., Carey, T.E., Vintermyr, O.K., Gabriel, S.B., Barrera-Saldaña, H.A., Melendez-Zajgla, J., Getz, G., Salvesen, H.B., Meyerson, M., 2014. Landscape of genomic alterations in cervical carcinomas. Nature 506, 371–375.
Roman, A., Munger, K., 2013. The papillomavirus E7 proteins. Virology 445, 138–168.
Schiller, J.T., Lowy, D.R., 2012. Understanding and learning from the success of prophylactic human papillomavirus vaccines. Nature Reviews Microbiology 10, 681–692.
Vande Pol, S.B., Klingelhutz, A.J., 2013. Papillomavirus E6 oncoproteins. Virology 445, 115–137.
de Villiers, E.-M., 2013. Cross-roads in the classification of papillomaviruses. Virology 445, 2–10.

Relevant Websites

American Cancer Society (ACS) information on Anal Cancer—http://www.cancer.org/cancer/analcancer/.
American Cancer Society (ACS) information on Cervical Cancer—http://www.cancer.org/cancer/cervicalcancer/.
American Cancer Society (ACS) information on Oral Cavity and Oropharyngeal Cancer—https://www.cancer.org/cancer/oral-cavity-and-oropharyngeal-cancer/causes-risks-prevention/risk-factors.html.
Evaluation of carcinogenic risk of HPVs by the International Agency for Research on Cancer (IARC)/World Health Organization (WHO)—http://monographs.iarc.fr/ENG/Monographs/vol90/.
Institut Català d'Oncologie (ICO) Information Centre on HPV and Cancer information on HPV associated cancers—http://www.hpvcentre.net/.
International Papillomavirus Society—http://www.ipvsoc.org/.
National Cancer Institute (NCI) information on Anal Cancer—http://www.cancer.gov/cancertopics/types/anal.
National Cancer Institute (NCI) information on Cervical Cancer—http://www.cancer.gov/cancertopics/types/cervical.
National Cancer Institute (NCI) information on HPV and Cancer—https://www.cancer.gov/about-cancer/causes-prevention/risk/infectious-agents/hpv-fact-sheet.
PaVE (PapillomaVirus Episteme) provides in depth scientific information on papillomaviruses—http://pave.niaid.nih.gov/#home.
The HPV and Anal Cancer Foundation—https://www.analcancerfoundation.org/

Parathyroid Cancer: Pathology and Genetics

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Glossary

Parathyroid carcinoma A malignant neoplasm derived from the parathyroid parenchymal cells.
Hungry bone syndrome Rapid profound and prolonged hypocalcemia associated with hypophosphatemia and hypomagnesemia that is exacerbated by suppressed parathyroid hormone levels after parathyroidectomy in patients with severe primary hyperparathyroidism such as with parathyroid carcinomas.
Hyperparathyoidism jaw-tumor syndrome Autosomal dominant inherited condition associated with parathyroid adenomas, parathyroid carcinomas and benign bone tumors of the mandible and maxilla.
Multiple endocrine neoplasia type 1 Autosomal dominant inherited condition associated with hyperplasia and tumors of the pituitary, parathyroid, pancreas and gastric acid hypersecretion.
Multiple endocrine neoplasia type 2 Autosomal dominant inherited condition associated with hyperplasia and tumors of the parathyroid, thyroid C-Cell, and adrenal medulla.
Nuclear pleomorphism A feature of parathyroid carcinoma in which there is variation in the size and shape of the nuclei in parathyroid carcinoma cells.
Oxyphilic/oncocytic cells Parathyroid carcinoma cells with granular pink cytoplasm after hematoxylin and eosin histochemical staining. The eosinophilia is caused by the abundant mitochondria in the cytoplasm of the tumor cells.

Nomenclature

Serum Calcium
Ionized calcium in serum 1–1.4 mmol/L (SI)
Total serum calcuim 2.2–2.6 mmol/L (SI)

Definition

A malignant neoplasm capable of metastasizing is derived from various types of parathyroid parenchymal cells. These can include chief cells, oncocytic or oxyphilic cells and clear cells.

Etiology

Most parathyroid carcinomas are sporadic while a small percentage of carcinomas are familial.

Sporadic Parathyroid Carcinoma

Some cases of parathyroid carcinoma may arise secondary to prior radiation to the head and neck region and secondarily involving the parathyroid glands. In this setting the latency period between radiation and parathyroid tumors is relatively long and may be more than 20–25 years. Post radiation parathyroid neoplasms are mainly adenomas, but rare cases of carcinomas have been reported. Rare cases of parathyroid carcinomas in patients with secondary and tertiary hyperparathyroidism following renal failure and chronic dialysis have been noted. In rare cases patients with celiac disease may also develop sporadic parathyroid carcinomas.

Genetic or Familial Parathyroid Carcinoma

Parathyroid carcinomas may develop in a familial setting in patients with hyperparathyroidism-jaw tumor syndrome (HPT-JT). This disorder is associated with mutations of the CDC73 gene and is autosomal dominant. The disorder was previously called HRPT2. In this condition patients have tumors of the parathyroid glands and about 10%–20% of these may be carcinomas. This syndrome is also associated with benign tumors of the jaw or osteomas. They may occur in the mandible or maxilla. Most tumors are unilocular, but they may occasionally be multifocal. Although the tumors are usually benign they may be infiltrative and recurrent. Female patients with this disorder may also develop uterine abnormalities including endometrial hyperplasia, uterine leiomyomas, adenofibromas and in a few cases adenosarcomas which are malignant uterine tumors. Rare cases of parathyroid carcinomas may also

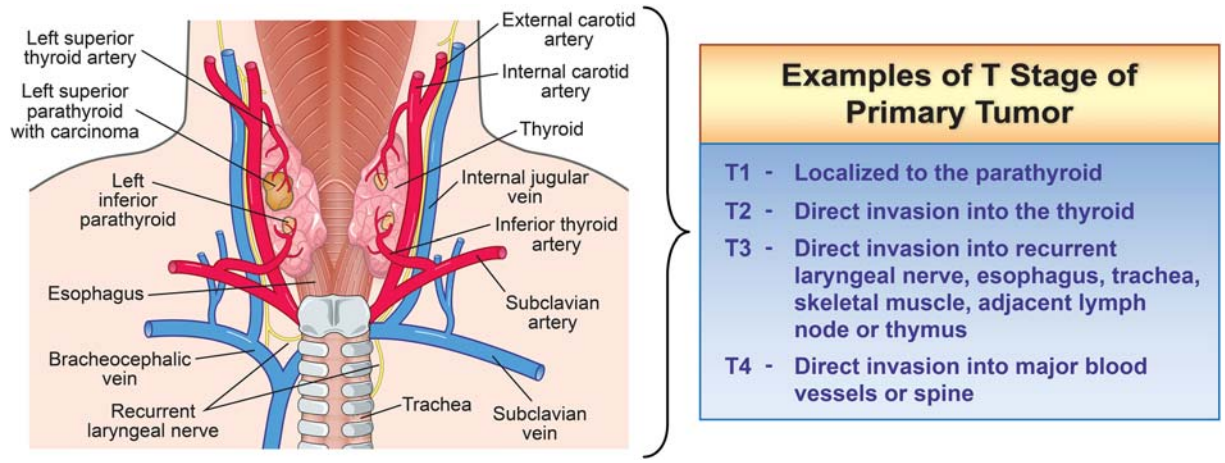

Fig. 1 Schematic diagram of the anatomy and pathology of the parathyroid glands including a parathyroid carcinoma from a posterior view. The carcinoma in the left upper parathyroid is markedly enlarged and is associated with the adjacent thyroid tissue. The right side shows examples of T stage classification of parathyroid carcinomas using the American Joint Commission on Cancer (AJCC) recommendation s. Most of the anatomic structures that could be invaded by the carcinoma and that are important in the T classification are shown in the diagram.

develop in patients with multiple endocrine neoplasia type 1 which usually affects the parathyroid glands, anterior pituitary gland and the pancreatic islets cells. Patients with multiple endocrine neoplasia type 2 or 2a may also develop parathyroid carcinomas rarely. These patients usually have familial tumors affecting the parathyroids as well as the C-cells of the thyroid and adrenal medullary cells.

Epideimiology

Parathyroid carcinomas are extremely uncommon. They are rarely encountered in a large surgical pathology laboratory (Fig. 1). In a large series of patients with primary hyperparathyroidism, which means that the disease originated in the parathyroid gland, less than 1% of these patients will have a parathyroid carcinoma that causes elevation of the serum calcium and parathyroid hormone level. Both sexes are equally affected by parathyroid carcinomas which are different from benign parathyroid tumors in which there is a female preponderance of cases. There has been an increase in the incidence of parathyroid carcinomas over the past few decades. One reason for this increase in both adenomas and carcinomas has been attributed to increased clinical screening that has enabled earlier detection of parathyroid tumors. Most patients with sporadic parathyroid carcinomas are in their 50s, but patients with familial disease are usually one or two decades younger.

Imaging of Parathyroid Carcinoma

Preoperative imaging for parathyroid carcinomas include ultrasonography (US), computed tomography (CT), magnetic resonance imaging and scintigraphy. The sensitivity of technetium-99 m sestamibi scanning is relatively high compared to ultrasound localization. A combination of sestamibi and ultrasound increases sensitivity for smaller lesions that are difficult to localize. Sestamibi scans can have a fairly high false positive rate, so newer methods such as single-photon emission CT (SPECT) and SPECT in combination with CT scanning (SPECT-CT) can improve the anatomic details and increase the rate of detection of smaller lesions. Recent studies have found that four-dimensional CT for parathyroid tumors that were previously considered nonlocalizable on US and single-photon emission CT with SPECT-CT increases sensitivity and specificity 4-D CT may be considered as first or second-line imaging for localizing parathyroid tumors. Fludeoxyglucose positron emission tomography may be used for detecting parathyroid carcinomas both in the primary site and for metastatic disease.

Clinical Features

Patients with parathyroid carcinomas usually have higher serum levels of parathyroid hormone and serum calcium than patients with parathyroid adenomas. Serum calcium is often greater than 3.5 mmol/L. Many patients may have renal disease at the time of presentation. The most common findings are renal stones or nephrolithiasis. The second most common fining would include bone diseases such as osteitis fibrosa cystica. Bone resorption, diffuse osteoporosis may be common in these patients. Some asymptomatic patients may present with spontaneous bone fractures. A combination of renal and bone diseases is not uncommon in patients

with parathyroid carcinomas. On physical examination patients often have a palpable neck mass, especially with larger parathyroid carcinomas. Some patients may present with hoarseness due to paralysis of the recurrent laryngeal nerves. Parathyroid adenomas are a lot more common than parathyroid carcinomas, but the possibility of carcinomas should be considered in patients with severe hyperparathyroidism, a palpable neck mass, hoarseness and evidence of local invasion at the time of surgery.

Gross Findings

Parathyroid carcinomas are quite variable in their gross appearance. They may be very small and weigh a little more than a gram or be extremely large and weigh up to 50 g or more. Many parathyroid carcinomas appear to have a thin capsule on gross examination. In very large tumor the capsule may appear extremely thin because of massive expansion by the tumor (Fig. 1). The cut surface of parathyroid carcinomas usually shows a firm tan to pink tumor mass that may look lobulated and hemorrhagic. Brown-tan firm fibrous bands may be appreciated on close gross examination. In large frank carcinomas with extensive invasion the tumor may be seen to permeate into the adjacent adipose tissue, skeletal muscle or into the nearby thyroid tissue that may have been excised with the parathyroid gland.

Microscopic Findings

Although the surgeon may suspect a parathyroid carcinoma at the time of surgery, it is usually the pathologist that makes the ultimate diagnosis of parathyroid carcinomas. A common reported finding by the surgeon is that the tumor is adherent to the adjacent thyroid. However some parathyroid adenomas are also adherent to the thyroid gland especially after fine needle aspiration biopsy in the vicinity of the parathyroid glands.

The most useful microscopic feature of malignancy is invasive growth of the tumor (Figs. 2–4). Invasion may involve the adjacent thyroid, adjacent skeletal muscle or fibroadipose tissue that may be appreciated on gross examination. More often the findings are more subtle such as vascular invasion or perineural invasion. Vascular invasion should involve vessels in the capsule or in the adjacent tissues. Invasion in the center of the parathyroid tumor does not qualify as true vascular invasion.

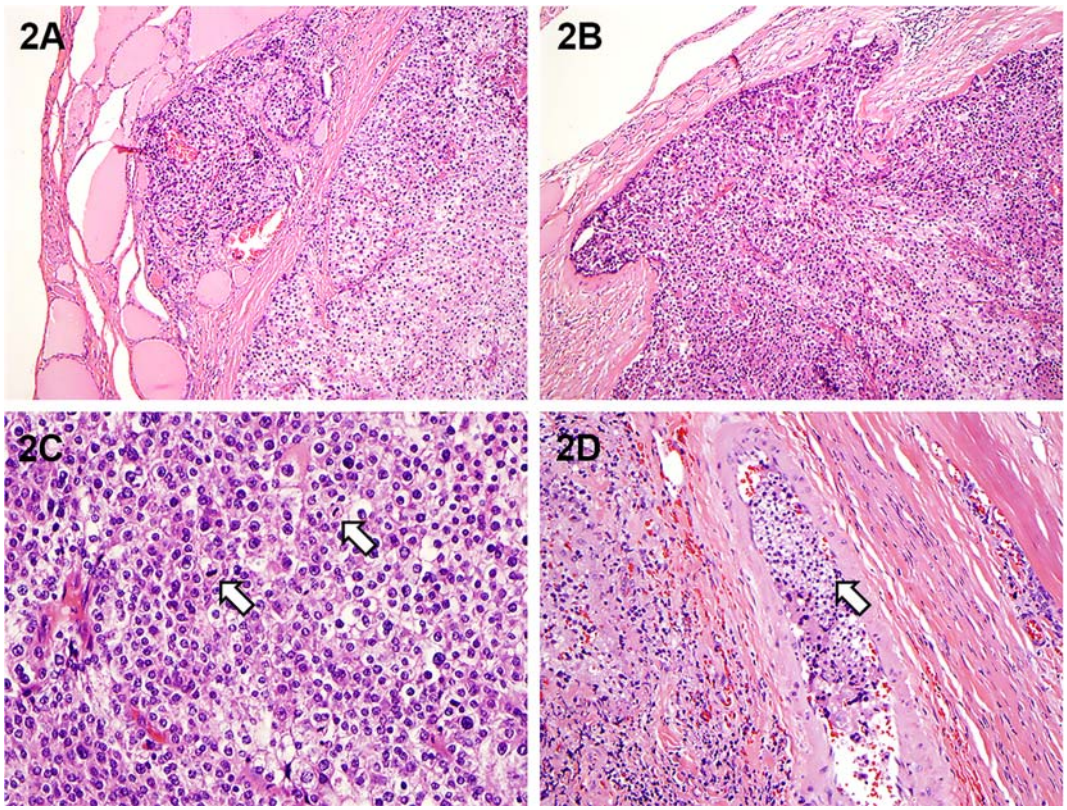

Fig. 2 Histopathological features of parathyroid carcinoma. (A) The parathyroid carcinoma shows invasion into the adjacent thyroid tissue. (B) The parathyroid carcinoma shows invasion into the thickened tumor capsule. (C) There is increased mitotic activity in the parathyroid carcinoma. Two mitotic figures are shown (*arrows*). (D) Vascular invasion in a parathyroid carcinoma. A vessel in the capsule (*arrow*) shows vascular invasion.

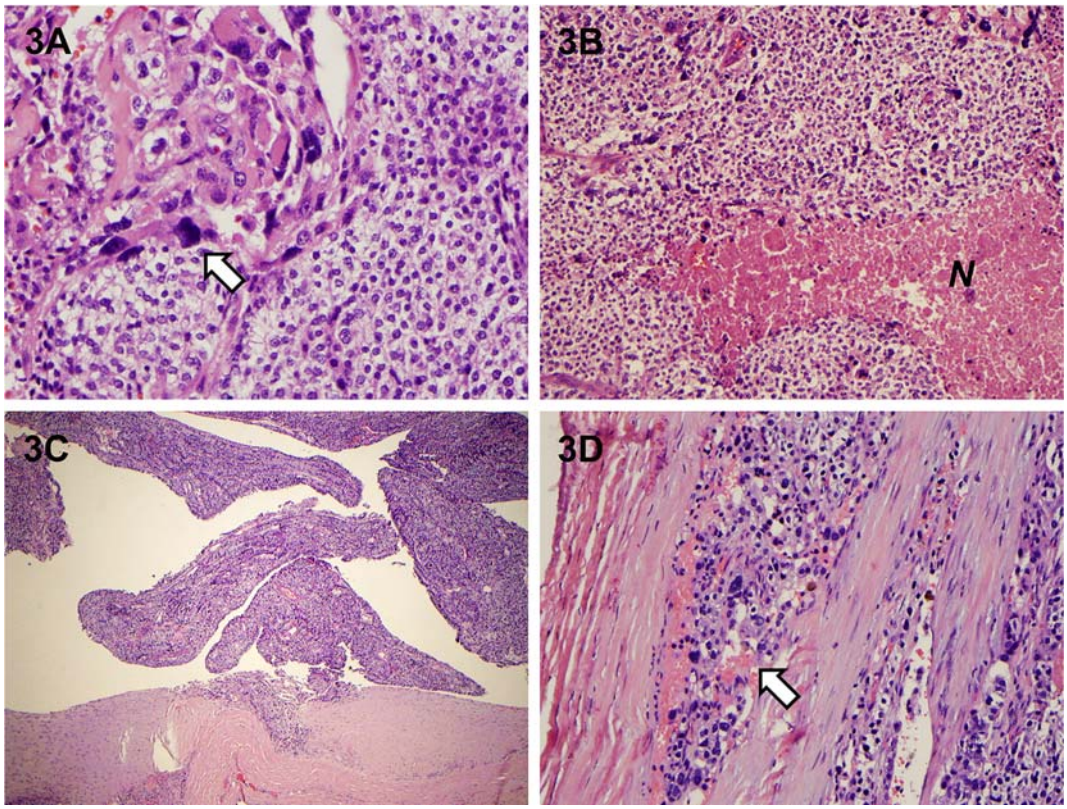

Fig. 3 Histopathological features of parathyroid carcinoma. (A) There is nuclear pleomorphism in the parathyroid carcinoma (*arrow*). (B) Extensive areas of necrosis (N) are present in this parathyroid carcinoma. (C) Hyperparathyroidism-jaw tumor (HPT-JT) syndrome in a 17 year old boy with parathyroid carcinoma. The cystic appearance of the parathyroid tumor is usually seen in about a third of patients with HPT-JT syndrome. (D) Another portion of the parathyroid carcinoma from the patient in C showing vascular invasion in the capsule (*arrow*).

A common finding seen in parathyroid carcinomas is the presence of broad fibrous bands. These bands consist of fibrous connective tissues which extend from the peritumoral capsule towards the center of the tumor. Although helpful in the diagnosis of parathyroid carcinomas, they are not specific for this disease, since they may also be seen in parathyroid adenomas and in atypical parathyroid adenomas. The type of cells in parathyroid carcinomas reflects the cell types seen in the normal parathyroid and in parathyroid adenomas. The chief cell is the most common constituent of parathyroid carcinomas. Oncocytic or oxyphilic parathyroid carcinomas were once thought to be nonfunctioning tumors, but studies in the literature have shown that most of these tumors are functional. There are only rare instances of nonfunctioning parathyroid carcinomas. Parathyroid carcinomas composed mostly of clear cells are very uncommon tumors. Nuclear pleomorphism is often seen in some areas of parathyroid carcinomas. Although some parathyroid carcinomas are composed of uniform population of tumor cells in many areas, nuclear pleomorphism is often seen focally. The pleomorphic nuclei may be quite large and have prominent nucleoli. The sizes of the pleomorphic cells may be two to three times larger than adjacent cells that are not pleomorphic. Ultrastructural examination of parathyroid adenomas shows similar features as seen in parathyroid adenomas. Cells usually have variable numbers of dense core secretory granules, moderate amounts of rough endoplasmic reticulum and Golgi complexes. The secretory granules vary in size from 100 to 600 nm. Oncocytic tumors usually contain abundant mitochondria on ultrastructural examination.

The proliferative index as evaluated by mitotic activity is another helpful diagnostic feature for parathyroid carcinomas. A minimum of 50 high power fields should be counted in the evaluation of parathyroid carcinomas. Most parathyroid adenomas may have 1 or 2 mitoses per 50 high power fields, but the number is usually much higher in parathyroid carcinomas. Mitotic figures may be found "in hot spots" which means that some areas may have abundant mitotic figures while many other areas may be devoid of mitotic figures. In recent years the proliferative index as assessed with Ki-67 or MIB1 may be helpful in the diagnosis of parathyroid carcinomas. The Ki-67 proliferative index is usually helpful in distinguishing adenomas from carcinomas, since the percentage is usually less than 4% in adenomas and greater than 6% in parathyroid carcinomas. Atypical mitoses are very helpful in the diagnosis of parathyroid carcinomas, since they are usually not seen in the normal parathyroid or in parathyroid adenomas. Immunohistochemical staining for Ki-67 may help in the detection of atypical mitoses.

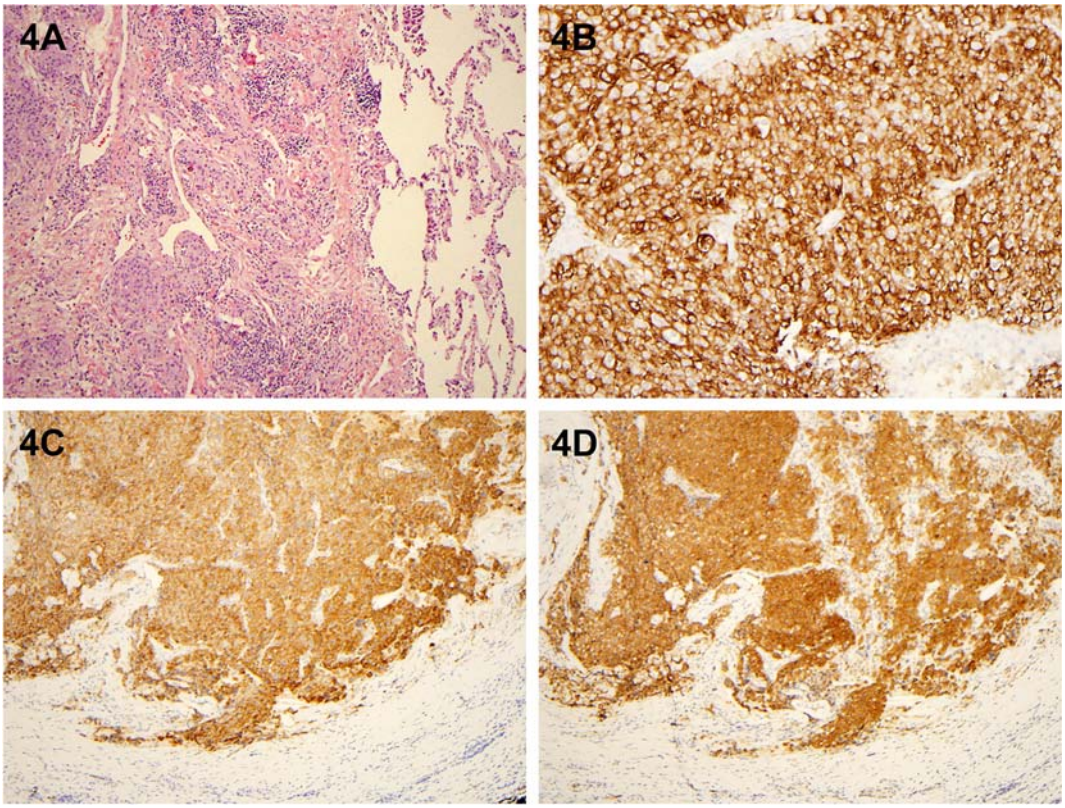

Fig. 4 Histological and immunohistochemical findings in parathyroid carcinomas. (A) Metastatic parathyroid carcinoma to the lung. Nest of malignant neuroendocrine cells are present in the left part of the field. (B) Immunohistochemical staining for parathyroid hormone is diffusely positive in this parathyroid carcinoma. (C) Immunohistochemical staining for chromogranin A is diffusely positive in the same parathyroid carcinoma as in B. (D) Immunohistochemical staining for synaptophysin is diffusely positive in the parathyroid carcinoma.

Immunohistochemical Staining

Immunohistochemical staing can be very helpful in the diagnosis of parathyroid carcinomas (Fig. 4). Immunohistochemical staining for Ki-67 has already been discussed under microscopic findings and its utility in the diagnosis of parathyroid carcinomas has already been emphasized. Immunohistochemical staining for parathyroid hormone can help to confirm the diagnosis of parathyroid carcinomas in more unusual cases. Immunopositivity for parathyroid hormone is typically weaker in parathyroid carcinomas compared to parathyroid adenomas or normal parathyroid gland tissues. General neuroendocrine markers such as chromogranin and synaptophysin can be useful in confirming the diagnosis of parathyroid carcinomas. Other broad spectrum neuroendocrine markers such as CD56 and neuron specific enolase are usually positive, but are not as specific as chromogranin and synaptophysin. Immunostaining for p27 (CDKN1B) has been studied in parathyroid carcinomas. Normal parathyroid tissues, parathyroid hyperplasia and parathyroid adenomas usually have higher levels of p27 than parathyroid carcinomas. Thus quantitation of p27 can be used as supplemental evidence in the diagnosis of parathyroid carcinomas, especially when used along with Ki-67 which shows the reverse relationship (highest in parathyroid carcinomas). Additional markers that are positive in parathyroid carcinomas include various keratins such as CAM5.2 and AE1/AE3. The mitochondrial keratin marker CK14 is surprisingly negative in most parathyroid carcinomas. RB protein which is a tumor suppressor gene is often negative in parathyroid carcinomas. The latter observation suggests that the RB gene may be important in the pathogenesis of parathyroid carcinomas. The transcription factor GATA3 is usually positive in parathyroid carcinomas. In addition, parathyroid carcinoma is usually positive for the regulatory gene that is important in the development of the parathyroid gland, GCM2.

Cyclin D1 is frequently overexpressed in parathyroid carcinomas. However like many markers some parathyroid adenomas and rare cases of normal parathyroid may express Cyclin D1, so this marker is not specific for parathyroid carcinomas. A molecule that is important for the pathogenesis of familial parathyroid carcinoma, parafibromin or CDC73 which was previously known as HPRT2 is often negative in a significant percentage of parathyroid carcinomas. However a significant percentage of parathyroid carcinomas may be positive for this marker, so it is not an absolute marker for parathyroid carcinomas. Some patients with parathyroid carcinomas and renal failure may retain expression of CDC73, so in this setting it is not a reliable marker. Another marker that is useful in the diagnosis of parathyroid carcinoma is the protein PGP9.5.

Some workers have shown that there is increased expression of PGP9.5 in parathyroid carcinomas, so combining the loss of parafibromin with increased expression of PGP9.5 would provide good immunohistochemical evidence supporting the diagnosis of parathyroid carcinoma.

Other markers that are frequently positive in parathyroid carcinomas include galectin 3, mTOR, COX1/2 and VEGFR-2. Markers that are frequently negative in parathyroid carcinoma include BCL2, CDKN1B, APC, RB protein and thyroglobulin.

Differential Diagnosis of Parathyroid Carcinoma

The differential diagnosis of parathyroid carcinomas includes parathyroid adenomas, atypical parathyroid adenomas, parathyromatosis and metastasis to the parathyroid. Parathyroid adenomas are the most common lesion that raises the possibility of parathyroid carcinomas in a few cases. Adenomas may be adherent to the thyroid gland, especially if the patient has a history of fine needle aspiration to the thyroid that is close to a parathyroid gland. Fibrous bands may occasionally be seen in parathyroid adenomas, but features such as increased mitotic activity, vascular invasion, perineural invasion, extension of parathyroid neoplasm into the thyroid or beyond the parathyroid gland to the fibroadipose or skeletal muscle tissue is usually not present. The mitotic activity and Ki-67 proliferative index are also helpful features that allow separation of parathyroid adenomas from parathyroid carcinomas.

Atypical parathyroid adenoma is an uncommonly used diagnosis that should be restricted for a specific entity. Atypical parathyroid adenomas usually have some of the features of parathyroid carcinoma, such as dense fibrous bands and capsular invasion. But the reliable diagnostic features of parathyroid carcinomas such as vascular and perineural invasion, invasion of the adjacent thyroid or invasion into the fibroadipose or skeletal muscle tissue is usually not present. Patients with atypical parathyroid adenomas should be followed closely with periodic measurement of serum calcium or parathyroid hormone levels to detect recurrent disease.

Parathyromatosis may be difficult to distinguish from parathyroid carcinomas, since there is often dispersement of nodules of neoplastic parathyroid gland tissue in the soft tissues of the neck. Primary parathyromatosis may result from persistent embryological remnants of parathyroid gland tissues in the soft tissues of the neck while secondary parathyromatosis is related to the surgical implantation of parathyroid gland tissues into the soft tissues of the neck during surgery for hyperparathyroidism. In this setting hyperplastic or neoplastic parathyroid tissues may be accidentally deposited in the soft tissues of the neck during surgery. The presence of a palpable neck mass is usually associated with a parathyroid carcinoma rather than primary or secondary parathyromatosis. Parathyromatosis may show increased mitotic activity on histological examination, but atypical mitosis is usually not seen. Other features diagnostic of carcinoma such as vascular invasion, perineural invasion, markedly elevated serum parathyroid hormone or calcium levels may help to make the distinction. Loss of parafibromin expression and increased expression of PGP9.5 may also be supportive evidence in favor of parathyroid carcinoma, although there have not been too many studies of parathyromatosis with respect to these biomarker expression.

Metastatic tumors to the parathyroid are very uncommon. It usually occurs in patients with a high tumor burden. Estimates in the literature have ranged from 0.2% to 12% of cases with disseminated malignancies. These are usually carcinomas from the most common organs that are likely to develop malignancies. The primaries sites of tumors reported as metastases to the parathyroid include breast, lungs, skin (melanoma), liver, kidney, and hematolymphoid malignancies such as chronic lymphocytic leukemia. Some of these metastases may be confused with a primary parathyroid carcinoma. An excellent example of this would be renal cell carcinoma because of the common finding of clear cells and the high vascularity as is seen in parathyroid carcinomas.

Treatment

Surgery

Surgery is the only potentially curative treatment for parathyroid carcinoma. According to the recently published guidelines by American Association of Endocrine Surgeons (AAES), complete resection of tumor with intact capsule should be attempted when there is clinical suspicion for carcinoma. It is often difficult to ascertain intraoperatively benign (adenoma) versus malignant (carcinoma) nature of the disease. Although an obviously invasive or metastatic tumor would indicate carcinoma, less specific signs such as enlarged size of the gland, fibrous capsule and adherence to the surrounding structures can occur in both adenomas and carcinomas. A piecemeal fashion of resection should therefore be avoided in parathyroid tumors with unknown pathology.

The optimal extent of resection for grossly confined tumors has been under debate. Traditionally, based on a large literature, extended en bloc resection including parathyroid tumor, ipsilateral thyroid lobe and other adherent structures has been believed to lead to better survival and reduced recurrence compared to simple parathyroidectomy. Nevertheless, more recent studies have found no outcome benefit in extensive resection over parathyroidectomy alone. The current AAES guidelines have no strong preference for either surgical approach.

Prophylactic cervical lymph node dissection in the absence of clinically suspicious nodes is controversial. The incidence of nodal metastasis is relatively low (up to 10%) in large series. Although nodal status was included in the current TNM staging system, multiple studies have found no association between survival and regional lymph node metastasis. Accordingly, the current AAES guidelines do not recommend prophylactic central or lateral neck dissection.

After tumor resection, patients should be monitored for development of hungry bone syndrome with acute, potentially life-threatening hypocalcemia, hypophosphatemia and hypomagnesemia due to abrupt drop of serum parathyroid hormone level

and reversal of bone resorption. Current AAES guidelines do not recommend adjuvant radiotherapy given that parathyroid carcinomas are typically radioresistant.

Nonsurgical Treatments

In patients with unresectable disease, treatment options are very limited. Radiotherapy and chemotherapy are both relatively ineffective. Patients with symptomatic hypercalcemia may be treated with generous hydration and calciuretic agents such as loop diuretics. Hypocalciuretic agents such as thiazides should be avoided. Sensipar (cinacalcet), a calcimimetic that acts on the calcium-sensing receptors on parathyroid chief cells to reduce parathyroid hormone secretion, was recently approved by the United States Food and Drug Administration for the treatment of hypercalcemia in patients with parathyroid carcinoma. The approval was based on a single-arm, open-label trial that included 29 patients. Calcitonin and bisphosphonates may also reduce bone resorption. Hemodialysis may be warranted in patients with renal failure.

Antiparathyroid hormone immunotherapy has been reported with promising results in three cases. These patients were immunized with human and bovine parathyroid hormone peptides with Freund's adjuvant and developed detectable anti-human parathyroid hormone antibodies within 4 weeks. All three patients experienced decreases in serum calcium; however, the sustainability of such improvement was heterogeneous. In one patient, the immunotherapy led to normalization of serum calcium and parathyroid hormone levels and 39.2%–71.4% shrinkage of pulmonary metastases for more than 2 years. In another patient, serum calcium decreased in the first week after injection but re-escalated in less than 3 weeks, with metastatic tumors continuing to develop. More research is needed to clarify the utility of immunotherapy. No molecular targeted therapy is currently available.

American Joint Commission on Cancer (AJCC) TNM Staging of Parathyroid Carcinomas

T Stage

Staging of parathyroid carcinomas is relatively new in risk assessment models, because of the rarity of these carcinomas (Fig. 1). There is no generally accepted staging system for parathyroid carcinomas at this time. A working TNM system has been proposed by the AJCC Cancer Staging Manuel and is summarized in this section. The staging of the primary tumor includes: TX or primary tumor cannot be assessed; T0 when the primary tumor cannot be localized; Tis refers to atypical parathyroid neoplasms or neoplasms of uncertain malignant potential. These are tumors that are histologically or clinically worrisome, but do not fulfill the exact criteria of malignancy for carcinomas such as invasion and metastasis. The atypical parathyroid adenoma would fall into this category. T1 refers to a carcinoma localized to the parathyroid gland with extension limited to adjacent soft tissues.T2 includes tumors with direct invasion of the adjacent thyroid gland. T3 includes carcinomas with direct invasion of the recurrent laryngeal nerve, esophagus, trachea, skeletal muscle, adjacent lymph nodes or the thymus gland. T4 stage involves parathyroid carcinomas with direct invasion into major blood vessels or spine.

N Stage

The N stage for parathyroid carcinoma ranges from no involvement of regional lymph nodes to extensive involvement of lymph nodes. NX indicates that lymph nodes cannot be assessed. N0 indicates that there are no lymph node metastases while N1 refers to lymph node metastases. The N1 category is divided into N1a in which there are metastases to level VI (Pretracheal, paratracheal and prelaryngeal/Delphian) lymph nodes. N1b refers to metastases to unilateral, bilateral or contralateral cervical (levels I, II, III, IV or V) or retropharyngeal or superior mediastinal lymph nodes (level VII).

M Stage (Distant Metastasis)

As with other tissues M0 refers to no distant metastasis, while M1 refers to cases with distant metastasis of parathyroid carcinoma.

Genetics/Molecular Biology

The major molecular driver for parathyroid carcinoma has been shown to be the *CDC73* gene which was previously known as *HPRT2* gene. This gene maps to 1q25-q31. The gene contains 17 exons spanning 18.5 kb of genomic distance and is predicted to express a 2.7 kb transcript. It encodes the 531 amino acid protein CDC73. CDC73 is ubiquitously expressed in many organs including lungs, brain, kidney, liver and pancreas. The gene is a tumor suppressor gene and is involved in the regulation of p53 and is a component of the PAF protein complex controlling RNA polymerase II-mediated general transcription. CDC73 protein has an important role in regulating gene expression and inhibiting cell parathyroid cell proliferation. Sporadic parathyroid carcinomas often have somatic inactivating mutations of the CDC73 gene. Germline mutation of the *CDC73* gene may be detected in some cases of patients who appear to have sporadic parathyroid carcinomas, which suggest that they may have a subtle manifestation of HPT-JT that may be more difficult to detect clinically without genetic testing. Other genetic abnormalities in

parathyroid carcinomas include loss of chromosome 13q. This may be associated with a genetic abnormality close to the RB1 gene. The MEN1 gene has also been associated with parathyroid carcinoma, but this is a very uncommon association, Next generation sequencing has uncovered other genes that may be important for the pathogenesis of parathyroid carcinomas including PIK3CA, ADCK1, KMT2D, PRUNE2, MTOR, UCHL1 which encodes the gene for PGP9.5 and CDH1. The general feeling of experts in this area is that parathyroid carcinomas do not usually develop by tumor progression from hyperplastic or adenomatous parathyroid disease.

Hyperparathyroidism-jaw tumor syndrome (HPT-JT) is an autosomal dominant tumor disorder. It has been linked to mutations in the *CDC73* gene. Patients usually develop parathyroid adenomas or carcinomas, ossifying fibromas of the mandible and maxilla, renal cysts, renal adenomas and carcinomas and females develop endometrial hyperplasia, uterine leiomyomas, adenofibromas. Patients with this condition are usually diagnosed by biochemical finding of primary hyperparathyoroidism, radiologic detection of ossifying fibromas, by family history and by molecular genetic analysis to uncover the CDC73 genetic mutations. Patients usually have solitary parathyroid lesions, but involvement of two or more glands may develop over time. About 80% of patients with HPT-JT present with hyperparathyroidism, but in some families the syndrome may be limited to the parathyroid abnormality without bone or renal lesions. Parathyroid disease usually becomes manifested in the second or third decade of life. The development of parathyroid carcinoma is much more common with HPT-JT syndrome compared to MEN1 and MEN2 diseases.

Prognosis

Parathyroid carcinomas tend to be indolent, persistent tumors. Postoperative recurrence is common, occurring in 30%–100% of cases. Distant metastasis occurs in up to 25% of cases, common metastatic sites include lung, bone, and liver. Recurrent and metastatic tumors are often treated by resection, embolization or chemical/heat ablation to reduce the metabolic effect of tumors, but these measures are rarely curative. Overall survival is estimated at 78%–85% at 5 years and 49%–70% at 10 years. The majority of mortalities are secondary to complications of hypercalcemia such as renal failure and cardiac arrhythmia (12). Adverse outcome predictors include older age, male gender, larger tumor size, incomplete resection, nonfunctional tumors and distant metastasis. It remains controversial whether regional lymph node metastasis has prognostic significance, and whether more radical surgery such as extended en bloc resection is more advantageous than simple parathyroidectomy.

Prospective Vision

The study of parathyroid carcinoma is rapidly advancing in several areas. Imaging studies will be developing newer and more sensitive methods to detect primary tumors that may be too small to detect with currently available techniques before surgery. Intraoperative techniques to try to distinguish between benign and malignant parathyroid tumors are being developed with mass spectrophotometric methods. New biomarkers including proteins, microRNAs and long noncoding RNAs are being examined as possible diagnostic and prognostic tools for parathyroid carcinomas. Finally whole genome sequencing and new molecular techniques are being used for gene discovery and for developing new methods to target recurrent and metastatic disease in patients with parathyroid carcinomas.

Further Reading

Abdelgadir, M.M., Untch, B.R., Olson Jr., J.A., 2010. Parathyroid carcinoma: Current understanding and new insights into gene expression and intraoperative parathyroid hormone kinetics. The Oncologist 15, 61–72.

Asare, E.A., Sturgeon, C., Winchester, D.J., et al., 2015. Parathyroid carcinoma: An update on treatment outcomes and prognostic factors from the National Cancer Data Base (NCDB). Annals of Surgical Oncology 22, 3990–3995.

Betea, D., Bradwell, A.R., Harvey, T.C., et al., 2004. Hormonal and biochemical normalization and tumor shrinkage induced by anti-parathyroid hormone immunotherapy in a patient with metastatic parathyroid carcinoma. The Journal of Clinical Endocrinology and Metabolism 89, 3413–3420.

Bradwell, A.R., Harvey, T.C., 1999. Control of hypercalcaemia of parathyroid carcinoma by immunization. Lancet (London, England). 353, 370–373.

DeLellis, R.A., Arnold, A., Bilezikian, J.P., et al., 2017. Parathyroid carcinoma. In: Lloyd, R.V., Osamura, R.Y., Kloppel, G., Rosai, J. (Eds.), WHO classification of tumors of endocrine organs, 4th edn. International Agency for Research on Cancer: World Health Organization, Lyon, pp. 147–152.

Harari, A., Waring, A., Fernandez-Ranvier, G., et al., 2011. Parathyroid carcinoma: A 43 year outcome and survival analysis. The Journal of Clinical Endocrinology and Metabolism 96, 3679–3686.

Hundahl, S.A., Fleming, I.D., Fremgen, A.M., Menck, H.R., 1999. Two hundred eighty-six cases of parathyroid carcinoma treated in the U.S. between 1985 and 1995: A National Cancer Data Base Report. The American College of Surgeons Commission on cancer and the American Cancer Society. Cancer 86, 538–544.

Koea, J.B., Shaw, J.H., 2016. Parathyroid cancer: Biology and management. Surgical Oncology 8, 155–165.

Landry, C.S., Wang, T.S., Asare, E.A., et al., 2017. Parathyroid. In: Amin, M.B., Edge, S.B., Greene, F.L., et al. (Eds.), AJCC Cancer staging manual, 8th edn. Springer Nature, Switzerland, pp. 903–910.

Lloyd, R.V., Arnold, A., Gill, A., Morreau, H., 2017. Hyperparathyoidism-jaw tumor syndrome. In: Lloyd, R.V., Osamura, R.Y., Kloppel, G., Rosai, J. (Eds.), WHO classification of tumors of endocrine organs, 4th edn. International Agency for Research on Cancer: World Health Organization, Lyon, pp. 255–256.

Lloyd, R.V., 2010. Endocrine pathology: Differential diagnosis and molecular advances. Springer Science and Business Media, New York.

Silverberg, S.J., Rubin, M.R., Faiman, C., et al., 2007. Cinacalcet hydrochloride reduces the serum calcium concentration in inoperable parathyroid carcinoma. The Journal of Clinical Endocrinology and Metabolism 92, 3803–3808.

Wei, C.H., Harari, A., 2012. Parathyroid carcinoma: Update and guidelines for management. Current treatment options in oncology 13, 11–23.
Wilhelm, S.M., Wang, T.S., Ruan, D.T., et al., 2016. The American Association of Endocrine Surgeons Guidelines for definitive Management of Primary Hyperparathyroidism. JAMA Surgery 151, 959–968.
Villar-del-Moral, J., Jimenez-Garcia, A., Salvador Egea, P., et al., 2014. Prognostic factors and staging systems in parathyroid cancer: A multicenter cohort study. Surgery 156, 1132–1144.

Relevant Websites

http://www.cancer.ca/en/cancer-information/cancer-type/parathyroid/prognosis-and-survival/?region=bc—Canadian Cancer Society.
https://www.cancer.gov/types/parathyroid/hp/parathyroid-treatment-pdq—Parathyroid Cancer Treatment (PDQ®)–Health Professional Version.
https://www.cancer.gov/types/parathyroid/patient/parathyroid-treatment-pdq—Parathyroid Cancer Treatment (PDQ®)–Patient Version.
http://endocrinediseases.org/parathyroid/cancer_parathyroid.shtml—American Association of Endocrine Surgeons.
http://www.mayoclinic.org/diseases-conditions/hyperparathyroidism/home/ovc-20319885—Mayo Clinic.
https://medlineplus.gov/ency/article/007264.htm—MedlinePlus.
https://my.clevelandclinic.org/health/articles/parathyroid-cancer—Cleveland Clinic.
http://www.parathyroid.com/parathyroid-cancer.htm—Norman Parathyroid Center in Tampa, Florida.

P-Glycoprotein-Mediated Multidrug Resistance☆

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Glossary

Apoptosis A process of programmed cell death stimulated by natural factors or anticancer drugs.

ATP binding cassette A protein sequence found in a related family of proteins that allows the cellular source of energy, ATP, to bind to these proteins and be utilized for various transport processes.

Extracellular space The space between cells in a tissue, or outside of a cell growing tissue culture, from which drugs are taken up by a cell.

Hydrophobic Of or relating to a property of molecules that prevents them from dissolving in water and enables their solution in cell membranes.

Induction A form of regulation of gene expression in which an environmental alteration (frequently the presence of a specific chemical) leads to the expression of a gene. In the case of drug resistance, the cytotoxic compound may turn on expression of a protective detoxifying mechanism. Induction is contrasted with mutations that are permanent, inherited changes in genes, which may result in drug resistance.

Lipid bilayer The collection of lipids that makes up the plasma (outer) membrane of cells. The phospholipids are arranged in two layers so that their polar, water-soluble heads are exposed either to the inner (cytoplasmic) or outer (extracellular) surface.

Multidrug resistance The development of cross-resistance to many different drugs as the result of a single biochemical change or a single genetic alteration. In its strictest sense, cross-resistance should refer to resistance to drugs with different structural features and different cytotoxic targets.

Multifactorial multidrug resistance Resistance that results from a change in many different resistance mechanisms, not a single mechanism as is the case in classical multidrug resistance.

Natural product A pharmacological agent that comes from the natural environment and is not synthesized *de novo* in the laboratory.

P-glycoprotein An energy-dependent plasma membrane pump responsible for multidrug resistance to many natural product drugs and their derivatives. In the human, P-glycoprotein is the 1280-amino acid product of the *MDR1* (*ABCB1*) gene.

Pharmacogenomics A term coined to indicate the study of those inherited changes in proteins and in the factors that regulate their expression and account for inherited differences in metabolism, uptake, distribution, and excretion of drugs.

Photoaffinity labeling The use of chemical compounds that can be activated by light so as to form chemical linkages with other macromolecules to which they are bound. When such photoaffinity labels are radioactive, they can be used to identify such binding macromolecules.

Plasma membrane The lipid bilayer and associated proteins and other molecules that make up the outer surface of cells.

Substrate A molecule that is acted upon by a macromolecule so as to change its chemical composition, or to transport it within, into, across, or out of cells.

Transgenic mice Mice whose genes have been altered in the laboratory to enable the study of the function of a specific gene or genes. Transgenic mice may carry new genes, or have altered genes, including alterations that totally knock out the expression of specific genes.

Transmembrane domains Those parts of proteins inserted into the lipid bilayer of cell membranes.

Xenobiotics Chemical materials, usually natural products, that are toxic to cells. Some of these are present in food and microorganisms and are ingested inadvertently, whereas others are used in the treatment of cancer and other diseases.

The Role of the Multidrug Efflux Pump, P-Glycoprotein, In Multidrug Resistance in Cancer Cells

Multidrug resistance is the phenomenon by which cancer cells display simultaneous resistance to many different anticancer drugs that are chemically dissimilar and that do not have the same cytotoxic target within the cancer cell. It is now known that multidrug resistance can have many different causes, including failure of cancer cells to accumulate drugs, to metabolize them to toxic products, to activate cell death pathways, and the ability of cancer cells to activate alternative growth-promoting pathways. For many years this

☆*Change History:* March, 2014. M. Gottesman introduced small edits and updates to the text. More references were added to the reference list. Figure 1 was re-done. Figures 2 and 3 were replaced with previously published figures that were slightly adapted for this article.
This article is an update of M.M. Gottesman,Multidrug Resistance I: P-Glycoprotein, In Encyclopedia of Cancer (Second Edition) edited by Joseph R. Bertino, Academic Press, 2002, Pages 247–254.

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phenomenon of broad drug resistance was mysterious to investigators who studied resistance to anticancer drugs. The first major breakthrough in this field was made in 1985 when three groups published the sequence of an energy-dependent plasma membrane transporter, or P-glycoprotein, the product of the *MDR1* (*ABCB1*) gene. P-glycoprotein could detect many different lipid-soluble anticancer drugs and pump them out of the cell so as to prevent accumulation to toxic levels (see Figure 1). The relative lack of specificity of this transport pump, and the fact that many anticancer drugs are lipid-soluble natural products, accounted for its ability to confer multidrug resistance to cancer cells. Table 1 summarizes some of the known substrates for the P-glycoprotein pump, which include not only anticancer drugs, but also many other important pharmacologic agents in common use in the clinic. Subsequently, other energy-dependent multidrug efflux pumps were discovered, including the *MRP* family of transporters and ABCG2.

Once antibodies and molecular probes were available for detection of P-glycoprotein in cancer cells, it was possible to ask whether cancers from patients, with or without exposure to anticancer drugs, expressed this efflux pump. It was known by the late 1980s that many different kinds of cancers express P-glycoprotein at levels high enough to confer resistance to anticancer drugs, and that the presence of P-glycoprotein frequently correlated with resistance to drugs known to be substrates for transport by P-glycoprotein. For example, epithelial cancers of the colon, small intestine, pancreas, adrenal, and kidney, derived from tissues that normally express P-glycoprotein, usually express high levels of this transporter. Although P-glycoprotein pump activity is not the only reason for the multidrug resistance of these epithelial tumors, expression of P-glycoprotein does appear to be a barrier to effective therapy in these cancers with many different standard anticancer drugs.

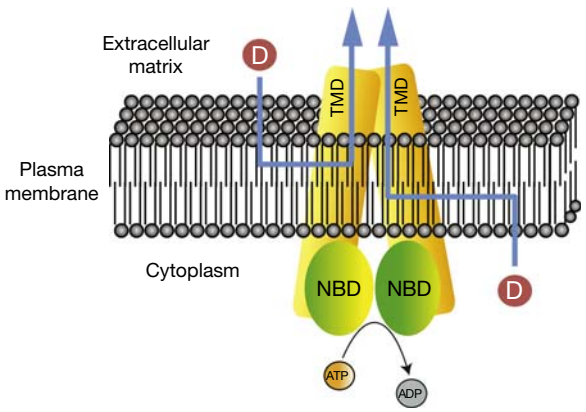


Figure 1 Diagram illustrating the potential mechanism of action of P-glycoprotein as it removes drugs (D) from the lipid bilayer, either as they are entering the cell, or once they have accumulated intracellularly. The solid lines represent likely pathways of drugs through the transporter. The molecule consists of two homologous halves each with a nucleotide-binding domain (NBD) which act together to hydrolyze ATP.

Table 1 Selected substrates of P-glycoprotein

Class	*Drug*
Anticancer drugs	Vinca alkaloids (vincristine, vinblastine)
	Anthracyclines (doxorubicin, daunorubicin, epirubicin)
	Epipodophyllotoxins (etoposide, teniposide)
	Paclitaxel (taxol)
	Actinomycin D
	Topotecan
	Mithramycin
	Mitomycin C
Other cytotoxic agents	Colchicine
	Emetine
	Ethidium bromide
	Puromycin
Cyclic and linear peptides	Gramicidin D
	Valinomycin
	N-Acetyl-leucyl-leucyl-norleucine
	Yeast **a**-factor pheromone
HIV protease inhibitors	Ritonavir
	Indinavir
	Saquinavir
Fluorescent compounds	Hoechst 33342
	Rhodamine 123
	Calcein-AM

Some cancers, which did not express high levels of P-glycoprotein initially, began to express high levels after many cycles of selection in anticancer drugs. Examples include leukemias, lymphomas, myelomas, and ovarian cancer. In these cases, it is presumed that the toxic drug kills most of the sensitive cells in the original population, and only those cells that express higher levels of P-glycoprotein, as a result of a mutation in the cancer cell affecting regulation of expression of this pump, are able to survive and multiply. Evidence suggests that, to some extent, cytotoxic drugs can directly turn on the expression of P-glycoprotein, and that this phenomenon might contribute to clinical multidrug resistance in some cases. However, in cultured cells, the survivors of drug selection appear to express P-glycoprotein in a stable manner, thereby arguing in favor of selection of a pre-existing mutant cancer cell, rather than an induction mechanism that would not result in stable, long-term expression of P-glycoprotein.

Finally, it appears that the malignant transformation process itself can result in turning on expression of the *MDR*1 gene. Examples include some leukemias (especially the blast transformation of cells, which occurs in chronic myelogenous leukemia), and neuroblastomas in children. It is not known why this occurs, but presumably the malignantly transformed state itself results in induction of the P-glycoprotein gene, and the resulting tumor cells are multidrug-resistant. Another possibility is that expression of P-glycoprotein enhances the malignant phenotype. There is preliminary evidence in some model systems that expression of P-glycoprotein has an antiapoptotic effect. These observations are consistent with the idea that P-glycoprotein may facilitate cell survival in tumors.

An important unanswered question is whether inhibiting the function of P-glycoprotein in tumor cells that express it will result in better responses to chemotherapy, i.e., is P-glycoprotein ever limiting in determining response to chemotherapy? Despite many clinical trials that have addressed this issue, the answer is not yet available. Until there are potent, specific inhibitors of P-glycoprotein available, and these are tested in cancers known to express P-glycoprotein, we will not have this answer. Preliminary studies for some leukemias and myelomas suggest that improved response is possible when P-glycoprotein is inhibited, but response in epithelial tumors seems minimal. Studies attempting to correlate resistance to chemotherapy in epithelial cancers with expression of a single multidrug resistance gene have so far suggested that in clinical cancers multidrug resistance is multifactorial, whereas resistance which occurs in cancers selected with anticancer drugs is more likely to be due to a single cause. However, despite the fact that inhibiting P-glycoprotein in epithelial cancers does not sensitize these cancers to natural product anticancer drugs, pharmaceutical companies have kept in mind the presence of this drug efflux pump, because drugs that do not accumulate in cells cannot kill them. Consequently, new anti-cancer drugs are now routinely screened to determine if they are P-glycoprotein substrates.

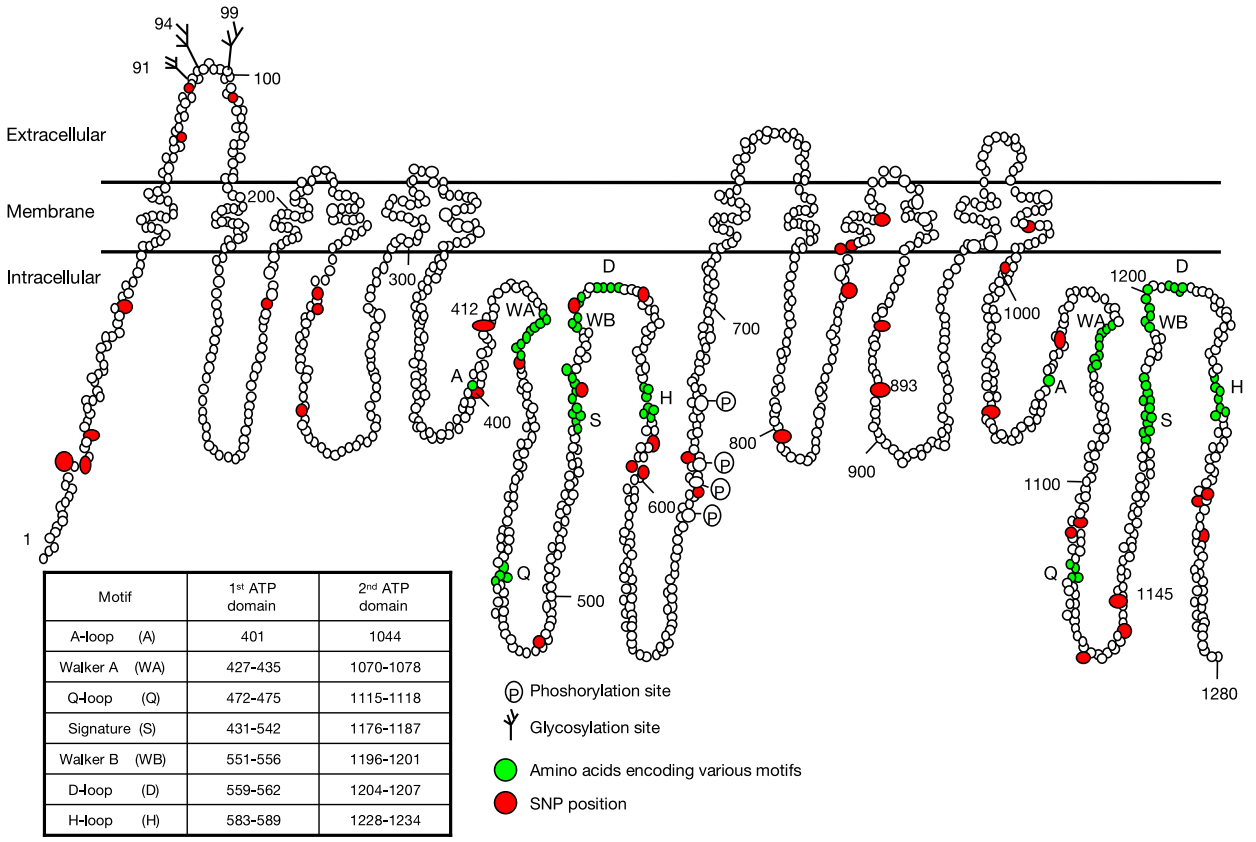
One new strategy to deal with P-glycoprotein-expressing tumors is to take advantage of the fact that expression of P-glycoprotein is an Achilles' heel that sensitizes cancers to other toxic small molecules. Efforts to develop such P-glycoprotein-targeted therapeutics are in progress.

Structure and Mechanism of Action of P-Glycoprotein

The discovery of a pump that can recognize and extrude many different anticancer drugs led naturally to the question of how so many different substrates could be recognized by one protein, and how the energy of ATP was harnessed to the pump process. The first step was to create a model of P-glycoprotein based on its known amino acid sequence. In the human, P-glycoprotein is encoded by 1280 amino acids. There are a total of 12 segments consisting mostly of water-insoluble amino acids that would be expected to cross the plasma membrane of the cell; 6 of these are in the amino-terminal half of the pump and 6 are in the carboxyl half. In addition, there are two regions that are very similar to ATP-binding regions of other proteins, and in particular appear to have some sequence characteristics seen in a large family of proteins (probably 80 are encoded in the human genome) involved in energy-dependent transport across cellular membranes. This family is known as the ATP-binding cassette (ABC) family of proteins. A hypothetical planar structure of P-glycoprotein based simply on the amino acid sequence and supported by data using specific antisera is shown in **Figure 2**.

Based on photoaffinity labeling studies using analogs of known substrates for P-glycoprotein, and on mutational analyses in which individual amino acids in the transmembrane domains of P-glycoprotein are mutated, it appears that the major drug interaction sites on P-glycoprotein are in the transmembrane segments. More recently, a low-resolution X-ray crystallographic structure of mouse P-glycoprotein with a drug bound has confirmed this prediction. This information, plus the knowledge that P-glycoprotein is able to decrease the rate of influx of drugs as well as increase their rate of efflux, and some studies showing a direct interaction of drugs dissolved in the plasma membrane with P-glycoprotein, has led to a model of P-glycoprotein as a 'hydrophobic vacuum cleaner.' In other words, P-glycoprotein detects and ejects drugs while they are still in the lipid bilayer (see **Figure 1**). The specific drug interaction sites on P-glycoprotein appear to be quite complex and consist of a series of overlapping hydrophobic sites within the parts of the protein that transit the plasma membrane. This model explains the rather broad substrate specificity of P-glycoprotein, as the important interactions are hydrophobic and not ionic. The general finding that recognition of drugs depends more on hydrophobicity and size and shape rather than specific chemistry is also consistent with this hypothesis.

The presence of two ATP-binding/utilization sites on P-glycoprotein has also been puzzling. Data suggest that ABC cassette proteins require hydrolysis of two molecules of ATP to transport a single molecule of substrate, and data based on transport of vinblastine by P-glycoprotein are consistent with this generalization. Why two molecules of ATP? Hydrolysis of one molecule of



Motif	1st ATP domain	2nd ATP domain
A-loop (A)	401	1044
Walker A (WA)	427-435	1070-1078
Q-loop (Q)	472-475	1115-1118
Signature (S)	431-542	1176-1187
Walker B (WB)	551-556	1196-1201
D-loop (D)	559-562	1204-1207
H-loop (H)	583-589	1228-1234

Figure 2 A hypothetical two-dimensional model of human P-glycoprotein based on hydropathy analysis of the amino acid sequence and its functional domains. In this schematic diagram, each circle represents an amino acid residue. Red circles represent the amino acids affected by known single nucleotide polymorphisms (SNPs) that alter the substrate specificity of P-gp. The regions that encode A-loops, D-loops, H-loops, Q-loops, signature motifs, Walker-A and Walker-B motifs are colored in green, and the amino acid positions of these motifs are summarized in the table. Phosphorylation sites and glycosylations are also shown. Adapted from Fung, KL and Gottesman MM (2009), A synonymous polymorphism in a common MDR1 (ABCB1) haplotype shapes protein function, *Biochimica et Biophysica Acta* **1794**, 860-71.

ATP appears to be sufficient to reduce the affinity of P-glycoprotein for its substrates, which probably corresponds to the change in shape that the pump undergoes as it extrudes drug from the lipid bilayer into the extracellular space. Evidence points to the need for hydrolysis of a second molecule of ATP so that the pump can return to its initial high affinity binding state and begin the pumping cycle all over again. Additional experimental evidence points to the need for two functional ATP sites (inactivation of one site results in loss of pump function), the asymmetry of the two sites, and the alternating action of these two sites so that ATP hydrolysis cannot occur at both sites at the same time.

Structural studies on other ATP-binding cassette (ABC) transporters indicate that the two ATPs each bind to sites formed from each half of the transporter. The binding of ATP brings the two ABCs together and activates the transport process.

Normal Cellular Function of P-Glycoprotein

The discovery of P-glycoprotein and its ability to recognize dozens, and perhaps hundreds of structurally dissimilar compounds, raised questions not only about its mechanism of action, but about how it had evolved and what its normal function might be. The finding that all living organisms so far studied, from bacteria to man, have pumps similar to P-glycoprotein, suggested that free living cells could not survive without a broad-spectrum pump for hydrophobic compounds. Whether this reflects the ubiquitous presence of toxic xenobiotics in the environment (most of which are themselves the products of life forms and may be part of the armaments of battle used as organisms strive to gain a selective advantage in their biological niches), or an intrinsic requirement for *MDR* genes to maintain cellular integrity, is still not known with certainty. However, genetically engineered organisms such as transgenic mice lacking *mdr*1 genes (in the mouse, there are two *mdr*1 genes, called *mdr*1a and *mdr*1b or *mdr*1 and *mdr*3), and other mammalian cells lacking *MDR*1 expression, survive with normal lifespans, suggesting that there is no absolute requirement for *MDR*1 expression in cells. Moreover, mice without functioning *mdr*1 genes are exquisitely sensitive to certain xenobiotics, but carry on other cellular functions with reasonable efficiency. Because many transport functions of P-glycoprotein in mammals are

redundant with other ABC transporters, especially the *MRP* system, it is still possible that every living cell needs one or another member of this class of transporters to survive.

What sort of evidence exists suggesting cellular functions for P-glycoprotein, other than to protect cells from toxic effects of xenobiotics? In general, overexpression of P-glycoprotein after gene transfer into various cell types or after selection in cytotoxic drugs, has been used to study the effect of P-glycoprotein on cellular processes. Under these conditions, it is possible to show a reduction in sensitivity to apoptosis in lymphoid cells, resistance to certain enveloped viruses, including HIV, and altered plasma membrane lipid composition. Early studies of P-glycoprotein-expressing cells suggested a correlation with metastatic potential of cancer cells. Use of inhibitors of P-glycoprotein results in altered cellular functions, but there are probably no totally specific P-glycoprotein inhibitors, which can give definitive results about the function of P-glycoprotein alone. Use of ribozymes and anti-sense technologies to eliminate P-glycoprotein expression have so far not totally eliminated expression of P-glycoprotein, although some reductions in P-glycoprotein levels have been possible using this approach.

Once again, the use of mice lacking functioning *mdr1* genes has proved useful in determining the normal function of P-glycoprotein. As noted above, *mdr1* knockout mice live normal lifespans and are sensitive to xenobiotics. More detailed studies of the function of individual tissues that normally express P-glycoprotein suggests subtle alterations in immune system function, not yet clearly defined, and relatively normal function of epithelial tissues of the gastrointestinal tract, liver, kidney and pancreas.

Role of P-Glycoprotein in Drug Pharmacokinetics

The use of inhibitors of P-glycoprotein and the study of *mdr*1 knockout mice has confirmed suspicions from the initial histochemical localization studies that *MDR*1 played a major role in uptake, distribution, and excretion of toxic xenobiotics in mice and men. Lack of expression of functional P-glycoprotein in the gastrointestinal tract dramatically enhances the absorption of several different drugs, including anticancer drugs such as taxol, the cardiac glycoside digoxin, and the antihistamine fexofenadine. This is manifested as much higher blood levels of these compounds after oral dosing. Furthermore, distribution of such drugs in the body is altered in the absence of functional P-glycoprotein, manifested as increased brain levels of compounds such as the anti-helminthic ivermectin, the anticancer drug vinblastine, and the anti-diarrheal narcotic analog loperamide. This is attributed to the abrogation of the blood-brain barrier for these compounds owing to the absence of P-glycoprotein in capillary endothelial cells in the brain. Similar effects of loss of P-glycoprotein at the placental barrier would be expected to result in increased teratogenicity of certain compounds that are P-glycoprotein substrates, as is seen in mice lacking P-glycoprotein, and perhaps toxic and/or mutagenic effects on germ cells in the testis and ovary.

Circulating cells, such as T cells and macrophages that normally express P-glycoprotein, might become sensitized to drugs and xenobiotics if their P-glycoprotein levels are altered. Because most of the HIV protease inhibitors are P-glycoprotein substrates, this phenomenon has led to the suggestion that cellular availability of drugs such as this could be manipulated by altering levels of functional P-glycoprotein. Conversely, resistance to certain drugs could occur at the cellular level because of variations in expression of P-glycoprotein that might be genetically determined (see below).

Finally, excretion by the liver (in bile) and kidney (in urine) of P-glycoprotein substrates is substantially reduced for many drugs in the mice lacking P-glycoprotein. This results in a decreased rate of clearance for drugs, and increased accumulation of drugs in the bloodstream and tissues. Thus, use of inhibitors of P-glycoprotein, or alterations in levels of functional P-glycoprotein that might be genetically determined, could have fairly profound effects on the blood and tissue levels of many different drugs, by increasing absorption, decreasing excretion, and altering distribution into tissues protected by P-glycoprotein barriers, or into cells expressing P-glycoprotein. These effects of P-glycoprotein are summarized in **Figure 3**.

Evidence is beginning to be published suggesting that levels of P-glycoprotein in the gastrointestinal tract may vary considerably from individual to individual, perhaps accounting for differences in absorption of drugs, which are primarily P-glycoprotein substrates. One study suggests genetic linkage with a single nucleotide polymorphism that does not change the sequence of P-glycoprotein. This polymorphism, though silent, changes the structure of P-glycoprotein mRNA, resulting in changes in the rate of mRNA translation and altered folding of P-glycoprotein, resulting in different substrate specificity. Several different coding polymorphisms of P-glycoprotein have also been described, and a few appear to alter the ability of P-glycoprotein to pump drugs. Much more work is needed in this important area of pharmacogenomics.

Implications of Studies on P-Glycoprotein for Treatment of Cancer

As we assemble a more complete picture of the biochemistry, pharmacology, and physiology of P-glycoprotein function, we can begin to make better guesses as to the role that manipulation of the *MDR1* gene is likely to have in future treatment of cancer. There are some conclusions that seem quite firm, and others that are still speculative. First, it seems clear from the correlative human studies and animal studies in which mouse cancers selected for drug resistance turn on expression of P-glycoprotein that P-glycoprotein is capable of conferring resistance to many anticancer drugs to cancer cells. Thus, as new drugs are developed, it will be necessary to determine whether cellular sensitivity to these drugs is affected by the presence of P-glycoprotein. As we continue to be dependent for the treatment of cancer on the existing hydrophobic natural products, and as new, more targeted

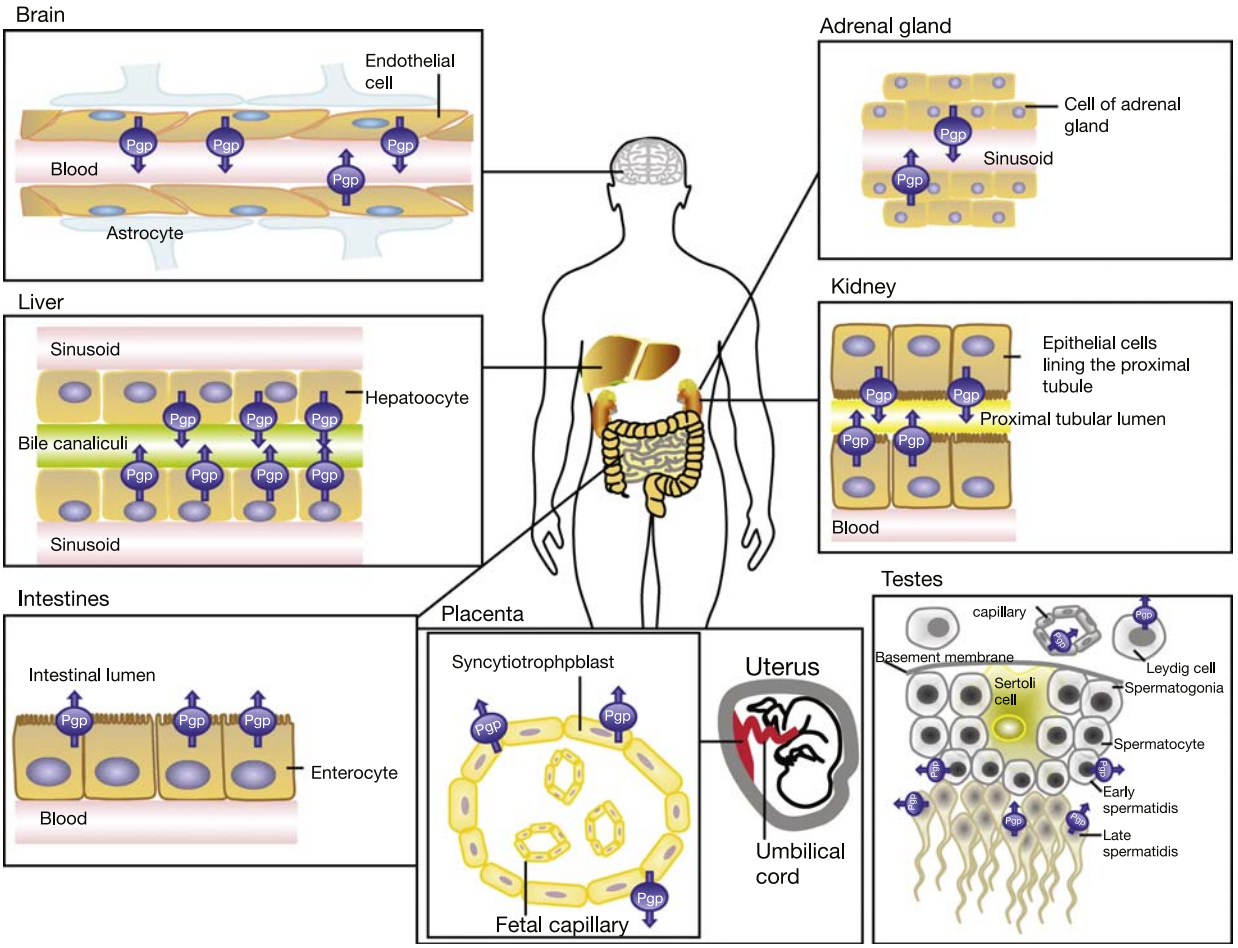


Figure 3 The effect of P-glycoprotein on pharmacokinetics and drug distribution is illustrated schematically. The multidrug transporter affects drug distribution throughout the body. Adapted from Wu CP, Ohnuma S and Ambudkar SV (2011) Discovering natural product modulators to overcome multidrug resistance in cancer chemotherapy. *Current Pharmaceutical Biotechnology* **12**, 609-620. Used by permission.

anti-cancer drugs are developed that may be P-glycoprotein substrates, then we will have to learn to inhibit P-glycoprotein, and its many molecular analogs, as we try to cure and palliate cancer.

Second, despite earlier concerns, it seems likely that more potent, more specific inhibitors of P-glycoprotein, or inhibitors of related ABC transporters, can be developed, and that their toxicity would be relatively limited and manageable. This conclusion comes from the studies with transgenic mice lacking P-glycoprotein, which have normal lifespans under controlled laboratory conditions. These inhibitors will play a role not only in sensitizing P-glycoprotein-expressing cancers to anticancer drugs, but also in altering uptake, excretion, and cellular distribution of many different drugs. Using P-glycoprotein inhibitors, it may be possible to give drugs orally that previously required intravenous administration, or to deliver drugs to the brain which previously were only active outside of the central nervous system.

Third, it appears that alterations in levels of expression of P-glycoprotein in different tissues, which may result from environmental exposures or from inherited alterations in pathways that regulate expression of the *MDR*1 gene, or inherited polymorphisms within the coding region of the *MDR*1 gene, could account for variations among individuals in the way they respond to drugs. Such changes could have substantial effects on drug uptake, distribution, and excretion. It will become necessary in the future to catalog these variations in the *MDR*1 gene and other genes, which affect metabolism, absorption, and excretion of drugs in order to predict individual responses to different drugs.

Finally, the cloning of the *MDR*1 cDNA encoding P-glycoprotein has made it possible to transfer this gene into drug-sensitive cells, thereby conferring multidrug resistance on all recipient cells. Because toxicity of anticancer drugs to sensitive tissues, such as epithelia and bone marrow, is a major dose-limiting problem in cancer chemotherapy, the ability to protect normal cells from this toxicity using transferred multidrug resistance genes has been the subject of much laboratory and clinical investigation. Although such gene transfer studies are limited by the low efficiency of existing vector systems and the safety of gene transfer of multidrug resistance genes is yet to be established, it is conceivable that multidrug resistance genes, such as *MDR1*, may prove useful in protecting normal tissues from cytotoxic effects of anticancer treatment.

Further Reading

Aller, S.G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P.M., Trinh, Y.T., Zhang, Q., Urbatsch, I.L., Chang, G., 2009. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323, 1718–1722.

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M., 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annual Review of Pharmacology and Toxicology 39, 361–398.

Fung, K.L., Gottesman, M.M., 2009. A synonymous polymorphism in a common MDR1 (ABCB1) haplotype shapes protein function. Biochimica et Biophysica Acta 1794, 860–871.

Gottesman, M.M., Fojo, T., Bates, S.E., 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nature Reviews Cancer 2, 48–58.

Gottesman, M.M., Hrycyna, C.A., Schoenlein, P.V., Germann, U.A., Pastan, I., 1995. Genetic analysis of the multidrug transporter. Annual Review of Genetics 29, 607–649.

Hall, M.D., Brimacombe, K.R., Varonka, M.S., Pluchino, K.M., Monda, J.K., Li, J., Walsh, M.J., Boxer, M.B., Warren, T.H., Fales, H.M., Gottesman, M.M., 2011. Synthesis and structure-activity evaluation of isatin-beta-thiosemicarbazones with improved selective activity toward multidrug-resistant cells expressing P-glycoprotein. Journal of Medicinal Chemistry 54, 5878–5889.

Kimchi-Sarfaty, C., Oh, J.M., Kim, I.W., Sauna, Z.E., Calcagno, A.M., Ambudkar, S.V., Gottesman, M.M., 2007. A “silent” polymorphism in the MDR1 gene changes substrate specificity. Science 315, 525–528.

Ling, V., 1997. Multidrug resistance: molecular mechanisms and clinical relevance. Cancer Chemotheraphy and Pharmacology 40 (Suppl.), S3–S8.

Schinkel, A.H., 1997. The physiological function of drug-transporting P-glycoproteins. Seminars in Cancer Biology 8, 161–170.

Senior, A.E., Gadsby, D.C., 1997. ATP hydrolysis cycles and mechanism in P-glycoprotein and CFTR. Seminars in Cancer Biology 8, 143–150.

Wu, C.P., Ohnuma, S., Ambudkar, S.V., 2011. Discovering natural product modulators to overcome multidrug resistance in cancer chemotherapy. Current Pharmaceutical Biotechnology 12, 609–620.

Physical Inactivity and Cancer

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Introduction

It is well-documented that moderate- or vigorous-intensity physical activity (MVPA) provides vast health benefits including lowering risk of developing or dying from chronic diseases such as cardiovascular disease, type 2 diabetes mellitus, stroke, and some cancers (Leitzmann et al., 2007; Nocon et al., 2008; Paffenbarger Jr et al., 1986; Blair and Morris, 2009; Moore et al., 2016). Furthermore, an insufficient physical activity level (referred to as physical inactivity) has been estimated to account for 6%–10% of the world's burden of noncommunicable diseases (Lee et al., 2012), yet nearly half of all adults in the United States do not regularly engage in recommended levels of physical activity. Various public health guidelines (Haskell et al., 2007; Nelson et al., 2007; Kushi et al., 2012) state adults should engage in at least 150 min of moderate- (such as walking at a pace of 20 min/mile or 3 miles/h) or 75 min of vigorous-intensity (such as jogging or running) physical activity per week. For additional protection against cancer, the American Cancer Society further recommends approaching or exceeding twice the minimum recommended amount by engaging in 300 min of moderate- or 150 min of vigorous-intensity activity per week (Kushi et al., 2012).

To appropriately account for both amount and intensity of physical activities, many epidemiologic studies ask individuals to report the amount of time spent engaging in activities of different intensities (such as walking, jogging, cycling). Summary MVPA measures are then calculated by summing the frequency of each activity multiplied by the metabolic equivalent of task (MET) value for that activity (MET-hours) (Ainsworth et al., 1993, 2000). A MET is the ratio of energy expended during a specific activity compared to that while at rest. One MET is equal to the energy expenditure of an individual while seated at rest. Moderate-intensity activities are defined as those with MET values between 3 and 6, and vigorous intensity activities have MET values greater than 6. For reference, walking 1 h at an average pace of approximately 20 min/mile (or 2.8–3.2 miles/h) is equal to 3.3 MET-hours (Ainsworth et al., 2000). Thus, 7.5 MET-hours/week is equal to the minimum recommended amount of MVPA.

Studies have shown that engaging in even relatively small amounts of MVPA are associated with health benefits compared to inactivity. One recent pooled analysis demonstrated that engaging in MVPA well below recommended levels (0.1–3.74 MET-hours/week vs. no activity) was associated with approximately 20% lower risk of all-cause mortality (RR = 0.81, 95% CI 0.79–0.83) and 1.8 (95% CI 1.6–2.0) years of life gained compared to inactivity (Moore et al., 2012). While meeting the minimum recommended level of MVPA (7.5 to < 15.0 MET-hours/week) was further associated with 32% lower risk of overall mortality (95% CI 0.66–0.69) (Moore et al., 2012). Similarly, a more recent pooled study suggested that engaging in any level of physical activity less than the recommended amount was also associated with 13% lower risk of cancer mortality compared to engaging in no MVPA (95% CI 0.83–0.90), and meeting recommended levels was associated with 21% lower risk (95% CI 0.75–0.82) of cancer mortality (Arem et al., 2015). Increasing MPVA to approximately three times recommended levels (22.5+ MET-hours/week) was associated with 41% lower mortality risk (95% CI 0.57–0.61), and other studies suggest that the risk continues to decline up to 10 times recommended levels (75+ MET-hours/week) (RR = 0.69, 95% CI 0.55–0.87) (Arem et al., 2015; Matthews et al., 2012a).

Distinct from physical inactivity, the amount of time spent sitting has increased significantly over the past few decades. This increase in sedentary behavior, characterized by very low energy expenditure (≤1.5 METs) while in a sitting, reclining, or lying position (Tremblay et al., 2010), is largely attributed to technologic advancements (e.g., computers in the workplace), increased dependence on automobiles for transportation, and engagement in more sedentary activities during leisure time (e.g., watching television or playing video games). For example, in 1950, approximately 15% of jobs were classified as sedentary compared to 25% in 2000 (US Bureau of Labor Statistics), and the average amount of time spent watching television, the most common leisure-time sedentary activity, increased from 4.75 to 7.5 h/day (Nielsen Report, 2007). Recent evidence suggests that prolonged time spent sitting (such as while watching television or other screen-based entertainment) may have deleterious health effects independent of physical inactivity. In fact, there has been a growing body of evidence to support that prolonged time spent sitting is associated with total, cardiovascular disease, and cancer mortality (Matthews et al., 2012a; Ekelund et al., 2016; Chau et al., 2013; Patel et al., 2010), and a recent large meta-analysis (Chau et al., 2013) reported 34% higher mortality risk for adults sitting 10 h/day (vs. 0–3 h) after taking physical activity into account.

There is sufficient biologic rationale and mounting scientific evidence to support the role of physical inactivity and sedentary time in the development and progression of various types of cancer. This article will describe the associations observed between specific cancer types, physical inactivity, and sitting time, and provide an overview of the biologic plausibility for these associations. Finally, future directions for research will be discussed.

Biologic Mechanisms

Physical activity and sitting time may have indirect and direct effects on cancer risk. Indirectly, physical activity and reduced sitting time are important in weight management and may be associated with lower cancer risk due to obesity-mediated effects. The most recent International Agency for Research on Cancer (IARC) report on obesity and cancer concluded that obesity was causally

associated with 13 different cancer sites (Lauby-Secretan et al., 2016): esophageal adenocarcinoma, gastric cardia, colorectum, liver, gallbladder, pancreas, breast (postmenopausal), corpus uteri, ovary, kidney (renal cell), meningioma, thyroid, and multiple myeloma. Through lowering or maintaining body weight, it is plausible that physical activity and/or reduced sitting time could lower risk of these obesity-related cancers.

Observational and intervention studies have convincingly shown that physically active participants are less likely to gain weight compared to less active participants (World Cancer Research Fund/American Institute for Cancer Research Report, 2007). As reviewed in the World Cancer Research Fund/American Institute for Cancer Research Report (2007), randomized trials and prospective cohort studies have shown that physical activity is beneficial for both weight maintenance or change in adults. For example, one prospective study reported a 0.77% decrease in annual percentage change in weight (95% CI 0.53–1.01) per one MET-hour per day increase in MVPA (Macdonald et al., 2003). Another (randomized controlled trial) study examined a one-year aerobic exercise program where post-menopausal women exercised an average of 178 min/week and observed 1.8 kg loss for body weight and 2.0 kg loss for total body fat compared to the control group (Friedenreich, 2011).

Similarly, greater time spent sitting (especially while watching television) has been shown to be associated with the development of obesity. For example, in the Nurses Health Study, sitting between 2 and 5 hours per day while watching television was associated with 22% higher risk of becoming obese during the 6-year follow-up period compared to <2 hours per day of TV viewing (Hu et al., 2003). There was a strong dose–response relationship culminating with a nearly twofold higher risk among women who spent >40 hours per week sitting while watching television (RR = 1.94, 95% CI 1.51–2.49, trend $P < 0.001$). This association persisted, albeit was attenuated, even among physically active individuals (Hu et al., 2003).

Regardless of accompanying changes in weight or body composition, physical activity is directly associated with numerous benefits that may be related to cancer risk. In particular, aerobic exercise has been shown to improve lipid metabolism and facilitate glucose uptake (McTiernan, 2008; Kerr et al., 2017). Regular physical activity is also associated with a decrease in circulating sex hormones, including estrogens and androgens. More recent evidence has confirmed that exercise elicits a favorable immune response, including an increase in macrophage phagocytosis, and a decrease in systemic inflammation, as evidenced by a decrease in inflammatory biomarkers (for example, IL-6, TNF-alpha, and CRP) (Ashcraft et al., 2016).

Human mechanistic studies exploring the independent effect of sedentary time on cancer risk are sparse, however early bed rest studies provide insight on the biologic consequences of excess sitting (Bergouignan et al., 2011). The complete lack of skeletal muscle activation associated with sedentary behavior inhibits glucose uptake by the skeletal muscles, inducing an eventual decrease in insulin sensitivity (McTiernan, 2008; Bergouignan et al., 2011). Chronic excess sitting time has also been associated with various markers of chronic disease risk, such as high cholesterol, high fasting insulin levels, and other biomarkers of chronic disease (Fung et al., 2000; Healy et al., 2008a,b,c; Jakes et al., 2003). Emerging evidence suggests that even relatively short active "breaks" in sedentary time may be beneficial for metabolic function, particularly glucose regulation and triglyceride metabolism (Healy et al., 2008a; Owen et al., 2010).

Physical Activity and Cancer Prevention

Hundreds of studies have examined the association between physical activity and cancer prevention (World Cancer Research Fund/American Institute for Cancer Research Report, 2007; Kerr et al., 2017). The most recent consensus report published in 2007 evaluated the evidence for these associations (World Cancer Research Fund/American Institute for Cancer Research Report, 2007) and concluded that there is sufficient evidence suggesting that physical activity is associated with lower risk of colorectal, postmenopausal breast, and endometrial cancer. More recent studies have provided further evidence that physical activity may also be associated with lower risk of various additional cancers (Moore et al., 2016). In a recent large pooled analysis including 12 prospective cohorts with over 1.44 million participants and over 186,000 incident cancer cases, leisure-time physical activity (top 10th percentile vs. bottom 10th percentile) was inversely associated with 16 of 26 individual cancer sites. The expected associations with colon, postmenopausal breast, and endometrial cancer were confirmed, and associations with the following additional cancers were noted: esophageal adenocarcinoma, gallbladder, liver, lung, kidney, small intestine, gastric cardia, myeloid leukemia, myeloma, head and neck, rectum, bladder, and non-Hodgkin lymphoma.

The association between physical activity and colon cancer has been one of the most commonly studied and most consistently associated of all cancer sites in observational studies. Based on one meta-analysis that included over 50 observational studies, the most active people had a 24% reduced risk of developing colon cancer, compared with the least active persons (relative risk, RR = 0.76; 95% confidence interval, CI: 0.72, 0.81) (Wolin et al., 2009). The association between physical activity and rectal cancer is less clear. In a recent meta-analysis including 11 studies (Robsahm et al., 2013), the relative risk of developing rectal cancer was 0.98 (95% CI = 0.88, 1.08) for the most active compared with the least active participants. Conversely, in the large pooled analysis of 12 cohorts described above, leisure-time physical activity was inversely associated with risk of rectal cancer (RR = 0.87, 95% CI 0.80, 0.95) (Moore et al., 2016). Thus, evidence for an association between physical activity and rectal cancer risk remains inconsistent.

More than 75 observational studies worldwide have examined some measure of physical activity in relation to breast cancer risk (Friedenreich, 2011). The majority of these studies focused on recreational MVPA and provide consistent and strong evidence that physical activity is associated with lower breast cancer risk. Studies suggest that, on average, the most physically active women have 25% lower risk of developing breast cancer compared to the least active. Studies have even examined physical activity across various

age and time periods, and consistently support that physical activity is beneficial across the life course (Friedenreich and Cust, 2008). Recent studies have specifically examined walking, as it is the most common moderate-intensity activity, in relation to breast cancer risk. In one of the largest studies to date (Hildebrand et al., 2013), women who reported walking at an average pace (approximately 3 miles/h or 20 min/mile) for an hour per day as their only form of recreational activity had 14% lower breast cancer risk compared to inactive women; this finding is consistent with associations previously reported for other types of moderate-intensity activity. However, vigorous-intensity physical activity appears to be associated with a greater breast cancer risk reduction (approximately 20% lower risk) than moderate-intensity physical activity (approximately 15% lower risk) when examining the "highest" versus "lowest" level of each activity intensity.

A recent meta-analysis (Schmid et al., 2015) including more than 30 observational studies examined the association between physical activity and endometrial cancer risk and found a 20% lower risk in the "highest" compared to the "lowest" physical activity category (95% CI 0.75, 0.85). Few studies have examined associations separately for moderate- and vigorous-intensity activity, but 10 studies have previously examined walking in relation to endometrial cancer risk. Across these studies, regular walking was associated with 18% lower risk of endometrial cancer (RR = 0.82, 95% CI 0.69, 0.97) compared to inactivity (Schmid et al., 2015). Limited evidence also suggests that physical activity may be more strongly associated with endometrial cancer risk in overweight or obese women compared to normal weight women.

As previously described, the largest study to date on physical activity and cancer was published in 2016 and harmonized data from 12 prospective cohort studies to evaluate the associations between the top and bottom 10% activity levels (Moore et al., 2016). The strongest association between physical activity and cancer was observed for esophageal adenocarcinoma (RR = 0.58, 95% CI 0.37–0.89), a finding largely supported by a recent meta-analysis including 24 studies which reported an inverse association with lesser magnitude (RR = 0.79, 95% CI 0.66–0.94) when examining the "highest" versus the "lowest" activity level in each study (Behrens et al., 2014). In both the pooled analysis (RR = 0.80, 95% CI 0.61–1.06) and meta-analysis (RR = 0.94, 95% CI 0.41–2.16), there was no evidence of an association between physical activity and squamous esophageal cancer.

An inverse association was also observed in the pooled analysis between physical activity and cancer of the gastric cardia (RR = 0.78, 95% CI 0.64–0.95) which was very similar to a recent large meta-analysis including 22 studies examining the "highest" versus "lowest" physical activity level (RR = 0.84, 95% CI 0.73–0.96) (Psaltopoulou et al., 2016). Associations between physical activity and bladder cancer were also similar between the pooled analysis (RR = 0.87, 95% CI 0.82–0.92) and a recent meta-analysis of 15 observational studies (RR = 0.85, 95% CI 0.74–0.98) (Keimling et al., 2014). Conversely, a different meta-analysis including 22 observational studies reported a modest inverse association between "highest" versus "lowest" level of physical activity in relation to pancreatic cancer risk (RR0.93, 95% CI 0.88–0.98) (Behrens et al., 2015), conflicting with the large pooled analysis which concluded physical activity was not associated with pancreatic cancer risk (RR = 0.95, 95% CI 0.83–1.08).

Based on a recent meta-analysis of 15 observational studies which examined the association between physical activity and various types of hematopoietic malignancies, physical activity was modestly but nonstatistically significantly associated with non-Hodgkin lymphoma (RR = 0.91, 95% CI 0.82–1.00) (Jochem et al., 2014). This finding was nearly identical to that reported in the large pooled analysis (RR = 0.91, 95% CI 0.83–1.00). The hematopoietic malignancies meta-analysis also concluded that physical activity was not associated with myeloid leukemia (RR = 0.97, 95% CI 0.84–1.13) or myeloma (RR = 0.86, 95% CI 0.68–1.09). In contrast, the large pooled analysis found that physical activity was inversely associated with both myeloid leukemia (RR = 0.80, 95% CI 0.70–0.92) and myeloma (RR = 0.83, 95% CI 0.72–0.95).

The reported associations between physical activity and lung cancer are also contradictory, largely due to differences in adjustment for residual confounding by smoking. One meta-analysis of 28 studies found a strong inverse association between physical activity and lung cancer risk (Brenner et al., 2016) in which "highest" versus "lowest" level of physical activity was associated with 24% lower lung cancer risk (95% CI 0.69–0.85). A significant limitation of this and other previous studies is the lack of stratification by smoking status. Individuals who smoke cigarettes are far less likely to be physically active, and even when active, they are likely less active than nonsmokers. Thus, the association of physical activity and lung cancer risk is likely significantly confounded by smoking. It is further possible that heavy smokers have impaired lung function resulting in inactivity which would lead to reverse causality. When stratifying on smoking status, an inverse association between physical activity and lung cancer risk was only evident among current (RR 0.77, 95% CI 0.72–0.83) and former (RR 0.77, 95% CI 0.71–0.90) smokers, while no association was observed among never smokers (RR = 0.96, 95% CI 0.79–1.18). Similarly, while an inverse association between physical activity and lung cancer was observed in the overall large pooled study population (RR = 0.74, 95% CI 0.71–0.77), the association was null among never smokers. Thus, the current evidence does not support an association between physical activity and lung cancer when smoking is appropriately accounted for.

A recent meta-analysis of 19 studies (Liu et al., 2011) found a modest, yet statistically significant, 10% lower risk for prostate cancer among the most physically active compared to the least active men. Because physically active men are also more likely to be screened for prostate cancer, it is possible that the association with non-advanced and advanced prostate cancer may differ (Patel et al., 2005). A subset of studies included in the meta-analysis examined aggressive and nonaggressive prostate cancer separately and found a stronger association of physical activity with aggressive (RR = 0.89, 95% CI 0.71–1.12) compared to nonaggressive prostate cancer (RR = 0.98, 95% CI 0.79–1.21). In contrast to the meta-analysis, the large pooled analysis by Moore et al. (2016) found a modest positive association between physical activity and prostate cancer overall (RR = 1.05, 95% CI 1.03–1.08), and further found that the association was driven by non-advanced prostate cancer (RR = 1.08, 95% CI 1.03–1.12), as no association was observed for advanced prostate cancer (RR = 0.99, 95% CI 0.88–1.10). Thus, evidence to date supports that physical activity is unlikely to lower risk of non-advanced prostate cancer, but may offer some benefit to more advanced disease.

In summary, as demonstrated by previous meta-analyses and large pooled analyses, there is ample evidence to support associations of physical activity and lower cancer risk. Evidence is strong for the benefits of physical activity in the prevention of colon, postmenopausal breast, and endometrial cancer. Evidence is growing to support the broader benefit of engaging in physical activity in the prevention of many other types of cancer.

Sedentary Time and Cancer Risk

Numerous studies have shown that prolonged time spent sitting (total, television viewing, leisure-time) is associated with total mortality independent of physical inactivity (Matthews et al., 2012a; Ekelund et al., 2016; Chau et al., 2013; Patel et al., 2010). In one large meta-analysis that included 13 studies, most of which were prospective cohorts (Biswas et al., 2015), prolonged sitting was associated with 24% higher mortality risk after taking physical activity into account (95% CI 1.09–1.41). Included in this meta-analysis was a review of the seven studies to date that have specifically examined leisure-time sitting in relation to cancer mortality (Biswas et al., 2015). The summary risk estimate was not as strong as total mortality, but prolonged sitting was statistically significantly associated with cancer mortality (RR = 1.16, 95% CI 1.10–1.22). In the large prospective NIH-AARP Diet and Health Study, both men and women who spent 7 or more hours per day watching television had 22% higher risk of dying from cancer compared to those who watched <1 h of television/day (95% CI 1.06–1.40) suggesting that associations with sitting while watching television may be more strongly associated with health than measures of overall sitting time (Matthews et al., 2012a).

While extensive research supports the role of physical activity in cancer prevention, far fewer studies have examined associations between sitting time and site-specific cancer incidence. A 2013 meta-analysis including 43 observational studies examined the associations between total sedentary time and television viewing in relation to cancer risk across various sites (Schmid and Leitzmann, 2014). The "highest" versus "lowest" level of television viewing time was associated with higher risk of colon cancer (RR = 1.54, 95% CI 1.19–1.98) and endometrial cancer (RR = 1.66, 95% CI 1.21–2.28). Although TV viewing time is often used as a proxy for measuring sedentary behavior, outcomes appear to be more strongly associated with TV viewing than with total sedentary time in most studies. It is not yet clear whether television viewing time is simply measured with greater precision compared to overall sitting, or if this is due to effect modification by unhealthy behaviors which may be concurrent with or promoted by television watching, such as excess snacking. Accordingly, the 2013 meta-analysis found that total sitting time was associated with 24% increased risk of colon cancer (RR = 1.24, 95% CI 1.09–1.41) and 21% increased risk of endometrial cancer (RR = 1.21, 95% CI 1.03–1.43). The same study additionally found a positive association between occupational sitting time and colon cancer (RR = 1.24, 95% CI 1.03–1.50) but not endometrial cancer (RR = 1.11, 95% CI 0.88–1.39). While there is evidence to support an association between sedentary time and endometrial cancer risk, some previous studies have shown that the association is largely attenuated after adjusting for body mass index (BMI) (Friberg et al., 2006; Gierach et al., 2009; Patel et al., 2015) suggesting that the association between sitting time and endometrial cancer incidence might be confounded or mediated by BMI.

Following this meta-analysis, one study comprehensively examined leisure-time spent sitting in relation to total and site-specific cancer incidence and found marked sex-specific differences. Women who reported sitting for more than 6 versus fewer than 3 h/day during their leisure-time had a 10% higher risk of all cancers combined (Patel et al., 2015). However, authors found that the overall association in women was driven by site-specific associations with invasive breast cancer (RR = 1.10, 95% CI 1.00, 1.21), ovarian cancer (RR = 1.43, 95% CI 1.10, 1.87), and multiple myeloma (RR = 1.65, 95% CI 1.07–2.54). Other prospective studies have similarly reported a modest higher risk with total sitting time (George et al., 2010) in relation to invasive breast cancer. Additionally, one case-control study (Zhang et al., 2004) reported a higher risk between leisure-time sitting and ovarian cancer, although it is worth noting that other studies examining domain-specific sitting time (not including leisure-time) were null (Xiao et al., 2013; Dosemeci et al., 1993).

On the contrary, leisure-time sitting was not associated with overall cancer risk in men, although among obese men there was an 11% higher risk associated with high levels of sitting time. Two prospective cohort studies have also reported an inverse association with total prostate cancer; however, there was no dose–response and results were attenuated when restricted to advanced disease (Patel et al., 2015; Lynch et al., 2014). A statistically significant association with multiple myeloma and borderline associations with head and neck and gallbladder cancer in men and women, esophageal cancer in women, and pancreatic cancer in men have also been documented (Patel et al., 2015). In general, physical activities of a moderate- to vigorous-intensity, BMI, and age have not been shown to modify associations of sitting time with cancer risk, with the exception of endometrial cancer.

The previously described meta-analysis by Schmid and Leitzmann (2014) concluded that sedentary behavior was unrelated to all other cancers examined including: breast, rectum, ovary, prostate, stomach, esophagus, testes, renal cell, and non-Hodgkin lymphoma. However, the evidence was limited for most individual cancer sites, and aggregated studies often examined different domains of sitting time (e.g., occupational, total, leisure-time, television time). Thus, the evidence is still emerging and data are likely too sparse to draw strong conclusions for an association between sedentary behavior and most site-specific cancers.

Prospective Vision (Future Directions)

While there is substantial evidence to support that physical activity is beneficial in the prevention of various types of cancer, there are numerous gaps in the evidence that warrant further research. The dose–response relationship between moderate- and/or

vigorous-intensity physical activity or sitting time in relation to cancer risk is not well known. While the association between physical activity and overall longevity has been well-characterized (Arem et al., 2015), the early evidence suggests that the association between physical activity and cancer incidence may be linear (Moore et al., 2016). Furthermore, some studies suggest that a greater dose of physical activity may be necessary for cancer prevention (Hildebrand et al., 2013). Future studies should aim to better understand this dose–response relationship as the nature of that relationship will inform public health recommendations for cancer prevention.

As previously described, early evidence suggests that certain types of physical activity, such as walking, and certain sedentary behaviors, such as television viewing, may be individually associated with cancer incidence, but additional research is needed. Walking is free, does not require special training or equipment, and can be done almost anywhere making it the most common moderate-intensity physical activity (U.S. Department of Health and Human Services, 2015). Thus, better understanding the cancer prevention value of walking can have tremendous value in helping to increase population physical activity levels. Similarly, there is a paucity of research on light-intensity physical activities, such as shopping, housework, and gardening, in relation to cancer risk. As television viewing is the most common leisure-time activity, it is important to understand if there are specific greater harms associated with sitting while watching television versus other types of sitting. More broadly, improving our understanding of the underlying biologic mechanisms for both physical activity and sedentary time in relation to cancer risk are necessary.

Fewer studies have examined whether physical activity and cancer associations are modified by other factors such as race, BMI, or family history of cancer. For some cancer sites, understanding whether associations differ by molecular (e.g., by hormone receptor status in breast cancer) or histologic (e.g., different subtypes of non-Hodgkin lymphoma such as diffuse large B-cell or follicular lymphoma) subtypes or subsite of disease (such as distal versus proximal colon) is also necessary. As these subgroup findings have varied in some previous studies, but not others, an improved understanding of associations across population subgroups will shed light on biologic pathways and further enhance cancer prevention messaging.

There is also a need to improve physical activity measurement. Most observational studies rely on self-reported data captured by questionnaires, which are easy to administer and inexpensive, but are prone to a greater amount of misclassification than more objective measures. Over the past several years, automated methods of capturing physical activity behaviors, such as internet-based tools, have been implemented (Matthews et al., 2012b). These tools have proven to be more valid than questionnaires with regard to measuring sitting time, intensity of different activities, and the context and domains of physical activity (Matthews et al., 2013; Gomersall et al., 2011). Evaluating the feasibility of integrating research-grade accelerometers or commercial monitors (such as Fitbit or Garmin™) in large-scale epidemiologic studies is also of potential value. Certain commercial devices have been shown to be more valid than survey-based physical activity assessment and of similar validity to research-grade devices (Evenson et al., 2015), and given the wide-scale uptake of commercially available devices in the consumer market, they offer potential for improved physical activity data collection.

See also: Obesity and Cancer: Epidemiological Evidence. Prevention and Control: Nutrition, Obesity, and Metabolism.

References

Ainsworth, B.E., et al., 1993. Compendium of physical activities: Classification of energy costs of human physical activities. Medicine and Science in Sports and Medicine 25, 71–80.

Ainsworth, B.E., et al., 2000. Compendium of physical activities: An update of activity codes and MET intensities. Medicine and Science in Sports and Exercise 32 (9 Suppl), S498–S504.

Arem, H., et al., 2015. Leisure time physical activity and mortality: A detailed pooled analysis of the dose–response relationship. JAMA Internal Medicine 175, 959–967.

Ashcraft, K.A., et al., 2016. Efficacy and mechanisms of aerobic exercise on cancer initiation, progression, and metastasis: A critical systematic review of in vivo preclinical data. Cancer Research 76 (14), 4032–4050.

Behrens, G., et al., 2014. The association between physical activity and gastroesophageal cancer: Systematic review and meta-analysis. European Journal of Epidemiology 29 (3), 151–170.

Behrens, G., et al., 2015. Physical activity and risk of pancreatic cancer: A systematic review and meta-analysis. European Journal of Epidemiology 30 (4), 279–298.

Bergouignan, A., et al., 2011. Physical inactivity as the culprit of metabolic inflexibility: Evidence from bed-rest studies. Journal of Applied Physiology 111 (4), 1201–1210.

Biswas, A., et al., 2015. Sedentary time and its association with risk for disease incidence, mortality, and hospitalization in adults: A systematic review and meta-analysis. Annals of Internal Medicine 162 (2), 123–132.

Blair, S., Morris, J., 2009. Healthy hearts–and the universal benefits of being physically active: Physical activity and health. Annals of Epidemiology 19 (4), 253–256.

Brenner, D.R., et al., 2016. Leisure-time physical activity and lung cancer risk: A systematic review and meta-analysis. Lung Cancer 95, 17–27.

Chau, J.Y., et al., 2013. Daily sitting time and all-cause mortality: A meta-analysis. PLoS One 8, e80000.

Dosemeci, M., et al., 1993. Occupational physical activity, socioeconomic status, and risks of 15 cancer sites in Turkey. Cancer Causes & Control 4 (4), 313–321.

Ekelund, E., et al., 2016. Does physical activity attenuate, or even eliminate, the detrimental association of sitting time with mortality? A harmonised meta-analysis of data from more than 1 million men and women. Lancet 388, 1302–1310.

Evenson, K.R., Goto, M.M., Furberg, R.D., 2015. Systematic review of the validity and reliability of consumer-wearable activity trackers. International Journal of Behavioral Nutrition and Physical Activity 12 (1), 159.

Friberg, E., Mantzoros, C.S., Wolk, A., 2006. Physical activity and risk of endometrial cancer: A population-based prospective cohort study. Cancer Epidemiology Biomarkers and Prevention 15 (11), 2136–2140.

Friedenreich, C.M., 2011. Physical activity and breast cancer: Review of the epidemiologic evidence and biologic mechanisms. Recent Results in Cancer Research 188, 125–139.

Friedenreich, C.M., Cust, A.E., 2008. Physical activity and breast cancer risk: Impact of timing, type and dose of activity and population subgroup effects. British Journal of Sports Medicine 42 (8), 636–647.

Fung, T.T., et al., 2000. Leisure-time physical activity, television watching, and plasma biomarkers of obesity and cardiovascular disease risk. American Journal of Epidemiology 152 (12), 1171–1178.
George, S.M., et al., 2010. Beyond recreational physical activity: Examining occupational and household activity, transportation activity, and sedentary behavior in relation to postmenopausal breast cancer risk. American Journal of Public Health 100 (11), 2288–2295.
Gierach, G.L., et al., 2009. Physical activity, sedentary behavior, and endometrial cancer risk in the NIH-AARP diet and health study. International Journal of Cancer, Journal international du cancer 124 (9), 2139–2147.
Gomersall, S.R., Olds, T.S., Ridley, K., 2011. Development and evaluation of an adult use-of-time instrument with an energy expenditure focus. Journal of Science and Medicine in Sport 14 (2), 143–148.
Haskell, W.L., et al., 2007. Physical activity and public health: Updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. Medicine and Science in Sports and Exercise 39 (8), 1423–1434.
Healy, G.N., et al., 2008a. Breaks in sedentary time: Beneficial associations with metabolic risk. Diabetes Care 31 (4), 661–666.
Healy, G.N., et al., 2008b. Television time and continuous metabolic risk in physically active adults. Medicine and Science in Sports and Exercise 40 (4), 639–645.
Healy, G.N., et al., 2008c. Objectively measured sedentary time, physical activity, and metabolic risk: The Australian diabetes, obesity and lifestyle study (AusDiab). Diabetes Care 31 (2), 369–371.
Hildebrand, J.S., et al., 2013. Recreational physical activity and leisure-time sitting in relation to postmenopausal breast cancer risk. Cancer Epidemiology, Biomarkers & Prevention 22 (10), 1906–1912.
Hu, F.B., et al., 2003. Television watching and other sedentary behaviors in relation to risk of obesity and type 2 diabetes mellitus in women. JAMA 289 (14), 1785–1791.
Jakes, R.W., et al., 2003. Television viewing and low participation in vigorous recreation are independently associated with obesity and markers of cardiovascular disease risk: EPIC-Norfolk population-based study. European Journal of Clinical Nutrition 57 (9), 1089–1096.
Jochem, C., et al., 2014. Physical activity in relation to risk of hematologic cancers: A systematic review and meta-analysis. Cancer Epidemiology, Biomarkers & Prevention 23 (5), 833–846.
Keimling, M., et al., 2014. The association between physical activity and bladder cancer: Systematic review and meta-analysis. British Journal of Cancer 110 (7), 1862–1870.
Kerr, J., Anderson, C., Lippman, S.M., 2017. Physical activity, sedentary behaviour, diet, and cancer: An update and emerging new evidence. The Lancet Oncology 18 (8), e457–e471.
Kushi, L., et al., 2012. American Cancer Society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. CA: A Cancer Journal for Clinicians 62, 30–67.
Lauby-Secretan, B., et al., 2016. Body fatness and cancer—Viewpoint of the IARC Working Group. New England Journal of Medicine 375 (8), 794–798.
Lee, I., et al., 2012. Impact of physical inactivity on the world's major non-communicable diseases. Lancet 380, 219–229.
Leitzmann, M.F., et al., 2007. Physical activity recommendations and decreased risk of mortality. Archives of Internal Medicine 167, 2453–2460.
Liu, Y., et al., 2011. Does physical activity reduce the risk of prostate cancer? A systematic review and meta-analysis. European Urology 60 (5), 1029–1044.
Lynch, B.M., et al., 2014. Sedentary behavior and prostate cancer risk in the NIH–AARP diet and health study. Cancer Epidemiology Biomarkers & Prevention 23 (5), 882–889.
Macdonald, H.M., et al., 2003. Longitudinal changes in weight in perimenopausal and early postmenopausal women: Effects of dietary energy intake, energy expenditure, dietary calcium intake and hormone replacement therapy. International Journal of Obesity and Related Metabolic Disorders 27 (6), 669–676.
Matthews, C.E., et al., 2012a. Amount of time spent in sedentary behaviors and cause-specific mortality in US adults. The American Journal of Clinical Nutrition 95 (2), 437–445.
Matthews, C.E., et al., 2012b. Best practices for using physical activity monitors in population-based research. Medicine and Science in Sports and Exercise 44 (1 Suppl 1), S68–S76.
Matthews, C.E., et al., 2013. Validation of a previous-day recall measure of active and sedentary behaviors. Medicine and Science in Sports and Exercise 45 (8), 1629–1638.
McTiernan, A., 2008. Mechanisms linking physical activity with cancer. Nature Reviews. Cancer 8 (3), 205–211.
Moore, S., et al., 2012. Leisure time physical activity of moderate to vigorous intensity and mortality: A large pooled cohort analysis. PLoS Medicine 9 (11), e1001335.
Moore, S., et al., 2016. Association of leisure-time physical activity with risk of 26 types of cancer in 1.44 million adults. JAMA Internal Medicine 176 (6), 816–825 epub ahead of print.
Nelson, M.E., et al., 2007. Physical activity and pyblic health in older adults: Recommendation from the American College of Sports Medicine and the American Heart Association. Circulation 116, 1094–1105.
Nielsen Report, Nielsen reports television tuning remains at record levels. 2007.
Nocon, M., et al., 2008. Association of physical activity with all-cause and cardiovascular mortality: A systematic review and meta-analysis. European Journal of Cardiovascular Prevention and Rehabilitation 15 (3), 239–246.
Owen, N., et al., 2010. Too much sitting: The population-health science of sedentary behavior. Exercise and Sport Sciences Reviews 38 (3), 105–113.
Paffenbarger Jr., R.S., et al., 1986. Physical activity, all-cause mortality, and longevity of college alumni. New England Journal of Medicine 314, 605–613.
Patel, A.V., et al., 2005. Recreational physical activity and risk of prostate cancer in a large cohort of U.S. men. Cancer Epidemiology, Biomarkers & Prevention 14 (1), 275–279.
Patel, A.V., et al., 2010. Leisure time spent sitting in relation to total mortality in a prospective cohort of US adults. American Journal of Epidemiology 172, 419–429.
Patel, A.V., et al., 2015. Leisure-time spent sitting and site-specific cancer incidence in a large US cohort. Cancer Epidemiology Biomarkers and Prevention.
Psaltopoulou, T., et al., 2016. Physical activity and gastric cancer risk: A systematic review and meta-analysis. Clinical Journal of Sport Medicine 26 (6), 445–464.
Robsahm, T.E., et al., 2013. Body mass index, physical activity, and colorectal cancer by anatomical subsites: A systematic review and meta-analysis of cohort studies. European Journal of Cancer Prevention 22 (6), 492–505.
Schmid, D., Leitzmann, M.F., 2014. Television viewing and time spent sedentary in relation to cancer risk: A meta-analysis. JNCI: Journal of the National Cancer Institute 106 (7), dju098.
Schmid, D., et al., 2015. A systematic review and meta-analysis of physical activity and endometrial cancer risk. European Journal of Epidemiology 30 (5), 397–412.
Tremblay, M., et al., 2010. Physiological and health implications of a sedentary lifestyle. Applied Physiology, Nutrition and Metabolism 35, 725–740.
U.S. Department of Health and Human Services, 2015. Step it up! The surgeon general's call to action to promote walking and walkable communities. U.S. Dept of Health and Human Services, Office of the Surgeon General, Washington, DC.
Wolin, K.Y., et al., 2009. Physical activity and colon cancer prevention: A meta-analysis. British Journal of Cancer 100 (4), 611–616.
World Cancer Research Fund/American Institute for Cancer Research Report, 2007. Food, nutrition, physical activity and the prevention of cancer: A global perspective. AICR, Washington, DC.
Xiao, Q., et al., 2013. Physical activity in different periods of life, sedentary behavior and the risk of ovarian cancer in the NIH-AARP Diet and Health Study. Cancer Epidemiology, Biomarkers and Prevention: A publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 22 (11), 2000–2008. https://doi.org/10.1158/1055-9965.EPI-13-0154.
Zhang, M., et al., 2004. Sedentary behaviours and epithelial ovarian cancer risk. Cancer Causes & Control 15 (1), 83–89.

Pituitary Tumors: Pathology and Genetics

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Abbreviations

ACTH Adrenocorticotropic hormone
CDX-2 Caudal type homeobox-2
CRH Corticotropin releasing hormone
FSH Follicle-stimulating hormone
GH Growth hormone
GHRH Growth hormone releasing hormone
GnRHR Gonadotropin-releasing hormone
LH Luteinizing hormone
MSH Melanocyte stimulating hormone
PRL Prolactin
SSRT2A Somatostatin receptor 2A
TRHR Thyrotropin releasing hormone
TSH Thyroid-stimulating hormone
TTF-1 Thyroid transcription factor-1

Epidemiology

Pituitary tumors account for up to 16% of all primary brain neoplasms and are the third most common intracranial tumor after gliomas and meningiomas, both in adults and in the pediatric age. The overall age-adjusted incidence rate of pituitary tumors has been reported by the Central Brain Tumor Registry of the United States (CBTRUS) as 2.94 cases per 100,000 persons between 2009 and 2013. In the last two decades the incidence has risen consistently, due to improvements in diagnostic technology that have increased sensitivity for detecting pituitary tumors. In addition, the greater use of imaging, particularly for indications such as sinusitis, trauma, and headache, has probably led to the increased detection of incidental pituitary tumors.

The risk of developing a pituitary tumor increases with age, with a peak incidence between 65 and 74 years, and is slightly higher in females, with a male/female ratio of 0.84. The male/female incidence rate ratio was 0.87, with incidence peaks in females at ages 30–34 and 70–74 years. In males, one large incidence peak at ages 75–79 years was found. The difference between sexes seems to be due to lactotroph adenoma, the most commonly diagnosed pituitary tumor, which occurs more frequently in women than in men and at earlier ages in women than in men. Lactotroph adenomas tend to present earlier in females because of an increased symptom burden from hyperprolactinemia, such as a galactorrhoea and disruption in the menstrual period. Among the various ethnicities, pituitary tumors are more frequent in black and Hispanic populations.

More than 90% of pituitary tumors have a benign behavior and are classified as pituitary adenomas. Pituitary carcinomas are very rare, accounting for only 0.12% of all pituitary tumors. Other rare tumors of the sellar region include craniopharyngioma, neuronal and paraneuronal tumors, tumors of the posterior pituitary, mesenchymal and stromal tumors, hematolymphoid tumors, germ cell tumors, and secondary neoplasms.

Classification

Pituitary tumors include a wide range of different neoplastic proliferations showing different morphological, immunohistochemical, molecular, clinical, and prognostic features. They can originate either in the anterior (adenohypophysis) or posterior (neurohypophysis) pituitary.

Most tumors arising in the *adenohypophysis* are adenomas; these show variable clinical behavior ranging from an incidentally detected small indolent clinically nonfunctioning tumor, to a biochemically active sellar mass giving rise to an endocrine syndrome, to rapidly growing large tumor invading adjacent structures with a high risk of local recurrence and causing visual disturbance and headache. Carcinomas are very rare neoplasms which can metastasize and have poor prognosis. Clinically, both adenomas and carcinomas can be either functioning (associated with specific symptoms related to hormone hypersecretion) or silent (lacking hormonal symptoms). Silent (or nonfunctioning) adenomas are usually larger and are more frequently associated with symptoms related to local growth, such as visual abnormalities and headache.

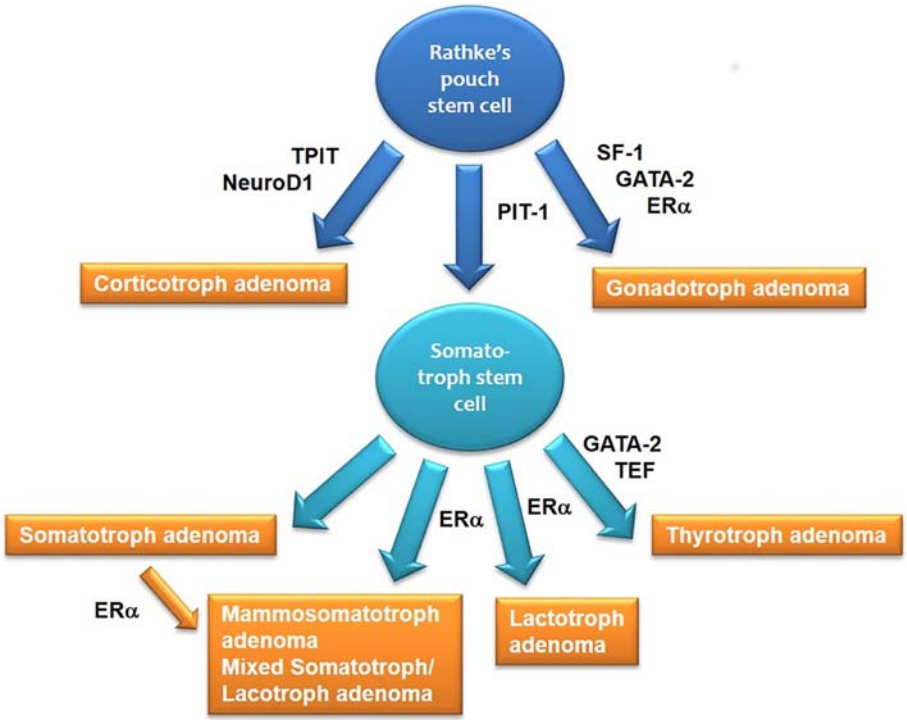

Fig. 1 Transcription factor-related pituitary cell differentiation and related pituitary adenomas.

The current classification of pituitary adenomas is mainly based on pituitary-cell lineage, which is characterized by expression of specific transcription factors involved in the differentiation of anterior pituitary cells (Fig. 1). Consequently, immunohistochemical staining to identify expression of such transcription factors, as well as of secreted hormones in tumor cells, is the basis of the current classification of pituitary tumors (Table 1). In addition, other immunohistochemical markers, including cytokeratins, are useful in identifying specific clinically relevant subtypes.

Tumors of the *neurohypophysis* are a heterogeneous group of low-grade neoplasms believed to derive from pituicytes and include pituicytoma and its variants: granular cell tumor of the sellar region, spindle cell oncocytoma, and sellar ependymoma.

In addition to tumors originating from pituitary cells, either of the anterior or posterior part of the gland, very rare neuronal, paraneuronal, and mesenchymal neoplasms can be observed in the pituitary glands.

Table 1 Classification of anterior pituitary adenomas

Cell type	*TS*	*Hormone*	*Tumor type(s)*
Corticotroph	TPIT Naro D1	ACTH, β-endorphin, MSH	DG corticotroph adenoma SG corticotroph adenoma Crooke cell adenoma
Somatotroph	PIT-1	GH	DG somatotroph adenoma SG somatotroph adenoma
Mammosomatotroph	PIT-1, ERα	GH, PRL	Mammosomatotroph adenoma Mixed somatotroph/lactotroph adenoma
Lactotroph	PIT-1, ERα	PRL	SG lactotroph adenoma DG lactotroph adenoma Acidophil stem cell adenoma
Thyrotroph	PIT-1, TEF, GATA-2	TSH	Thyrotroph adenoma Silent subtype 3 adenoma
Gonadotroph	SF-1, ERα, GATA-2	FSH, LH	Gonadotroph adenoma
Null cells	None	None	Null cell adenoma

TS, transcription factor; *DG*, densely granulated; *SG*, sparsely granulated.

Tumors of the Adenohypophysis

Pituitary Adenoma

Pathogenesis

Sporadic pituitary adenomas

Pituitary adenomas, regardless of cell type, are benign. Moreover, although they may invade parasellar structures, an overt malignant phenotype with documented extracranial metastases has never been reported. It is well established that pituitary adenomas are monoclonal proliferations which makes it unlikely that they are arisen in hyperplastic lesions as a consequence of hormonal signals, such as hypothalamic hormone excess or estrogen excess. This notion is supported by the absence of features of hyperplasia of the pituitary tissue surrounding the adenoma, and of activating mutations of hypothalamic hormone receptors in pituitary adenomas (GHRHR, CRHR, GnRHR, TRHR), as well as of inactivating mutations of hypothalamic inhibitory factor receptors (SSTR1–5; D2R).

Despite their benign phenotype, pituitary adenomas are remarkably aneuploid as a consequence of marked chromosomal instability, leading to both dysregulated hormone gene transcription and cell proliferative activity. On the other hand, mutational events implicated in pituitary tumorigenesis are few and apply to specific adenoma subtypes. The common mutations associated with neoplastic transformation, including activation of *RAS* or *BRAF* genes and loss of the tumor suppressor *TP53* or *RB1* genes, are not found in pituitary adenomas and have only been rarely reported in pituitary carcinomas. However, several growth factors, hormones and cell cycle genes have a pivotal role in the development of these neoplasms.

Cell cycle regulators

Heterozygote Rb+/− mutant mice develop pituitary adenomas with high penetrance, usually arising from the intermediate lobe. Although Rb is expressed (i.e., not mutated) in most nonfunctioning (silent) pituitary adenomas, approximately 25% of somatotroph adenomas show hypermethylation of the *RB* gene promoter with associated loss of expression. Rb activity is regulated by cyclin-dependent kinases (CDKs), which are, in turn, under the control of specific CDK inhibitors. In pituitary adenomas, inactivation of one or more of the members of two families of CDK inhibitors—the INK4a/ARF (p16, p15, p18) and cip/kip inhibitors (p21, p27, p57)—is involved in tumorigenesis. For example, the gene that encodes p16, which normally inhibits Rb phosphorylation and inactivation by CDK4, is hypermethylated and transcriptionally silent in some human pituitary tumors. In mice, deletion of p18 leads to gigantism, organomegaly, and pituitary intermediate lobe hyperplasia and adenomas; p18 protein levels are also attenuated in human adenomas. In a mouse model, disruption of the gene that encodes p27 results in mice with increased body weight, organ hyperplasia and intermediate pituitary lobe tumors. Furthermore, loss of p27 cooperates with overexpression of cyclin E to facilitate corticotroph tumorigenesis.

Another cell cycle regulator, encoded by the pituitary tumor transforming gene 1 (PTTG1), controls correct separation of sister chromatids. PTTG1 is a mammalian securin, and its overexpression causes cell transformation in vitro. Pituitary-directed PTTG1 overexpression in transgenic mice results in focal pituitary hyperplasia and adenoma development. Maintenance of chromosomal stability requires tight control of intracellular cell-cycle dependent PTTG1 levels, as either PTTG1 overexpression or disruption results in dysregulated G2 to M phase cell cycling, aneuploidy and chromosomal instability in vitro and in transgenic mouse models.

Defective signaling pathways

The G protein α (Gα) subunit, encoded by *GNAS* located on chromosome 20q13.2, is a key component of signal transduction pathways. The GTPase function of the Gα subunit is inactivated by *GNAS* mutation, commonly known as *gsp* mutation, which is present in about 30% of somatotroph adenomas, and results in elevated cAMP levels and hypersecretion of GH. Thus, constitutively induced somatotroph proliferation and GH secretion may occur independently of GH releasing hormone, and may also be associated with activation of downstream signaling factors. An endocrine hypersecretory syndrome (McCune–Albright syndrome), which includes pigmented skin lesions, precocious puberty, hyperthyroidism, acromegaly and/or Cushing syndrome with fibrous dysplasia of bone, is associated with a *gsp* mutation. The syndrome is not inherited as it is due to an early postzygotic *gsp* mutation, which results in mosaicism and constitutively active Gα subunit and cAMP activation in the affected cells. Patients with McCune–Albright syndrome usually exhibit selective pituitary hyperplasia, rather than focal adenomas. Furthermore, *gsp* mutation has also been described in the transition of an aggressive lactotroph adenoma to a somatotroph adenoma.

Several growth factors and their receptors, including selective members of the fibroblast growth factor, epidermal growth factor, nerve growth factor, bone morphogenetic protein and vascular endothelial growth factor families, and Akt signaling pathways, have been implicated in the pituitary tumorigenic cascade. Wnt pathway inhibitors are also differentially expressed in pituitary adenomas in comparison to normal pituitary tissue. Fibroblast growth factor receptor variants may underlie disrupted chromatin access and interfere with cell adhesion molecules in pituitary adenomas. Bone morphogenetic protein 4, which normally acts as a suppressor of corticotroph adenoma growth, is abundantly expressed in lactotroph adenomas. Disruption of other signaling molecules associated with pituitary tumorigenesis includes transforming growth factor β and gp130 cytokines. Studies in estrogen receptor β null mice have highlighted the requirement of estrogen receptor α for gonadotroph adenoma development. In fact, estrogen receptor α seems to be more abundantly expressed in large than small pituitary tumors.

Transcriptional regulators

In transgenic mice, overexpression of an HMGA2 protein results in both somatotroph and lactotroph pituitary adenoma formation, which appears to be mediated by enhanced E2F action. In humans, re-arrangement and amplification of *HMGA2* gene expression seems to occur predominantly in lactotroph adenomas, and HMGA2 mRNA abundance correlates with pituitary tumor size and proliferation markers. These tumor growth effects may result from *CCNB2* promoter induction, resulting in enhanced cyclin B2 levels and subsequent activation of the E2F transcription factor.

Expression of several putative pituitary-selective tumor suppressor genes have been shown to be lost or downregulated in adenomas in comparison to normal pituitary tissue. Several of these factors are inactivated by epigenetic mechanisms, which involve Runt-related transcription factor 1, which upregulates galectin-3, a pituitary tumor marker. Expression of the zinc finger protein PLAGL1, a transcriptional coregulator, appears to be lost in a subset of pituitary tumors and somatostatin analogs may in fact mediate anti-proliferative actions through PLAGL1. GADD45-γ and maternally imprinted MEG3 have been identified as pituitary-derived suppressor genes, and are silenced by methylation mechanisms especially in nonfunctioning pituitary tumors. A dominant negative isoform of the DNA-binding protein Ikaros is present in almost half of all pituitary adenomas and this molecule has been shown to regulate pituitary tumor cell chromatin remodeling and cell survival. Ikaros also activates the POMC promoter and may serve as a systemic integrator of hypothalamic–pituitary signaling.

Pituitary tumor microRNAs

The pathogenetic role of miRNAs and their target genes in the pathogenesis of pituitary adenomas remains largely unknown; however, the altered expression of some miRNAs has been associated with tumor diameter, invasiveness, and therapy efficacy. For example, miR-15a and miR-16-1, located in a region which is frequently deleted in pituitary tumors, were reported to have lower expression in somatotroph and lactotroph adenomas than in normal tissue. Moreover, each subtype of pituitary adenoma tends to be characterized by a specific miRNA profile. In corticotroph tumors miR21, miR15 and miR16 are downregulated, while miR122 and mi493 are upregulated in ACTH-expressing carcinomas. Suppression of let-7 miRNA family members, as well as of miR15a/miR16, is conserved across most pituitary tumor subtypes. In nonfunctioning and in somatotroph adenomas, Wee1-like protein kinase, which acts as a tumor suppressor protein, was shown to be regulated by miR128a, miR155 and miR516a-3p.93. MiR-126 and miR-381 have PTTG1 as a target and were shown to be downregulated in GH-secreting pituitary adenomas.

Familial pituitary adenomas

Multiple endocrine neoplasia type 1(MEN1) syndrome

MEN1 syndrome is an autosomal dominant disease with high penetrance characterized by the occurrence of multiple endocrine proliferations: pituitary adenomas, parathyroid hyperplasia, and pancreatic neuroendocrine tumors. It is caused by one of hundreds of inactivating mutations of the *MEN1* gene, which acts as a tumor suppressor gene. Pituitary tumors may include lactotroph, somatotroph, corticotroph and, very rarely, thyrotroph adenomas. MEN1 affects both sexes equally but the pituitary manifestations have a female preponderance.

Multiple endocrine neoplasia type 4 (MEN4) syndrome

Multiple endocrine neoplasia type 4 (MEN4) syndrome is the latest member to join the family of MEN syndromes. It has been defined as a *MEN1* mutations-negative MEN1-like syndrome, with a *CDKN1B* germline mutation. This syndrome may present with somatotroph or corticotroph adenomas. Evidence suggests that p27, encoded by *CDKN1B*, is a tissue-specific target for both the tumor suppressor menin and RET proto-oncogene-mediated cell proliferative signaling. As increasing numbers of *CDK* inhibitor mutations are being identified, it is conceivable that specific clinical phenotypes of these syndromes will become clearer in the future.

Carney syndrome

This autosomal dominant disorder is caused by activated protein kinase A activity, due largely to a *PRKAR1A* mutation on chromosome 17q22–24, and comprises spotty skin pigmentation, myxomas, testicular tumors, and adrenal and/or pituitary adenomas or hyperplasia.

Familial isolated pituitary adenoma (FIPA)

FIPA syndrome is defined as a familial presentation of any type of pituitary adenoma in the absence of clinical and genetic evidence for MEN1 and Carney complex. It represents about 2% of all pituitary adenomas. Genealogical information suggests that FIPA is inherited in an autosomal dominant pattern with variable penetrance. Based on the tumor phenotype in the individual families, FIPA can be divided into two almost equal subgroups: *homogeneous*, when all affected family members experience the same adenoma type, and *heterogeneous*, with different pituitary tumors within the family. Lactotroph and somatotroph adenomas comprise more than 70% of all cases, and although all types of tumors can be seen in heterogeneous FIPA, there is at least one prolactin- or growth hormone-secreting adenoma in almost all affected families. Females tend to be more frequently affected (62%). Gonadotroph, corticotroph, and thyrotroph adenomas are rare and account for 4%, 4%, and 1% of FIPA tumors, respectively. They are usually associated with other adenoma types in heterogeneous families, although individual families with a homogeneous presentation have been reported. Germline mutations in the aryl-hydrocarbon interacting protein gene are identified in

around 25% of familial isolated pituitary adenoma kindreds. Tumors associated with *AIP* mutations are large and generally diagnosed by the age of 25 years and hence patients present with gigantism.

McCune–Albright syndrome

McCune–Albright syndrome is a rare disorder associated with early embryonic postzygotic somatic activating mutations in the *GNAS1* gene. The disorder is characterized clinically by the classic triad of polyostotic fibrous dysplasia (POFD), cafe-au-lait skin pigmentation, and peripheral precocious puberty. However, the disorder is clinically heterogeneous and can include various other endocrinological anomalies such as thyrotoxicosis due to nodular or diffuse goiter, pituitary gigantism or hyperprolactinemia due to pituitary adenoma, and adrenal Cushing syndrome due to nodular adrenal hyperplasia.

Hereditary pheochromocytoma and paraganglioma syndrome

Individuals with this syndrome, associated with succinate dehydrogenase (*SDH*) genes, can also rarely be affected by pituitary adenomas in which the same SDHX mutation is detected.

X-linked acrogigantism (XLAG)

X-linked acrogigantism (XLAG) syndrome is a recently characterized genomic form of pediatric gigantism due to aggressive pituitary tumors, caused by submicroscopic chromosome Xq26.3 duplications that include the *GPR101* gene. Affected patients generally have a normal size at birth after unremarkable pregnancy, and develop mixed growth hormone (GH)- and prolactin-secreting pituitary hyperplasia and/or adenomas within the first 12–36 months of life.

Morphology and immunophenotype

Pituitary adenomas are neoplastic proliferations derived from adenohypophyseal cells. As such, they are composed of epithelial cells with neuroendocrine differentiation and must be distinguished from pituitary hyperplasia. For correct classification, the cell lineage population responsible for the proliferation must be established. Finally, a number of morphological, prognostic and predictive parameters have to be identified.

Hyperplasia may be clinically indistinguishable from adenoma, usually in patients with acromegaly or Cushing's disease, and radiological examination may also not be particularly informative. For microscopic evaluation, reticulin stain is very useful in distinguishing pituitary adenoma from pituitary hyperplasia or normal pituitary tissue. The normal adenohypophysis is composed of small acini of pituitary cells surrounded by a network of reticulin fibers. In hyperplasia, the acinar architecture is maintained and the reticulin network is preserved, but the acini are larger in size. In contrast, pituitary adenomas are characterized by complete disruption of the reticulin fiber network. Pituitary adenomas share morphological features with other well differentiated neuroendocrine proliferations: the cells show granular cytoplasm and round nuclei with finely dispersed chromatin. They generally express both general neuroendocrine markers (synaptophysin, chromogranin) and cytokeratins. However, they may present with a wide range of morphological features depending on their hormonal subtype or associated gene abnormalities, or as a result of a treatment effect. Although benign, pituitary adenomas can be locally invasive, with involvement of the adjacent vascular and bony structures.

The 2017 WHO classification of pituitary tumors is based on the recognition of the adenohypophyseal cell lineage composing the various adenomas, with subclassification of histological variants according to hormone content and specific histological and immunohistochemical features (see section "**Classification**"). Consequently, this new classification approach utilizes immunohistochemistry as the main ancillary tool for diagnosis, leaving the need for ultrastructural analysis only to very rare and unusual instances. Immunohistochemistry for the following general neuroendocrine markers and hormones is routinely performed: synaptophysin, chromogranin A, GH, PRL, ACTH, beta subunits of TSH, FSH and LH, and alpha subunit. In addition, immunohistochemistry for transcription factors, including PIT1, SF1 and TPIT is recommended even though the necessary antibodies are not generally available and their diagnostic value is still under investigation. Immunoreactivity for low molecular weight cytokeratin (CK 7/8 and/or CK 18) may be useful in evaluating specific types of adenomas. For example, it can help to discriminate between sparsely granulated and densely granulated somatotroph adenomas: dot like or globular staining corresponds to the fibrous bodies seen ultrastructurally in sparsely granulated somatotroph adenomas. MIB-1 antibody is used to establish Ki67-based proliferative index, which should always be reported, as it represents a relevant prognostic indicator in individual adenomas. The prognostic value of p53 expression is still under discussion, whereas useful predictive immunohistochemical markers are the somatostatin receptors 2 and 5 (SSTR2 and SSTR5), methyl-guanine methyl transferase (MGMT), and MSH6.

Somatotroph adenoma

Somatotroph adenoma is a pituitary adenoma, arising from PIT1-lineage cells, which expresses and secretes GH and clinically associated with gigantism and/or acromegaly. Somatotroph adenomas represent about 10%–15% of all pituitary adenomas, do not show sex predilection, and can occur at any age, with a mean age at diagnosis of 47 years. Most cases are endocrinologically symptomatic, even if rare silent (nonfunctioning) cases have been reported. Some tumors can co-secrete GH and PRL, resulting in acromegaly and/or gigantism associated with amenorrhoea and galactorrhoea. At radiological investigation, somatotroph adenomas are generally clearly visible and most of them are macroadenomas (1–4 cm).

All somatotroph adenomas show nuclear expression of PIT-1. On the basis of the density of GH-containing secretory granules and of their low-molecular-weight cytokeratin expression, pure GH-producing somatotroph adenomas are divided in two clinically

different subtypes: densely granulated (DG) somatotroph adenomas and sparsely granulated (SG) somatotroph adenomas. In addition, cases co-expressing PRL can be divided into three different categories: mammosomatotroph adenomas, mixed somatotroph and lactotroph adenomas, and plurihormonal adenomas. The distinction of these different subtypes is of clinical importance since they respond differently to available therapies.

- *DG somatotroph adenomas* are acidophilic tumors (Fig. 2A) with a trabecular, sinusoidal, or diffuse histological pattern. They show nuclear immunoreactivity for PIT-1, diffuse and strong cytoplasmic positivity for GH (Fig. 2B), and are also positive for α-subunit of glycoprotein hormones. Low-molecular-weight cytokeratin expression shows a perinuclear distribution.
- *SG somatotroph adenomas* are larger and more invasive tumors, showing a more aggressive behavior than the DG variant. These particular macroscopic and clinical features seem to be related to the delay of diagnosis due to the lower GH levels, with as a consequence a less conspicuous endocrine syndrome. Tumor cells grow in a solid fashion, are chromophobe (Fig. 2C) and show considerable nuclear and cellular pleomorphism. Although nuclear PIT-1 immunoreactivity is strong, GH expression is usually faint and focal. PRL and α-subunit are usually not expressed. The most characteristic feature of these tumors is juxtanuclear globular positivity for low-molecular-weight cytokeratins (fibrous bodies) (Fig. 2D).
- *Mammosomatotroph adenomas* are plurihormonal tumors in which the same neoplastic cells produce GH, PRL, and α-subunit (Fig. 3). They are usually strongly acidophilic tumors that resemble DG somatotroph adenomas with intense nuclear staining for PIT-1 and cytoplasmic positivity for estrogen receptors and GH, variable immunoreactivity for PRL, α-subunit, and a predominant perinuclear pattern of staining for low-molecular-weight cytokeratins. Occasionally they may also produce β-TSH and can be associated with hyperthyroidism and goiter.
- *Mixed somatotroph–lactotroph adenomas* are bihormonal tumors which, instead of being composed of a monomorphous population of GH and PRL producing cells, are composed of two distinct mature GH and PRL cells (Fig. 3). The most common association is DG somatotrophs and SG lactotrophs, but other combinations can be seen.

Most somatotroph adenomas are sporadic, but some cases are associated with a genetic predisposition, either as part of a syndromic disease or as an isolated pituitary disease. Somatotroph adenomas have been described in the context of MEN1, SDH-related familial paraganglioma syndromes, Carney complex, McCune–Albright syndrome, neurofibromatosis type 1, familial isolated pituitary adenoma, and X-linked acrogigantism syndrome.

Surgery is the first treatment choice, although it may not be curative in macroadenomas with extrasellar extension and invasion of the cavernous sinus. Risk stratification of patients is based on different parameters, including histologic subtype, Ki67 proliferative index and invasiveness. Somatostatin antagonists can be used, although they are less active on SG adenomas.

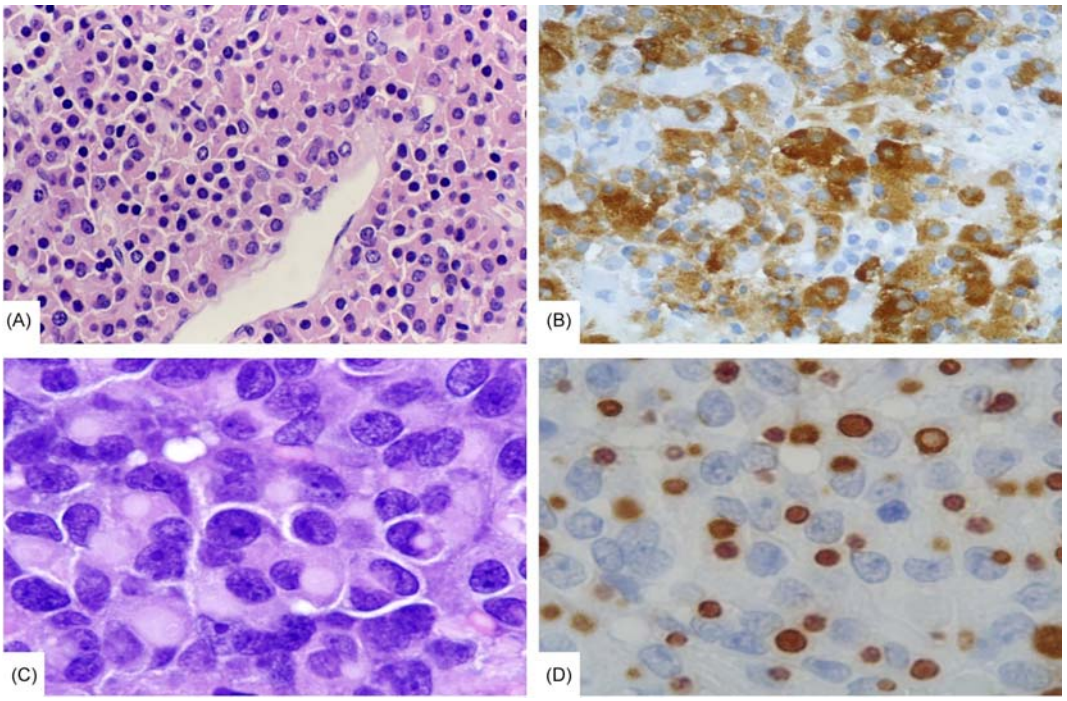

Fig. 2 Somatotroph adenomas: densely granulated somatotroph adenoma is composed of eosinophilic cells growing in a diffuse pattern (A), with intense expression of GH (B). Sparsely granulated somatotroph adenoma shows chromophobe cells with a juxta-nuclear pale cytoplasmic globules (C), which are intensely immunoreactive for low weight cytokeratin (D).

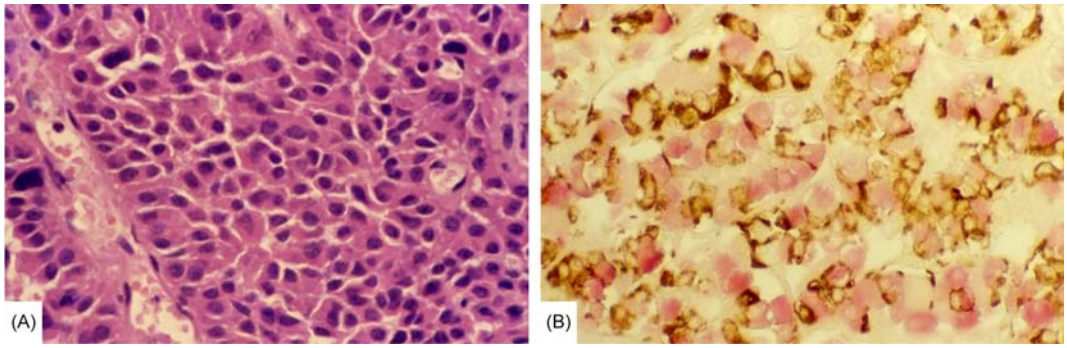

Fig. 3 Mixed somatotroph–lactotroph adenoma composed of a double population of acidophilic cells (A), with immunohistochemical expression of either GH (*brown*) or PRL (*red*) (B).

Lactotroph adenoma

Lactotroph adenomas are pituitary adenomas arising from PIT1-lineage cells, which express and secrete PRL. Lactotroph adenomas are the most common pituitary adenoma type, accounting for about 30%–50% of cases. They show a female predominance, and occur more frequently in young adults, with a peak incidence between 21 and 40 years. In most cases arising in women the adenoma is small, while in men they tend to be larger. Clinical manifestations of lactotroph adenomas include reproductive and sexual dysfunction, which is different between genders. In females, lactotroph adenomas are generally associated with galactorrhea, ovulatory disorders, and amenorrhea, while males present with decreased libido and impotence.

Most lactotroph microadenomas arise in the lateral and posterior part of the gland and are frequently discovered in females. In males they tend to be larger (macroadenomas) and widely invasive into adjacent structures.

Lactotroph adenomas can show prominent vascularity and some cases present abundant fibrous stroma and even occasional amyloid deposition (Fig. 4). On the basis of morphology and PRL immunoreactivity, they can be divided into three distinct histological subtypes.

- *SG lactotroph adenomas* are the most common type of pituitary adenoma. They are chromophobic tumors with variable architecture including trabecular, papillary, or solid patterns of growth. Cells are basophilic and show characteristic juxtanuclear globular positivity for PRL, depending on the abundance of hormone in the Golgi complex rather than in secretory granules,

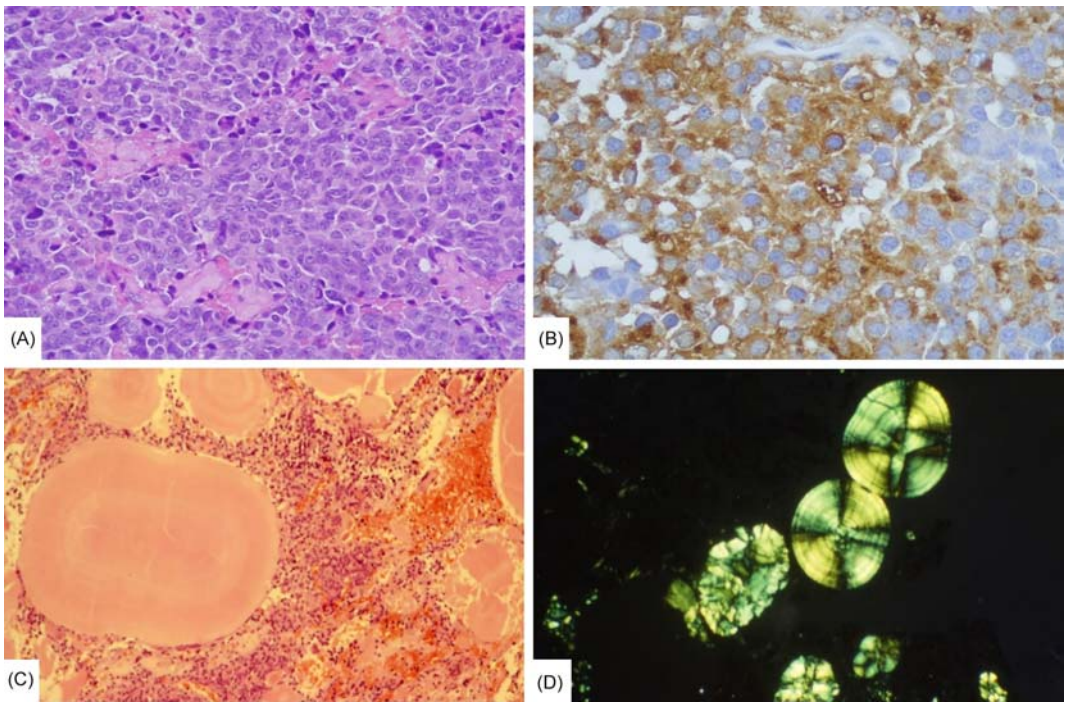

Fig. 4 Sparsely granulated lactotroph adenoma is composed of acidophilic cells (A) with diffuse cytoplasmic immunoreactivity for PRL (B). Stromal amyloid deposits which stain with Congo Red (C) and give an apple-green birefringence at polarized light (D) are observed in this case.

which are scarce. The tumor cells also express PIT-1 and estrogen receptors. They are generally negative for other pituitary hormones, although occasionally they can be positive for α-subunit.

- *DG lactotroph adenomas* are very rare and are composed of acidophilic cells with an abundant amount of PRL-storing secretory granules in the cytoplasm. These tumor cells strongly resemble resting non-neoplastic lactotrophs. The immunohistochemical profile is the same as that of the SG variant, with the exception of the pattern of PRL staining, which is diffuse throughout the cytoplasm.
- *Acidophil stem cell adenomas* are also rare PRL producing tumors, composed of oncocytic cells with abundant acidophilic granular cytoplasm, which may be punctuated by clear cytoplasmic vacuoles that represent giant dilated mitochondria. These adenomas show a diffuse histological architecture and are positive for PIT-1, estrogen receptors, PRL and occasionally for GH.

Only a minority of lactotroph adenomas are associated with familial predisposition and rare cases can be found in the context of MEN1, MEN4, SDH-related familial paraganglioma syndromes, Carney complex, McCune–Albright syndrome, familial isolated pituitary adenoma, and X-linked acrogigantism syndrome.

Pharmacotherapy with dopamine agonists is the first-line of treatment and morphological changes induced by preoperative dopamine agonists are dramatic and can create diagnostic pitfalls in the absence of appropriate clinical history. Most SG lactotroph adenomas undergo significant tumor mass reduction within weeks of initiating therapy and the tumors appear histologically more cellular because of shrinkage in cell size, due to the reduction of the cytoplasmic volume through involution of the rough endoplasmic reticulum and Golgi complexes. The nuclei can become irregular and heterochromatic and the number of secretory granules increases as a consequence of reduced PRL secretion. After prolonged therapy, perivascular, interstitial fibrosis, focal hemorrhage, and hemosiderin deposition are frequently observed. All these morphological changes are usually rapidly reversible on discontinuation of therapy.

Corticotroph adenoma

Corticotroph adenoma is a pituitary adenoma arising from TPIT-lineage cells which express and secrete ACTH (Fig. 5) and proopiomelanocortin-derived peptides. Corticotroph adenomas represent about 15% of all pituitary adenomas. All ages can be affected, but the peak incidence is between 30 and 50 years. In the pediatric population males predominate, while in adult patients corticotroph adenomas are more frequent in females. The majority of patients with corticotroph adenomas show high serum levels of ACTH and cortisol, and present symptoms of Cushing syndrome. However, about 20% of cases are clinically silent, lacking signs of ACTH and cortisol excess. These latter are called silent corticotroph adenomas and are an incidental finding in patients presenting visual or neurological symptoms related to tumor mass.

Corticotroph adenomas associated with Cushing syndrome are classified in three different subtypes: DG corticotroph adenoma, SG corticotroph adenoma, and Crooke cell adenoma.

- *DG corticotroph adenomas* are the most common tumor type in patients with Cushings disease. They have a sinusoidal architecture and are composed of basophilic cells, strongly positive with PAS stain. Tumor cells are immunoreactive for TPIT (nuclear staining), CAM5.2, cytokeratin 20, galectin-3, ACTH and other POMC derivatives including β-endorphin and MSH.
- *SG corticotroph adenomas* are less frequent than the DG counterpart, usually larger presenting as a macroadenoma with more subtle clinical features of Cushing syndrome. Tumor cells are faintly basophilic or chromophobic, with focal and weak PAS positivity and ACTH immunoreactivity. However, they retain a strong nuclear TPIT and cytoplasmic keratin and galectin-3 positivity.
- *Crooke cell adenomas* are rare variants in which tumor cells show Crooke hyaline changes, consisting of a homogeneous hyaline material composed of filaments occupying large areas of the cytoplasm and displacing organelles and secretory granules to the cell periphery. Tumor cells are large and have a homogeneous glassy, slightly acidophilic cytoplasm with granular basophilia limited to the cell periphery and juxtanuclear region. Nuclei may be highly atypical. Immunohistochemistry identifies nuclear

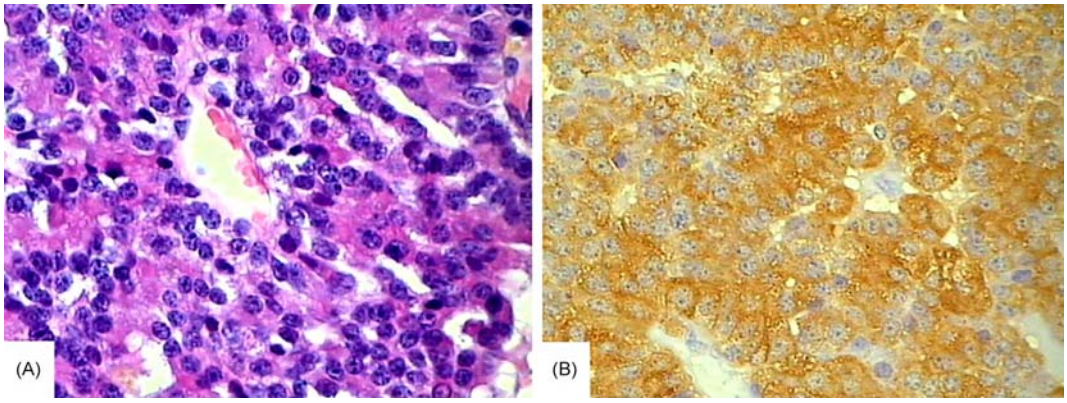

Fig. 5 Corticotroph adenoma showing a sinusoidal growth of basophilic cells (A) with intense immunostaining for ACTH (B).

TPIT positivity and ACTH reactivity limited to the cell periphery and juxtanuclear region, while low-molecular-weight cytokeratins exhibit intense ring-like cytoplasmic positivity. These tend to be more aggressive than the usual corticotroph adenomas.

In addition to corticotroph adenomas associated with Cushing syndrome there are some cases of clinically silent or nonfunctioning adenomas showing cytoplasmic immunoreactivity for ACTH and nuclear expression of TPIT. They are divided into two subtypes: *type 1* (densely granulated) and *type 2* (sparsely granulated).

Thyrotroph adenoma

Thyrotroph adenomas are rare pituitary tumors arising from PIT1-lineage cells, which express and secrete TSH. They account for $<2\%$ of all pituitary adenomas. In most cases the etiology is unknown, but rare cases have been described to arise in the context of MEN1 syndrome or in patients with long-standing untreated primary hypothyroidism. In general, thyrotroph adenomas are a rare cause of hyperthyroidism.

Thyrotroph adenomas are usually large and invasive lesions at the time of diagnosis. Microscopically, thyrotroph adenomas can be solid or may exhibit a sinusoidal or nesting pattern. Stromal fibrosis and calcification are common. Tumor cells are usually polygonal or elongated, with chromophobic cytoplasm and indistinct cell borders. Immunohistochemistry identifies uniform nuclear positivity for PIT-1 and variable cytoplasmic reactivity for α-subunit and β-TSH that highlights the angular morphology of the tumor cells. They frequently coexpress GH and PRL and are also positive for GATA2 and SSRT2A. Ki67 is generally $<3\%$.

Surgery is the first choice for therapy, but it is worth noting that thyrotroph adenomas are exceedingly sensitive to somatostatin analogs which reduce TSH secretion in more than 90% of cases.

Gonadotroph adenoma

Gonadotroph adenomas are derived from SF1-lineage cells of the adenohypophysis and produce β-FSH, β-LH and α-subunit. Functioning forms of these tumors are rare and present with elevated gonadotropins serum levels and signs or symptoms of gonadal dysfunction. More frequently, gonadotroph adenomas are diagnosed because of their mass effect, causing visual abnormalities, hypopituitarism or headaches. Functioning gonadotroph adenomas are frequently macroadenomas and have distinctive clinical manifestations, such as menstrual irregularities and ovarian hyperstimulation in premenopausal women, testicular enlargement in males, and isosexual precocious puberty in children. It is to be kept in mind that gonadotropin excess in children is almost never caused by a pituitary adenoma. True gonadotropin-dependent precocious puberty is usually caused by germ cell tumors, hypothalamic hamartomas, or other CNS tumors or tumor-like lesions that interfere with physiological suppression of the hypothalamic–pituitary–gonadotropic axis. In most men with gonadotroph adenoma, FSH is elevated; in some, both FSH and LH are high. Only in rare cases they produce LH only. In women, the elevation of one gonadotropin alone, or elevated gonadotropins in the presence of hypopituitarism, would suggest the diagnosis of a gonadotropin-producing adenoma.

Macroscopically, gonadotroph adenomas are usually large macroadenomas with significant suprasellar or parasellar invasion at the time of presentation. They are usually well-vascularized and may exhibit areas of hemorrhage or necrosis. Microscopically (Fig. 6), these tumors have a sinusoidal, trabecular, or papillary architecture and form prominent pseudorosettes around blood vessels. The cells are usually chromophobic, but oncocytic change is common and usually focal. Nuclear pleomorphism is not prominent. Tumor cells may contain a few PAS-positive cytoplasmic granules. Immunohistochemistry identifies strong nuclear SF-1 staining and focal, variable ERα. The cytoplasm contains variable positivity for α-subunit, β-FSH, and β-LH; FSH is usually more abundant than LH. It is worth noting that variation in fixation, signal amplification techniques and immunohistochemical methods may result in wide differences in immunostaining of gonadotropins. In addition, the immunostaining for α-subunit, β-FSH, and β-LH may be heterogeneous within the same tumor. For these reasons, many tumors diagnosed as null cell adenomas are in fact gonadotroph adenomas. Recently, it has been reported that SF1 may help to diagnose a gonadotroph adenoma in bona fide hormone-negative tumors.

The most reliable predictor of biological behavior in gonadotroph adenomas is the Ki67-based proliferative index. It is reported to be predictive for progression risk in tumor remnants, even if it may not be predictive for recurrence risk.

The first line therapy for these tumors is surgery, usually with a trans-sphenoidal approach. Gonadotroph adenomas express dopamine and somatostatin receptors. However, bromocriptine, as well as somatostatin analogs, suppress gonadotropin release

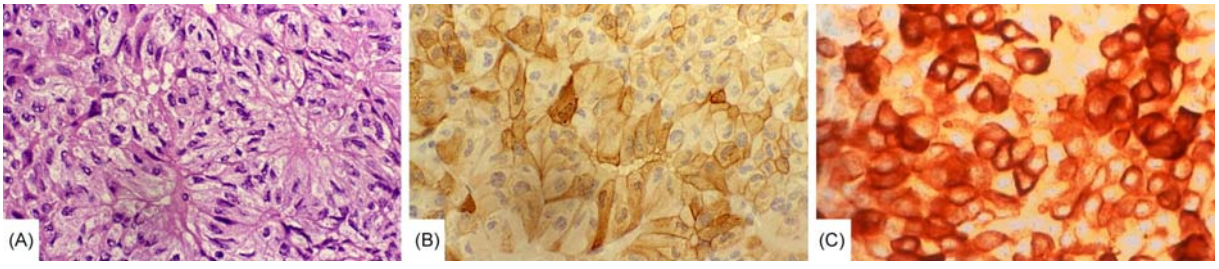

Fig. 6 Gonadotroph adenoma composed of eosinophilic elongated cells with pseudorosette formation (A). Immunostains show positivity for LH (B) and alpha-subunit (C).

in vitro and in vivo but have no effect on tumor size. Recurrence is common, occurring in up to 50% of patients at 10 years after surgery, but most patients are treated with repeat surgery and radiation therapy is reserved for those with rapid and aggressive tumor recurrence.

Null cell adenoma

Null cell adenomas are defined as pituitary adenomas with no evidence of specific cell lineage differentiation by immunohistochemistry for pituitary hormones and transcription factors. When restricted to these criteria, null cell adenomas are rare, representing $<5\%$ of clinically nonfunctioning adenomas. They are tumors that exhibit a somewhat more aggressive clinical behavior, consistent with their lack of differentiation.

The clinical presentation of null cell adenomas is that of a macroadenoma that causes symptoms of an intracranial mass lesion: headache, visual field defects, other cranial nerve deficits, or cavernous sinus syndrome. Some patients present with pituitary apoplexy or hypopituitarism, often with hyperprolactinemia due to pituitary stalk compression. Hypopituitarism may involve only GH or may also feature gonadotropin insufficiency. Reduced gonadotropin levels may be the result of hyperprolactinemia rather than tissue destruction. Hypothyroidism or adrenocortical insufficiencies are uncommon, but dynamic testing may elicit impaired corticotroph or thyrotroph responses.

Microscopically, null cell adenomas are composed of chromophobic cells, with a negative PAS reaction, that grow in chords or in solid sheets. Immunohistochemically, these adenomas lack all hormones and transcription factors of differentiated cells but are positive for cytokeratins.

The prognosis of null cell adenomas is still good after surgical resection, despite their somewhat more aggressive biological behavior than that of other nonfunctioning subtypes, as they are generally large, with extrasellar extensions and infiltration of adjacent vascular and bony structures. Radiotherapy may be of use in post-surgical residuals.

Plurihormonal and double adenomas

Plurihormonal adenomas are adenohypophyseal tumors producing two or more hormones. They represent $<1\%$ of all pituitary adenomas. The most common form of this adenoma derives from the PIT-1 lineage and produces various combinations of GH, PRL, and TSH. However, there are plurihormonal adenomas with unusual hormonal combinations, which cannot be related to a single cell lineage. Plurihormonal adenomas may be monomorphous, i.e. composed of a single cell type producing more than one hormone, such as mammosomatotroph adenomas, or they may be plurimorphous, i.e. composed of two or more different cell types, such as the mixed somatotroph–lactotroph adenomas. PIT-1 positive plurihormonal adenomas are frequently macroadenomas with extrasellar extensions and vascular and bony invasion. After surgical treatment, the persistence of residual tumor is common and the recurrence rate is around 30%. Medical treatment may include somatostatin analogs and temozolomide, as about 40% of these adenomas are reported to have MGMT promoter methylation and absent or low MGMT protein expression at immunohistochemistry.

Double adenomas are rare tumors (0.4%–1.3% of all adenomas in surgical series), composed of two different and separate neoplastic masses, possibly representing collision tumors. Multihormonal adenomas, with more than two tumor masses have rarely been described.

Pituitary Carcinoma

Pituitary carcinomas are malignant neoplasms composed of anterior pituitary cells showing cerebrospinal and/or distant metastasis, and this definition is independent of the morphological features. It is worth noting that the local growth and invasiveness is not sufficient to define an anterior pituitary tumor as a carcinoma. The presence of metastasis at the time of the first diagnosis is very rare, so most cases are first diagnosed as adenomas and the diagnosis is changed during follow-up when metastatic dissemination becomes evident, sometimes years after the original diagnosis.

Pituitary carcinomas account for about 0.12% of all anterior pituitary tumors; its annual incidence in Europe has been estimated to be <0.1 cases per 100.000 population. However, considering that metastases are frequently clinically occult, their incidence is probably underestimated. The median age at diagnosis is in the sixth decade and a slight female predominance has been reported. Pituitary carcinomas have been described to produce almost any type of pituitary hormone. Although pituitary carcinomas are usually a disease of adults, at least one pediatric case has been reported and clinically silent cases present at a younger age than their benign counterparts. The etiology of pituitary carcinomas is unknown.

Tumors generally present as invasive macroadenomas and the macroscopic appearance of a pituitary carcinoma is the same as that of a pituitary adenoma. There are no morphological features of a pituitary tumor that can predict the development of metastases. The tumors have the same morphological and immunohistochemical features of the various pituitary adenomas. Although pituitary carcinoma may exhibit nuclear pleomorphism, mitoses, and necrosis (Fig. 7) these parameters are not per se diagnostic of malignancy. Some parameters, including high microvascular density, high Ki-67 proliferative index, high p53 immunoreactivity, loss of p27 expression, and overproduction of HER2/Neu, have been proposed as markers of malignancy but none of them is specific.

Several molecular alterations have been documented in pituitary carcinomas, but there are no data supporting a specific pattern of molecular alterations. Point mutations of *HRAS* have been demonstrated in metastases, but not in the relative primaries, although the number of cases investigated is still not sufficient to demonstrate a role of this gene in pituitary carcinoma

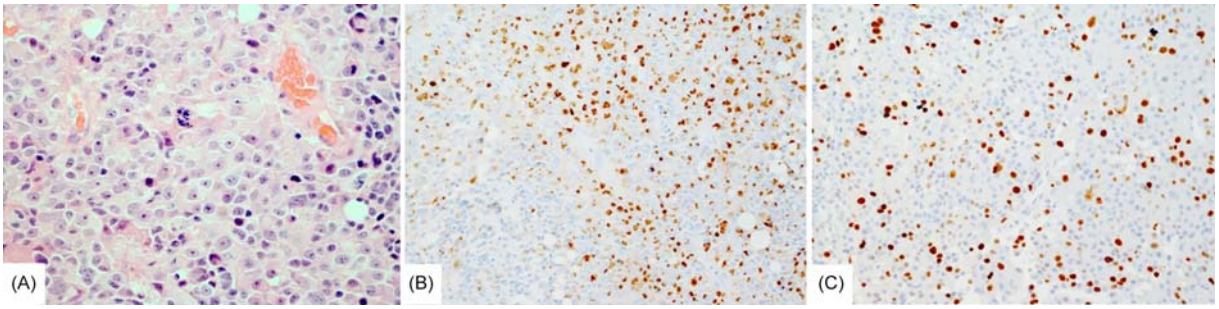

Fig. 7 Pituitary carcinoma composed of atypical cells with abundant eosinophilic cytoplasm and nuclei with prominent nucleoli. An atypical mitosis is visible at the center of the image (A). Tumor cells are positive for prolactin (B) and show high Ki67 proliferative index (C). Courtesy from Dr. Kathreena M Kurian, Institute of Clinical Neurosciences, Bristol Medical School: Translational Health Sciences, Bristol, UK.

tumorigenesis. Using CGH analysis several chromosomal alterations have been identified, including gains of chromosomes 5, 7p, 14q, 13q22. Loss of heterozygosity of 1p, 3p, 10q26, 11q13, and 22q12 has been described, while rearrangements of the *TP53* locus, as well as mutations of *TP53* have rarely been found. Finally, a pathogenetic role of microRNAs has recently been proposed, but these results need to be confirmed. Pituitary carcinomas have been described in the context of MEN1 syndrome but there are not sufficient data to support an association between carcinoma and MEN1 syndrome.

The survival of patients with pituitary carcinoma is poor: about 80% of patients die of metastatic disease. The survival rates at 1 year is 57%, and this decreases to 29% at 2 years but then remains stable. Surgical resection of metastatic deposits within the CNS is recommended, whereas radiation therapy and chemotherapy do not seem to be effective in improving patient survival.

Pituitary Blastoma

Pituitary blastoma is a rare primitive malignant neoplasm of the anterior pituitary gland, composed of cells resembling primordial Rathke epithelium, small folliculo-stellate cells, and partially differentiated adenohypophyseal cells. It occurs in children younger than 24 months of age (median 8 months), with a slight female predominance. In the majority of the cases, signs and symptoms of Cushing syndrome are present, followed by visual alterations. The overall prognosis is poor with 40% death in the first 2 years (median 8 months) after diagnosis.

Pituitary blastomas are consistently associated with mutation of the *DICER1* gene. Most of the cases are seen in the context of the DICER1 syndrome, or pleuropulmonary blastoma (PPB)-familial tumor and dysplasia syndrome, caused by heterozygous germline mutations in the *DICER1* gene. It is possible that some cases bear two somatic *DICER1* mutations, rather than one germline and one somatic mutation.

The tumors are histologically composed of three cell populations, variably admixed: (1) Rathke-type epithelial cells, arranged in glands with rosette-like formations, (2) small primitive appearing cells with a blastema-like appearance, and (3) larger secretory epithelial cells resembling adenohypophyseal cells. Most cases express ACTH, and a few pituitary blastomas were reported to express GH in a subset of cells. The Ki-67 based proliferative index varies from low to high, although a prognostic value for Ki-67 index has not been established.

Tumors of the Neurohypophysis

Tumors of the neurohypophysis are low grade neoplasms believed to derive from pituicytes, which are characterized by TTF1 immunoreactivity. Although different entities including pituicytoma, granular cell tumor of the sellar region, spindle cell oncocytoma, and sellar ependymoma have been described, they are currently thought to represent a morphological spectrum of a single nosologic entity. Pituicytomas are rare low-grade spindle cell tumors composed of pituicytes. Spindle cell oncocytomas, originally thought to derive from folliculo-stellate cells of the anterior pituitary, are now considered as oncocytic variants of pituicytomas. Granular cell tumors, also known as choristomas, granular cell myoblastomas, and granular cell schwannomas, are actually considered to be granular cell pituicytomas. Finally, a fourth variant of pituicytoma, reflecting the ependymal pituicyte, has also been reported.

Pituicytoma and Its Variants

These tumors are rare and their annual incidence is not known. Pituicytomas account for $< 0.1\%$ of sellar tumors, spindle cell oncocytomas for about 0.4%, and granular cell tumors for about 0.5%. Data on sellar ependymomas are lacking, as only seven cases have been described. Most cases occur in adults with a median age at diagnosis around 60 years. Pituicytomas and sellar ependymomas show a male predominance, while granular cell tumors are more common in females. The etiology of these neoplasms is unknown.

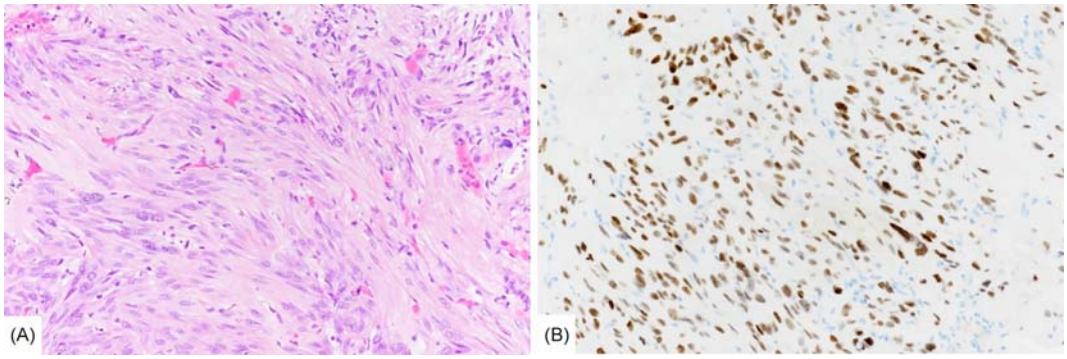

Fig. 8 Pituicytoma is composed of spindle cells that are arranged in interlacing fascicles with storiform pattern. Tumor cells have eosinophilic cytoplasm and nuclei showing minimal atypia (A) and strongly immunoreactivity for TTF1 (B). Courtesy from Dr. Jean-Philippe Brouland, Institut Universitaire de Pathologie, CHUV, Lausanne, Switzwerland.

Most are sellar and/or suprasellar neoplasms, although granular cell tumors show an exclusive suprasellar location. Clinically, they mimic a non-functioning pituitary macroadenoma often showing symptoms related to compression of the pituitary stalk with mild hyperprolactinemia. In addition, visual defects are quite frequent.

Macroscopically, pituicytomas and their variants are highly vascular proliferations with a propensity to bleed. Pituicytomas are composed of elongated spindle-shaped cells arranged in interlacing fascicles with a storiform pattern forming solid sheets (Fig. 8A). The tumor cell cytoplasm is eosinophilic with well-defined cell borders. Oncocytic and granular pituicytomas have more abundant granular cytoplasm, which may result in areas of polygonal or epithelioid morphology in the tumor cells. Spindle cell oncocytomas are characterized by a proliferation of cells forming poorly defined lobules. As a general rule, these tumors show minimal nuclear atypia and mitoses are inconspicuous. Immunohistochemistry is mandatory for a correct diagnosis. Indeed, these tumors are negative for pituitary hormones, cytokeratins, synaptophysin, chromogranin A, neurofilaments, CD34, BCL-2, smooth muscle actin, and desmin, but are positive for vimentin. However, the hallmark of these entities is nuclear reactivity for TTF-1 (Fig. 8B), which is produced in the developing and mature infundibulum, supporting their purported origin from infundibular pituicytes. Tumor cells also show positivity for galectin-3 although it does not seem to be of diagnostic utility, since other intracranial tumors including meningiomas, peripheral nerve sheath tumors and pituitary adenomas can also be galectin-3 positive. The granular variant has been found to be positive for CD68, α_1-antitrypsin, and cathepsin B suggesting histiocytic differentiation. The Ki-67 proliferative index is usually low. The differential diagnosis varies depending on tumor cell morphology and includes different entities. Pituicytomas mimic pilocytic astrocytomas while oncocytic and granular cell variants mimic pituitary adenomas. Immunohistochemistry is indispensable for the differential diagnosis. These low-grade tumors are associated with a favorable outcome when surgically resected.

Neuronal and Paraneuronal Tumors

Neuronal and paraneuronal tumors of the pituitary gland are extremely rare neoplasms including gangliocytoma, neurocytoma, paraganglioma, and neuroblastoma.

Gangliocytomas are well-differentiated, slowly growing intrasellar neoplasms composed of mature neurons resembling hypothalamic ganglion cells and producing hypothalamic peptides. These tumors may cause hypopituitarism alone or hypopituitarism with deafferentation hyperprolactinemia and visual field defects when they are large with suprasellar extension. Some of these tumors are hormonally active, producing hypothalamic regulatory peptides such as acromegaly due to GHRH secretion. They may also cause precocious puberty through GnRH production, or Cushing's disease with excess CRH. They may also be associated with pituitary adenomas (Fig. 9). Radiologically these tumors resemble pituitary adenomas and their gross appearance is non-specific. Morphologically they are composed of randomly oriented large mature ganglion cells of variable size and shape, with large nuclei and prominent nucleoli, sometimes binucleated or multinucleated. Mitoses are generally absent. Gangliogliomas are distinguished by the presence of glial stroma with Rosenthal fibers and eosinophilic granular bodies. Neurons and neuropil are intermingled with adenohypophyseal cells that may be normal, hyperplastic or adenomatous and may represent the dominant component of the tumor mass.

Pituitary neurocytomas are extraventricular neurocytomas (WHO grade II) involving the pituitary gland. These tumors are extremely rare. Pituitary neurocytomas are composed of small to medium-sized round monotonous cells forming solid nests and sheets within a reticulin-rich, fibrovascular stroma. There is abundant fibrillar neuropil, and rosettes may be present. The tumor cells have granular, pale eosinophilic or chromophobic cytoplasm with round to oval nuclei with finely granular chromatin, with or without prominent nucleoli. Tumor cells are positive for synaptophysin, chromogranin, neuron-specific enolase, and CD56 and lack expression of cytokeratins, EMA, glial markers (GFAP, OLIG2), and pituitary transcription factors and hormone expression. Vasopressin (ADH) expression has been reported. The therapy of choice is surgery and the prognosis depends on complete surgical

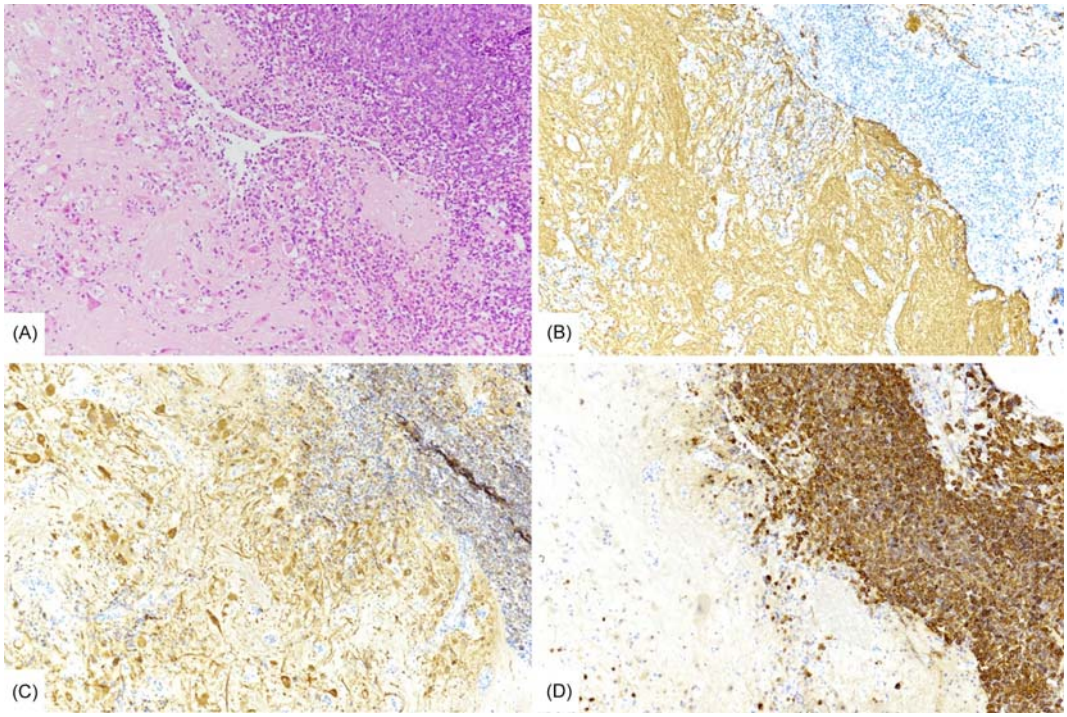

Fig. 9 Mixed pituitary gangliocytoma-adenoma. The tumor is composed of mature ganglionic cells distributed in abundant neuropil (A, left) and of a pituitary adenoma (A, right). The neuropil is positive for neurofilaments (B). Ganglion cells are strongly positive for microtubule-associated protein 2 (Map2) (C), while adenomatous cells are positive for GH (D).

resection and on histological features, as presence of necrosis, microvascular proliferation, increased mitotic activity ($\geq$3 mitoses $\times$ 10HPF) and a Ki67 index higher than 3% are associated with poorer prognosis. Radiation therapy has been reported to improve local control, but its impact on long-term survival is unknown.

Paragangliomas of the pituitary gland are extremely rare tumors arising from chief cells of the dispersed paraganglia of the sellar region. These tumors clinically resemble silent (nonfunctioning) pituitary adenomas, and are associated with headache, visual disturbances, hypopituitarism, and mild hyperprolactinemia due to stalk compression. Morphologically, they are very difficult to distinguish from hormone-negative pituitary adenomas. However, similar to paragangliomas of other sites, they are composed of large, irregular polyhedral or elongated cells with pale eosinophilic cytoplasm and bland nuclei with rare mitoses, which form solid nests called *zellballen* within a highly vascular stroma. Tumor cells are positive for synaptophysin, chromogranin A, and tyrosine hydroxylase, while sustentacular cells are positive for S100. The differential diagnosis with pituitary adenomas is based on immunohistochemistry for cytokeratins, particularly low-molecular-weight cytokeratins, since adenomas are positive, while paragangliomas are negative. Conversely, immunoreactivity for neurofilaments supports a diagnosis of paraganglioma. Most paragangliomas are benign and can be surgically resected. A diagnosis of paraganglioma strongly suggests to search for germline mutations of the *VHL* (the von Hippel–Lindau tumor suppressor), *SDHB*, *SDHC*, and *SDHD*, and *NF1*and *NF2* genes, which predispose patients to the disease.

Neuroblastomas of the pituitary gland or sellar region are neuroectodermal neoplasms similar to olfactory neuroblastoma (esthesioneuroblastoma). They are very rare, accounting for less than 20 cases reported in the literature, and are generally associated with symptoms of tumor mass, visual field loss, headache, and hormone deficiencies. Tumors show lobular architecture separated by fine fibrovascular septae that may contain stromal neurofibrillary material. Tumor cells are uniformly small and round with scant cytoplasm, positive for synaptophysin and chromogranin, but negative or only focally and weakly positive for cytokeratins. In addition, they are also negative for pituitary transcription factors and hormones. These lesions usually have a higher Ki67 proliferative index (10%–20%) than pituitary adenomas. S100-positive sustentacular cells are generally present at the periphery of tumor nests. Surgical resection is usually not curative and is usually followed by radiotherapy associated with chemotherapy in advanced cases.

Miscellaneous Tumors

Rare tumors of the pituitary gland include mesenchymal and stromal tumors, hematolymphoid tumors, germ cell tumors, secondary tumors, and mixed craniopharyngioma-pituitary adenoma (MiNEN).

Mesenchymal and stromal tumors developing in the pituitary region are mostly represented by meningiomas, however benign and malignant soft tissue tumors may occur in this site.

Meningiomas represent about 5% of sellar tumors and the most common meningioma types in this region are meningothelial, fibrous or fibroblastic, and transitional variants. They commonly occur in patients in their 5th and 6th decades of life and most frequently in females. The majority of meningiomas in this site are suprasellar, originating from the planum sphenoidale, the diaphragma sellae or the tuberculum sellae. Clinical signs and symptoms are that of a sellar mass, with visual alterations and headache. Meningiomas usually express EMA and vimentin, some are immunoreactive for S100 protein, and they are characteristically positive for estrogen and progesterone receptors, as well as for SSTR2A. Most are well-differentiated grade I lesions with rare mitoses and low a Ki-67 proliferative index (below 1%). Grade II tumors include those with ≥ 4 mitoses/10 high-power fields ($\times 40$) or three of the following features: increased cellularity, sheeting architecture, small cells with high nuclear/cytoplasmic ratio, macronucleoli, focal necrosis, or brain invasion. Chordoid and clear cell variants are also defined as grade II. The rare grade III or anaplastic meningiomas include rhabdoid and papillary subtypes, and meningiomas with a high proliferation index (>20 mitoses per 10 high power fields).

Sellar *schwannomas* are exceedingly rare and are derived from cranial nerves in and around the sella. They can be clinically misdiagnosed as non-functioning pituitary adenomas, causing hypopituitarism with or without hyperprolactinemia and other symptoms of a mass lesion. Macroscopically, they are well circumscribed, pseudoencapsulated and firmer than pituitary adenomas. Microscopically, schwannomas are composed of spindle-shaped cells, characteristically arranged in compact Antoni type A and loose Antoni type B patterns. The immunohistochemical profile includes strong S100 protein reactivity, with variable Leu-7, calretinin, and GFAP staining; they are usually negative for EMA, unlike meningiomas. Sellar schwannomas are usually benign and are amenable to surgical resection unless they involve critical structures in the parasellar region.

Chordomas are rare midline neoplasms that may rarely arise in the clivus and involve the sellar region. Clinically, they mimic nonfunctioning pituitary adenomas, presenting with signs and symptoms of a sellar mass, and usually arise in young patients (mean age 30 years). Macroscopically, they appear as a gelatinous mass, histologically composed of chords of physaliphorous cells surrounded by polysaccharide-rich matrix. Immunohistochemical markers of chordomas are S100, EMA, calretinin, cytokeratins and vimentin. Nuclear expression of the transcription factor brachyury proves derivation from the embryonic notochord. Chordomas are aggressive neoplasms, frequently invasive and with a high tendency to recur. The chondroid variant of chordoma shows focal cartilaginous differentiation, lacks EMA and cytokeratin reactivity, and may actually represent a well differentiated chondrosarcoma. This variant is less aggressive than the usual clival chordoma.

Hematological disorders, namely, *lymphoma*, *myeloma* and *leukemia*, may very rarely involve the sellar region and the pituitary as a consequence of a systemic dissemination. In immunocompromised patients, primary pituitary lymphomas have been anecdotally reported. Plasmacytoma or lymphoma involving the sellar region may clinically mimic nonfunctioning pituitary adenoma. Conversely, bromocriptine-treated prolactin-producing adenomas and lymphocytic hypophysitis, may be misdiagnosed as lymphoma. Immunohistochemical analysis is clearly pivotal in making a correct diagnosis in these cases. The majority of pituitary-involving lymphomas are diffuse large B-cell lymphomas (DLBCL), but also Burkitt lymphoma, marginal zone lymphomas, solitary plasmacytomas, and T-cell lymphomas have been described.

Among hematological disorders, Hand–Schüller–Christian disease belongs to the group of Langerhans' cell histiocytosis and its classical presentation involves the hypothalamus and, in some cases, the pituitary gland itself. Also non-Langerhans cell histiocytoses, including Rosai–Dorfman disease, Erdheim–Chester disease, hemophagocytic lymphohistiocytosis, and juvenile xanthogranuloma, as well as rare malignant histiocytic tumors, may involve the pituitary and the hypothalamus.

Germ cell tumors may be found outside the gonads along the midline, such as in the mediastinum and in the cranium, where they occur mainly in the pineal and suprasellar region. They represent $<1\%$ of intracranial neoplasms in adults, but are more common in children, where they constitute 6.5% of intracranial tumors. The most common germ cell tumors found in the sellar region are teratomas and mixed germ cell tumors, but all histotypes have been described. The prognosis of these neoplasms strictly depends on the histotype, germinomas having the best prognosis (5-year survival rates over 80%), compared to cases with a non-germinomatous component (5-year survival rates around 40%–50%).

Secondary tumors, i.e. metastasis to the pituitary gland, are uncommon but as they represent about 1% of pituitary tumors cannot be neglected (**Fig. 10**). The posterior pituitary is more frequently involved, whereas the anterior lobe seems to be protected by the portal nature of its vasculature. Pituitary metastases derive most commonly from primary carcinomas of the lung, breast, and colon. The presentation almost always includes diabetes insipidus, a feature that highlights the involvement of the hypothalamus and distinguishes even small lesions from pituitary adenoma. Large tumors cause headache, visual and oculomotor defects, along with anterior pituitary hormone deficiencies. Metastatic carcinoma to a pituitary adenoma has been reported and may cause a sudden increase of pituitary size with worsening of symptoms of a known tumor. Metastatic neuroendocrine carcinoma may represent a diagnostic challenge, because in the absence of immunoreactivity for pituitary hormones and transcription factors, and being immunoreactive for general neuroendocrine markers, it can be misdiagnosed as a null cell adenoma. Unusual features, such as high mitotic activity, high Ki-67 proliferative index, or any history of a primary neuroendocrine tumor elsewhere, are clues for a metastatic origin of the neuroendocrine proliferation. Additional immunostaining, including transcription factors (TTF-1 and CDX-2) and non-pituitary hormones may be helpful in the differential diagnosis with an adenoma and in finding an occult primary. The most complex examples have been reported in patients with acromegaly or Cushing's disease caused by ectopic expression of GHRH or ACTH and/or CRH, respectively. Aggressive neuroendocrine tumors of various sites commonly produce ACTH; a metastatic lesion in the pituitary producing ACTH could easily be mistaken for a primary pituitary adenoma. In this situation,

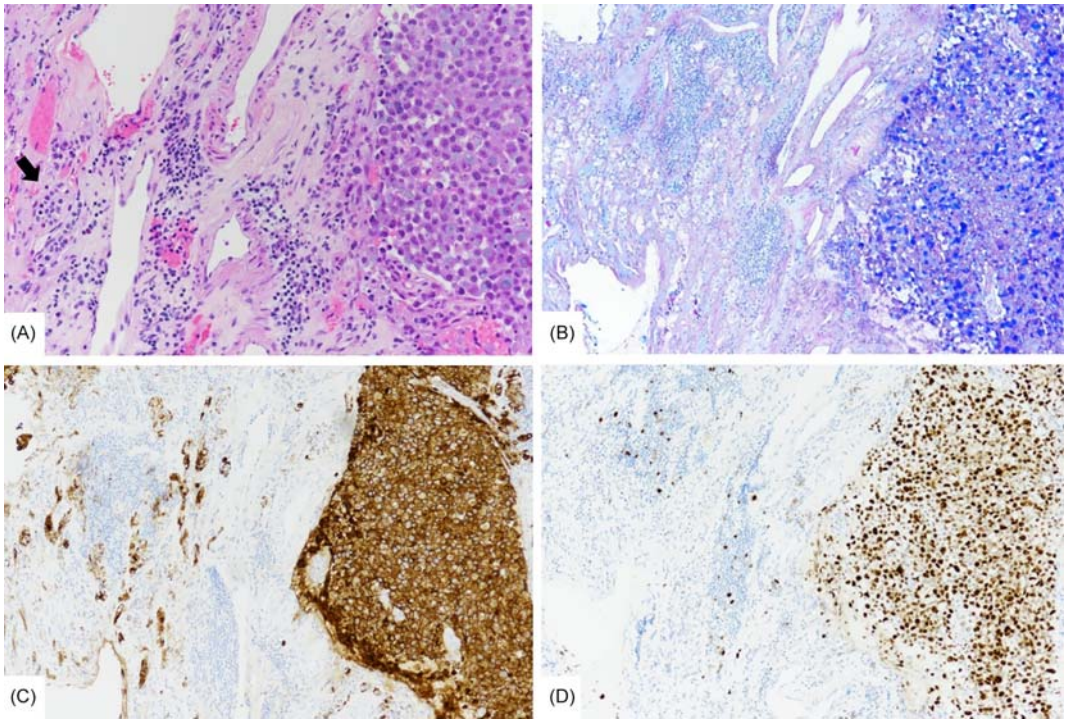

Fig. 10 Pituitary metastasis from a signet-ring cell carcinoma. Neoplastic tumor cells growth forming solid sheets (right side of the images), while residual pituitary tissue is found in the left side (A, *arrow*). Tumor cells show intracytoplasmic Alcian-Blue positive mucus (B), are strongly positive for cytokeratin (C), and show a high Ki67 proliferative index (D).

there is usually evidence of widespread disease; examination of TPIT may be helpful, since extrapituitary neuroendocrine tumors that produce ACTH do not express TPIT.

MiNENs arising in the sellar region are very rare and the cases described in the literature are composed of admixed craniopharyngioma and pituitary adenoma. Some of these cases represented concomitant or collision tumors in which separate pituitary adenomas and craniopharyngiomas were present in the gland. However, other reported cases were examples of true mixed neoplasms consisting of areas of pituitary adenoma strictly admixed with areas of craniopharyngioma. As a group, these tumors occurred more frequently in males with an average age of 48 years. The nonneuroendocrine component of sellar MiNENs was always represented by an adamantinomatous craniopharyngioma, whereas the neuroendocrine component consisted of different types of pituitary adenomas including gonadotroph, somatotroph, corticotroph and thyrotroph adenomas. The pathogenesis of mixed pituitary neoplasms composed of craniopharyngioma and pituitary adenoma is still not clear. Morphologically, they are characterized by an intimate admixture between the two components and hybrid cells, and features of both craniopharyngioma and pituitary adenoma have been demonstrated. This suggests that these tumors may originate from a common precursor, which undergoes divergent differentiation. However, molecular analyses confirming this hypothesis are lacking.

This entity needs to be recognized for clinical and prognostic reasons and should be considered among the potential differential diagnoses of neoplasms arising in the sellar region.

See also: Pituitary Tumors: Diagnosis and Treatment.

Further Reading

Asa, S.L., 2011. Tumors of the pituitary gland. In: AFIP atlas of tumor pathology, series 4. America Registry of Pathology, Washington, DC.

Asa, S.L., Mete, O., 2016. The pituitary gland. In: Mete, O., Asa, S.L. (Eds.), Endocrine pathology. Cambridge University Press, Cambridge, pp. 315–397.

El Hussein, S., Vincentelli, C., 2017. Pituicytoma: Review of commonalities and distinguishing features among TTF-1 positive tumors of the central nervous system. Annals of Diagnostic Pathology 29, 57–61.

La Rosa, S., Sessa, F., Uccella, S., 2016. Mixed neuroendocrine-nonneuroendocrine neoplasms (MiNENs): Unifying the concept of a heterogeneous group of neoplasms. Endocrine Pathology 27, 284–311.

Lloyd, R.V., Osamura, R.Y., Klöppel, G., Rosai, J., 2017. WHO classification of tumours of endocrine organs, 4th edn. IARC Press, Lyon.

Melmed, S., 2011. Pathogenesis of pituitary tumors. Nature Reviews Endocrinology 7, 257–266.

Mete, O., Lopes, M.B., 2017. Overview of the 2017 WHO classification of pituitary tumors. Endocrine Pathology 28, 228–243.
Ostrom, Q.T., Gittleman, H., Xu, J., et al., 2016. CBTRUS statistical report: Primary brain and other central nervous system tumors diagnosed in the United States in 2009-2013. Neuro-Oncology 18 (Suppl. 5), v1–v75.
Raverot, G., Vasiljevic, A., Jouanneau, E., Trouillas, J., 2015. A prognostic clinicopathologic classification of pituitary endocrine tumors. Endocrinology Metabolism Clinics of North America 44, 11–18.
Rotondo, F., Kovacs, K., Lloyd, R.V., 2018. The role of molecular pathology in the classification and clinicopathologic evaluation of pituitary neoplasms. Diagnostic Histopathology.

Pituitary Tumors: Diagnosis and Treatment

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Glossary

Acromegaly A disease characterized by growth hormone (GH) excess in adults resulting in enlargement of the extremities.
Adenohypophysis The front (anterior) section of the pituitary gland that is composed of epithelial neuroendocrine cells.
Corticotroph Pituitary cell dedicated to the production of a pro-hormone pro-opiomelanocortin (POMC) from which the adrenal stimulating hormone, adrenocortictrophic hormone (ACTH), is cleaved.
Crooke cell A pituitary corticotroph cell characterized by accumulations of cytoplasmic filaments that reflect inhibition of that cell by adrenal steroid hormone excess.
Cushing disease A disease characterized by inappropriate hypersecretion of the adrenal stimulating hormone, adrenocorticotrophic hormone (ACTH).
Gigantism A disease characterized by growth hormone excess before puberty resulting in increased stature.
Gonadotroph A dedicated pituitary cell responsible for producing the gonad stimulating hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH).
Lactotroph A pituitary cell that is responsible for producing the breast milk-generating hormone prolactin.
Mammosomatotroph A pituitary cell with the capacity to produce both prolactin and growth hormone (GH).
Neurohypophysis The back (posterior) portion of the pituitary gland that is composed of the axons of hypothalamic neurons and their supporting stromal cells, modified glial cells known as pituicytes.
Pituitary A gland at the base of the brain, within the bony structure known as the sella turcica; the pituitary is responsible for sensing the body's hormones and responding by producing hormones that control the body's hormone-producing glands.
Somatotroph Dedicated pituitary cell responsible for producing growth hormone (GH).
Thyrotroph Dedicated pituitary cells responsible for producing thyroid stimulating hormone (TSH).

Nomenclature

αSU Alpha subunit of glycoprotein hormones
ACTH Adrenocorticotrophic hormone
FSH Follicle stimulating hormone
GH Growth hormone
LH Luteinizing hormone
MRI Magnetic resonance imaging
Pit-1 Pituitary transcription factor-1
PitNET Pituitary neuroendocrine tumor
POMC Pro-opiomelanocortin hormone
PRL Prolactin
SF-1 Steroidogenic factor-1
Tpit T-box pituitary transcription factor
TSH Thyroid stimulating hormone

Introduction

Pituitary tumors are common neoplasms that have been recognized with increasing frequency (Ezzat et al., 2004). The vast majority arise from the hormone-secreting cells of the anterior pituitary gland, the adenohypophysis, and are therefore epithelial neuroendocrine tumors. They are usually insidious in their initial presentation and have variable biological behaviors. Some are small incidental lesions that are of no major clinical significance, others are small tumors that produce hormones in excess but can be managed by medical therapy and do not progress. Those that come to surgery may be hormonally active, proliferative, and/or invasive, causing significant morbidity and ultimately can result in mortality due to tumor growth or invasion of critical brain structures. In this article, we provide an overview of their epidemiology and classification, as well as the fundamentals of the diagnosis and management of patients with these endocrine neoplasms.

Epidemiology of Pituitary Tumors

Epidemiologic data from prior to 1969 indicated that pituitary tumors were rare, with an annual incidence of 1.85 per 100,000 population (Gold, 1981). Surgical literature indicated that they represented 10%–25% of intracranial neoplasms (Scheithauer, 1984) however, this was considered to reflect a referral bias to publishing academics. Radiology and pathology data identified common incidental findings which were reviewed in a meta-analysis that resulted in a prediction of pituitary tumors in 17% of the population (Ezzat et al., 2004). Subsequent population studies identified that the prevalence of clinically diagnosed pituitary tumors ranges from approximately 78 to 116 cases per 100,000 people (Daly et al., 2006; Fernandez et al., 2010; Agustsson et al., 2015, Fontana and Gaillard, 2009) with only one study from Sweden finding a lower prevalence of 3.9/100,000 people (Tjornstrand et al., 2014).

These data indicate that pituitary tumors are much more common than previously thought and point to the importance of clinical awareness and education to ensure early diagnosis.

Clinico-Pathological Classifications of Pituitary Tumors

The classification of these tumors as "adenomas" belies the extreme variability of their clinical behaviors and the impact that they can have on patients. Relegation to the benign category has hampered collection of data on incidence, prevalence, and outcomes. Pituitary patients with significant morbidity are denied assistance that is available to cancer patients. For these reasons and more, and by analogy with other endocrine tumors that have variable biological outcomes, a change in terminology has been proposed by the International Pituitary Pathology Club to avoid the use of the term "adenoma" (Asa et al., 2017a). Instead, these tumors should be classified as "pituitary neuroendocrine tumors," or PitNETs, and when referring to specific subtypes, the cell type should be followed by "tumor" rather than "adenoma." In this review we will apply this new terminology.

The spectrum of PitNETs includes tumors that reflect the six cell types of the normal adenohypophysis (Asa, 2011). Each type can have several morphologic subtypes that have different clinical behaviors; each tumor type has a distinct clinical presentation, response to therapy, and prognosis (Mete and Asa, 2012, 2013; Gomez-Hernandez et al., 2015). **Table 1** outlines the various tumor types that arise from adenohypophysial cells, based on the cell lineages defined by transcription factors, the hormone(s) they

Table 1 Clinico-pathologic classification of pituitary tumors

Lineage	*Cell type*	*Tumor type*	*Hormone(s)*	*Typical presentation*	*Unusual presentation*
Tpit	Corticotroph	Densely granulated corticotroph tumor	ACTH; other POMC-derived peptides	Florid Cushing disease; small tumor	Clinically nonfunctioning
	Corticotroph	Sparsely granulated corticotroph tumor	ACTH; other POMC-derived peptides	Subtle Cushing disease, large tumor	Clinically nonfunctioning
	Crooke cell[a]	Crooke cell tumor	ACTH; other POMC-derived peptides	Cushing disease that may be cyclical or atypical	Clinically nonfunctioning
Pit-1	Somatotroph	Densely granulated somatotroph tumor	GH; αSU	Florid acromegaly	
	Somatotroph	Sparsely granulated somatotroph tumor	GH	Subtle acromegaly	Clinically nonfunctioning
	Mammosomatotroph	Mammosomatotroph tumor	GH; PRL; αSU	Florid acromegaly with hyperprolactinemia	
	Mammosomatotroph precursor	Acidophil stem cell tumor	PRL; GH; αSU	Hyperprolactinemia	Hyperprolactinemia with subtle acromegaly
	Lactotroph	Sparsely granulated lactotroph tumor	PRL	Hyperprolactinemia	
	Lactotroph	Densely granulated lactotroph tumor	PRL	Hyperprolactinemia	
	Thyrotroph	Thyrotroph tumor	TSH[b] (βTSH and αSU)	Hyperthyroid-ism	Clinically nonfunctioning
	Poorly differentiated Pit-1 lineage cell	Poorly differentiated Pit-1 lineage tumor	αSU; GH; PRL; βTSH	Clinically nonfunctioning	Acromegaly, hyperthyroidism, hyperprolactinemia or any combination
SF-1	Gonadotroph	Gonadotroph tumor	FSH;LH[b] (αSU; βFSH, βLH)	Clinically nonfunctioning	Gonadotropin excess
None	Null cell	Null cell tumor	None	Clinically nonfunctioning	
Multiple	Atypical	Unusual plurihormonal tumor	Multiple	Any or multiple hormone excess	Clinically nonfunctioning

[a]Crooke cells are suppressed corticotrophs that are usually found in the nontumorous gland of patients with cortisol excess from any etiology.
[b]The glycoprotein hormones TSH, FSH, and LH are composed of two subunits, the common αSU and a unique β subunit.

produce and their clinical features. Morphologic classification is based on immunoreactivity for transcription factors and hormones as well as other biomarkers, most importantly keratins (especially cytokeratin 18, usually identified using the Cam 5.2 antibody) that offer additional valuable information (Asa, 2011; Asa and Mete, 2016) (**Fig. 1**).

The clinical presentations of a pituitary tumor are classified based on the hormonal manifestations (Gomez-Hernandez and Ezzat, 2016). *Acromegaly* is a multisystem disorder with enlargement of the hands and feet due to a growth hormone (GH)-secreting tumor. Unfortunately, this clinical manifestation is usually only detected late in the course of the disease, by which time the prolonged excess of GH and its target, hepatic insulin-like growth factor 1 (IGF-1), have resulted in enlargement and dysfunction of most internal

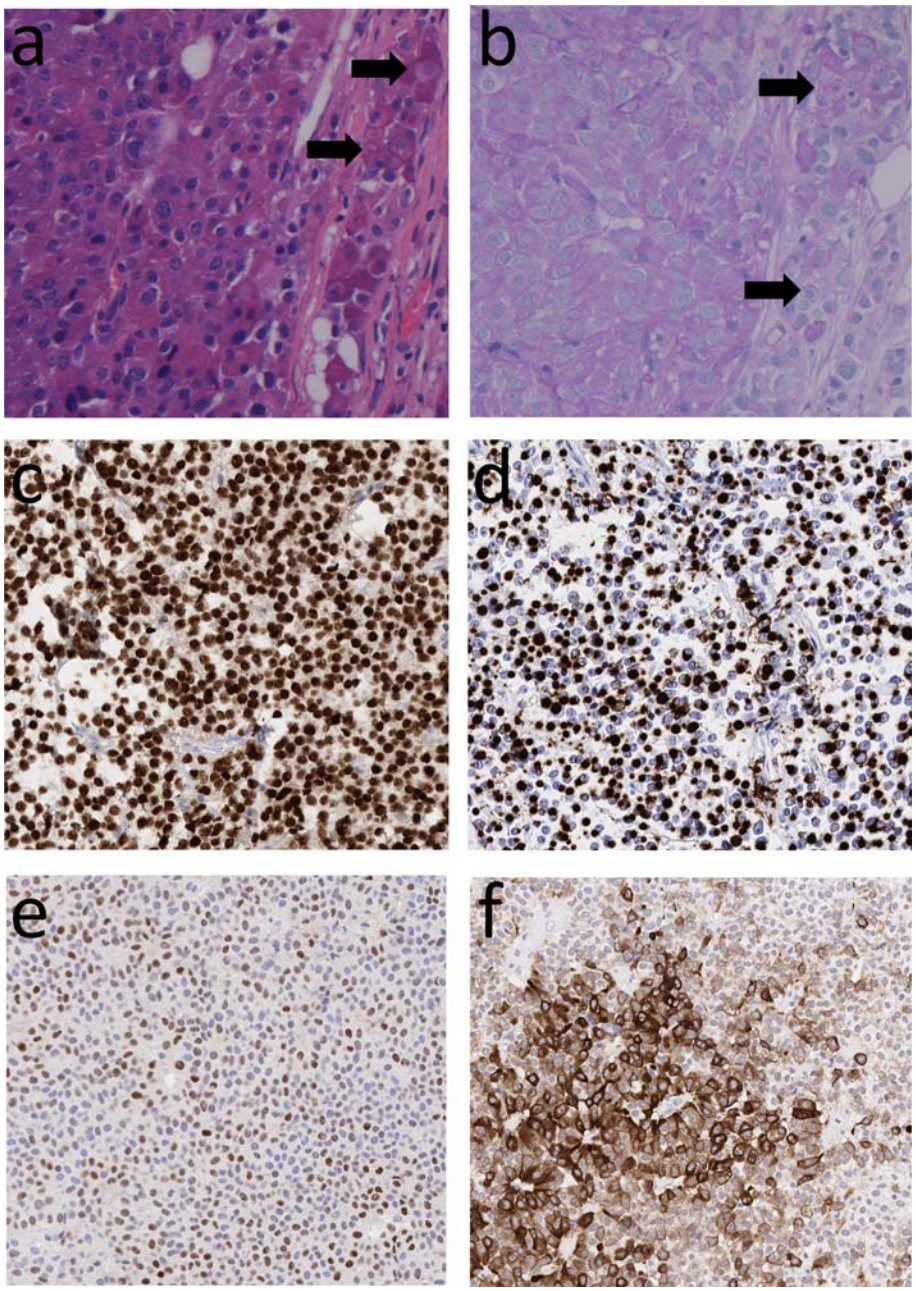

Fig. 1 Morphologic features of pituitary adenohypophysial tumors. (A and B) A densely granulated corticotroph tumor seen in the *left* of these photomicrophs with hematoxylin and eosin staining (A) and the periodic acid Schiff stain (B) has basophilic and PAS-positive cytoplasm that reflects ACTH content; the nontumorous tissue in the *right* of each photo contains corticotrophs with accumulation of pale glassy filaments induced by the negative feedback of excess cortisol (*arrows*), a feature known as Crooke's hyaline change. Crooke cells rarely comprise pituitary tumors. (C) Nuclear staining for the transcription factor Pit-1 identifies a tumor of this lineage that may produce GH, PRL, and/or TSH. (D) The tumor known as a sparsely granulated somatotroph tumor is characterized by juxtanuclear keratin aggresomes known as "fibrous bodies" identified with antibodies to cytokeratin 18. (E). Gonadotroph lineage is identified by nuclear staining for steroidogenic factor-1 (SF-1). (F) Some gonadotroph tumors have strong expression for gonadotropins, usually FSH as seen here, but they may also have faint, focal or no gonadotropin staining.

organs. The delay in diagnosis, typically nearly a decade, is associated with invasive tumors and incomplete surgical resection, necessitating long-term medical therapy. *Gigantism* is the result of early onset GH excess prior to epiphysial fusion; the young patients are tall but again, this diagnosis is often made only after many years of excessive growth and the disease progresses to acromegaly. *Cushing disease* presents with clinical features that result from cortisol excess; these range from weight gain, diabetes and hypertension to neurocognitive mood and behavioral changes. As with acromegaly, the slow, insidious and seemingly common manifestations often elude early diagnosis and patients eventually develop osteoporosis and immunosuppression. *Hyperprolactinemic disorders* present differently depending on the age at the time of presentation. In the young, failure to manifest pubertal features can be a warning sign. In middle aged men and women, sexual dysfunction is typical; women usually present earlier with menstrual irregularities. Infertilty in both genders often uncovers the underlying problem which significantly alters management. In the elderly, accelerated bone loss and/or muscle mass can be a sign of long-standing unrecognized prolactin-producing pituitary tumors. *Hyperthyroidism* presents with the typical hypermetabolic features of heat intolerance, palpitations, weight loss, and anxiety. Inappropriately "normal" levels of TSH can sometimes be overlooked, resulting in ablation of the thyroid gland without attention to the pituitary tumor as the true underlying cause. *Clinically nonfunctioning pituitary tumors* present with signs and symptoms attributable to the mass. These include headaches as well as visual field defects due to compression of the optic chiasm. Depending on the firmness and invasiveness of the pituitary tumor, loss of anterior pituitary hormones is another major presenting feature leading to the diagnosis.

The aggressiveness of a pituitary tumor is determined by the cellular differentiation, the proliferative rate of the tumor and its invasiveness. In general, tumors composed of well differentiated cells, including sparsely granulated lactotrophs, densely granulated somatotrophs, and mammosomatotroph, tend to be responsive to medical therapies that target their normal receptor regulation (Tindall et al., 1982; Bhayana et al., 2005; Asa and Ezzat, 2009). Proliferation rates, assessed by Ki67 labeling using the MIB-1 antibody, can give some indication of the rate of growth of a tumor; in general, tumors with low proliferative rates tend to regrow slower if incompletely resected than do those with higher proliferation indices (Asa and Ezzat, 2016; Mete et al., 2012). Tumors that grow upwards, pushing the diaphragma sellae up rather than infiltrating laterally into the cavernous sinuses or downwards into bone and paranasal sinuses, are more likely to be surgically resectable and therefore have a better prognosis (Micko et al., 2015; Knosp et al., 1993). The factors underlying invasive potential remain to be clarified (Mete et al., 2012, 2013) however it is interesting that among clinically nonfunctioning tumors, gonadotroph tumors tend to have low proliferation and upwards growth, whereas silent corticotroph, poorly differentiated Pit-1 lineage and undifferentiated null cell tumors are more likely to be locally invasive (Mete et al., 2013, 2016; Nishioka et al., 2015).

All three factors are important in determining the management and prognosis of a patient with a pituitary tumor. The application of proliferation alone or with p53 to define "atypical adenomas" (DeLellis et al., 2004) has been abandoned (Mete and Lopes, 2017). While some groups stratify pituitary tumors into three categories as "noninvasive," "invasive," and "aggressive-invasive" based principally on imaging features (Wierinckx et al., 2007), this categorization fails to include the likelihood of response to medical therapy. As examples, there are some giant lactotroph tumors that are widely invasive of many structures but respond well to dopamine agonist therapies with longterm shrinkage and normalization of hormone excess, and in contrast, some patients with corticotroph microadenomas that are not invasive of dura or bone and have low proliferation indices recur due to clinically and pathologically undetected local invasion of the adjacent adenohypophysis.

The classification of a pituitary tumor as a "carcinoma" requires the presence of discontinuous growth in the form of metastatic disease in other parts of the central nervous system or spread to lymph nodes, liver and other organs (DeLellis et al., 2004; Asa, 2011). This occurrence is exceptionally rare. There are no specific morphologic or other biomarkers that predict metastatic behavior and the vast majority of patients with pituitary carcinoma have had an initial diagnosis of a pituitary "adenoma," providing yet another rationale for changing the terminology to PitNET (Asa et al., 2017a).

Rarely, other types of tumor arise in the pituitary. These include *craniopharyngiomas* (**Fig. 2**) that arise from embryological remnants of the developing pituitary gland from the oral cavity, *neuronal and glial tumors* of the hypothalamus and neurohypophysis (the posterior neural portion of the pituitary gland), as well as *mesenchymal, hematologic, and germ cell tumors* that can arise in the region of the sella turcica (Asa, 2011). *Metastatic tumors* can involve the pituitary gland and there are also tumor-like lesions, including *cysts, inflammatory conditions*, and rare *hyperplasias* of adenohypophysial cells that can mimic a primary pituitary tumor.

Assessment of Patients With Pituitary Tumors

Biochemical Evaluation

Biochemical assessments are driven by the clinician's suspicions and rest on two major concerns. Hormone loss due to compromise of anterior pituitary function requires measures of serum pituitary trophic hormones and their targets. These include corticotroph secreted ACTH and its target cortisol, thyrotroph secreted TSH and its target thyroxine (T4), somatotroph secreted growth hormone (GH), and its target insulin like growth factor 1 (IGF-1), gonadotroph secreted luteinizing hormone (LH), and its target sex steroids, estradiol in women and testosterone in men. Follicle stimulating hormone can be measured directly but typically clinicians rely on the presence or absence or loss of LH from the same gonadotroph, or in the case of premenopausal women, the absence or presence of normal menstrual function. Finally, prolactin can be measured directly in the blood, where levels generally correlate with the size of the pituitary tumor if it is a lactotroph tumor; discrepancies between tumor size and prolactin values should raise the suspicion of a "stalk effect" where a nonlactotroph tumor grows to interrupt delivery of the hypothalamic prolactin inhibitor dopamine.

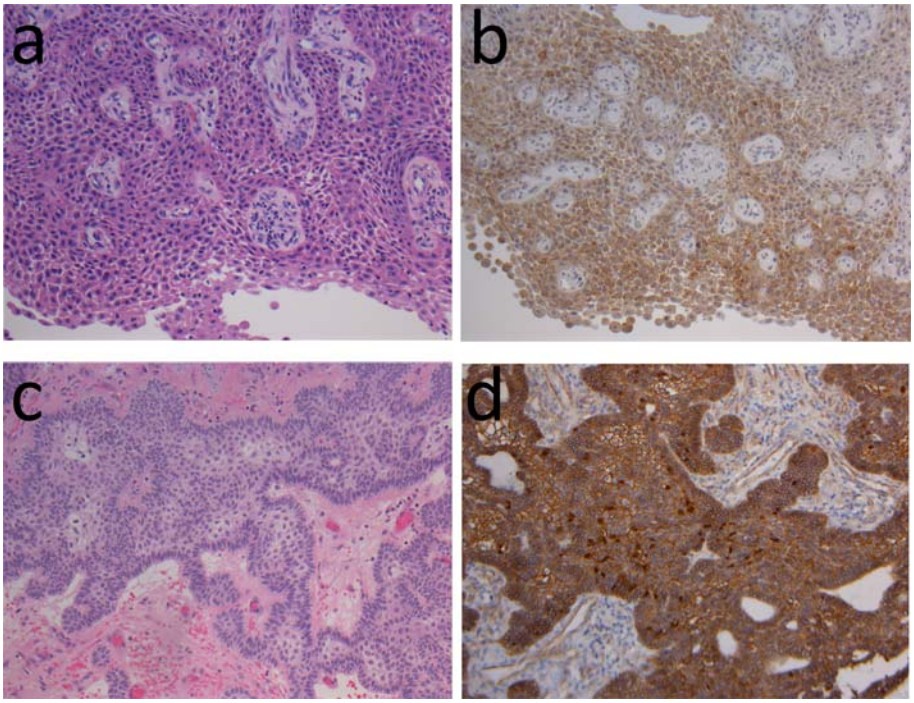

Fig. 2 Morphologic features of craniopharyngiomas. Craniopharyngioma is a tumor derived from the oral endoderm and the morphologic features reflect this squamoid origin. The papillary type (A and B) is composed of cords or islands of epithelial cells in a loose fibrous stroma; it may harbor the activating BRAFV600E mutation as identified by the VE1 antibody (B). The adamantinomatous type (C and D) has a morphology that resembles the ameloblastic organ; it may have mutation of beta-catenin that results in focal nuclear translocation (D).

Radiologic Imaging

As with biochemical studies, imaging is driven by clinical suspicion further supported by biochemical findings. The rationale behind this sequence of management helps avoid mis-diagnosis of incidental nonfunctional lesions, and to distinguish those of modest clinical relevance from small occult lesions of paramount importance. Magnetic resonance imaging (MRI) is the preferred method of examination (Bakir et al., 2016) (**Fig. 3**), as it affords detection of small lesions which require separation from the normal gland, as is typically required in patients with Cushing disease. It can also identify extension beyond the sella turcica and invasion into surrounding structures. This includes superior extension to involve the optic chiasm leading to visual field defects, lateral invasion into the cavernous sinuses where rapid growth can result in cranial nerve palsies leading to diplopia, and inferior growth into the sphenoid sinus leading to congestion and rarely cerebrospinal fluid leakage. More recently there has been a growing interest in how MR signals on T2-weighted images correlate with hormone granularity, in particular, hypointensity on T2 imaging appears to correlate with a densely granulated morphology which in the case of growth hormone tumors can be associated with favorable response to somatostatin analogue therapy (Asa et al., 2017b). While functional nuclear studies remain promising using isotopes that can detect pituitary tumor cell surface receptors (Opalinska et al., 2017), the limits of resolution remain problematic compared with current MRI 3T technology.

Genetic Testing

PitNETs are most often sporadic tumors (Asa and Ezzat, 2009); some are associated with somatic mutations such as *GNAS* in densely granulated somatotroph tumors and *USP8* mutations in densely granulated corticotroph tumors (Asa and Mete, 2018). The majority have no known genetic mutations and are thought to be due to epigenetic alterations (Ezzat et al., 2018).

There are, however, a number of familial syndromes that predispose to the development of PitNETs (Asa and Ezzat, 2009; Asa and Mete, 2018). These include Multiple Endocrine Neoplasia (MEN) Type 1 due to mutations in the *MEN1* gene, Carney complex due to *PRKAR1A* mutations, MEN Type 4 due to mutations in the genes encoding p27 or p18, familial isolated pituitary adenoma (FIPA) syndrome due to *AIP* mutations, and the 3PA syndrome (Pituitary Adenoma with Paraganglioma/Pheochromocytoma) due to SDH complex mutations. Other germline events associated with pituitary tumor development include McCune Albright syndrome due to mosaic germline *GNAS* mutation, X-linked acrogigantism (X-LAG) characterized by early childhood onset of gigantism due to Xq26 microduplications and *GPR101* mutation, and the very rare pituitary blastoma due to *DICER* mutations.

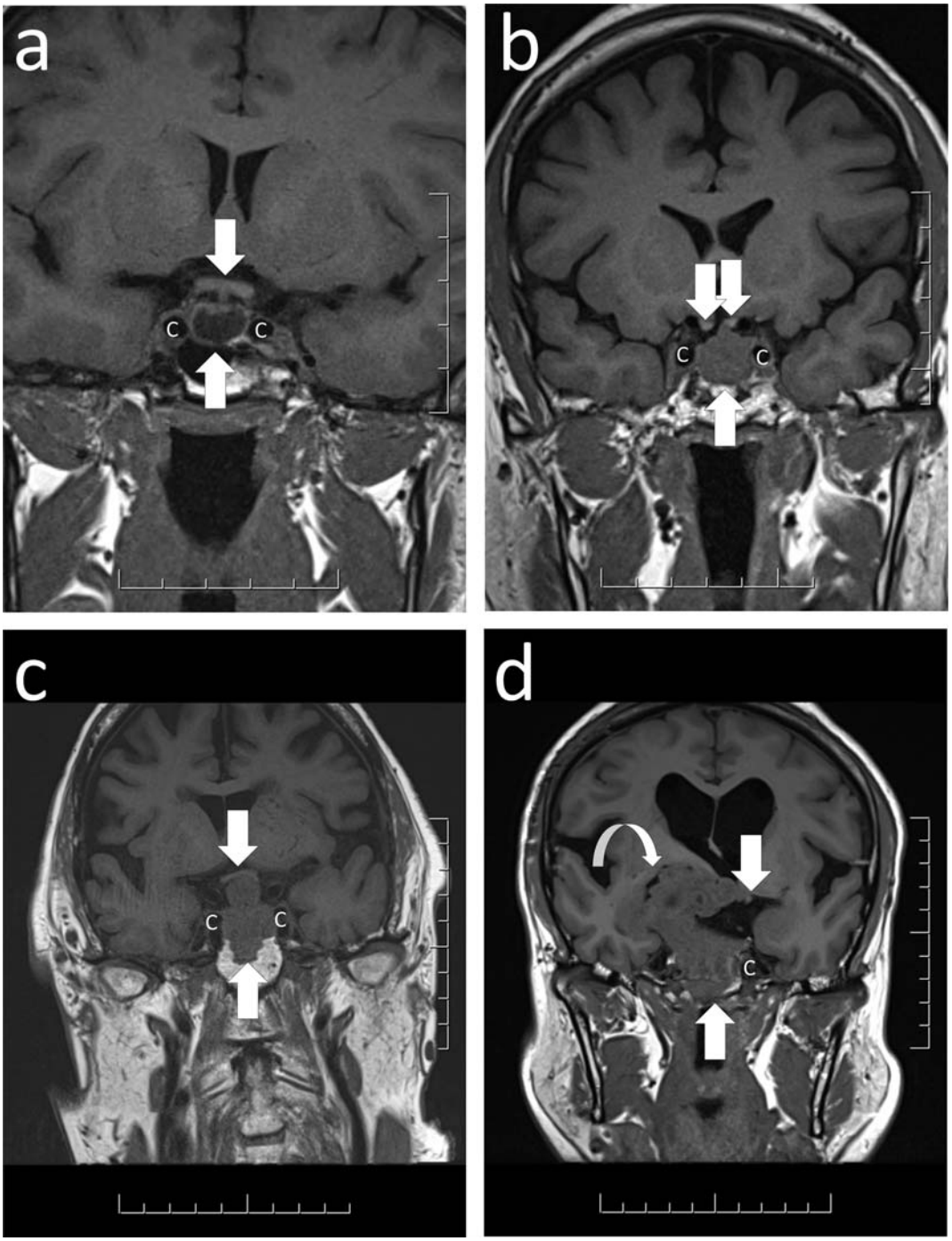

Fig. 3 Magnetic resonance imaging of pituitary tumors. The pituitary is located at the base of the brain in the bony structure of the sella turcica (bottom *arrows*) that lies between the cavernous sinuses (C) and below the optic chiasm (top *arrows*). Pituitary tumors can be (A) small, within the pituitary and cystic, (B) slightly invasive into the cavernous sinuses, (C) large and soft, ballooning upwards with a "snowman" shape due to compression by the diaphragm sellae, or (D) giant and aggressively invasive of the brain (curved *arrow*), obstructing cerebrospinal fluid flow and causing hydrocephalus, obliterating the lateral cavernous sinus, and infiltrating inferiorly though bone and into the paranasal sinuses.

Management of Patients With Pituitary Tumors

Medical Therapy

The purpose of medical therapy is to control if not reverse disease progression in terms of hormone excess, and where relevant, reduce tumor size, and invasiveness. Pharmacological agents have been developed based on recognition of endogenous pituitary

hormone inhibitors. These objectives have been met with varying degrees of success. Classically, dopamine agonists have proven their benefits in terms of reducing prolactin levels and diminishing the size of lactotroph tumors (Cuny et al., 2015), such that they are the mainstay of treatment for this tumor type. The same dopamine agonists have proven to be relatively less effective in GH-secreting tumors (Cuny et al., 2015) and even less in clinically nonfunctioning gonadotroph tumors (Greenman et al., 2005). First generation somatostatin analogues have proven to be effective in lowering GH levels and reducing the size of tumors causing acromegaly or gigantism (Cuny et al., 2015; Grasso et al., 2013; Iacovazzo et al., 2016). In some institutions, this medical therapy is used initially, to enhance the outcome of surgical resection, whereas in other institutions this medical therapy is only used for surgical failures. Response is usually better in patients with densely granulated somatotroph tumors than in those with sparsely granulated somatotroph tumors (Bhayana et al., 2005; Asa et al., 2017b), consistent with the presence of *GNAS* mutations that characterize the densely granulated tumors. More recently, second generation analogues, that bind with more somatostatin receptor subtypes, have also been used in managing inoperable Cushing disesase (Pivonello et al., 2014); again the response has been shown to be better in densely granulated tumors that may harbor *USP8* mutations (Hayashi et al., 2015). Analogues of the GnRH hypothalamic peptides have not shown to be of benefit in shrinking gonadotroph tumors (Cuny et al., 2015) and, therefore, are rarely used in managing patients with pituitary tumors.

Aggressive PitNETs and pituitary carcinomas can be quite challenging to treat. Use of the DNA methylation inhibitor temozolomide has gained momentum, given its utility in primary CNS tumors (Vieira et al., 2013; Annamalai et al., 2012; Raverot et al., 2010, 2012; Bush et al., 2010; Fadul et al., 2006). Unfortunately, such therapy continues to be associated with the emergence of resistance (Batisse et al., 2013), highlighting the need for more effective agents in restraining rapidly proliferative and metastatic pituitary neoplasms.

Whether cured of their pituitary tumor or not, patients may require ongoing medical follow up to ensure appropriate hormone replacement and to deal with visual field loss, cardiac dysfunction, bone loss, sleep apnea, and obesity that can be complications of the hormone excess or from hormonal loss due to anterior pituitary failure.

Surgery

As with medical approaches, the aim of surgical intervention is to reduce, if not normalize hormone levels, and eliminate the responsible tumor. Naturally, the obvious advantage would be the more definitive nature of a surgical procedure. To a great extent, these objectives are met when the tumor is detected early and its limits are confined within the sella turcica. Surgical success rates, however, decline sharply as tumor invasiveness extends into surrounding structures (Micko et al., 2015). Despite improvements in current surgical techniques, frank invasion into the lateral walls of the carvenous sinuses precludes complete endoscopic pituitary microsurgery. With these limitations in mind, there continues to be debate on the optimal approach to the integration of medical and surgical management of pituitary tumors. It is anticipated that prospective controlled studies will assist in the selection and design of optimal management approaches to difficult pituitary tumors.

Radiation Therapy

The role of external beam radiation has evolved over the last decades (Lillehei et al., 1998; Tsang et al., 1994). In general, with improvement in medical and surgical approaches, the role of radiation has declined. In most centres, radiation is reserved for those cases where other therapeutic options have failed. In contrast, the role of more focused, gamma-knife radiotherapy has emerged as a suitable option where the tumor target is confined to a limited region within or outside the sella turcica (Jagannathan et al., 2008; Oyesiku, 2007). Long-term outcomes of gamma knife radiation continue to emerge, helping provide better evidence for integration of this therapeutic modality in the growing armamentarium of management options.

Psychosocial Assistance

Patients with pituitary tumors are known to have emotional disturbances that result from hormone excess, hormone deficiency or the effects of the tumor on adjacent brain structures (Sobrinho, 1998; Flitsch et al., 2000; Starkman et al., 2007; Sievers et al., 2009; Tiemensma et al., 2010a,b). It is also possible that there are common predisposing factors to endocrine and emotional dysfunction (Kiehl et al., 2008). The psychosocial alterations including depression, anxiety, loneliness, anger, and other reactions to changes in body image and sexuality require supportive management to enhance the quality of life of patients with pituitary disorders.

Prospective Vision

Pituitary tumors represent one of the more common endocrine neoplasms. They vary widely in terms of endocrine manifestations and structural impact. A successful management approach rests fundamentally on heightened clinical suspicion and early detection. Delayed diagnosis is associated with the potential for significant endocrine, neurologic, and multisystem dysfunctions. Fortunately, advances in biochemical, radiographic, and histomorphologic examinations are leading to refinements in risk stratification and management approaches.

See also: Pituitary Tumors: Pathology and Genetics.

References

Agustsson, T.T., Baldvinsdottir, T., Jonasson, J.G., Olafsdottir, E., Steinthorsdottir, V., et al., 2015. The epidemiology of pituitary adenomas in Iceland, 1955–2012: A nationwide population-based study. European Journal of Endocrinology 173 (5), 655–664.

Annamalai, A.K., Dean, A.F., Kandasamy, N., Kovacs, K., Burton, H., et al., 2012. Temozolomide responsiveness in aggressive corticotroph tumours: A case report and review of the literature. Pituitary 15 (3), 276–287.

Asa, S.L., 2011. In: Silverberg, S.G. (Ed.), Tumors of the pituitary gland, AFIP Atlas of Tumor Pathology, Series 4, Fascicle 15. ARP Press, Silver Spring, MD.

Asa, S.L., Ezzat, S., 2009. The pathogenesis of pituitary tumors. Annual Review of Pathology 4, 97–126.

Asa, S.L., Ezzat, S., 2016. Aggressive pituitary tumors or localized pituitary carcinomas: Defining pituitary tumors. Expert Review of Endocrinology and Metabolism 11 (2), 149–162.

Asa, S.L., Mete, O., 2016. The pituitary gland. In: Mete, O., Asa, S.L. (Eds.), Endocrine pathology, 12. Cambridge University Press, Cambridge, pp. 315–397.

Asa, S.L., Mete, O., 2018. What's new in pituitary pathology? Histopathology 72 (1), 133–141.

Asa, S.L., Casar-Borota, O., Chanson, P., Delgrange, E., Earls, P., et al., 2017a. From pituitary adenoma to pituitary neuroendocrine tumor (PitNET): An international pituitary pathology club proposal. Endocrine-Related Cancer 24 (4), C5–C8.

Asa, S.L., Kucharczyk, W., Ezzat, S., 2017b. Pituitary acromegaly: Not one disease. Endocrine-Related Cancer 24 (3), C1–C4.

Bakir, B., Chan, M., Cruz, J.P., Kartal, M.G.D., Gulenchyn, K.Y., et al., 2016. Radiological imaging. In: Mete, O., Asa, S.L. (Eds.), Endocrine pathology, vol. 5. Cambridge University Press, Cambridge, pp. 109–214.

Batisse, M., Raverot, G., Maqdasy, S., Durando, X., Sturm, N., et al., 2013. Aggressive silent GH pituitary tumor resistant to multiple treatments, including temozolomide. Cancer Investigation 31 (3), 190–196.

Bhayana, S., Booth, G.L., Asa, S.L., Kovacs, K., Ezzat, S., 2005. The implication of somatotroph adenoma phenotype to somatostatin analog responsiveness in acromegaly. The Journal of Clinical Endocrinology and Metabolism 90 (11), 6290–6295.

Bush, Z.M., Longtine, J.A., Cunningham, T., Schiff, D., Jane Jr., J.A., et al., 2010. Temozolomide treatment for aggressive pituitary tumors: Correlation of clinical outcome with *O*(6)-methylguanine methyltransferase (MGMT) promoter methylation and expression. The Journal of Clinical Endocrinology and Metabolism 95 (11), E280–E290.

Cuny, T., Barlier, A., Feelders, R., Weryha, G., Hofland, L.J., et al., 2015. Medical therapies in pituitary adenomas: Current rationale for the use and future perspectives. Annales d'Endocrinologie 76 (1), 43–58.

Daly, A.F., Rixhon, M., Adam, C., Dempegioti, A., Tichomirowa, M.A., Beckers, A., 2006. High prevalence of pituitary adenomas: A cross-sectional study in the province of Liege, Belgium. The Journal of Clinical Endocrinology and Metabolism 91 (12), 4769–4775.

DeLellis, R.A., Lloyd, R.V., Heitz, P.U., Eng, C., 2004. Pathology and genetics of tumours of endocrine organs. IARC Press, Lyons.

Ezzat, S., Asa, S.L., Couldwell, W.T., Barr, C.E., Dodge, W.E., et al., 2004. The prevalence of pituitary adenomas: A systematic review. Cancer 101 (3), 613–619.

Ezzat, S., Cheng, S., Asa, S.L., 2018. Epigenetics of pituitary tumors: Pathogenetic and therapeutic implications. Molecular and Cellular Endocrinology 469, 70–76.

Fadul, C.E., Kominsky, A.L., Meyer, L.P., Kingman, L.S., Kinlaw, W.B., et al., 2006. Long-term response of pituitary carcinoma to temozolomide. Report of two cases. Journal of Neurosurgery 105 (4), 621–626.

Fernandez, A., Karavitaki, N., Wass, J.A., 2010. Prevalence of pituitary adenomas: A community-based, cross-sectional study in Banbury (Oxfordshire, UK). Clinical Endocrinology 72 (3), 377–382.

Flitsch, J., Spitzner, S., Ludecke, D.K., 2000. Emotional disorders in patients with different types of pituitary adenomas and factors affecting the diagnostic process. Experimental and Clinical Endocrinology & Diabetes 108 (7), 480–485.

Fontana, E., Gaillard, R., 2009. Epidemiology of pituitary adenoma: Results of the first Swiss study. Revue Médicale Suisse 5 (223), 2172–2174.

Gold, E.B., 1981. Epidemiology of pituitary adenomas. Epidemiologic Reviews 3, 163–183.

Gomez-Hernandez, K., Ezzat, S., 2016. Clinical presentations of endocrine diseases. In: Mete, O., Asa, S.L. (Eds.), Endocrine pathology, vol. 1. Cambridge University Press, Cambridge, pp. 1–55.

Gomez-Hernandez, K., Ezzat, S., Asa, S.L., Mete, O., 2015. Clinical implications of accurate subtyping of pituitary adenomas: Perspectives from the treating physician. Türk Patoloji Derneği 31 (Suppl 1), 4–17.

Grasso, L.F., Pivonello, R., Colao, A., 2013. Investigational therapies for acromegaly. Expert Opinion on Investigational Drugs 22 (8), 955–963.

Greenman, Y., Tordjman, K., Osher, E., Veshchev, I., Shenkerman, G., et al., 2005. Postoperative treatment of clinically nonfunctioning pituitary adenomas with dopamine agonists decreases tumour remnant growth. Clinical Endocrinology 63 (1), 39–44.

Hayashi, K., Inoshita, N., Kawaguchi, K., Ardisasmita, A.I., Suzuki, H., et al., 2015. The USP8 mutational status may predict drug susceptibility in corticotroph adenomas of Cushing's disease. European Journal of Endocrinology 174 (2), 213–226.

Iacovazzo, D., Carlsen, E., Lugli, F., Chiloiro, S., Piacentini, S., et al., 2016. Factors predicting pasireotide responsiveness in somatotroph pituitary adenomas resistant to first-generation somatostatin analogues: An immunohistochemical study. European Journal of Endocrinology 174 (2), 241–250.

Jagannathan, J., Sheehan, J.P., Pouratian, N., Laws Jr., E.R., Steiner, L., Vance, M.L., 2008. Gamma knife radiosurgery for acromegaly: Outcomes after failed transsphenoidal surgery. Neurosurgery 62 (6), 1262–1269.

Kiehl, T.R., Fischer, S.E., Ezzat, S., Asa, S.L., 2008. Mice lacking the transcription factor Ikaros display behavioral alterations of an anti-depressive phenotype. Experimental Neurology 211 (1), 107–114.

Knosp, E., Steiner, E., Kitz, K., Matula, C., 1993. Pituitary adenomas with invasion of the cavernous sinus space: A magnetic resonance imaging classification compared with surgical findings. Neurosurgery 33 (4), 610–617.

Lillehei, K.O., Kirschman, D.L., Kleinschmidt-DeMasters, B.K., Ridgway, E.C., 1998. Reassessment of the role of radiation therapy in the treatment of endocrine-inactive pituitary macroadenomas. Neurosurgery 43 (3), 432–438.

Mete, O., Asa, S.L., 2012. Clinicopathological correlations in pituitary adenomas. Brain Pathology 22 (4), 443–453.

Mete, O., Asa, S.L., 2013. Therapeutic implications of accurate classification of pituitary adenomas. Seminars in Diagnostic Pathology 30 (3), 158–164.

Mete, O., Lopes, M.B., 2017. Overview of the 2017 WHO classification of pituitary tumors. Endocrine Pathology 28 (3), 228–243.

Mete, O., Ezzat, S., Asa, S.L., 2012. Biomarkers of aggressive pituitary adenomas. Journal of Molecular Endocrinology 49 (2), R69–R78.

Mete, O., Hayhurst, C., Alahmadi, H., Monsalves, E., Gucer, H., et al., 2013. The role of mediators of cell invasiveness, motility, and migration in the pathogenesis of silent corticotroph adenomas. Endocrine Pathology 24 (4), 191–198.

Mete, O., Gomez-Hernandez, K., Kucharczyk, W., Ridout, R., Zadeh, G., et al., 2016. Silent subtype 3 pituitary adenomas are not always silent and represent poorly differentiated monomorphous plurihormonal Pit-1 lineage adenomas. Modern Pathology 29 (2), 131–142.

Micko, A.S., Wohrer, A., Wolfsberger, S., Knosp, E., 2015. Invasion of the cavernous sinus space in pituitary adenomas: Endoscopic verification and its correlation with an MRI-based classification. Journal of Neurosurgery 122 (4), 803–811.

Nishioka, H., Inoshita, N., Mete, O., Asa, S.L., Hayashi, K., et al., 2015. The complementary role of transcription factors in the accurate diagnosis of clinically nonfunctioning pituitary adenomas. Endocrine Pathology 26 (4), 349–355.

Opalinska, M., Hubalewska-Dydejczyk, A., Sowa-Staszczak, A., 2017. Radiolabeled peptides: Current and new perspectives. The Quarterly Journal of Nuclear Medicine and Molecular Imaging 61 (2), 153–167.
Oyesiku, N.M., 2007. Stereotactic radiosurgery for Cushing disease: A review. Neurosurgical Focus 23 (6), E14.
Pivonello, R., Petersenn, S., Newell-Price, J., Findling, J.W., Gu, F., et al., 2014. Pasireotide treatment significantly improves clinical signs and symptoms in patients with Cushing's disease: Results from a phase III study. Clinical Endocrinology 81 (3), 408–417.
Raverot, G., Sturm, N., de Fraipont, F., Muller, M., Salenave, S., et al., 2010. Temozolomide treatment in aggressive pituitary tumors and pituitary carcinomas: A French multicenter experience. The Journal of Clinical Endocrinology and Metabolism 95 (10), 4592–4599.
Raverot, G., Castinetti, F., Jouanneau, E., Morange, I., Figarella-Branger, D., et al., 2012. Pituitary carcinomas and aggressive pituitary tumours: Merits and pitfalls of temozolomide treatment. Clinical Endocrinology 76 (6), 769–775.
Scheithauer, B.W., 1984. Surgical pathology of the pituitary: The adenomas. Part I. Pathology Annual 19 (1), 317–374.
Sievers, C., Ising, M., Pfister, H., Dimopoulou, C., Schneider, H.J., et al., 2009. Personality in patients with pituitary adenomas is characterized by increased anxiety-related traits: Comparison of 70 acromegalic patients with patients with non-functioning pituitary adenomas and age- and gender-matched controls. European Journal of Endocrinology 160 (3), 367–373.
Sobrinho, L.G., 1998. Emotional aspects of hyperprolactinemia. Psychotherapy and Psychosomatics 67 (3), 133–139.
Starkman, M.N., Giordani, B., Gebarski, S.S., Schteingart, D.E., 2007. Improvement in mood and ideation associated with increase in right caudate volume. Journal of Affective Disorders 101 (1–3), 139–147.
Tiemensma, J., Biermasz, N.R., van der Mast, R.C., Wassenaar, M.J., Middelkoop, H.A., et al., 2010a. Increased psychopathology and maladaptive personality traits, but normal cognitive functioning, in patients after long-term cure of acromegaly. The Journal of Clinical Endocrinology and Metabolism 95 (12), E392–E402.
Tiemensma, J., Kokshoorn, N.E., Biermasz, N.R., Keijser, B.J., Wassenaar, M.J., et al., 2010b. Subtle cognitive impairments in patients with long-term cure of Cushing's disease. The Journal of Clinical Endocrinology and Metabolism 95 (6), 2699–2714.
Tindall, G.T., Kovacs, K., Horvath, E., Thorner, M.O., 1982. Human prolactin-producing adenomas and bromocriptine: A histological, immunocytochemica, ultrastructural and morphometric study. The Journal of Clinical Endocrinology and Metabolism 55, 1178–1183.
Tjornstrand, A., Gunnarsson, K., Evert, M., Holmberg, E., Ragnarsson, O., et al., 2014. The incidence rate of pituitary adenomas in western Sweden for the period 2001–2011. European Journal of Endocrinology 171 (4), 519–526.
Tsang, R.W., Brierley, J.D., Panzarella, T., Gospodarowicz, M.K., Sutcliffe, S.B., Simpson, W.J., 1994. Radiation therapy for pituitary adenoma: Treatmen outcome and prognostic factors. International Journal of Radiation Oncology, Biology, Physics 30, 557–565.
Vieira, N.L., Chimelli, L., Pereira, P.J., Gasparetto, E.L., Bines, J., et al., 2013. The role of temozolomide in the treatment of a patient with a pure silent pituitary somatotroph carcinoma. Endocrine Practice 19 (6), e145–e149.
Wierinckx, A., Auger, C., Devauchelle, P., Reynaud, A., Chevallier, P., et al., 2007. A diagnostic marker set for invasion, proliferation, and aggressiveness of prolactin pituitary tumors. Endocrine-Related Cancer 14 (3), 887–900.

Further Reading

Asa, S.L., 2011. In: Silverberg, S.G. (Ed.), Tumors of the pituitary gland, AFIP Atlas of tumor pathology, Series 4, Fascicle 15. ARP Press, Silver Spring, MD.
Asa, S.L., Ezzat, S., 2009. The pathogenesis of pituitary tumors. Annual Review of Pathology 4, 97–126.
Asa, S.L., Ezzat, S., 2016. Aggressive pituitary tumors or localized pituitary carcinomas: Defining pituitary tumors. Expert Review of Endocrinology and Metabolism 11 (2), 149–162.
Asa, S.L., Mete, O., 2016. The pituitary gland. In: Mete, O., Asa, S.L. (Eds.), Endocrine pathology, 12. Cambridge University Press, Cambridge, pp. 315–397.
Asa, S.L., Mete, O., 2018. What's new in pituitary pathology? Histopathology 72 (1), 133–141.
Asa, S.L., Casar-Borota, O., Chanson, P., Delgrange, E., Earls, P., et al., 2017a. From pituitary adenoma to pituitary neuroendocrine tumor (PitNET): An international pituitary pathology club proposal. Endocrine-Related Cancer 24 (4), C5–C8.
Asa, S.L., Kucharczyk, W., Ezzat, S., 2017b. Pituitary acromegaly: Not one disease. Endocrine-Related Cancer 24 (3), C1–C4.
Ezzat, S., Cheng, S., Asa, S.L., 2018. Epigenetics of pituitary tumors: Pathogenetic and therapeutic implications. Molecular and Cellular Endocrinology 469, 70–76.
Gomez-Hernandez, K., Ezzat, S., 2016. Clinical presentations of endocrine diseases. In: Mete, O., Asa, S.L. (Eds.), Endocrine pathology, vol. 1. Cambridge University Press, Cambridge, pp. 1–55.
Laws, E.R., Ezzat, S., Asa, S.L., Rio, L.M., Michel, L., Knutzen, R., 2013. Pituitary disorders: Diagnosis and management. Wiley-Blackwell, West Sussex.
Mete, O., Asa, S.L., 2012. Clinicopathological correlations in pituitary adenomas. Brain Pathology 22 (4), 443–453.
Mete, O., Asa, S.L., 2013. Therapeutic implications of accurate classification of pituitary adenomas. Seminars in Diagnostic Pathology 30 (3), 158–164.
Mete, O., Lopes, M.B., 2017. Overview of the 2017 WHO classification of pituitary tumors. Endocrine Pathology 28 (3), 228–243.
Mete, O., Ezzat, S., Asa, S.L., 2012. Biomarkers of aggressive pituitary adenomas. Journal of Molecular Endocrinology 49 (2), R69–R78.
Wierinckx, A., Auger, C., Devauchelle, P., Reynaud, A., Chevallier, P., et al., 2007. A diagnostic marker set for invasion, proliferation, and aggressiveness of prolactin pituitary tumors. Endocrine-Related Cancer 14 (3), 887–900.

Relevant Websites

https://pituitary.org/—Pituitary Network Association.
https://www.pituitary.org.uk/information/pituitary-conditions/—The Pituitary Foundation.
https://www.cancer.org/cancer/pituitary-tumors.html—Pituitary Tumors—American Cancer Society.
http://www.cancerresearchuk.org/about-cancer/brain-tumours/types/pituitary-tumours—Pituitary Tumors—Cancer Research UK.
http://www.cancer.ca/en/cancer-information/cancer-type/pituitary-gland-tumour/pituitary-gland-tumours/?region=on—Pituitary gland tumors—Canadian Cancer Society.
AACE, 2004. AACE medical guidelines for clinical practice for the diagnosis and treatment of acromegaly. Endocrine Practice 10 (3), 213–225.

Polyomaviruses in Human Cancer

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Glossary

Large T (LT) LT is a polyomavirus replicase that possesses DNA helicase activity, but also acts as viral oncoprotein by inhibiting multiple tumor suppressors such as the retinoblastoma protein and p53 protein.

Merkel cell polyomavirus (MCV) A first human oncogenic polyomavirus discovered in 2008 that is strongly linked to human Merkel cell carcinoma.

Polyomavirus (Greek: poly many, multiple; oma: tumors) A small circular double-stranded DNA virus originally identified in animals as an infectious agent that induces tumors in nonpermissive hosts.

Small T (sT) sT is another oncoprotein encoded by T antigen gene that commonly inhibits the host protein phosphatase 2A tumor suppressor.

Tumor (T) antigen A polyomavirus gene required for viral genome replication. This is a major polyomavirus oncogene that produces multiple oncogenic proteins such as large T and small T antigen proteins by the alternative splicing.

Polyomavirus research has been central to cancer research. Animal polyomaviruses, in particular, murine polyomavirus (MPyV) and simian vacuolating virus 40 (SV40), are well-studied tumor viruses that have been used to model cellular transformation and carcinogenesis in rodents. Research on the SV40 T antigen led to the discovery of p53 and uncovered the functions of a retinoblastoma tumor suppressor protein (pRb) in cell cycle regulation. On the other hand, molecular analysis of the MPyV oncogene identified the importance of tyrosine phosphorylation and phosphoinositide 3-kinase signaling in cell transformation.

Because of their strong tumorigenicity, polyomaviruses have been suspected to play a role in human cancer. However, for a long time until 2006, the JC polyomavirus (JCV) and BK polyomavirus (BKV) were the only known human polyomaviruses, and neither of these viruses had been strongly linked to human cancer. In this century since 2007, the developments in all fields of genetics and genetic technology facilitated the discovery of human polyomaviruses. As a result, 12 new polyomaviruses joined the human polyomavirus family. Notably, the discovery of the Merkel cell polyomavirus (MCV or MCPyV) from human Merkel cell carcinoma (MCC), as well as the identification of the rare BKV integration event in urological cancers, proved our assumptions of polyomavirus involvement in human tumorigenesis to be true.

This article provides an overview of basic polyomavirus virology, revisits hallmarks of polyomavirus-induced malignancy, and describes common molecular mechanisms of the T antigen-mediated oncogenic transformation. With an updated list of 14 new human polyomaviruses, their discoveries and potential roles in human carcinogenesis are discussed. Finally, the first human oncogenic polyomavirus MCV is described with a particular focus on its transformation mechanisms.

Genome Structure and Basic Virology of Polyomaviruses

Polyomaviruses are unenveloped, double-stranded DNA viruses with a closed-circular genome of approximately 5000 base pairs (bp), which can be divided into the viral regulatory region and the early and late coding sequences (Fig. 1A). The regulatory region, or noncoding control region (NCCR), contains the DNA replication origin and two bidirectional promoters that drive early and late gene expression. The early gene is transcribed prior to viral DNA replication and expresses the Tumor (T) antigen, also known as a viral oncogene. The T antigen gene expresses at minimum the large tumor antigen (LT) and small tumor antigen (sT) proteins from a single multiply spliced mRNA, which are common across the polyomavirus family. In addition to LT and sT, each polyomavirus expresses unique isoforms of the T antigen such as the middle T (MT) antigen from murine polyomavirus (MPyV) and the multiply spliced 17kT from simian vacuolating virus 40 (SV40). While the oncogenic function of T antigens is better described, these two viral antigens play important roles in polyomavirus replication. LT is the viral replicase protein with an origin-binding domain (OBD) and DNA helicase domain, which directly binds to the replication origin (Fig. 1B). In fact, the cell-free SV40 replication system has served as a model for understanding mammalian DNA replication, because SV40 DNA replication exploits the host eukaryotic cellular replication machinery except for the MCM helicase, whose function is fulfilled by the LT. Recent studies demonstrated that the architecture, dimensions, and assembly of LT and MCM2–7 double hexamers on their cognate origin DNAs are closely related, and the host protein machinery at viral and host replication forks is remarkably similar. The LT helicase directly binds to the pentanucleotide sequences (GAGGC) in the replication origin through OBD and assembles into two

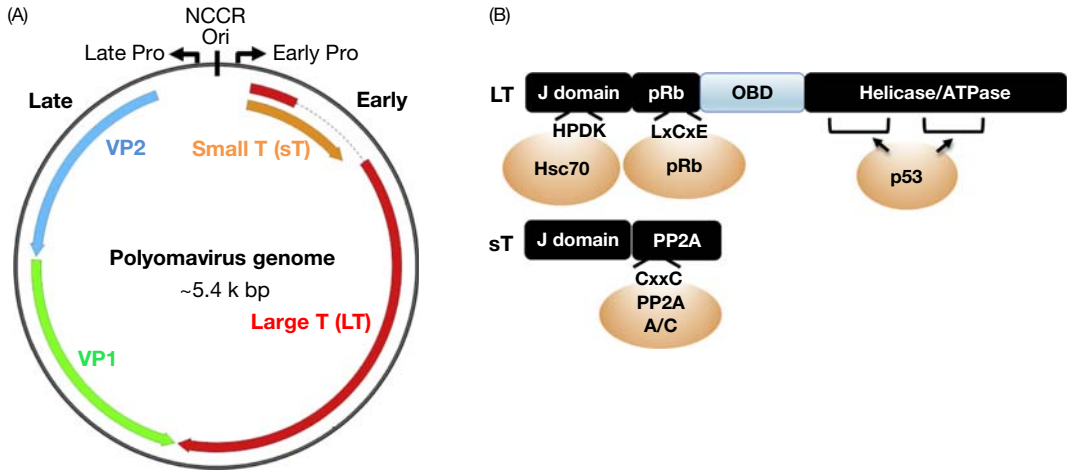

Fig.1 (A) The genome structure of polyomavirus. (B) LT and sT antigen proteins. Host proteins and their interacting domains on T antigen proteins are indicated.

ring-shaped hexamers. Upon binding to ATP, the LT double hexamers separate from each other, and the individual hexamers move in opposite directions on single-stranded DNA in a 3′-to-5′ direction to initiate bidirectional DNA replication. Thus, LT is the protein to orchestrate the replication of the viral mini-chromosome in infected cells.

The sT protein is also important for virus replication. However, in contrast to LT, the effect of sT is indirect, because the sT protein does not enhance the LT-mediated viral replication in vitro. In SV40, the sT deletion mutant is infectious, but virus production is significantly reduced. It has been shown that the exogenous expression of SV40 sT in cells infected with a sT-defective mutant virus stimulates viral DNA replication. Because sT promotes cell cycle progression from G0/G1 to S phase, which coincides with viral replication enhancement, sT is suggested to create an optimal environment for viral DNA replication. Similar to the SV40 sT, human JC polyomavirus (JCV) sT also activates viral replication through its protein phosphatase 2A (PP2A) and Rb binding domains, which are critical for cell cycle progression. In contrast, human Merkel cell polyomavirus (MCV) sT activates virus replication in a PP2A-independent mechanism. MCV sT harbors a functional domain that stabilizes MCV LT, which is described later in this article.

In addition to the LT and sT proteins, the T antigen gene also expresses other isoforms that are unique to each polyomavirus. These T antigen isoforms are produced by the unique alternative splicing of T antigen transcripts or by the alternative translation initiation that occurs on the LT transcript. The early gene region is expressed in polyomavirus-induced cancers and has been shown to be the polyomavirus oncogene. The oncogenic roles of LT and sT oncoproteins will be described in the following section.

The late gene transcription occurs after DNA replication to produce VP proteins—components of the viral capsid. The polyomavirus capsid is an icosahedral shell with a diameter of approximately 40–50 nm and consists of pentameric capsomeres, which are composed of the major capsid protein VP1. The viral capsid also contains minor capsid proteins VP2 and VP3 that are not always required for the self-assembling process of the capsid, but rather for infectivity, egress, and for viral genome transport into the nucleus.

Hallmarks of Polyomavirus-Induced Malignant Transformation

MPyV, the founder of the polyomavirus family, was discovered in 1953 as a filterable infectious agent from mouse leukemia tissue extracts. Extracts of the tumor contained a virus that induced the formation of a variety of solid tumors in newborn mice. It was thus termed polyomavirus for its ability to induce multiple tumors in mice. SV40, which naturally infects rhesus macaque, was the second member of this family isolated in 1959. It was identified as a contaminant of Salk poliovirus vaccines prepared using Macacus monkey renal cell cultures. SV40 infection in nonpermissive rodents developed tumors, while infection in permissive monkey cells produced progeny viruses instead of inducing transformation. Because of the strong tumorigenic activities in nonpermissive hosts and in cultured cells, SV40 provided a new model for cell transformation and tumorigenesis.

Polyomavirus is a direct carcinogen, delivering its T antigen oncogene into cells to induce malignant transformation, and each transformed tumor cell contains a clonally integrated viral genome. Importantly, although the T antigen is required for viral replication, the loss of viral replication activity in a nonpermissive host is associated with increased transformation activity of animal polyomaviruses. The functional domain of LT for viral replication can be separated from the domain responsible for cell transformation. Replication-defective MPyV, for instance, shows better transformation efficiency in vitro and induces more tumors in vivo than wild type MPyV. Similarly, SV40, with mutations disrupting viral replication and helicase activity, shows enhanced transforming activity. Loss of LT helicase expression is also observed when examining MPyV-induced tumors in hamsters, while sT and MT expression is maintained. Furthermore, in transformed tumor cells, these animal polyomaviruses produce neither late capsid proteins nor progeny viruses. Taken together, the loss of viral replication capacity in transformed host cells resulting from LT

helicase mutation, viral genome integration, and the loss of capsid protein expression are the hallmarks of polyomavirus-induced tumors. Consequently, tumor cells become the terminal host for the oncogenic polyomaviruses.

The Molecular Mechanism of Polyomavirus Transformation

T Antigen: The Polyomavirus Oncogene

The strategy of polyomaviruses for inducing cellular proliferation and transformation is generally the direct inhibition of tumor suppressors and the activation of oncogenic pathways. T antigen is the primary polyomavirus oncogene that mediates these processes and plays essential roles in cell transformation and tumorigenesis. Mutational analysis of the T antigen uncovered independent domains that contribute to transformation. The reverse genetic studies on SV40 T antigen molecules revealed three cellular tumor suppressors: retinoblastoma protein (pRb), p53 protein, and protein phosphatase 2A (PP2A). Inhibition of these tumor suppressors by T antigen results in aberrant cell cycle progression, leading to oncogenic transformation. While each polyomavirus targets unique oncogenes and tumor suppressors, this section focuses on three tumor suppressors pRb, p53, and PP2A that are commonly targeted by oncogenic polyomaviruses and required for human cell transformation by SV40.

Retinoblastoma family

Most polyomavirus LTs have a well-conserved pRb-binding motif LxCxE (Leu-X-Cys-X-Glu), which is responsible for cell transformation and tumorigenesis. pRb, originally described in a rare childhood eye tumor—retinoblastoma—in which this protein is defective, regulates cell cycle progression from G1 to S phase. The pRb is a member of retinoblastoma protein family that also includes p107 (pRb1) and p130 (pRb2). pRb is hypophosphorylated in resting G0 cells, increasingly phosphorylated during cell cycle progression through G1 by cyclin-dependent kinases cyclinD1/cdk4 and cyclin E/cdk2, and maintained in a hyperphosphorylated state until mitosis. Furthermore, Rb family proteins control S phase progression by modulating E2F-response genes. E2F is a family of transcription factors that consist of eight members. E2F1, E2F2, and E2F3 (E2F3a and E2F3b) are potent transcriptional activators that interact exclusively with pRb and are periodically expressed throughout the cell cycle. On the other hand, E2F4, E2F5, E2F6, E2F7 (E2F7a and E2F7b), and E2F8 are transcriptional repressors. Unlike activator E2Fs, E2F4 interacts with all three Rb family proteins, while E2F5 associates predominantly with p130, and these suppressor complexes exert their influence in the large multiprotein complex called DREAM (*d*imerization partner (DP), *R*b-like, *E*2F *a*nd *M*uvB) during G0 phase quiescence. The DREAM complex directly binds to the E2F-regulated gene promoter through the suppressor E2Fs and p130 or p107. This transcriptional suppression is suggested to play important roles in the maintenance of cellular quiescence, senescence, and tumor suppression. Other suppressor E2Fs, including E2F6, E2F7, and E2F8, do not interact with Rb family members and are thought to function independently. E2Fs regulate transcription of the genes required for cell cycle progression and DNA replication such as cyclin A, cyclin D1, cyclin E, dihydrofolate reductase, cdk2, survivin, thymidine kinase, c-myc, c-myb, cdk1, cdc6, and DNA polymerase α. Other targets also include gene products involved in DNA repair, cellular differentiation, and apoptosis. To regulate E2F activities, hypophosphorylated pRb suppresses the activities of E2F transcription factors by recruiting chromatin remodeling factors, histone deacetylases (HDAC), and histone methyltransferases. Moreover, pRb binds within the transactivation domains of E2F1-E2F3 and prevents the assembly of the transcription initiation complex. Phosphorylation of pRb results in the disassembly of the Rb-E2F complex, leading to the activation of E2F1-E2F3, while the phosphorylation of p130 and p107 also results in the dissociation of the DREAM repressor complex. Thus, the inactivation of Rb family proteins is critical for natural cell cycle progression.

Shortly after the pRb protein was discovered, SV40 LT was identified as a direct binding partner to pRb and later to p107 and p130 as well. This interaction is mediated by the LT LxCxE motif that is conserved in most polyomaviruses (**Fig. 1**B). For pRb inhibition, the LxCxE domain cooperates with the N-terminal J domain (His-Pro-Asp-Lys (HPDK) motif) of LT that binds Hsc70, a chaperone with weak ATPase activity. The J domain is conserved in the DnaJ protein chaperone family and activates the ATPase activity of Hsc70 upon its binding. The energy produced from the ATP hydrolysis is exploited by LT to dissociate the pRb/E2F complex and to liberate E2F from the pRb regulation. Another mechanism for LT to target the Rb-E2F pathway is by affecting the expression levels of the Rb family proteins. SV40 LT decreases p130 protein levels by promoting proteasomal degradation of p130, but not pRb. While LT interaction with Rb family proteins is a highly conserved phenotype among polyomaviruses, the binding affinity to each Rb family protein varies across different polyomavirus LTs. Nevertheless, human polyomavirus LTs from JCV, BK polyomavirus (BKV), and MCV have been shown to interact with Rb family proteins and promote E2F-target gene transcription. BKV LT downregulates the protein expression and phosphorylation of all Rb family proteins. JCV LT also reduces p130 protein expression, and re-expression of p130 protein is shown to overcome JCV LT-mediated tumorigenesis both in vitro and in vivo. Thus, the inhibition of Rb family proteins by LT is likely to play critical roles in polyomavirus-associated human cancers.

p53 protein

In 1979, several groups independently stumbled upon p53. When sera from animals with SV40-induced tumors were used to immunoprecipitate SV40 LT, a host cellular protein with an apparent molecular weight around 53 kDa was identified as a binding partner. p53 is a transcription factor that suppresses tumor growth through regulation of dozens of target genes involved in antiproliferative and apoptotic responses, and its mutation is associated with $>50\%$ of all human cancers. p53 is a transcription factor whose activity is controlled at the protein level by MDM2. MDM2 is a ubiquitin E3 ligase that inhibits p53-mediated gene expression through direct binding to p53 and ubiquitin-mediated degradation via its E3 ligase activity. The protein kinases ATM and ATR,

which signal DNA damage, phosphorylate the N-terminal of p53 protein to dissociate p53 from MDM2 and stabilize the p53 protein. On the other hand, to prevent oncogene-induced transformation, ARF protein ($p14^{ARF}$ in mouse and $p19^{ARF}$ in human) induced in response to oncogene activation promotes MDM2 degradation and stabilizes the p53 protein. As a tumor suppressor, p53 plays a central role in cell cycle checkpoints that limit cell division or that induce cell suicide in response to DNA damage or oncogene activation. In particular, p53 is involved in cell cycle arrest in the G1/S and G2 phases. G1/S phase arrest is achieved by the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ (CDKN1A), a negative regulator of cyclinD1/cdk4 and cyclin E/cdk2 that phosphorylate and inactivate pRb as mentioned in the previous section. Part of the mechanism by which p53 blocks cells at the G2 checkpoint involves inhibition of cdk1, the cyclin-dependent kinase required to enter mitosis. Cdk1 is simultaneously inhibited by three p53 transcriptional targets: Gadd45, p21, and 14-3-3 sigma.

Polyomaviruses including SV40, JCV, and BKV contain the p53-binding domain that overlaps with the C-terminal helicase/ATPase domain (Fig. 1B). The binding of SV40 LT to p53 blocks the ability of p53 to bind to its consensus sequence and inhibits transcription of its targets such as $p21^{Waf1/Cip1}$. This drives cell cycle progression even in the presence of oncogene activation and DNA damage and contributes to transformation.

Moreover, oncogenic polyomavirus T antigens also inhibit p53 function independently from their direct interaction with this protein. For instance, none of the T antigen proteins encoded by oncogenic MPyV associate with p53, nor do they inhibit p53 DNA binding. MPyV LT, however, blocks p53-mediated growth suppression. Even a SV40 T antigen defective in p53 binding suppresses p53-mediated gene transcription. Several reports suggest that the J domain and Rb-binding motif of LT are also required, or even essential, to override p53-induced growth inhibition. MPyV sT also inhibits p53 function by preventing $p14^{ARF}$-mediated activation of p53. Thus, even if LT lacks p53-binding activities, oncogenic polyomaviruses exploit different components of their viral machinery to escape from the antiproliferative activity of p53. Similar to MPyV LT, human MCV LTs expressed in MCC tumors do not associate with p53 nor inhibit p53 activities, and the intact p53 protein is generally expressed in MCV-positive MCC tumors. MCV T antigen may yet have unknown mechanisms to counteract p53-mediated tumor suppressor activities.

Protein phosphatase 2A

Protein phosphatase 2A (PP2A) serves as a tumor suppressor by its phosphatase activity. This protein phosphatase contains an active core dimer composed of a catalytic C subunit (PP2A C) and a scaffolding A subunit (PP2A A). The scaffolding subunit PP2A A mediates the formation of heterotrimeric holoenzymes by interacting with regulatory B subunits that determine substrate specificity, localization, and function. Specific PP2A holoenzymes containing B56γ and B56α regulatory subunits control the dephosphorylation of oncoproteins Akt and c-Myc to negatively regulate their activities. The B56 holoenzyme complex also downregulates Wnt/β-catenin signaling by destabilizing the β-catenin protein; however, the molecular mechanism for this still remains unclear.

PP2A was identified to be one of the binding partners of SV40 sT and MPyV MT. To inhibit PP2A activity, SV40 sT binds to PP2A A and PP2A C subunits by displacing the regulatory B subunit. Structural studies have demonstrated that the conserved C-terminal zinc-binding motifs CxxC (Cys-X-X-Cys) and the N-terminal J domain of the sT protein are critical for interaction with the PP2A A subunit (Fig. 1B). The PP2A interaction domain is well-conserved in the sT protein of most polyomaviruses. sT proteins from human polyomaviruses JCV, MCV, HPyV6 and MWPyV have also been shown to interact with PP2A.

Studies on SV40 sT mutants demonstrated that the sT interaction and inhibition of PP2A activity are essential for its transforming activity in human cells. Human cells are generally more resistant to malignant transformation. While the coexpression of SV40 LT with oncogenic H-Ras is sufficient to transform several types of rodent cells, human cell transformation requires additional expression of human telomerase reverse transcriptase (hTERT), oncogenic H-Ras, and SV40 sT. This model has served as a system to study the transformation activities of the SV40 sT antigen. SV40 sT has been shown to displace the aforementioned B56γ and B56α subunits from the PP2A A-C core dimer, leading to the increased phosphorylation of Akt and c-Myc oncoproteins as a result of the phosphatase inhibition. Increased Akt phosphorylation persistently activates downstream Akt-mTOR pathway signaling, while the phosphorylation on c-Myc inhibits its turnover and activates its function. SV40 sT also activates Wnt/β-catenin signaling, consistent with its inhibition of PP2A isoforms containing B56γ and B56α subunits. Activation of these oncogenic pathways is strongly implicated in SV40 sT-induced human cell transformation. The inhibition of Akt, β-catenin, or c-Myc expression by shRNA significantly reduces the transformation-associated soft agar colony formation in SV40 sT-transformed human cells, and the expression of gain-of-function Akt or c-Myc mutants substitutes for SV40 sT in human cell transformation.

The Rapidly Expanding Family of Human Polyomaviruses and Their Potential Roles in Human Malignancy

As of 2017, 14 human polyomaviruses have been identified (Table 1). For nearly 35 years, however, BKV and JCV had been the only known human polyomaviruses until 2006, presumably due to the lack of techniques needed to sensitively detect low copy polyomavirus infection in healthy and diseased tissues. New genetic technologies such as next generation sequencing and rolling circle amplification developed in this century broke this barrier and discovered 12 more human polyomaviruses (Table 1). Because of the expanding number of new polyomaviruses, the International Committee on Taxonomy of Viruses (ICTV) updated the taxonomy of the *polyomaviridae* family in 2016 and divided it into four genera (alpha-, beta-, gamma-, and delta-polyomaviruses) based on the observed phylogenetic distances between large T antigen amino acid sequences (Fig. 2). Human polyomaviruses are a diverse family of small DNA viruses that usually cause asymptomatic, lifelong infections in healthy individuals. This is consistent with relatively high seroprevalence of human polyomaviruses (Table 1). Some of the human polyomavirus infections have been associated with

Table 1 Human polyomaviruses

Name (ICTV designation)	*Abbreviation*	*Year of discovery (reference)*	*Ref. seq. accession*	*Methods of discovery*	*Source of isolation*	*Disease association*	*% seroprevalence*[a]
BK polyomavirus (HPyV1)	BKPyV, BK, BKV	1971 (Gardner et al. *Lancet*)	NC_001538.1	Viral particle identified by EM	Urine	Nephlopathy, hemorrhagic cystits	82 92
JC polyomavirus (HPyV2)	JCPyV, JC, JCV	1971 (Padgett et al. *Lancet*)	NC_001699.1	Viral particle identified by EM	Brain	Progressive multifocal encephalopathy	40–55
Karolinska Institute polyomavirus (HPyV3)	KIPyV or KI	2007 (Allander et al. *J. Virol*)	NC_009238.1	Random PCR	Nasopharyngeal aspirates	Unknown	55 70 90
Washington University polyomavirus (HPyV4)	WUPyV or WU	2007 (Gaynor et al. *PLoS Pathog.*)	NC_009539.1	Shotgun sequencing	Nasopharyngeal aspirates	Bronchitis	70 80 90
Merkel cell polyomavirus (HPyV5)	MCV or MCPyV	2008 (Feng et al. *Science*)	NC_010277.1	NGS	Merkel cell carcinoma	lesion	60 65
Human polyomavirus 6 (HPyV6)	HPyV6	2010 (Schowalter et al. *Cell Host Microbe*)	NC_014406.1	RCA	Normal skin	Pruritic dermatosis	70
Human polyomavirus 7 (HPyV7)	HPyV7	2010 (Schowalter et al. *Cell Host Microbe*)	NC_014407.1	RCA	Normal skin	Pruritic dermatosis	35
Trichodysplasia spinulosa-associated polyomavirus (HPyV8)	TSPyV	2010 (van der Meijden et al. *PLoS Pathog.*)	NC_014361.1	RCA	Trichodysplasia spinulosa, pilomatrix dysplasia	lesion	70–80
Human polyomavirus 9 (HPyV9)	HPyV9	2011 (Scuda et al. *J. Virol*; Sauvage et al. *Emerg. Infect. Dis.*)	NC_015150.1	Degenerate PCR/Shotgun sequencing	Skin, Blood, Urine	Unknown	25–50
Malawi polyomavirus (HPyV10)	MWPyV	2012 (Siebrasse et al. *J. Virol*; Buck et al. *J. Virol.*; Yu et al. *PLoS One*)	NC_018102.1	RCA NGS	Stool, Wart	Unknown	42
St Louis polyomavirus (HPyV11)	STLPyV	2013 (Lim et al. *Virology*)	NC_020106.1	NGS	Stool	Unknown	68–70 93
Human polyomavirus 12 (HPyV12)	HPyV12	2013 (Korup et al. *PLoS One*)	NC_020890.1	Degenerate PCR	Liver	Unknown	17–23 97
New Jersey polyomavirus (HPyV13)	NJPyV	2014 (Mishra et al. *J. Infect. Dis.*)	NC_024118.1	NGS	Muscle biopsy	Unknown	58
Lyon IARC PyV (HPyV14)	LIPyV	2017 (Gheit et al. *Virology*)	KY404016.1	NGS/Degenerate PCR	Normal Skin	Unknown	Unknown

NGS, next generation sequencing; *RCA*, rolling circle amplification.

[a]Each number indicate the results from different groups.

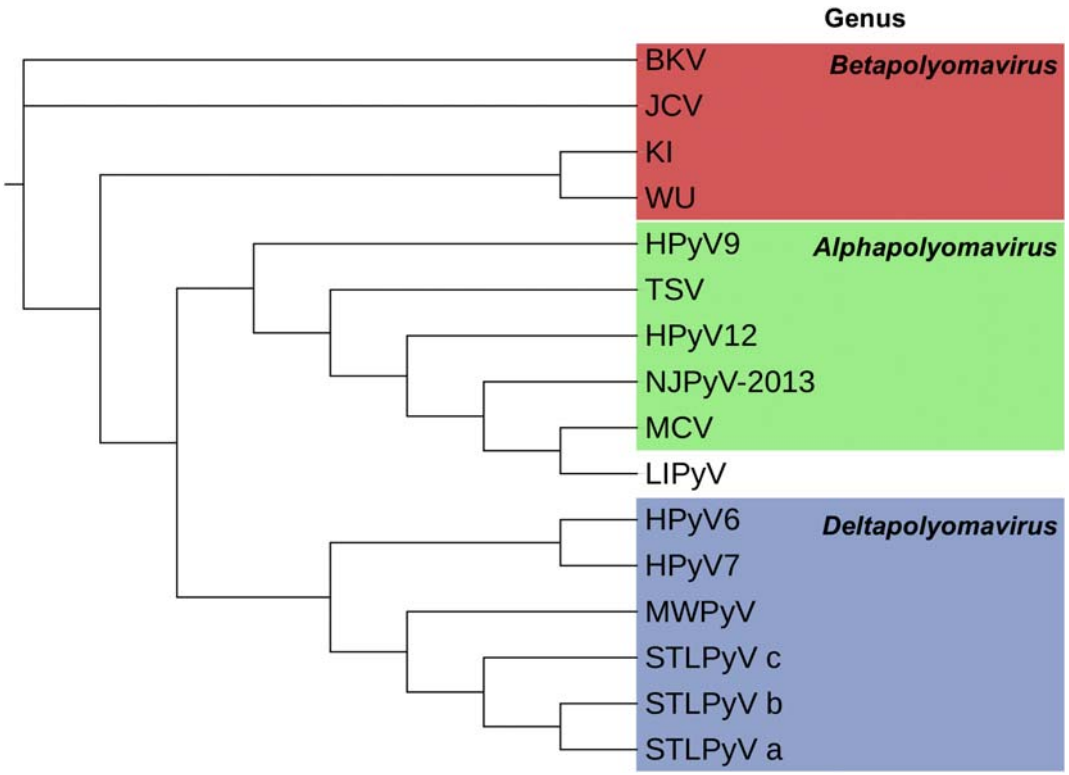

Fig. 2 Bayesian chronogram deduced from the alignment of human polyomavirus 14 large T protein sequences. Due to the reported variations, STLPyV are divided into three substrains.

human disease, including cancers. An important commonality is that all polyomavirus-mediated pathogenesis reported so far is associated with immune suppression. This section focuses on the discovery of human polyomaviruses, disease associations, and their potential roles in human cancer.

BK Polyomavirus (BKV/BKPyV/HPyV1) and JC Polyomavirus (JCV/JCPyV/HPyV2)

In 1971, two independent groups discovered the first human polyomaviruses, BK polyomavirus (BKV or BKPyV) and JC polyomavirus (JCV or JCPyV) in specimens from immunocompromised patients. BKV was identified from the urine of a renal transplant patient and JCV from the brain of a patient with a history of Hodgkin's lymphoma and progressive multifocal leukoencephalopathy. Both diseases show strong association with immune suppression. Subclinical infection with BKV and JCV occurs in early childhood, and these viruses coexist within the human population, as approximately 82%–95% and 39%–54% of adults in United States exhibit BKV and JCV seroreactivity, respectively. Despite their close association with acute diseases in the immunocompromised population, BKV and JCV have been suspected as human tumor viruses because of their strong oncogenic potential. Based on their well-documented transforming potential in experimental models, BKV and JCV are categorized as "possible carcinogens (group 2b)" by the International Agency for Research in Cancer.

BKV in human cancer

BKV is highly tumorigenic in newborn hamsters and mice, inducing several different types of tumors. When injected into hamsters intracerebrally or intravenously, BKV efficiently induces tumors in 82% of the inoculated animals. The most frequent tumors are cerebral ependymomas (72%), followed by pancreatic islet tumors (12%), and osteosarcoma (10%). BKV is frequently integrated into the tumor cell genome and expresses T antigen in tumors. Generation of transgenic mice expressing the BKV early region revealed that BKV T antigen expression induces renal tumors in line with viral tissue tropism. In addition, hepatocellular carcinoma and thymoproliferative disorder (ranging from extreme hyperplasia to thymomas and lymphomas) were also observed in the transgenic mice. BKV T antigen expression also transforms normal cells such as embryonic fibroblasts and cells cultured from the brain and kidneys of hamsters, mice, rats, rabbits, and monkeys consistent with its tumorigenic effect in vivo. In human cells, however, BKV T antigen is insufficient to induce transformation. Although BKV-infected or transfected normal human cells show increased lifespan, they do not display complete transformed phenotypes such as anchorage-independent cell proliferation and tumorigenicity in nude mice. BKV T antigen expression contributes partially to human cell transformation, and additional oncogene expression such as adenovirus E1A is required for full transformation.

Because BKV shows oncogenic tropism for the ependymal tissue, pancreas, and bones in rodents, BKV DNA was initially explored in human ependymomas, other brain tumors, and insulinomas. The search was extended to Kaposi's sarcoma, bone tumors, urinary tract tumors, genital tumors, neuroblastomas, Hodgkin's disease, acute lymphoblastic leukemia, and neuroblastomas. Through southern hybridization and PCR techniques, multiple groups detected BKV DNA in human brain tumors, neuroblastomas, insulinomas, Kaposi's sarcoma, urinary tract tumors, and genital tumors, as summarized in a review article. However, in most studies, BKV DNA in tumors has been detected at a low copy number, less than a single copy per cell, and the results from different groups are not consistent within the same types of tumors.

On the other hand, a rare clonal integration event of BKV DNA is reported. Monini et al. identified two cases of bladder carcinoma, which contain 1–50 copies of viral DNA per cell. Southern blot hybridization combined with two-dimensional gel electrophoresis identified that the first tumor case harbors clonally integrated BKV genome, while the second case contains both integrated BKV and episomal viral genomes. In both cases, the late region was disrupted. This was recently confirmed by two groups. Tang et al. detected predominant expression BKV LT-host fusion transcript in 1 out of 96 bladder carcinoma cases from The Cancer Genome Atlas Research Network (TCGA) transcriptome database. Kenan et al. reported a case of urothelial carcinoma arising in a renal allograft that expresses BKV LT expression. Genomic DNA sequencing of this tumor identified that BKV is clonally inserted into the *MYBPC1* gene with a breakpoint in the VP1 gene. Recently, Kenan et al. also identified a high-grade renal cell carcinoma that harbors clonally integrated BKV in the *BRE* gene from chromosome 2. Similar to the first case, viral breakpoints were found at the integration site, disrupting the late gene encoding VP1 and VP2/3. Despite its rare occurrence, BKV is clonally integrated in bladder carcinoma, urothelial carcinoma, and renal cell carcinoma, losing the viral replication capability due to clonal integration and late gene ablation. These BKV-associated tumors are terminal hosts for BKV infection, reflecting the hallmarks of polyomavirus-induced cancers established in the animal models. These results strongly indicate that BKV plays etiological roles in rare cases of bladder, urothelial, and renal cell carcinomas.

JCV in human cancer

Similar to BKV, JCV also induces tumors in newborn hamsters, particularly in the tissues of neural origin, including medulloblastomas, undifferentiated neuroectodermal tumors, glioblastomas, pineocystomas, neuroblastomas, and meningiomas. Productive infection of JCV has not been observed in JCV-induced tumors. However, the tumors retain T antigen gene expression. Furthermore, in owl and squirrel monkeys, astrocytoma, glioblastoma and neuroblastoma are also developed after intracerebral JCV inoculation. In accordance with the observations in hamster models, neither the expression of VP1 protein nor viral replication is detected in tumor tissues, which only express the JCV T antigen proteins. These results are consistent with the fact that the JCV-induced tumor cells are nonpermissive for JCV replication. JCV transgenic mice, generated to express JCV early T antigen by its own promoter, also develop neural tumors including adrenal neuroblastoma, pituitary adenoma, malignant peripheral nerve sheath tumor, and medulloblastoma. Above all, JCV-infected animals or mice expressing JCV T antigen are predisposed to develop tumors of neural origin. JCV-mediated transformation can also be seen in cell culture. JCV has the ability to infect and transform primary cells of neural origin, including human cells such as fetal glial cells and vacuolar endothelial cells as well as rodent cells such as baby hamster kidney cells and rat fibroblasts.

Because JCV is a neurotropic virus and induces tumors of neural origin in these animal models, viral DNA and T antigen protein expression were intensively searched for in various central nervous system tumors by PCR-based techniques and T antigen immunohistochemistry. After JCV was identified as the etiologic agent of progressive multifocal leukoencephalopathy (PML), investigations were focused on CNS tumors that concomitantly developed in PML patients. The studies covered a wide variety of CNS tumors: gangliocytomas, choroid plexus papillomas, pilocytic astrocytomas, subependymomas, pleomorphic xanthoastrocytomas, oligodendrogliomas, all subtypes of astrocytomas, ependymomas, oligoastrocytomas, glioblastomas, medulloblastomas, pineoblastomas, gliosarcomas, and primitive neuroectodermal tumors, as summarized in the recent review article. Despite the accumulating evidence of an association between JCV infection and CNS tumors, there is a lack of consistency between studies. Several reports also demonstrated that JCV DNA or even a fragment of JCV is present without gene expression in brains from healthy immunocompetent individuals, with neither PML nor CNS tumors. The nature of ubiquitous, latent JCV infection also makes it difficult to affirm whether JCV is the etiological factor or a simple bystander in human CNS tumorigenesis. Until now, no clear case that displays clonal JCV integration into the tumor cell genome, like BKV integration in bladder tumors, has been identified in human malignancies. Taken together, a causative role of JCV in human cancers is still to be evidenced while, based on its strong tumorigenicity in animal models and the presence of JCV in multiple human tissues, it is reasonable to hypothesize that JCV could play a role as a possible cofactor during human tumorigenesis.

Karolinska Institute Polyomavirus (KI/KIPyV/HPyV3) and Washington University Polyomavirus (WU/WUPyV/HPyV4)

In 2007, groups from Karolinska Institute (KI) and from Washington University (WU) independently discovered KI polyomavirus and WU polyomavirus from human respiratory tract samples by exploiting random PCR and by shotgun sequencing, respectively. Phylogenetic analysis in the LT sequence revealed that these two viruses coincidentally fall into the same clade, belonging to *betapolyomavirus* (**Fig. 2**). High seroprevalence suggest that KI and WU are common human infections (**Table 1**). Since their initial discovery, both of these viruses have been detected in respiratory specimens worldwide. One interesting feature of LI and WU infections is their high rate of coinfection with other respiratory viruses. A coinfection rate of 74% has been detected for KI and rates ranging from 68% to 79% for WU. While the clinical significance of WU and KI infection remains unclear, clinical presentation

in individuals with specimens positive for WU or KI appears to be similar to that of other respiratory viruses, including fever, cough, and other upper and lower respiratory symptoms. To find their involvement in human cancer, presence of KI and WU has been investigated in multiple malignant tissues, including tumors of the CNS including neuroblastoma, non-UV-associated melanoma, kidney/urinary bladder tumors, and various types of lung cancers by PCR-based methodology and serological analysis to detect KI and WU infection. However, none of the studies shows direct involvement of KI and WU in human cancers.

Merkel Cell Polyomavirus (MCV/MCPyV/HPyV5)

In 2008, a group at the University of Pittsburgh discovered a novel member of the human polyomavirus family, named Merkel cell polyomavirus (MCV or MCPyV) by next generation sequencing in the genome of Merkel cell carcinoma (MCC), a rare skin cancer associated with immunosuppression. Their initial report and several studies from different geographic regions confirmed that ~80% of MCCs are associated with MCV, while ~20% are virus-negative MCCs linked to an ultraviolet (UV)-damage pathway associated with a high mutation rate with a signature of UV mutagenesis and inactivation of RB1 and p53. As seen in animal polyomavirus-induced rodent tumors, MCV is clonally integrated into the tumor genome and loses replication capacity in tumor tissues as a result of a tumor-specific mutation that ablates C-terminal viral helicase activity. In addition, the expression of late gene has never been detected in MCC tumors and virus-positive MCC cell lines, consistent with tumor cells being a terminal host for MCV infection. Despite being a rare cancer, MCC is associated with the virus that naturally exists as a component of the human skin microbiome. Showalter et al. demonstrated that MCV virions are chronically shed from the surface of healthy human skin. Seroepidemiological analysis using the seroreactivity to the MCV VP1 protein as a marker of past MCV infection indicates that seropositivity for MCV is 60%–65% (**Table 1**). These results are consistent with MCV being a virus commonly present in the human population. MCV has also been investigated in other cancers to identify secondary MCV-associated cancers. Non-small cell lung cancer (NSCLC) is one of cancers in which MCV DNA is frequently detected by several groups, while contradictory results are also reported. Hashida et al. reported MCV genome integration into the host genome and the LT truncation mutation in several NSCLC cases as seen with MCC. These results suggest the possibility of MCV contribution to a subset of NSCLC tumors. However, a more detailed characterization of MCV-positive tumors is necessary, such as Southern blotting for clonality and histopathological characterization with reliable biomarkers for NSCLC.

While evidence in humans and animal models is still limited, based on the strong mechanistic evidence in humans, IARC classified MCV as "probable carcinogen to human (group 2A)" in 2012. The details of MCV-mediated MCC carcinogenesis will be described later in a following section.

Human Polyomavirus 6 and Human Polyomavirus 7 (HPyV6 and HPyV7)

Rolling cycle amplification (RCA) is a robust molecular technique for new virus discovery that does not require prior viral genetic information. If a sample contains small circular DNA viruses, such as polyomaviruses and papillomaviruses, the bacteriophage phi29 DNA polymerase and random primer efficiently amplify long, concatenated linear DNA, exploiting its unique 5′-to-3′ strand-displacement properties and 3′-to-5′ proofreading exonuclease activity. Cleavage of the amplified DNA with a single-cut restriction enzyme results in the generation of a single genome unit of the circular DNA. A group from National Cancer Institute applied RCA to identify circular viruses in skin swabs from healthy adults and discovered two polyomaviruses human polyomavirus 6 (HPyV6) and human polyomavirus 7 (HPyV7), along with MCV. ICTV classified these viruses as *deltapolyomavirus*. Phylogenic study classified these viruses into the same clade (**Fig. 2**). The same study also demonstrated that MCV, HPyV6, and HPyV7 are shed from healthy, normal skin and suggested that these viruses are a part of the normal human virome in the skin. Recently, however, two groups demonstrated that HPyV6 and HPyV7 are closely associated with pruritic dermatoses in immunosuppressed individuals. Selective B-RAF inhibitor therapy is associated with the development of malignant and benign cell growths. Schrama et al. has reported that HPyV6 is frequently present in the proliferative epithelial cells from the skin of the cancer patients treated with mutation-specific B-RAF inhibitor. HPyV6 may play a role in inducing pathological skin proliferations in combination with specific chemotherapeutic drugs.

Trichodysplasia Spinulosa Polyomavirus (TSV/TSPyV/HPyV8)

A polyomavirus from trichodysplasia spinulosa (TS) was also discovered by RCA, and the virus was named trichodysplasia spinulosa-associated polyomavirus (TSV or TSPyV). TS is a rare skin disease characterized by the development of follicular papules and keratin spines, which are strongly associated with immune suppression. Since 1999, viral etiology has been suspected since affected hair follicle cells contain icosahedral virus-like particles. TS is likely to be associated with the lytic TSV replication, because affected lesions express both viral VP1 capsid protein and T antigen proteins. While the TSPyV T antigen gene is characterized and known to express five different T antigen isoforms in the skin of TS patients, the role of T antigen in cell transformation remains unknown. Because of its skin tropism, TSV was searched for in uncommon skin cancers, including mycosis fungoides, Sezary syndrome, primary cutaneous T-cell lymphoma, primary cutaneous B-cell lymphoma, and non-UV-associated mucosal melanoma, as well as benign skin cancers including spitz nevi and keratoacanthoma. One study also examined benign nonmalignant tonsillar tissues and tonsillar carcinoma for TSV detection. By and large, the results from these studies do not support an etiological role of TSV in the examined human cancers.

Human Polyomavirus 9 (HPyV9)

Serological studies have suggested the presence of cross-reactive antibodies in human sera that recognize the viral capsid protein VP1 of the African green monkey polyomavirus, also known as monkey B-lymphotropic papovavirus or LPV. LPV by itself could explain the reactivity because the LPV DNA sequence could be detected in peripheral blood mononuclear cells (PBMCs) from the human immunodeficiency virus-positive (HIV+) patients with different leukoencephalopathies. On the other hand, in 2011, Scuda et al. identified human polyomavirus 9 (HPyV9) from the sera and urine of kidney transplant patients under immunosuppressive treatment. HPyV9 is a novel polyomavirus that resembles LPV, showing 76% of pairwise nucleic acid identity. In the same year, by using next generation sequencing, Sauvage et al. also identified HPyV9 from the skin surface of a MCC patient together with MCPyV, HPyV6, and HPyV7. While discovered from the skin surface of the MCC patient, HPyV9 was rarely detectable in skin swab specimens from healthy individuals or non-MCC patients, suggesting that HPyV9 is not a component of normal skin virome unlike HPyV6, HPyV7, and MCV. On the other hand, HPyV9 viremia was reported in sera from kidney transplant patients, consistent with its discovery from a kidney transplant patient. In this report, 21% of kidney transplant patients showed increased HPyV9 viral DNA load and seroprevalence after transplantation, suggesting that HPyV9 infection is more common in kidney transplant patients. However, the question of the nature of HPyV9 infection and its pathogenic potential remains unanswered.

Malawi Polyomavirus (MWPyV/HPyV10/MXPyV)

In 2012 Siebrasse et al. identified a new polyomavirus in the stool from a healthy 15-month-old child from Malawi and named it Malawi polyomavirus (MWPyV). To discover a new virus in feces, they used virus-like particles (VLP) recovered from a fecal sample, amplified the circular VLP-derived DNA by RCA, and applied it to next generation shotgun sequencing. A nearly identical virus was actually reported by two additional groups in the same year. Buck et al. discovered a polyomavirus, termed human polyomavirus 10 (HPyV10) by RCA from anal warts derived from a rare genetic disorder called warts, hypogammaglobulinemia, infection, and myelokathexis (WHIM) syndrome. This syndrome is a congenital immunodeficiency disease in which HPV infections cannot be controlled. Thus, WHIM patients may not be able to control human polyomavirus as well as HPV infections. In addition, a third group from Mexico also identified the new polyomavirus from stool samples of a Mexican child presenting with diarrhea and named it Mexico PyV (MXPyV). An analysis on nucleotide sequences of MWPyV, HPyV10, and MXPyV demonstrated 95%–99% nucleotide sequence similarities, indicating that these viruses are variants of the same virus. In 2015, ICTV classified these viruses as *deltapolyomavirus* (Fig. 2). While the presence of MWPyV DNA was searched for in several human cancers including mucosal melanoma, lung, and tonsillar cancers, no significant correlation has been observed. The oncogenic activity of MWPyV T antigen has been also studied in vitro. While MWPyV T antigens interact with known human tumor suppressors such as p53 and pRb proteins, its effect on cell proliferation was minimal due to the faster turnover of MWPyV LT than that of SV40 or MCV LT, suggesting that MWPyV T antigen has lower transforming potential.

STL Polyomavirus (STLPyV/HPyV11)

In 2013, a group from Washington University further identified a new 11th polyomavirus from the stool of a healthy 15-month child in Malawi and named it STL polyomavirus (STLPyV). STLPyV was also detected in clinical stool specimens from the United States and The Gambia at ~1% frequency. STLPyV shares ~64.2% nucleotide sequence similarity to MWPyV, appears to share an ancestral recombination origin with MWPyV, and is classified as *deltapolyomavirus* (Fig. 2B). The comparison of two full-length virus isolates demonstrates significant divergence (~5%) in their nucleotide sequence. The prevalence of STLPyV infection was tested on samples collected in US cities by measuring seroreactivity to STLPyV VP1 capsid protein. So far, no human diseases including cancers have been convincingly linked to STLPyV infection, although its DNA has been detected in both cancerous and noncancerous tonsillar and lung tissues.

Human Polyomavirus 12 (HPyV12)

The 12th human polyomavirus, HPyV12, was discovered in 2013 from liver specimens by degenerate PCR targeting the VP3 gene. In the initial study, while polyomaviruses were searched for in various gastrointestinal samples including liver, gall bladder, esophagus, stomach, colon, rectum as well as spleen and lymph node samples, HPyV12 was found only in 4 out of 124 liver specimens, but not in other samples. These results suggest HPyV12 is a liver tropic polyomavirus, although the presence of this virus has not been investigated in non-gasterointestinal samples. Viral sequence analysis revealed that HPyV12 belongs to a group of primate polyomavirus or genus *Alphapolyomavirus* in the updated ICTV taxonomy in 2015 (Fig. 2). Seroprevalence of HPyV12 was also examined by the VP1 capsomer-based ELISA assay, and the prevalence was determined at 23% and 17% in sera from healthy adults and a pediatric group of children, respectively. This data indicates that HPyV12 naturally infects humans and that the primary infection of HPyV12 occurs during childhood.

As of 2017, the role of HPyV12 infection in human cancers and diseases has not yet been elucidated. HPyV12, however, is the first human polyomavirus that lacks the Rb-binding LxCxE motif in the LT protein. Since Rb-targeting is a critical step for polyomavirus-induced transformation, HPyV12 is likely to exhibit a weaker transforming potential.

New Jersey Polyomavirus (NJPyV-2013/HPyV13)

In 2014, a novel polyomavirus was identified from the muscle tissue of the pancreatic transplant patient in New Jersey who developed weakness, retinal blindness, and necrotic plaques on face, scalp, and hands. The new polyomavirus, named New Jersey polyomavirus (NJPyV-2013), was identified by next generation sequencing, and shares 80.7% overall nucleotide homology to chimpanzee polyomaviruses. In 2015, ICTV classified NJPyV-2013 as a member of the genus *Alphapolyomavirus* (Fig. 2). In situ hybridization studies demonstrated that NJPyV-2013 is present in endothelial cells located in endomysium and in the superficial dermis. The high copy viral infections in both muscle and skin lesions were also confirmed by qPCR analysis. Thus, an initial study revealed that NJPyV-2013 is the human polyomavirus that infects endothelial cells in muscle and skin, and its viral replication is closely associated with immunosuppression. However, the recently found NJPyV-2013 requires further investigation, and whether or not NJPyV-2013 plays a role in malignant transformation and human cancers remains unclear.

Lyon IARC PyV (LIPyV/HPyV14)

Lyon IARC PyV (LIPyV) is the newest human polyomavirus discovered by a group at the International Agency for Research on Cancer (IARC), World Health Organization at Lyon. A DNA fragment of LIPyV was discovered by next generation sequencing performed on crude PCR products amplified from cutaneous skin swabs by degenerate primers targeting the conserved LT antigen sequence. Analysis of the complete genome sequence revealed the typical 5269 bp polyomavirus genome that encodes early LT, sT, and ALTO open reading frames (ORFs) and late VP1, VP2, and VP3 ORFs. While not yet classified by ICTV, LIPyV falls into the same clade as MCV according the large T protein sequence similarities among 14 human polyomaviruses (Fig. 2). Extended search for LIPyV using skin swabs, eyebrow hair samples, and oral gargles demonstrated that approximately 2% of skin and oral gargles are positive for LIPyV DNA. Nonetheless, no information is available to predict the role of LIPyV in human cancer. However, an analysis of viral sequences demonstrated that most of the previously defined transforming domains, including the p53 binding motif, LFCDE Rb-binding motif, and J domain on LT as well as the PP2A binding motif on sT, remain intact in LIPyV T antigen. Seroprevalence has not been determined yet, but an evaluation of a multiplex immunoassay to measure serum IgG reactivity to 13 polyomavirus VP1 proteins suggests that seroreactivity to LIPyV is low. Further molecular epidemiological studies and cell biological evaluation of LIPyV T antigen oncogenic activities may reveal its potential involvement in human cancers.

Merkel Cell Polyomavirus-Mediated Merkel Cell Carcinogenesis

Cells Targeted by MCV for MCC Genesis

The discovery of MCV revealed that ~80% of MCC is linked to MCV. MCC, a small neuroendocrine skin cancer described by Toker in 1972, is an uncommon skin cancer associated with immunosuppression. Despite the similarities between MCC and Merkel cells, MCV infection in normal Merkel cells in the skin has not been evidenced. Recent reports suggest that MCV preferentially infects dermal fibroblasts in human skin. In addition, cells of origin for MCC are still debated. Several groups suggest that MCCs do not arise from Merkel cells, but instead arise from epidermal stem cells, dermal stem cells, or pre-/pro-B cells. Although MCV is a common virus present in the human skin virome, the origin of MCC tumor cells that were transformed by MCV as a result of viral clonal integration has not yet been identified.

MCC Tumor Cells Are the Dead-End Host for MCV Infection

Among the 14 known human polyomavirus, MCV is the only human polyomavirus clearly linked to human cancer. Similar to the animal polyomavirus-induced tumors described in the previous section, approximately ~80% of MCC tumors harbor clonally integrated MCV and express the early T antigen gene. In contrast, expression of the late gene has never been detected in MCC tumors and MCV-positive cell lines, consistent with MCC tumors being nonpermissive to MCV infection. Viral sequence analysis revealed that tumor-derived viral LT antigens always harbor mutations that prematurely truncate the C-terminal helicase domain of LT, while the N-terminal tumor suppressor targeting Rb-binding and Hsc70-binding J domains remain intact. The tumor-specific viral mutations render MCV replication-defective in MCC tumors, ablating helicase function of LT. This is consistent with the hallmarks of polyomavirus-induced cancers.

MCC Is Addicted to Viral T Antigen Expression

The integrated MCV early gene expresses truncated LT and sT antigens in MCC tumors. The expression of these antigens is essential for MCC tumors. Several studies have shown that the knockdown of viral T antigen transcripts in MCV-positive MCC cancer cell lines ablates cell proliferation, indicating that MCC tumors require viral T antigen oncogene expression. Targeting of individual T antigen isoforms further demonstrated that both MCV LT and sT play important roles in promoting MCC cell proliferation whereas one group suggests that LT targeting of pRb is solely responsible for promoting MCC cell proliferation.

Mechanism of MCV-Induced Carcinogenesis

Similar to other polyomaviruses, the MCV T antigen locus encodes two major overlapping transcripts for the large T (LT) and small T (sT) antigen oncoproteins. In contrast to other oncogenic polyomaviruses, MCV sT is highly oncogenic, and the expression of sT alone is sufficient to transform immortalized rodent fibroblast cell lines such as NIH3T3 and Rat-1, indicating that MCV sT possesses unique oncogenic properties. Surprisingly, MCV sT-mediated rodent cell transformation is not mediated by its PP2A-binding activity unlike SV40 sT. This has been supported by a report showing that the displacement of the PP2A B regulatory subunit by MCV sT is more restricted than that of SV40 sT. This suggests that PP2A inhibition of MCV sT is not as potent as that of SV40 sT. If PP2A inhibition is not the major oncogenic mechanism, what is the host signaling pathway involved in MCV sT transformation? As aforementioned, SV40 sT activates the Akt-mTOR pathway by preventing PP2A dephosphorylation of Akt, and this activity is essential for human cell transformation. MCV sT, however, has a minimal effect at this node of the Akt-mTOR signaling pathway and instead, acts downstream of mTOR to increase levels of hyper-phosphorylated 4E-BP1, a negative regulator of cap-dependent mRNA translation. Activation of cap-dependent translation by eIF4E plays important roles in tumorigenesis, and 4E-BP1 is a tumor suppressor that negatively regulates eIF4E. Phosphorylation of 4E-BP1 by upstream Akt-mTOR signaling disables its tumor suppressor function, leading to active cap-dependent translation, cell proliferation, and tumorigenesis. Expression of a constitutively active, phosphorylation-defective 4E-BP1 mutant prevents sT-mediated rodent cell transformation, and thus, 4E-BP1 phosphorylation is critical for MCV sT transformation.

As discussed in the section "**The Molecular Mechanism of Polyomavirus Transformation**", polyomavirus T antigens promote cell transformation by directly interacting with host tumor suppressor proteins. MCV sT also binds to host proteins including PP2A, Fbw7, Cdc20, PP4C, NF-κB essential modulator (NEMO), and L-Myc. A structural prediction suggests that the MCV sT protein possesses two molecular surfaces that allow the interaction of sT with host cell molecules. One molecular surface binds to PP2A and shares significant structural similarity to that of SV40 sT. In contrast, the second surface is quite unique to MCV and contains a disordered loop, which is predicted to bind host proteins. The second surface of MCV sT contains a unique functional domain that stabilizes LT and enhances MCV replication. The large T stabilization domain (LSD) is also responsible for cell transformation; mutation of this domain not only ablates sT-mediated LT stabilization but also transformation. This LSD domain interacts with Fbw7 and inhibits the SCF^{Fbw7} E3 ubiquitin ligase that targets MCV LT for proteasomal degradation. Actually, SCF^{Fbw7} is known as a tumor suppressor E3 ligase that degrades multiple oncogenes, such as c-Myc, c-Jun, cyclin E, and mTOR proteins. Interestingly, MCV sT binds to multiple ring finger ubiquitin ligases, including the anaphase promoting complex/Cdc20 (APC/C^{Cdc20}), APC/C^{cdh1}, and β-TrCP. The promiscuous targeting of these E3 ligases has been shown to induce mitotic 4E-BP1 phosphorylation and chromosomal instability. Thus, MCV sT activates oncogenic pathways and promotes transformation primarily by hijacking host oncoprotein degradation machinery. A recent study further revealed that the second surface of MCV sT molecule also binds to L-Myc, Max, and EP400 histone acetyltransferase complexes. L-Myc is one of the Myc isoforms predominantly expressed in MCV-positive MCC, and the EP400 histone acetyltransferase complex is involved in multiple biological events including transcription, stem cell maintenance, and DNA damage response. Cheng et al. have shown that MCV sT recruits L-Myc/Max to the EP400 complex to activate specific gene expression, promote cellular transformation, and contribute to its oncogenic potential.

Although MCV LT alone does not show strong transforming activity in a rodent fibroblast transformation model, MCV LT is a strong growth promoter, and the conserved N-terminal J domain and Rb targeting domain mostly mediate the oncogenic property of MCV LT. In MCV-positive MCC cells, MCV LT maintains expression of the genes downstream of the E2F transcription factor by inhibiting the function of pRb protein. While SV40 LT inhibits p53 by the C-terminal p53-binding domain, truncated MCV LT proteins expressed from MCC lose the corresponding p53 binding domain and do not interact with p53. Instead, MCV LT uniquely binds to the host vesicle trafficking factor Vamp6p, and this interaction has been shown to inhibit MCV virus production. The functional significance on transformation, however, has been unclear.

Some studies suggest that host molecules targeted by MCV LT could be druggable targets for MCC. One of them is Survivin (BIRC5), an E2F downstream factor activated by LT. The inhibition of Survivin by shRNA and its transcriptional inhibitor YM155 has been shown to inhibit MCV-positive MCC cell proliferation in mouse xenografts. Furthermore, the Hsp70 ATPase inhibitor MAL3-101, which is required for SV40 LT-mediated Rb-E2F disassembly, efficiently prevents xenograft MCC growth and induces apoptosis. Now that MCV has been shown to be a causal agent of most MCCs, rational targeting of cellular pathways perturbed by MCV may lead to the identification of additional treatments that may be more effective than current therapies.

Conclusions and Outlook

By virtue of the new genetic techniques for sensitive virus detection, 12 new human polyomaviruses were identified during the last 13 years. In particular, the discovery of MCV and BKV clonal integrations in human Merkel cell carcinoma and urological cancers have renewed the interest in the roles of human polyomaviruses in human cancer. The available information for cancer association with human polyomaviruses is predominantly from old JCV and BKV studies, while the knowledge on the recently discovered human polyomaviruses and their association with human cancer remains limited.

The fast pace of new polyomavirus discoveries suggest that a plethora of undiscovered human polyomavirus exist. It would not be surprising if these previously unknown human polyomaviruses play roles in other human cancers.

See also: Cervical Cancer: Screening, Vaccination, and Preventive Strategies. Papillomaviruses.

Further Reading

Chen, H.Z., Tsai, S.Y., Leone, G., 2009. Emerging roles of E2Fs in cancer: An exit from cell cycle control. Nature Reviews. Cancer 9 (11), 785–797.
Damania, B., Pipas, J.M., 2009. DNA tumor viruses. Springer Science+Buisiness Media, New York.
DeCaprio, J.A., 2017. Merkel cell polyomavirus and Merkel cell carcinoma. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 372 (1732).
DeCaprio, J.A., Garcea, R.L., 2013. A cornucopia of human polyomaviruses. Nature Reviews. Microbiology 11 (4), 264–276.
Delbue, S., et al., 2008. Presence and expression of JCV early gene large T antigen in the brains of immunocompromised and immunocompetent individuals. Journal of Medical Virology 80 (12), 2147–2152.
Feng, H., Shuda, M., Chang, Y., Moore, P.S., 2008. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319 (5866), 1096–1100.
Ho, J., et al., 2015. Human polyomavirus 7-associated pruritic rash and viremia in transplant recipients. The Journal of Infectious Diseases 211 (10), 1560–1565.
Kenan, D.J., Mieczkowski, P.A., Burger-Calderon, R., Singh, H.K., Nickeleit, V., 2015. The oncogenic potential of BK-polyomavirus is linked to viral integration into the human genome. The Journal of Pathology 237 (3), 379–389.
Knipe, D.M., Howley, P.M., 2013. Fields virology, 6th Ed. Wolters Kluwer/Lippincott Williams & Wilkins Health, Philadelphia.
Levine, A.J., Oren, M., 2009. The first 30 years of p53: Growing ever more complex. Nature Reviews. Cancer 9 (10), 749–758.
Moore, P.S., Chang, Y., 2010. Why do viruses cause cancer? Highlights of the first century of human tumour virology. Nature Reviews. Cancer 10 (12), 878–889.
Nguyen, K.D., et al., 2017. Human polyomavirus 6 and 7 are associated with pruritic and dyskeratotic dermatoses. Journal of the American Academy of Dermatology 76 (5), 932–940.e3.
Pipas, J.M., 2009. SV40: Cell transformation and tumorigenesis. Virology 384 (2), 294–303.
Sadasivam, S., DeCaprio, J.A., 2013. The DREAM complex: Master coordinator of cell cycle-dependent gene expression. Nature Reviews. Cancer 13 (8), 585–595.
Tognon, M., Corallini, A., Martini, F., Negrini, M., Barbanti-Brodano, G., 2003. Oncogenic transformation by BK virus and association with human tumors. Oncogene 22 (33), 5192–5200.

Relevant Website

https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsdna-viruses-2011/w/dsdna_viruses/129/polyomaviridae—International Committee on Taxonomy of Viruses Polyomaviridae Taxonomy: 2017 release.

Prevention and Control: Nutrition, Obesity, and Metabolism

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Glossary

Adipocytes Fat cells that compose adipose tissue, or body fat mass.

Adipose tissue The body's fat-storing tissue, made up of adipocytes that have further paracrine roles for example, through adipokines and steroid hormone synthesis; generally measured as a percentage (%) of total body mass

Alpha-linolenic acid (ALA) An *n*-3 polyunsaturated fatty acid (18:3); essential fatty acid; precursor to *n*-3 very long-chain polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA); generally measured in milligrams (mg) or grams (g).

Arachidonic acid (ARA) An *n*-6 very long-chain polyunsaturated fatty acid (20:4); synthesized in the body from dietary linoleic acid (18:2); and is a precursor in the biosynthesis of eicosanoids, including prostaglandins, thromboxanes, and leukotrienes that have major roles as tissue hormones, that is, on inflammation and immune function; generally measured in grams (g).

Body fat The total mass of fat/adipose tissue in the body; generally measured as a percentage (%) of total body weight.

Body mass index (BMI) A commonly used calculated measure of height-proportionate weight, reported as kg m^{-2}. A healthy range is 18.5–25 kg m^{-2}, while 30 kg m^{-2}, and above represents obesity.

Cancer mortality shift The epidemiological turning point whereby cancer mortality surpassed that from heart disease.

Complex carbohydrate Polymeric carbohydrate molecule composed of long chains of monosaccharide units bound together by a variety of glycosidic linkages, and include starch, glycogen, and soluble and insoluble fiber; generally measured in grams (g).

Dietary energy density The amount of energy (or calories) per gram of food.

Eicosapentaenoic acid ((EPA) 20:5 *n*-3) and docosahexaenoic acid ((DHA) 22:6 *n*-3) *n*-3 very long-chain polyunsaturated fatty acids; mostly attained from marine sources; slightly synthesized in the body, especially during pregnancy and breast feeding, from dietary alpha-linoleic acid (18:3)—DHA can also be synthesized from dietary EPA—and are precursors in the biosynthesis of *n*-3 eicosanoids, competing with *n*-6 very-long polyunsaturated fatty acids for same desaturase enzyme and having opposing effects, including with antiinflammation, anticoagulation, and anticarcinogenesis; generally measured in milligrams (mg).

Gender gap The difference between men's and women's parameters, for example, life expectancy and mortality.

Gender-specific nutrition The dichotomy between men's and women's requirements for nutrients and dietary composition, physiological responses, and associated socio-cultural factors.

High-density lipoprotein (HDL) cholesterol Cholesterol carried within small lipoprotein particles distinguished by a high proportion of proteins to lipids; generally measured in mmol L or mg dL

Linoleic Acid (LA) An *n*-6 polyunsaturated essential fatty acid (18:2), a source of very long *n*-6 polyunsaturated fatty acids, attained from fatty plants (nuts, seeds) and animals fed high *n*-6 feed; essential for immune, inflammation, blood coagulation and skin health; generally measured in grams (g).

Lipoprotein Complex lipid-protein particles transporting fat molecule micelles in the blood/extracellular fluid; include high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL); generally measured in mmol L or mg dL.

Long-chain polyunsaturated fatty acid (LCPUFA) A polyunsaturated fatty acid with 20 or more carbons; generally measured in grams (g) or milligrams (mg).

Low-density lipoprotein (LDL) cholesterol Cholesterol carried within large lipoprotein particles distinguished by a low proportion of proteins to lipids; generally measured in mmol L or mg dL

Monounsaturated fatty acid (MUFA) A fatty acid with one double bond between carbon atoms at a specific location in the molecule, with the rest of the carbon atoms being linked with single bonds; generally measured in grams (g) or milligrams (mg).

Obeso-carcinogenic Tending to cause obesity-related cancer.

Obesogenic Tending to cause obesity, for example, nutritional and environmental factors.

Polyunsaturated fatty acids (PUFA) A fatty acid with double bonds between carbon atoms in multiple locations in the molecule, with the rest of the carbon atoms being linked with single bonds; generally measured in grams (g) or milligrams (mg).

Saturated fatty acid (SFA) A fatty acid with single bonds between all carbon atoms in the molecule; generally measured in grams (g).

Simple carbohydrate The most basic units of carbohydrates; in the context of diet, this can refer to monosaccharides or disaccharides, and are usually present as purified/refined sugars with little-to-no fiber; generally measured in grams (g).

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Trans-fat A type of fat produced either naturally by biological process, for example, in cow rumen, or industrially from unsaturated vegetable fats through hydrogenation or heating; generally measured in grams (g).
Waist circumference The girth measured at a level midway between the lowest rib and the iliac crest, generally measured in centimeters (cm) or inches (in); it is a principle criterion of the metabolic syndrome.

Introduction

"Cancer Mortality Shift" Over Heart Disease Risk

This term was coined in Israel in 1999 to describe the epidemiological turning point, led by women, whereby cancer mortality surpassed that from heart disease. This trend became global, with risk of death declining more steeply for heart disease than cancer. If current trends continue, cancer will become the leading cause of death by 2020.

This shift is especially prominent among females, given their innate tendency toward fat accumulation, especially in obesogenic environments and due to nutrient-exhausting menstruation, pregnancy and lactation, and resultant deficiencies. Gender-differential effects of the obesogenic environment on women can also explain the recent decline in the gender gap in life expectancy (LE) and in healthy LE (HLE), gradually narrowing the gender gap.

According to the American Cancer Society (ACS), the rising global cancer epidemic causes more than 8 million cancer deaths worldwide annually; in 2012 there were 6.1 million new cancer cases in developed countries compared to 8 million new cancer cases in developing countries and predicted to rise to 13.1 million by 2030. Many of these occurrences are considered avoidable through lifestyle modifications.

It was predicted that from 1969 through 2020, the number of heart disease deaths would decrease 21.3% and 13.4% among men and women while cancer deaths would increase 91.1% and 101.1% respectively, suggesting that cancer would become the leading cause of death.

The Global Obesity Epidemic

Worldwide obesity has nearly tripled since 1975. The World Health Organization (WHO) reported that In 2016, 39% of adults aged 18 years and older were overweight (BMI ≥ 25 kg m^{-2}), translating to 1.9 billion in absolute numbers, with approximately 13% categorized as obese (BMI ≥ 30), over 650 million; among children under the age of 5, 41 million were overweight or obese, and for older children and adolescents aged 5–19, the number was over 340 million.

The obesity epidemic has become the leading lifelong risk factor for disability and mortality, and a high level of obesity is contributing to reduced LE. It is increasingly recognized as a potentially modifiable factor associated with poor prognosis in several cancers, such as those of the breast, colon, and endometrium.

Obeso-Carcinogenic Environmental Pressure

Conditions of early human evolution included food scarcity, which drove the need for efficient transformation of available nutrition to daily living activities, metabolic energy, tissue-building, reproduction, building energy reserve and health of ensuing generations. However, the enabling physiology that was once advantageous may now be a liability in the modern obesogenic environment that is increasingly shown to be carcinogenic.

The increase in dietary energy intake over the past several decades is due to increased availability/accessibility, portion size, dietary energy density (DED) and eating opportunities—with reduced nutrient density in processed foods and associated metabolic implications—linking obesity to cancer.

The "obeso-carcinogenic" diet, most common in modernized cultures, is characterized by excessive energy intake, high intake of saturated fatty acids (SFA) and *n*-6 polyunsaturated fatty acids (PUFA), reduced *n*-3 PUFA and fiber intake, overuse of salt and refined sugar, and inadequate exposure to beneficial microorganisms, a combination believed to contribute to obesity, as well as to the type of immune dysfunction that enable cancer development and progression.

Obesity and Cancer Relationships

One-fourth of all cancer cases related to excess BMI in 2012 could be attributed to the rising BMI since 1982, especially in women. Overweight and obesity are clearly associated with increased risk for developing many cancers, including breast cancer in postmenopausal women, endometrium, adenocarcinoma of the esophagus, and kidney. Evidence is highly suggestive that obesity also increases risk for cancers of the pancreas, gallbladder, thyroid, ovary, and cervix, and for multiple myeloma, Hodgkin lymphoma, and aggressive prostate cancer. Hepatocellular carcinoma, the fifth most common cancer in the world and the second leading cause of cancer-related mortality, has been linked obesity both directly and indirectly through nonalcoholic fatty liver disease (NAFLD) or steatosis, particularly prevalent in obesity.

Obesity has also been associated with a higher risk of cancer recurrence, progression, and poorer prognosis. Increased risk of progression was shown in patients who gained weight from the time they finished chemotherapy, and possibly in ovarian cancer.

The longer the duration and the higher the degree of overweight/obesity, the greater the risk of developing several forms of cancer, especially endometrial cancer. Therefore, preventing overweight/obesity or meliorating it from early onset may be important to reducing cancer risk. In women, however, managing excess body weight was shown to be more critical regardless of age.

Obesity-Related Cancer Mechanisms

Overweight and obesity have been shown to affect risk of cancers through a variety of mechanisms, some of which are specific to particular cancer types. These mechanisms include insulin resistance (hyperinsulinemia and dysglycemia); inflammation; oxidative stress; immune dysfunction; fat (adipose and lipid/lipoprotein) and sugar (including carbohydrate) metabolism; microbial balance; altered adipose-derived cytokines (adipokines, e.g., higher leptin and lower adiponectin), sex hormones (estrogens, androgens, and testosterone); factors that regulate cell proliferation and growth, such as insulin-like growth factor-1 (IGF-1); and carrier proteins that make hormones more or less available to tissues, such as sex hormone-binding globulin (SHBG) (Fig. 1). These alterations impact biological processes implicated in tumor development, progression and invasion, and are affected by nutrition-related factors, providing potential routes for preventive dietary actions.

Insulin Resistance and Metabolic Syndrome

Elevated insulin or C-peptide (cleaved from proinsulin) is associated with increased risk of numerous cancers, including those of the breast and colon. In the context of obesity-associated hyperinsulinemia, the insulin receptor and its associated signaling pathways can activate numerous growth-promoting and proliferative intracellular signaling pathways, potentially driving the adverse effects of obesity in cancers. This is particularly evident in breast: more than 90% of breast cancer tumor specimens in two different patient cohorts were found to express the insulin receptor; and insulin receptor expression and activation in tumors have been linked to poor survival in patients with breast cancer.

Insulin resistance and hyperinsulinemia are features of the metabolic syndrome, defined by at least three of five metabolic factors: high plasma levels of glucose (>110 mg/100 mL), high levels of triglycerides (>150 mg/100 mL), low levels of high-density lipoprotein (HDL) cholesterol (<50 mg/100 mL), large waist circumference (>88 cm), and hypertension (systolic blood pressure >130 mmHg or diastolic blood pressure >85 mmHg). A systematic review and meta-analysis (97,277 adult females)

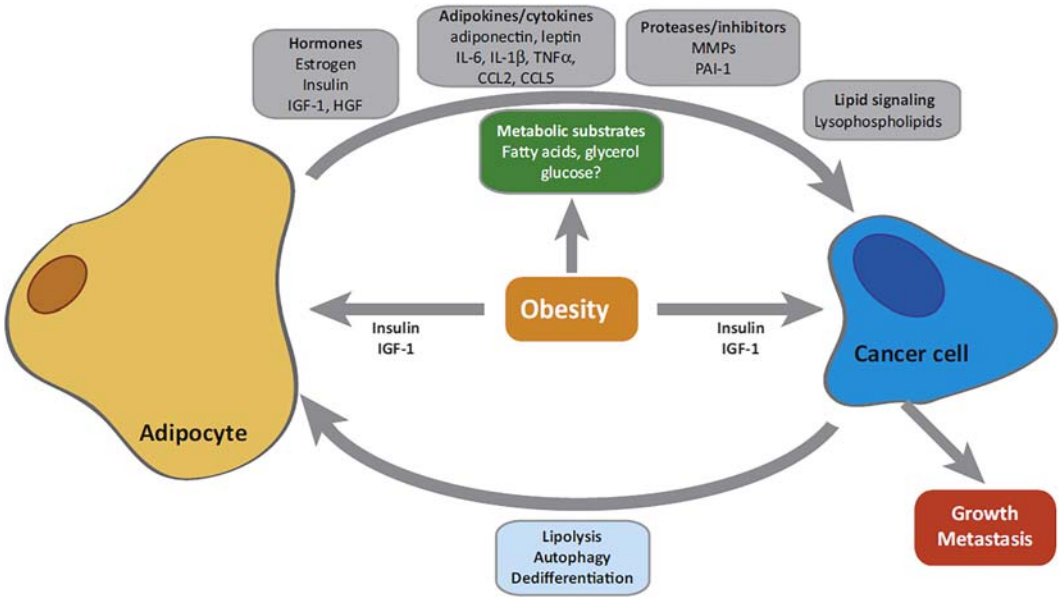

Fig. 1 Within the cancer microenvironment, tumor cells and adipocytes are in close proximity and can exert a variety of reciprocal effects on each other. Cancer cells induce the production of endocrine and paracrine signaling mediators, proteolytic enzymes, and bioactive lipids, along with metabolic substrates by adipocytes. These in turn drive increased growth and invasion of tumor cells along with therapeutic resistance. Obesity leads to the increased production of some signaling factors, such as hormones and adipokines and/or cytokines, and increased availability of metabolic substrates, accentuating in turn, cancer cell growth and metastasis. *CCL2*, C-C motif chemokine ligand 2; *CCL5*, C-C motif chemokine ligand 5; *HGF*, hepatocyte growth factor; *IGF-1*, insulin-like growth factor 1; *IL-1β*, interleukin 1β; *IL-6*, interleukin 6; *MMP*, matrix metalloproteinases; *PAI-1*, plasminogen activator inhibitor-1; *TNFα*, tumor necrosis factor α. From Hoy, A. J., Balaban, S., Saunders, D. N. (2017). Adipocyte-tumor cell metabolic crosstalk in breast cancer. *Trends in Molecular Medicine* **23** (5), 381–392. https://doi.org/10.1016/j.molmed.2017.02.009, with permission.

showed a modest positive association between the metabolic syndrome and breast cancer risk (RR = 1.47), confirmed for postmenopausal women (HR = 1.80). Cholesterol's connection was recently emphasized, through the observed agonist effect of its metabolite 27-hydroxycholesterol on estrogen receptors and postmenopausal breast cancer pathogenesis. Moreover, breast cancer patients with the metabolic syndrome have shown worse prognoses, especially if associated with increased androgenic activity; together suggesting that beyond individual factor associations with breast cancer, their combination may elevate the risk by activating molecular pathways through endocrine, metabolic, and immune functions, which influence breast tumorigenesis.

High serum insulin and insulin resistance associated with obesity are also risk factors for developing prostate cancer. Along with hyperinsulinemia, insulin growth factor (IGF) elevation is also observed in obese men. Insulin stimulates the expression of growth hormone receptors and inhibits liver production of insulin-like growth factor binding protein (IGFBP)-1 and 2, thus further increasing the bioavailability of insulin-like growth factor (IGF)-1.

In type 2 diabetics and obesity, insulin treatment in a dose-dependent and time-varying manner has demonstrated a significant association with increased risk of the most prevalent malignancies in men and women with hemoglobin A_{1c} (HbA_{1c}) $\geq 8.5\%$—colorectal cancer (16.3%) and breast cancer (20.7%), respectively—while metformin use was associated with lower risk. This suggests that in an outpatient setting, the obese patients with poorly controlled insulin-treated type 2 diabetes mellitus should be rigorously assessed toward malignancies, particularly breast cancer in women and colorectal cancer in men.

Adipokines and Obesity-Related Cancers

Alterations in adipokines, particularly leptin and adiponectin, are commonly observed in obesity, and may influence tumorigenesis in obese individuals. Adiponectin and leptin are produced by adipose tissue, but their biosynthesis and secretion are affected differently in the context of obesity—where leptin levels are elevated in the obese state, adiponectin secretion is reduced, resulting in disruptions in a variety of critical processes implicated in affecting whole body homeostasis, as well as cancer.

Correspondingly, binding of adiponectin negatively regulates proliferation, and inhibition of factors that stimulate cell division and growth, while leptin receptor causes activation of these same cellular networks. Thus, the imbalance in circulating levels of adiponectin and leptin observed in the obese state may lead to signaling networks favoring cancer cell proliferation and growth, with implications for incidence and outcome of numerous obesity-related cancers.

Reproductive Hormones and Obesity-Related Cancer

Beyond adipose tissue playing a role of energy storage, it also acts as an endocrine organ. Multiple hormones secreted by the fat tissue can activate alternative pathways that effect physiological function of the body system or promote pathological procedure. Abnormal metabolism of sex hormones, including estrogens and androgens, has been associated with both BMI and the risk of breast cancer. Large increases in endogenous estrogen levels (estrone [E1] and estradiol [E2]) caused by excess body fat among postmenopausal women have been linked to higher risks of postmenopausal breast and endometrial cancer. A significant increase in penetrance of breast cancer-related genetic mutations over the years is thought to reflect increased western lifestyle risk factors, including overfatness, smoking, and low physical activity.

Postmenopausal overweight is associated with increased peripheral conversion of androgens into estrogens, decreased SHBG, and increased insulin levels. Insulin in turn stimulates the synthesis of androgens in the ovary and inhibits liver production of SHBG, thus increasing the bioavailability of sex hormones.

Increased levels of serum estradiol in men with obesity lead to an inhibition feedback of pituitary–hypothalamic axis, resulting in low levels of serum free and bioavailable testosterone, which have been significantly associated with higher prostate cancer risk, though some studies have suggested an opposite association with high grade and advanced tumors.

Obesity-Related Oxidative Stress

Oxidative stress is a physiological state of imbalance between the body's inherent antioxidant defenses and high pro-oxidant load from reactive oxygen species ([ROS], also referred to as "free radicals"). Oxidative stress is known to damage DNA, alter signaling pathways, and regulate progression of various cancers, including those of the breast, lung, liver, colon, prostate, ovary, and brain. Cancer initiation from diet-related oxidative stress has been attributed to alterations in epigenetic events and induction of genomic instability, which alter gene expression, increase the risk of mutagenesis, cause resistance to apoptosis, and induce tumor invasion and metastasis.

Intracellular ROS production in adipocytes can increase with substrate overload from dietary overconsumption. As visceral fat stores expand due to nutrient overconsumption, adipocytes generate increasing levels of ROS and thus oxidative stress that incites increased expression and secretion of inflammatory adipokines and leads to insulin resistance.

Cancer progression is aided by ROS, either by their activity as signaling elements or stimulation of alterations or damage to DNA. ROS also incapacitates tumor suppressors, due to the existence of the redox-sensitive cysteine residues that exist in their catalytic sites. Like those for normal stem cells, cancer stem cell renewal and differentiation are controlled by ROS levels. Liver and breast cancer stem cells tend to have low ROS levels. Since cancer stem cell growth is vital for tumor initiation, retaining low ROS levels may be essential for their proliferation in these organs.

The presence of high levels of oxidized low-density lipoprotein (LDL) cholesterol (ox-LDL), as well as increased LDL receptor expression in macrophages, may serve as a chemotaxis signal that recruits or stimulates the macrophages, and further supports tumor cell growth, as has been demonstrated in colon cancer cells. Accordingly, NAFLD has demonstrated a high association to colorectal, as well as breast cancers.

Obesity-Related Inflammation

Low-grade chronic inflammation and dysregulated adipose tissue inflammatory mediator/adipokine secretion are well-established in obesity, and these factors increase the risk of developing inflammation-associated cancer.

A growing body of evidence demonstrates links between visceral obesity in particular—the type associated with insulin resistance and the metabolic syndrome—and a related secretion pattern of adipokines and cytokines contributing to carcinogenic processes, including adiponectin, leptin and resistin, are able to cyclically increase the pro-inflammatory and insulin resistant state and thus promote tumorigenesis and cancer cell progression through the NFκB pathway, which controls transcription of DNA and cytokine production. NFκB is activated by toll-like receptors, essential components of the innate immune responses. Increased toll-like receptor levels in visceral adipose tissue have been shown to link obesity with inflammation and extracellular matrix remodeling, the latter being a key factor in cancer.

Breast cancer is a key example of how toll-like receptors drive pathogenesis and recurrence through NFκB, as increased inflammation within the subcutaneous mammary adipose tissue depot can alter the local tissue inflammatory microenvironment such that it resembles that of obese visceral adipose tissue. Therefore, in obese women with breast cancer, increased inflammatory mediators both locally and systemically can perpetuate inflammation-associated pro-carcinogenic signaling pathways, thereby increasing disease severity.

Visceral fat-induced inflammation has also been identified as an independent risk factor for esophageal adenocarcinoma, colorectal adenocarcinoma, and colorectal adenomas.

Obesity-Related Immune Response

Dietary factors that result in overweight and obesity influence inflammation, and in turn, immunological reactions. Hyperplastic and hypertrophic adipose tissue from obese individuals exhibits significant irregularities in production of hormones, adipokines, and cytokines contributing to altered immune cell infiltration. Among the immune cells in fat depots, macrophages are especially common, and their properties are altered by obesity. Adipose tissue macrophages in healthy fat depots are skewed toward the M2 antiinflammatory phenotype, but during obesity, pro-inflammatory M1 macrophages become more abundant. Pro-inflammatory adipose tissue macrophages produce tumor-promoting cytokines, including tumor necrosis factor (TNF) α, interleukin (IL)-6, and IL-1b, as well as monocyte chemo-attractants such as monocyte chemotactic protein (MCP-1 or CCL-2), and macrophage migration inhibitory factor (MIF)—orchestrated by the IκB kinase/NF-κB and c-Jun N-terminal kinase/activator protein-1 pathways involved in malignant transformation.

Effect of Local Adiposity on Cancer Risk

A mechanism making breast cancer more aggressive was elucidated in a recent study, and the lipogenic enzyme acetyl-CoA carboxylase (ACC) 1 is now suggested to be a key player in breast cancer metastasis. ACC1 is a key component of fatty acid synthesis, the first and speed-determining step in fatty acid synthesis. ACC1 function is impaired by the cytokines leptin and TGF-β, particularly in the blood of severely overweight subjects. *Thus, fatty acid precursors promote metastases* due to the described inhibition of ACC1 that leads to the accumulation of the fatty acid precursor acetyl-CoA. This precursor is transferred to certain gene "switches" that in turn increase the metastatic capacity of cancer cells by activating a specific gene program.

Effect of Blood Lipids on Cancer Risk

Lipid profile, including triglycerides and total, HDL, and LDL cholesterol, are modifiable factors sensitive to obesity. Recent studies suggest risk of prostate cancer may increase with obesity-related dyslipidemia, including a low HDL, high LDL and total cholesterol, and high triglycerides. Dyslipidemia may also be related to increased tumor grade, as evidenced by abnormal HDL level being a strong predictor of developing high-risk disease.

The association between cancer and lipid metabolic dysfunction in obesity is especially high in liver cancer, where 80% of cancer occurs in patients with underlying steatosis. Steatosis is accompanied by infiltration of macrophages, resulting in enhanced expression of wingless-type mammary tumor (Wnt) signaling and the activation of Wnt/β-catenin signaling, which in turn leads to the accumulation of tumor-initiating cells and tumorigenesis. Wnt signature genes were observed to predict 90% of tumors in a cohort of 558 patient samples.

Epithelial cells of normal prostate have higher cholesterol content compared with other normal cells of organs across the whole body and during the progression of prostate cancer, the cholesterol content gets even higher, indicating that the accumulation of cholesterol may contribute to the malignant conversion of prostate. HDL cholesterol can transport cholesterol from cells to the liver and other steroidogenic organs, and has antiinflammatory and antioxidant functions, and may reduce growth and progression

activity of prostate cancer. High level of triglycerides has also been demonstrated to be responsible for prostate cancer development by elevating oxidative stress and insulin resistance.

Effects of Hyperglycemia on Cancer Risk

Blood sugar metabolism results in highly reactive compounds called advanced glycation end-products (AGE). Excessive production from dietary sugar overconsumption and/or impaired glucose metabolism, with failure to remove them from the system can lead to protein damage, aberrant cell signaling, increased stress responses, and decreased genetic fidelity. AGEs offer a potential mechanistic link between excess sugar-derived metabolites and cancer, which may provide a molecular consequence of lifestyle choices.

Diabetics experience a roughly 20%–25% higher cancer incidence compared to nondiabetics, as well as increased early and late mortality compared to cancer patients without diabetes. Prediabetes is also related to an increased risk of developing and dying from cancer. In addition to hyperglycemia and related AGE generation, possible mechanisms include hyperinsulinemia, alterations of the insulin-like growth factor system, chronic subclinical inflammation, abnormalities in sex hormone metabolism, and adipokines.

Effects of the Microbiome on Cancer Risk

The most important aspect of host-microbiome interactions is host exposure to components of intestinal bacteria that stimulate inflammatory reactions. In particular, high-fat and high-energy diets have been shown to facilitate absorption of bacterial lipopolysaccharide (LPS) from intestinal bacteria.

The microbiome can be affected by changes in carcinogenic or tumor-suppressive metabolites and microbe-associated molecular patterns generated in the gut; endocrine factors, such as insulin, insulin-like growth factor, leptins and adiponectins; and inflammatory and immunological parameters, which are interrelated because the same leukocyte populations contribute to both inflammation and immune responses.

Obesity-induced alterations of gut microbiota have been observed to increase the levels of deoxycholic acid, a gut bacterial metabolite known to cause DNA damage. The enterohepatic circulation of deoxycholic acid provokes senescence-associated secretory phenotype in hepatic stellate cells, which in turn secrete various inflammatory and tumor-promoting factors in the liver, potentially facilitating hepatocellular carcinoma development, including in individuals with nonalcoholic steatohepatitis (NASH).

Carcinogenic Cell Initiation, Proliferation, and Growth

Dietary effects on stem cell mutations

Obesity has been shown to promote stem cell expansion during cancer initiation, and to enhance the conversion of adipose-derived stem cells into carcinoma-associated fibroblasts, leading to cancer cell proliferation and progression to an invasive form.

It was recently assessed that the majority of carcinogenic genetic mutations—66%—are caused by simple random errors occurring when self-renewing cells divide, accumulating damage over time, may be exacerbated by key extraneous inputs such as diet. However, increased stem cell divisions have been observed to correlate with feeding-induced growth of cancer cells.

Poor diet high in "empty calories" tends to be low in essential vitamins—particularly B6, folate, and choline—involved in the methylation pathways critical to DNA repair during cell division. As a result, abnormal cells proliferate unchecked, ultimately contributing to cancer cell growth. Further, choline insufficiency has been independently linked to fatty liver, adding a compounding mechanism.

Adiposity effect on cancer cell progression and invasiveness

Adipose tissue within the tumor microenvironment actively contributes to tumor growth and metastasis by functioning as an endocrine organ, through secretion of signaling molecules such as adipokines, pro-inflammatory cytokines, pro-angiogenic factors and extracellular matrix constituents, and acting as an energy reservoir for embedded cancer cells. Thus, the importance of adipose tissue as an integral contributor to cancer progression and recurrence is increasingly appreciated.

Hypertrophic expansion of adipose tissue in the context of obesity shares many features with solid tumor growth. For example, tumor hypoxia is a hallmark of cancer that is associated with poor patient outcomes and resistance to chemotherapy. The rapid cellular proliferation and expansion of adipose tissue also induces hypoxia, which triggers compensatory angiogenesis, so that limitations in nutrient and oxygen supply can be overcome. Similar to that seen in tumor growth, the hypoxia in adipose tissue that occurs in the context of obesity induces expression of the transcription factor HIF-1α, which upregulates a profibrotic pathway involving extracellular matrix proteins (such as collagens, matrix metalloproteinases and tissue inhibitors of metalloproteinases) and pro-inflammatory cytokines (such as IL-6, TNFα, and C–C motif chemokine (CCL) 2 (also known as monocyte chemo tactic protein-1)). The microenvironment within local adipose deposits clearly provides a tumor-permissive niche for transformed, infiltrating cells. Importantly, fibrotic and pro-inflammatory cytokines that are characteristic of adipose tissue in individuals with obesity, such as endotrophin (a C-terminal cleavage fragment of collagen 3α(VI) chain), IL-6, IL-8, and plasminogen activator inhibitor-1, have also been directly implicated in tumor growth and metastasis.

Excessive expansion of white adipose tissue in obesity is linked to increased aggressiveness of certain cancers. Adipose stromal cells can become mobilized from white adipose tissue, recruited by tumors and promote cancer progression. It was recently shown

that adipocytes from periprostatic adipose tissue support the directed migration of prostate cancer cells and that this event is strongly promoted by obesity. This process is dependent on the secretion of the chemokine CCL7 by adipocytes. In human prostate cancer tumors, expression of the CCR3 receptor is associated with the occurrence of aggressive disease with extended local dissemination and a higher risk of biochemical recurrence.

Gender Differential in Obesity

Obesity rates are higher in women, though varying widely by country. For example, an estimated 18% of women in France are obese, in Greece 26%, in Mexico 35%, and in Saudi Arabia 44%; in contrast, the percentage in both Japan and China is 3%.

In the United States, with overall prevalence of 68.3% overweight and 33.9% obesity, women show higher rates than men of severe obesity (BMI $\geq$35), 17.8% versus 10.7%, and morbid obesity (BMI $\geq$40) 7.2% versus 4.2%, differences of 78% and 71.4%, respectively. Between 1975 and 2014, obesity increased from 6.4% to 14.9% in women and from 3.2% to 10.8% in men. A representative sample of 13,066 men and non-pregnant women from the United States National Health and Nutrition Examination Survey (NHANES) found fat mass percentage—a clinically more relevant marker—in men was 28.1% ($n = 6559$) and 40.0% in nonpregnant women ($n = 6507$), both well above the recommended levels of 25% and 32%, respectively ($p < 0.001$ for sex differences). As several of the obesity-related cancer types only affect women, the growing number of people of both sexes who are severely overweight is likely to have a greater effect on incidence of the disease among women, according to a recent analysis by Cancer Research United Kingdom. Cases of ovarian, cervical and oral cancers are predicted to rise the most, approximately 0.5% for men and 3% for women.

Critical Periods

Rapid length, weight, and BMI growth from birth is associated with higher insulin levels in childhood. The health of young people, and that of the adults they will later become, is critically linked to the establishment of healthy behaviors in childhood, which are more likely to continue throughout life. About half of people who are overweight as children will remain overweight in adulthood; 70% of overweight adolescents will remain overweight as adults.

In adolescent girls, when adiposity shifts to higher metabolic risk—coupled with the increased eating opportunities noted as contributory to obesity—it may create precarcinogenic potential. High birth weight, early menarche, fast growth during adolescence, age at first full-term pregnancy, breast feeding, and menopause all are factors associated with critical periods in which hormonal and metabolic factors are mutually related to obesity and cancer risks.

Where overweight predicted an increased risk of colon cancer, the association of BMI $\geq$85th percentile was even more pronounced in analyses that were restricted to men followed until at least 40 years of age; this suggests that adolescent overweight may be significantly associated with colon cancer incidence in young to middle-aged adults, emphasizing the need for approaching adolescent obesity with its attendant increasing population impact.

Epidemiological studies show that the inclusion of vegetables, legumes, fruits, and whole grains in diets are associated with reduced cancer risk, with diet during early life (age $<$8 years) having the strongest apparent association with cancer incidence. For these reasons, efforts to establish healthy weight and eating patterns should begin in childhood.

Dietary Risk Factors

Dietary factors considered contributory to obesity are increasingly being studied for their associations with cancer. Overeating, an imbalance between consumption and need, is a principal driver. However, specific dietary components and metabolic characteristics can differentially affect each side of the equation.

Animal-Based Foods

Data from the adventist health study showed BMI to increase commensurately with the amount of animal foods in the diet. United States national cross-sectional data also show positive associations between meat consumption and risk for general and central obesity, due to higher energy and fat content (especially SFA), correlation to higher overall daily total energy intake, and displacement of less energy-dense, high-fiber, nutrient-rich plant foods associated with a superior health profile. Studies in Italian, Swedish, and Taiwanese populations revealed similar observations.

Higher total red meat, fresh red meat, and processed meat intake have also been found to be risk factors for several types of cancer, including breast and colorectal, for similar and additional reasons. Chymotrypsin levels are increased in individuals eating a high-meat diet, which could deplete innate tumor suppressors from cells of the gastrointestinal tract and immune cells in contact with it, as well as in other tissues. Heme iron in red meat may generate free radicals that damage DNA, and substances used to process meat (nitrates/nitrites) contribute to the formation of nitrosamines that can also damage DNA, possibly exacerbated by increases in secondary bile acids and other promoters of carcinogenic activity triggered by the fat content in meat. Further, cooking of meat can yield mutagens and carcinogens, including heterocyclic amines and polycyclic aromatic hydrocarbons.

Overeating

It is well-known that overnutrition may generate free radicals and subsequently elevate oxidative stress and ROS-mediated modulation of various molecular pathways. Additionally, overeating increases secretion of the serine protease chymotrypsin, which can induce carcinogenic mechanisms such as increased cell migration and decreased innate tumor suppressors, thereby contributing to obesity-related cancer.

Postprandial Risk Factors

The modern nutritional environment is replete with eating opportunities, resulting not only in increased obesity risk, but also increased likelihood of constantly being in a postprandial state most of the day—an estimated 18 h daily. Among the main postprandial risks of modern meal practices are hyperlipemia, hyperglycemia, and oxidative stress, as well as paradoxical lack of satiety, which can drive additional overconsumption.

Oxidative stress after carbohydrate, protein, and lipid intake results in a series of metabolic alterations in various tissues, including the liver, adipose tissue, pancreatic β-cells, and skeletal muscle. Such active but metabolically distressed tissues further augment oxidative stress, eventually resulting in an infinite vicious cycle. Obese individuals experience more pernicious and acute oxidative stress after a meal, compared to non-obese individuals.

Excessive caloric intake further abnormally increases concentrations of blood glucose, free fatty acids, and triglycerides circulating in the blood, outpacing the capacity of mitochondria for oxidative phosphorylation, the transfer from single electrons to molecular oxygen; consequently, ROS enter the circulation. ROS production by leukocytes is also induced by caloric overload, and conversely caloric restriction can lead to a reduction in ROS production from lipid peroxidation and protein carboxylation.

In obese individuals, hyperglycemia may impact tumor growth by providing cancer cells with an abundance of fuel and enabling them to maintain their rapid proliferative rates. Cancer cells are known to exhibit alterations in glucose metabolism, particularly their reliance on glycolysis even in the presence of oxygen, known as the Warburg effect. Although glycolysis is a less efficient form of energy production compared with mitochondrial oxidative phosphorylation, cancer cells surmount this shortcoming by upregulating glucose transporters, thus increasing their glucose uptake and potentially increasing their sensitivity to the elevated systemic glucose levels found in obesity.

Dietary Energy Density (DED)

High DED is considered to be a notable contributor to overconsumption leading to overweight and obesity. It has also been associated with a 10% increased risk of any obesity-related cancer compared to individuals of normal weight. High DED, increasing portion sizes, and increased eating opportunities—that multiply the incidences of postprandial oxidative and inflammatory stress—can complicate associated overconsumption and obesity, making the individual more susceptible to carcinogenic mechanisms.

Higher DED may be a contributing factor to obesity-related cancers. DED has been associated with statistically significantly greater risk of pancreatic, postmenopausal breast, endometrial, ovarian, and colorectal cancers among women, largely through its contribution to obesity. High DED is potentially modifiable through dietary interventions, suggesting a significant route of reducing obesity-associated cancer risk.

Dietary Fat

As fat is the highest-ED source of calories, it has been correlated with high DED and low satiety, and therefore with risk of "passive" overconsumption that may contribute to overweight and obesity. Excess weight has itself been associated with at least 11 types of cancer, suggesting an indirect effect of dietary fat. Early research on diet and cancer risk also suggested a direct link, since countries with low fat intake, such as Japan, had lower rates of cancer than countries with higher-fat diets, such as the United States.

Relative proportions of energy from dietary fat and carbohydrates have also been suspected to affect excess body fat storage and cancer, which in turn is related to refining of carbohydrate and ratios between major fat categories, for example, high SFA versus unsaturated fatty acids (UFA), and among unsaturated, high *n*-6 PUFA versus *n*-3 PUFA or *n*-9 monounsaturated fatty acids (MUFA).

The Western diet is characterized by excessive intake of energy, SFA and *n*-6 PUFA, sodium, and refined carbohydrates (including sugar), with low intake of *n*-3 PUFA that likely contribute to immune dysfunction, oxidative stress, augmented inflammatory and oncogenic cellular reactions, and altered tissue metabolism. Under unfavorable conditions, in which dietary factors act as inflammatory and pro-oxidant factors, oxygen derivatives can damage nucleic acids, lipids, and proteins, and most significantly, alter cell viability.

As low-fat dietary interventions can influence body weight and decrease breast cancer recurrence, a differential effect of diet on hormone-receptor-positive and -negative disease suggest that metabolic mechanisms involving insulin and insulin-like growth factor-1 may be involved in tumorigenesis indirectly through fat intake.

Saturated fat (SFA)

Some long-term research adjusting for other dietary and lifestyle choices ruled out total fat, and narrowed the *focus to fat from red meat, of which a significant percentage is saturated*. The mechanistic link between SFA and cancer in obese individuals was recently suggested to be through increasing lipopolysaccharides.

Gastric cancer has demonstrated a positive association with intakes of total fat and saturated fat, with an inverse association for PUFA and no effect of MUFA. High total, saturated, and trans-fats increase ovarian cancer risk, and different histological subtypes have different susceptibility to dietary fat. For example, trans-fats have been shown to increase risk of epithelial ovarian cancer overall and endometrioid tumors in particular, while *n*-3 PUFA was found to be protective.

Polyunsaturated fatty acids (PUFA)

PUFA have consistently demonstrated advantages in supporting weight loss and metabolic improvements when substituting for SFA in the diet. Though the *n*-6 PUFA linoleic acid ([LA] 18:2) is essential in the diet, high intake of *n*-6 PUFA has been associated with increased risk of various cancers, including in a cross-generational manner. The incorporation of fatty acids in cell membranes has been considered a pivotal event in modulating inflammatory processes.

n-6 long-chain PUFA (LCPUFA) arachidonic acid ([ARA] 20:4), a product of LA and precursor to inflammatory eicosanoids, induces DNA methylation with profiles similar to those described for palmitic acid and linked to atherosclerosis, diabetes, obesity, and autism, but relatively dissimilar from *n*-9 MUFA oleic acid (OA)-induced profiles. Human atherosclerosis grade-associated DNA methylation profiles were significantly enriched in ARA-induced profiles, with biochemical evidence pointing to β-oxidation, PPAR-α, and sirtuin 1 as important mediators of the changes.

In contrast, dietary *n*-3 PUFA—particularly LCPUFA such as eicosapentaenoic acid [(EPA] 20:5) and docosahexaenoic acid ([DHA] 22:6), synthesized from essential alpha-linolenic acid ([ALA] 18:3)—may have utility in mitigating the severity of obesity-associated inflammation and cancer. *n*-3 PUFA modulate inflammation and specifically the production of adipokines by increasing levels of antiinflammatory adiponectin, while decreasing levels of inflammatory mediators such as leptin and cytokines including TNFα, IL-6, and CCL2. Moreover, dietary *n*-3 PUFA have been found to reverse and/or improve obesity-associated hepatic steatosis and impairments in glucose metabolism and insulin sensitivity. Collectively, these antiinflammatory effects of *n*-3 PUFA alter the obesity-associated inflammatory environment and may meliorate related cancer risk.

The chemoprotective effects of *n*-3 LCPUFA decrease cell proliferation and increased apoptosis, ultimately resulting in reduced cancer tumor incidence, growth, multiplicity, and metastasis, as observed in rodent models. Similar *n*-3 PUFA-mediated antitumorigenic effects have been reported in overweight humans wherein *n*-3 PUFA supplementation upregulated the expression of several genes involved in cell cycle regulation. In rodents, a beneficial effect of *n*-3 PUFA shown on the breast cancer phenotype was related to their potential for reducing obesity-associated inflammation and consequent tumorigenic risk. In an obese postmenopausal model of breast cancer, *n*-3 PUFA supplementation reduced mammary adipose tissue inflammation and markers of inflammatory M1 macrophage infiltration, reflecting the inflammatory microenvironment promoting tumorigenesis. These studies clearly demonstrate that *n*-3 PUFA can independently modulate responsiveness to cell proliferative and/or apoptotic signaling.

Monounsaturated fatty acids (MUFA)

As with PUFA, MUFA substitution for SFA in the diet has been shown to reduce postprandial inflammatory responses and improve insulin sensitivity in obese individuals. This benefit was shown to be enhanced with co-consumption of dietary fibers, with a decrease in *E*-selectin, TNFα, interferon (IFN)γ, IL10, and IL17, compared to an increase in IFNγ and I6 with saturated fat intake. Weight maintenance with a MUFA-rich diet was shown to improve HOMA-IR and fasting proinsulin levels, while the latter effects have been more consistent and superior with PUFA, MUFA has demonstrated an advantage overall inflammatory responses linked to carcinogenic metabolic processes. In a large-scale European case-control analysis investigating colorectal cancer incidence, risk reduction was observed with the MUFAs OA (*n*-9, 18:1) and palmitoleic acid (*n*-7, 16:1), in contrast to pro-inflammatory PUFAs LA and ARA, as well as the SFA stearic acid.

OA has been observed to suppress over-expression of HER2 (erbB-2), and to react to invasive progression and metastasis in several human cancers; intracellular calcium signaling pathways have been observed to induce apoptosis in carcinoma cells, where its antiinflammatory effects could support the activation of various pathways of immune-competent cells.

Dietary Carbohydrates and Fiber

Dietary carbohydrate effects on glycemic and insulin metabolism have long been suspected to contribute to overweight and obesity, and have been linked to cancers as well.

While total carbohydrate intake itself has been associated with breast cancer risk in some population-based studies, many have found associations with dietary glycemic index (GI) and glycemic load (GL), which more directly reflect how foods impact metabolism. High intakes of staples, especially refined white rice, were positively associated with endometrial cancer, with the link to GI being more evident among lean and normal weight women, suggesting that intake of high GL or GI foods may be more relevant as a risk factor. A Mediterranean population characterized by traditionally high and varied carbohydrate intake demonstrated that a diet high in GL played a role in the development of breast cancer, colon cancer, and esophageal cancer risk. Caucasian American adults (1991–2013) demonstrated associations between carbohydrate intake and cancer that varied by cancer site, and healthier low-GI carbohydrate foods lowered the risk of adiposity-related cancers among women, for example, lower breast cancer risk (HR 0.51).

Fructose

Fructose was previously considered a beneficial or at least relatively innocuous dietary sugar, as it does not stimulate insulin secretion and demonstrates a low GI. However, it has more recently been closely linked to NAFLD and the related NASH, which is considered a precancerous condition. A very recent Australian study showed that even one portion daily of a beverage sweetened with fructose syrup significantly increased 13 types of cancer, and the greater the amounts and the longer the intake the higher the cancer risk.

Dietary Protein

A recent study evaluating links between protein intake and mortality found that in individuals aged 50–65 years, high protein intake was associated with a 75% increase in overall mortality and a fourfold increase in cancer death risk during the ensuing 18 years, though plant-based protein abolished or attenuated the effect, and reduced cancer and overall mortality. Studies in murine models confirmed the effect of high protein intake and GHR-IGF-1 signaling on the incidence and progression of breast and melanoma tumors. High-protein diets are often used to manage weight in obesity, including during middle age, when it may counteract the health benefits of losing weight, while a low-protein diet was found to be detrimental in individuals over 65 years of age.

A protein-restricted diet is thought to improve metabolism and attenuate cancer progression by modulating levels of associated adipokines and other endocrine signals, for example, elevated FGF21 and adiponectin and reduced leptin concentrations. Long-term consumption of a low-calorie, low-protein diet has been associated with reductions in the plasma growth factors, such as IGF-1, and hormones linked to increased risk of cancer, including independent of body fat mass.

Animal-based protein sources

In contrast to red meat, fish and poultry proteins were shown to be suitable to obesity- and cancer-preventive diets. Two 100-g portions of fatty fish per week provide 3.5 g of *n*-3 PUFA, that potentially reduce obesity and cancer, with suggested calculated potential of each additional 0.7 g of marine omega-3 polyunsaturated fat per week reduces risk by 5% of breast cancer.

Pescetarian diets, such as the Mediterranean and Okinawa diets that are high in fish and other seafoods as well as low-medium-fat dairy products—often fermented and containing "friendly" bacteria—have been shown to be among the best obesity- and cancer-protective diets.

Though dairy foods contain the potentially cancer promoting hormones estrogen and IGF-1, concerns are not supported by epidemiological evidence. This may be because these hormones are present in minute amounts (even with daily intake), and are counteracted by protective compounds in the same foods, including short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA), conjugated fatty acids (CFA), cysteine-rich whey proteins, calcium, and vitamin D. Fermented dairy products in particular have demonstrated protective contributions against obesity and cancer through supporting a healthy microbiome and producing intermediate products of fermentation.

Plant-based protein sources

Cancer risk has been found to decrease with intakes of soy food, other legumes, nuts, poultry, skim milk, and fish, leading some researchers to advocate vegetarian diets for prevention.

The EPIC-Oxford study, as well as many other studies, showed a more plant-based diet to be protective against weight gain, and that older adults and men were more likely to lose weight in response to a plant-based diet. Such a diet is also highly recommended against cancer risk due to its low DED and high nutritional density, including high amounts of vitamins, minerals, fiber, and phytonutrients such as carotenoids, polyphenols, protease-inhibitors, sulforaphanes, and more, which having demonstrated preventive potential against various aspects of cancer development.

Vegetarians have a 22% lower risk of colorectal cancer—in the colon by 19% and rectum by 29%—compared to nonvegetarians. In Japan, age-adjusted prostatic cancer mortality rose during the 50-year period from 1948 to 1998, concurrently with an increase in intake of eggs (sevenfold) and meat (ninefold). Legumes constitute a nonmeat, "plant-based" protein source that also yields nutrients shown to protect against cancer, for example, fiber, as well as micronutrients whose insufficiency is linked to increased cancer risk. They are especially rich in isoflavones, nutrients that may protect against cancer, including incidence of endometrial and gastrointestinal cancers, and mortality and recurrence of breast cancer.

A plant-based diet does not necessarily mean vegetarian or vegan, but can also be one that quantitatively emphasizes plant sources of nutrition with potentially protective qualities against obesity and cancer, complemented by animal-based proteins, which have high nutritional and biological value—being rich in micronutrients, especially iron, zinc, copper, and B12 and additional B vitamins—and are effective in enhancing satiety and building lean body mass.

Fiber

Fiber content is protective through mechanisms that are key in healthy weight management. In the gut, fibers promote the decomposition and excretion of inflammatory and carcinogenic substances, growth of beneficial and inhibition of pathogenic bacteria, phagocytosis of macrophages, inhibition of nitrosamine synthesis, reduction of estrogen levels, modulation of IGF-1 activity and inflammation, and production of short-chain fatty acids (SCFAs) by bacteria in the colon, all of which are associated with attenuation of tumor development. Balancing of the microbiome in particular is gaining attention for its important role in metabolic

regulation affecting carcinogenesis and body weight and composition. Further, fiber's contribution to satiety through retention of liquids from food and drinks and resultant gastric distension, and to improved glucose and insulin metabolism through slowed digestion, has been linked to lower risk for obesity and related hormonal and inflammatory processes linked to cancer. Both colon and breast cancers have been linked to both high BMI and their risks attenuated by weight management and high-fiber intake. A dose-response analysis showed that for every 10 g d^{-1} increase in dietary fiber intake, there was a 4% reduction in breast cancer risk, particularly in postmenopausal women.

Nutrient Deficiencies

The malnutrition that often accompanies obesity is attributed to poor diet high in "empty calories" and low in essential micronutrients, that is, those involved in the methylation pathways critical to DNA repair during cell division. As a result, damaged DNA leads to cell mutations that proliferate unchecked and ultimately contribute to cancer.

High prevalence of micronutrient deficiencies has been repeatedly observed in obese individuals, despite overconsumption of energy that often means low intake of micronutrient-rich foods such as fruits and vegetables, whole grains, nuts, legumes, dairy products, and fish that are major sources of most vitamins, minerals, and essential fatty acids. The nutrients most commonly affected include vitamins A (as well as precursor beta-carotene), B1, B12, and D, folate, and the essential minerals magnesium, selenium, zinc, and iron, with notable presence of nutritional anemia.

B-vitamins such as folate and choline, as well as betaine and other reactants may influence the methyl donor pool and ultimately, levels of DNA and histone methylation. Calorie restriction and excess have opposite effects on DNA methylation, where calorie excess and high BMI are risk factors for several types of cancer via multiple DNA methylation alterations.

Deficiencies of these nutrients can cause significant damage to DNA via single and double-strand breaks, oxidative lesions, or both, or limit the body's repair mechanisms for these processes. They may be a contributing factor to the twofold greater risk of most types of cancer (lung, larynx, oral cavity, esophagus, stomach, colon and rectum, bladder, pancreas, cervix) among individuals with the lowest consumption of fruits and vegetables compared to those with the highest. More than 200 studies showed an association between low consumption of fruits and vegetables and the incidence of cancer; unfortunately, $\geq$68% of Americans of all ages do not meet the intake recommendations of the National Cancer Institute and National Research Council.

However, the majority of supplementation studies indicate no effect on general mortality or cancer incidence, either positive or negative. The antioxidant supplements tested thus far seem to offer no improvement over a well-balanced diet, possibly because of the choice of the substances tested, inadequate or excessive dosage, or lack of synergistic variables present in foods. Despite the protective effects observed for dietary micronutrients—vitamins, minerals, and phytonutrients, for example, polyphenols, carotenoids, sulforaphanes—when they come in a whole food source, isolated and concentrated active compounds are still believed to potentially hold greater promise in some cases.

Nutritional Recommendations

Diet, physical activity, and body weight have been shown to play important roles in reduction of cancer risk, as well as recurrence and mortality. Optimal strategies to reduce pro-inflammatory and oxidative exposures linking obesity with cancer differ in each individual. Most research on energy imbalance and cancer focuses on increased risks associated with overweight and obesity, showing that losing weight may reduce the risk of cancer (**Table 1**). Even bariatric surgery to treat morbid obesity and short-term intentional weight loss has been shown to improve insulin sensitivity and biochemical measures of hormone metabolism, and to contribute to reduced obesity-related cancers. Efforts to reduce weight—and specifically fat weight, and primarily abdominal fat—are considered paramount in prevention of related cancers.

Table 1 2007 World Cancer Research Fund (WCRF) conclusions on diet and cancer

Food group/other	*Risk effect*	*Cancer types*	*Evidence strength*	*Recommendation*
Obesity (linked to energy-dense foods high in fat/sugar)	Increase	Breast Bowel Esophagus	Convincing	Maintain healthy bodyweight, avoid energy-dense foods (>250 kcal/100 g)
Physical activity	Decrease	Bowel	Convincing	30 min day^{-1} of moderate exercise
Alcohol	Increase	Bowel Breast Liver	Convincing	Limit drinks to 2 day^{-1} (men) or 1 day^{-1} (women)
Red meat	Increase	Bowel	Convincing	<500 g $week^{-1}$
Processed meat	Increase	Bowel	Convincing	Avoid
Fiber	Decrease	Bowel	Probable	More whole grains
Fruit and vegetables	Decrease	Digestive tract prostate	Probable	Five portions day^{-1}

Based on United Kingdom National Health Service (2009). *Statistics on Obesity, Physical Activity and Diet—England*. Accessed from: https://digital.nhs.uk/catalogue/PUB00188.

Reducing Energy Density

Reducing caloric intake through favoring low DED foods can provide a trifecta of benefits by managing body weight and fat, dyslipidemia and dysglycemia, and inflammatory and oxidative processes linking excess weight to cancer. DED is lowered by adding water and fiber and reducing intake of added sugars (particularly refined sugars), saturated and trans-fats, and alcohol, which all provide substantial calories but few or no essential nutrients, and yield low satiating potential because of lack of gut distension. Caloric intake can also be reduced by decreasing the size of food portions and frequency of eating opportunities.

High-DED foods and beverages high in refined ingredients are consistently recommended to be replaced with low-DED, nutrient-rich choices like vegetables and fruits, whole grains, beans, soups, and water or unsweetened beverages.

Increasing Nutrient and Phytonutrient Density

Meliorating micronutrient deficiencies through improved nutrition—such as greater consumption of vegetables, fruits and other whole foods—is a viable means of greatly reducing obesity and cancer risk. A major part of the protective effect of fruits and vegetables may be due to their micronutrient content, including plant phytoestrogens, fiber, and especially antioxidants such as vitamin C, phenolic acids, and carotenoids. This is emphasized the strong recommendation to consuming whole rather than processed/ultra-processed foods.

Recent large-scale studies have confirmed effectiveness of evidence-based recommendations for reducing cancer risk, which are the same corroborated as being effective against obesity and pathometabolic sequelae, promoted by health authorities around the world, as well as cancer-specific organizations such as the ACS and National Cancer Institute (NCI).

Optimizing Dietary Fatty Acid Profile

n-*6 PUFA*

High dietary intake of *n*-6 PUFA has been found to be associated with an increased risk of various cancer development. The incorporation of fatty acids in cell membranes has been considered a pivotal event in modulating inflammatory processes. Excessive *n*-6 PUFA can harm DNA, that is, by creating ethenoadducts, depurination, and mutations. Cross-generational effects shown by the total number of reproductive system tumors, pituitary tumors and metastases was increased in the offspring of dams exposed to a high dietary level of *n*-6 PUFA. The differential impact of ARA and OA on the DNA methylome, with ARA contributing to the epigenome of important metabolic diseases, supports and expands current diet-based therapeutic and preventive efforts to limit its overconsumption and excessive endogenous production.

n-*3 PUFA*

Marine-derived *n*-3 PUFA have well-established antitumorigenic effects in chemically induced, transgenic and xenograft rodent models of breast cancer. The traditional diets of populations with historically superior obesity and cancer profiles, that is, that of Japan, features 1%–2% of daily energy as *n*-3 LCPUFA, and Greenland Inuit typically consume 2.4%–6.3%, demonstrating influence against both inflammation and obesity.

n-*6*:n-*3 PUFA ratio*

A high dietary *n*-6:*n*-3 PUFA ratio has been shown to cause an increase in pro-inflammatory cascades contributing to both obesity and cancer. By decreasing *n*-6 and increasing *n*-3 in the diet, the proadipogenic and procarcinogenic pathways may be inhibited. Therefore, it has been recommended to return to the balanced dietary *n*-6:*n*-3 ratio of 1–2:1 based on data from evolutionary studies, observational studies of populations, and large-scale clinical studies, while diets of modern populations known to be relatively free from obesity and cancer, that is, Japan, tend to be in the range of 4–5:1.

n-*9 MUFA*

OA has been found to specifically regulate cancer-related oncogenes, and to exert antiinflammatory effects opposing those of *n*-6 LCPUFA ARA and SFA palmitic acid. It is the primary MUFA in olive oil, and a principle component of the traditional Mediterranean diet, a pattern linked to protection against obesity sequelae and cancer. To achieve these benefits, it has been recommended to use OA to replace a similar amount of SFA and *n*-6 PUFA, especially when *n*-6 PUFA intake exceeds daily needs.

Maximizing Plant Food Potential

Whole grains are considered to be an important part of an overall healthful diet for people without contraindications. Whole grain foods, which are those made from the entire grain seed, are relatively low in caloric density and can contribute to maintaining energy balance, are higher in fiber, polyphenols, vitamins and minerals than highly processed (refined) flour products, and have been associated with lower risk of cancer and other metabolic sequelae of obesity and dysglycemia.

Polyphenols have been reported to be efficacious in the prevention of cancer and the reversal of cancer progression. Polyphenols may affect epithelial-to-mesenchymal transition pathways, which are involved in cancer metastasis, including by increasing levels of epithelial markers while downregulating mesenchymal markers.

For example, the olive oil polyphenol hydroxytyrosol inhibits cell proliferation in colon cancer cells and enhances targeted chemotherapy, an effect presumably related to its antiinflammatory activity; epigallocatechin gallate, found in high amounts in green tea and touted for thermogenic benefits in weight management, has been found to inhibit the type of oncoprotein-induced angiogenesis that contributes to tumor growth and metastasis.

High intake of fruit, vegetables, and legumes has been associated with reduced risk of various cancers. They are complex foods, each containing numerous potentially beneficial vitamins, minerals, fiber, carotenoids, and other bioactive substances, such as flavonoids, terpenes, sterols, indoles, and phenols, which increase the efficacy of epigenetic mechanisms that help decrease the risk of obesity and its comorbidities and prevent cancer. Inhibition of serine proteases by compounds in fruit and vegetables could also account for some of the protective effects of a plant-rich diet. Greater consumption of produce is associated with decreased risk of lung, esophageal, stomach, and colorectal cancer in particular.

For other cancers, fruits and vegetables provide benefit by reducing DED, total energy intake, and weight gain and thus risk of developing obesity over time. Using produce to replace more calorically dense foods is a frequently recommended strategy for maintaining a healthy weight, but only when not deep-fat fried (e.g., French fries) or consumed with calorically dense dressings and sauces high in saturated fat and/or overly processed ingredients.

There is ongoing research on the potential benefits of particular plant foods or groups, including dark green and orange vegetables, cruciferous vegetables (e.g., cabbage, broccoli, cauliflower, Brussels sprouts), soy products, legumes, *Allium* vegetables (onions and garlic), and tomato products.

Attempts to isolate specific nutrients and administer them as supplements, sometimes in very high doses, have been mostly unsuccessful in preventing cancer or its precursor lesions, and in some cases, have had adverse effects. Notable examples were four randomized trials of beta carotene for prevention of lung cancer, based on epidemiological indications against lung cancer; in two of these trials, the smoking individuals taking high-dose beta carotene supplements developed lung cancer at higher rates than those taking a placebo, supporting the concept of whole foods being more protective than the isolated active compounds.

Despite recommendations for increasing intake of fruit and vegetables, it remains low among both adults and children. This may be partially due to lack of access to affordable options, preparation time, and taste preferences. For cancer risk reduction, the recommendation is to consume at least five servings of a variety of vegetables and fruits each day; however, for overall health, the ACS supports the recommendation to consume higher levels, depending on calorie needs, as stated in the US Department of Health and Human Services' Dietary Guidelines for Americans.

Advantageous Dietary Patterns

Better understanding of dietary interrelationships with cancer—as applied to food selection, combination, and preparation, and potential for recommended patterns—have increased public motivation and authoritative support for early, timely, and ultimately lifelong prevention and management of both obesity and cancer.

Traditional dietary patterns such as the Mediterranean and Okinawa have been associated with healthy weight and low cancer rates in observational studies, and improved metabolic health in interventional studies. Increasing rates of disease in those regions, concurrent with abandonment of original principles in favor of modern western obeso-carcinogenic dietary characteristics, have served to confirm the formers' advantage and encourage a return in order to regain health and extreme longevity. Several medical diets have been established based on key factors that traditional diets have in common, and have yielded outcomes corroborating the value of these factors and their relationships to specific risk factors.

- *Mediterranean diet*: A pescetarian diet, low in red meat and highly based on local plants—legumes, beans, whole grains, almonds, fresh and lightly-cooked vegetables, fruits, herbs and spices, olives and extra-virgin olive oil—plus eggs, light and fermented dairy, and moderate red wine with meals; incorporates social eating with family and friends.
- *Okinawan diet*: A pescetarian diet, with great variety of whole, fresh and/or lightly cooked or fermented local foods, high in plants and algae/seaweed, very low in meat, most carbohydrates from whole tubers, rice, and buckwheat, without highly processed foods; traditionally yields the longest longevity with lowest noncommunicable disease risk.

Both the Mediterranean and Okinawan diets are low in DED, GL, and fat—particularly SFA and artificial trans-fats—with much of the fat content consisting of *n*-9 MUFA and *n*-3 PUFA, yielding an *n*-6:*n*-3 PUFA ratio of 5:1 or lower. They are high in whole plant foods such as unrefined grains, fresh fruits, and fresh or lightly cooked vegetables, rich in fiber, micronutrients, and phytochemicals, many of which have antioxidant properties. Protein sources are largely lean, and include fish, eggs, dairy, legumes, nuts, seeds, and probiotic-rich fermented dairy, while red meat is greatly limited. Refined sugar and industrially processed foods are rarely—if at all—used. Many of the local traditional foods, herbs, and spices consumed on a regular basis are considered "functional foods," exerting protective benefits beyond basic nutrition.

In addition, traditional cultures with low rates of obesity and cancer were known to complement their dietary advantage with an active lifestyle that involved very little sedentariness.

Leading health authorities, such as the WHO, United States National Cancer Institute, ACS, and dietetics associations around the world, have adopted these principles in their formal recommendations for prevention and management of obesity and cancer. The following are shared recommendations for prevention of cancer through prevention and management of obesity:

- Achieve and maintain a healthy weight throughout life, as lean as possible without being underweight. Body weight should be maintained in the body mass index range of 18.5–25 kg m^{-2}.
- Engage in regular physical activity and limit consumption of high-calorie foods and beverages as key strategies for maintaining a healthy weight.

- For those who are currently overweight or obese, losing even a small amount of weight has health benefits and is a good place to start.
- Adults should engage in at least 150 min of moderate intensity or 75 min of vigorous intensity activity weekly, or an equivalent combination, preferably spread throughout the week.
- Children and adolescents should engage in at least 1 h of moderate or vigorous intensity activity each day, with vigorous intensity activity occurring at least 3 days each week.
- Limit sedentary behavior such as sitting, watching television, or other forms of screen-based entertainment.
- Reduce dietary energy density (DED) to increase specific satiety per calorie and thus limit risk of overconsumption linked to obesity and cancer.
- Consume a healthy diet, with an emphasis on whole foods and variety of plant foods.
- Limit consumption of red meat, choose light/slow cooking and avoid ultra-processed meat.
- Cook foods by steaming, sautéing, baking, or boiling, rather than deep-fat frying or charbroiling/grilling.
- Eat at least 2.5 cups (500 mL) or five servings of vegetables and fruits each day.
- Choose whole grains instead of refined/processed/ultra-processed grain products.
- If you drink alcoholic beverages, limit consumption—no more than 1 drink per day for women or 2 per day for men.

Prospective Vision

Appreciation of chronic, cumulative events linking obesity and cancer is leading researchers and practitioners toward comprehension of the cumulative effects of acute postprandial responses to characteristics of each meal, for example, size and composition, and ultimately to recommendations for early and lifelong optimization of diet and fitness, from birth through critical periods in the life cycle, as a preventive strategy against obesity and cancer. Measures include harnessing whole, fresh, minimally processed, home-cooked foods as a simple means of reducing DED and obesogenic and carcinogenic factors, which is attainable by wide-ranging populations.

Beyond population-based approaches, rapidly accumulating research is demonstrating how interactions between gender, genes and environmental factors such as dietary intake impact both obesity and cancer, including through mutual pathways that could ultimately lead to synergistic interventions. This is important to better understand obesity by individuals' genetic predisposition and to create a concept of "personalized nutrition" for the effective prevention and treatment of obesity.

See also: Cancer Risk Reduction Through Lifestyle Changes. Diet and Cancer. Obesity and Cancer: Epidemiological Evidence

Further Reading

Berger, N.A., 2014. Obesity and cancer pathogenesis. Annals of the New York Academy of Sciences 1311, 57–76.

Colditz, G.A., Emmons, K.M., 2018. Accelerating the pace of cancer prevention-right now. Cancer Prevention Research 11 (4), 171–184.

Colditz, G.A., Bohlke, K., Berkey, C.S., 2014. Breast cancer risk accumulation starts early: Prevention must also. Breast Cancer Research and Treatment 145, 567–579.

Duffey, K.J., Popkin, B.M., 2011. Energy density, portion size, and eating occasions: Contributions to increased energy intake in the United States, 1977–2006. PLoS Medicine 8, e1001050.

Giacosa, A., Barale, R., Bavaresco, L., Gatenby, P., Gerbi, V., Janssens, J., Johnston, B., Kas, K., La Vecchia, C., Mainguet, P., Morazzoni, P., Negri, E., Pelucchi, C., Pezzotti, M., Rondanelli, M., 2013. Cancer prevention in Europe: The Mediterranean diet as a protective choice. European Journal of Cancer Prevention 22, 90–95.

Kushi, L.H., Byers, T., Doyle, C., Bandera, E.V., McCullough, M., McTiernan, A., Gansler, T., Andrews, K.S., Thun, M.J., American Cancer Society 2006 Nutrition and Physical Activity Guidelines Advisory Committee, 2006. American Cancer Society Guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. CA: a Cancer Journal for Clinicians 56, 254–281.

Lohmann, A.E., Goodwin, P.J., Chlebowski, R.T., Pan, K., Stambolic, V., Dowling, R.J., 2016. Association of obesity-related metabolic disruptions with cancer risk and outcome. Journal of Clinical Oncology 34, 4249–4255.

Manni A, El-Bayoumy K, Thompson H (2017) Docosahexaenoic acid in combination with dietary energy restriction for reducing the risk of obesity related breast cancer. International Journal of Molecular Sciences 19: pii: E28.

Shapira, N., 2007. Israeli "cancer shift" over heart disease mortality may be led by greater risk in women with high intake of n-6 fatty acids. European Journal of Cancer Prevention 16, 486–494.

Shapira, N., 2012. Women's higher risk with N-6 PUFA vs. men's relative advantage: An "N-6 gender nutrition paradox" hypothesis. The Israel Medical Association Journal 14, 435–441.

Shapira, N., 2017. The potential contribution of dietary factors to breast cancer prevention. European Journal of Cancer Prevention 26, 385–395.

Signori, C., El-Bayoumy, K., Russo, J., Thompson, H.J., Richie, J.P., Hartman, T.J., Manni, A., 2011. Chemoprevention of breast cancer by fish oil in preclinical models: Trials and tribulations. Cancer Research 71, 6091–6096.

Simopoulos, A.P., 2004. The traditional diet of Greece and cancer. European Journal of Cancer Prevention 13, 219–230.

Prostate Cancer: Diagnosis and Treatment

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Abbreviations

ADT Androgen deprivation therapy
ASR Age-standardized incidence rates
CAB Complete/combined androgen blockade
DRE Digital rectal examination
EBRT External beam radiotherapy
FDA US Food and Drug Administration
IARC International Agency for Research on Cancer
ICD-O International Classification of Disease for Oncology
IMRT Intensity-modulated radiation therapy
LHRH luteinizing hormone-releasing hormone
PCA3 Prostate cancer gene 3
PCa Prostate cancer
PSA Prostate-specific antigen
RARP Robot-assisted radical prostatectomy
RT Radiotherapy

Definition and Classification

Prostate cancer (PCa) is a malignant tumor of the prostate gland. The most frequent PCa type is acinar adenocarcinoma (ICD-O code: 8140/3) which is an invasive carcinoma of the prostate consisting of neoplastic epithelial cells with secretory differentiation arranged in a variety of histomorphological patterns, including glands, cords, single cells and sheets. Basal cells are typically absent.

In addition to "usual" acinar prostate adenocarcinoma, there are eight histological prostate adenocarcinoma variants: atrophic, pseudohyperplastic, microcystic, foamy gland, mucinous (colloid; ICD-O code: 8480/3), signet ring-like cell (ICD-O code: 8490/3), pleomorphic giant cell, and sarcomatoid variant (ICD-O code: 8572/3).

The recommended grading system is the Gleason score ($2-10$) which takes into account the inherent morphological heterogeneity of prostate cancer and is of clear prognostic value.

Presentation and Diagnosis

Most prostatic adenocarcinomas are multifocal, with an average of two to three separate tumors per gland and are macroscopically variable.

Prostate cancer is usually suspected based on a digital rectal examination (DRE) and/or prostate-specific antigen (PSA) levels. DRE, however, is not specific for prostate cancer and misses 25%–50% of cases detected by serum PSA, especially smaller size and lower-stage carcinomas. Hence, PSA serum levels have become a largely accepted parameter in evaluating the risk of prostate cancer, with higher levels indicating a higher PCa risk. Still, some men may harbor preclinical PCa despite having low PSA levels. Several PSA derivatives, like PSA density, doubling time, and velocity, have been used to improve the specificity of PSA testing, although with a rather modest efficiency.

Transrectal prostate ultrasound is the first technique of choice to use in a diagnostic setting when a prostate cancer is suspected based on an abnormal DRE and/or elevated or rising serum PSA levels. Magnetic resonance imaging (MRI) is used for localizing prostate tumors and determining their size and invasiveness. Definitive PCa diagnosis is based on a histopathological verification of prostate biopsy cores, or specimens from transurethral resection of the prostate or prostatectomy for benign prostatic enlargement.

In countries where PSA measurements are standard clinical practice, most prostate cancers are detected at an asymptomatic stage. Clinical symptoms are usually a manifestation of locally advanced or metastatic disease. For locally advanced disease, these include urinary frequency and difficulty urinating which can stimulate benign hyperplasia, acute urinary retention and hematuria, whereas rectal invasion, priapism and uremia are late findings characteristic of highly advanced tumors.

Epidemiology and Risk Factors

Prostate Cancer Burden

Prostate cancer is the second most commonly diagnosed cancer in men. With an estimated 1.1 million diagnoses worldwide in 2012, it accounted for 15% of all cancers diagnosed. The frequency of autopsy-detected PCa is roughly the same worldwide. However, there are remarkable differences in the PCa incidence between different geographical areas, with 70% of cases diagnosed in more developed regions. The highest incidence rates were reported in Australia/New Zealand and Northern America (2012 age-standardized incidence rates (ASR) of 111.6 and 97.2 per 100,000, respectively), and in Western and Northern Europe. This is largely due to the use of prostate-specific antigen (PSA) testing in standard clinical practice and due to the aging population. Incidence rates are also relatively high in certain less developed regions, such as the Caribbean (79.8), Southern Africa (61.8), and South America (60.1), but remain low in Asian populations (10.5) as well as in Eastern and South-Central Asia (4.5; Fig. 1).

With an estimated 307,000 deaths in 2012, prostate cancer is the fifth leading cause of death from cancer in men (6.6% of all male deaths). As PSA testing has a lower effect on mortality than on incidence, there is relatively less variation in mortality rates worldwide (10-fold from approximately 3 to 30 per 100,000). Mortality rates are generally high in predominantly black populations (Caribbeans: ASR of 29 per 100,000 and sub-Saharan Africa: 19–24 per 100,000), very low in Asia and intermediate in the Americas and Oceania (Fig. 1).

Both PCa incidence and mortality rates are predicted to rise, with 1,853,391 new cases and 544,209 PCa-related deaths predicted for the year 2030.

Etiology and Risk Factors

Family history and racial/ethnic background are associated with an increased PCa incidence suggesting a genetic predisposition. Genome-wide association studies have identified 100 common susceptibility loci contributing to the PCa risk, explaining nearly 40% of the familial risk for this disease. Proposed sites of prostate cancer susceptibility loci include hereditary prostate cancer (HPC) 1 (1q24–q25, candidate gene: *RNASEL*), HPC2 (17p, candidate gene: *ELAC2*), PCAP (1q42.2–q43), HPCX (Xq27–q28), CAPB (1p36), HPC20 (20q13), and 8p21–q23 (candidate gene: *MSR1*). An increased prevalence of germline mutations in genes mediating DNA repair processes was found among patients with metastatic PCa. Moreover, male *BRCA1* and *BRCA2* mutation carriers have also been found to be at an increased risk of prostate cancer. However, only a small subpopulation of men with PCa (about 9%) have a true hereditary disease.

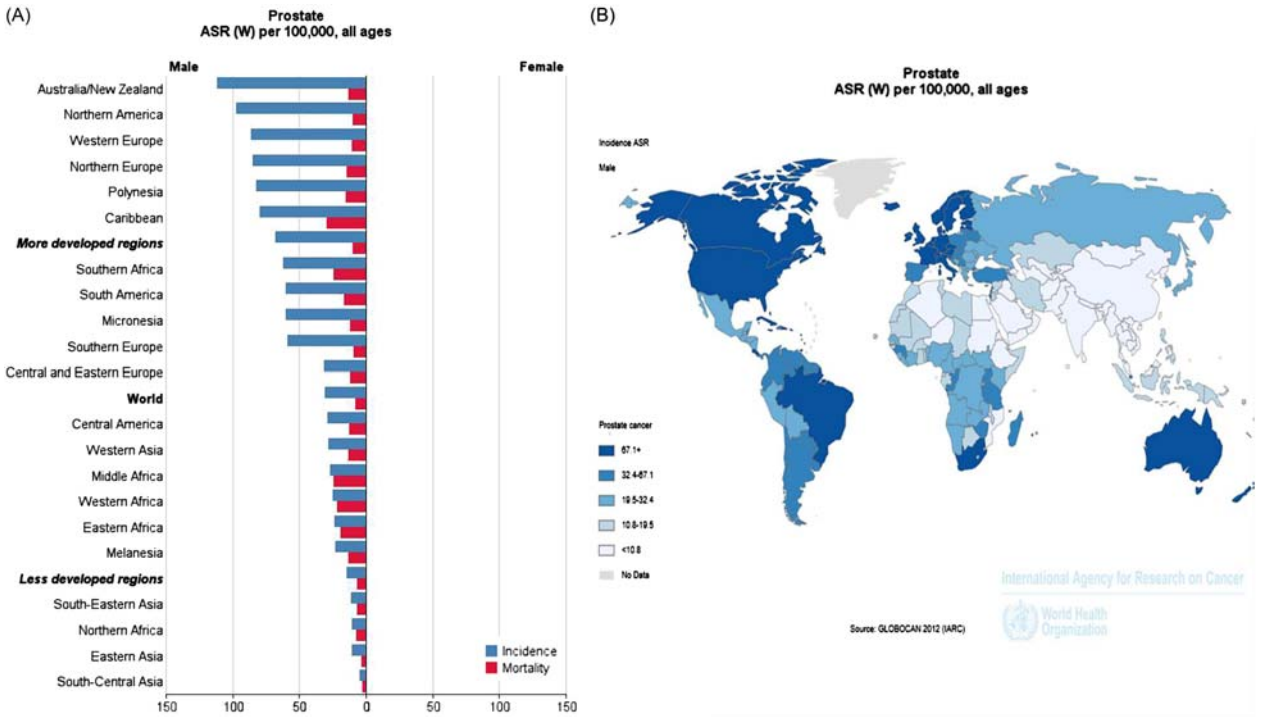

Fig. 1 Incidence and mortality of prostate cancer worldwide. (A) Age-standardized incidence and mortality rates (ASR) by gender and geographical area. (B) Incidence distribution worldwide. From Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. and Bray, F. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet].* Lyon, France: International Agency for Research on Cancer. Available from http://globocan.iarc.fr (accessed on February 20, 2018).

Table 1 Risk factors for prostate carcinoma

Carcinogenic agents classified by IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (volumes 1–120)
Agents of limited[a] evidence for prostate carcinogenicity in humans
Androgenic (anabolic) steroids
Arsenic and inorganic arsenic compounds
Cadmium and cadmium compounds
Malathion
Rubber production industry
Thorium-232 and its decay products
X-radiation, gamma-radiation
Red meat consumption
Other carcinogenic agents and lifestyle risk factors
Obesity
Animal fat consumption
Prostate inflammation due to sexually transmitted infections

[a]No agents with sufficient carcinogenicity evidence have been identified.

The risk of developing PCa increases with age. A number of lifestyle and environmental factors have been linked to an increased risk of prostate carcinoma. In particular, growing evidence points to dietary habits, such as consumption of animal fat and red meet, especially if cooked at high temperature or charbroiled, as risk factors. Obesity has been shown to be associated with an increased risk of high-grade PCa. Sexually transmitted infections (e.g. trichomoniasis, chlamydia) have been cited as potential initiators of predisposing prostate inflammation. However, pinpointing exact environmental carcinogens associated with an increased prostate cancer risk has proven to be difficult (**Table 1**).

Pathology and Genetics

Prostate tumors show considerable molecular heterogeneity. The most common genetic alteration are recurrent fusions involving *ETS* transcription factors, typically fusing the 5′ untranslated region of an androgen-regulated gene to an almost entire coding sequence of an *ETS* family member, which are found in approximately half of PSA-detected prostate cancers. *ETS* fusions have been shown to be associated with poor prognosis in population-based studies of watchful waiting cohorts. However, studies in radical prostatectomy patients have reported conflicting results. *ERG*, an *ETS* family member, has been identified as a potential therapeutic target in tumors with *ETS* fusions ("*ETS*-positive"). Inhibiting poly ADP-ribose polymerase I (PARPI), a protein which is critical for the ERG function, results in a decreased growth of *ETS*-positive prostate cancer xenografts.

The prostate cancer genome displays relatively few focal chromosomal gains or losses (most commonly focal loss at *PTEN*) and an overall low mutation rate compared with cancers at other sites. In localized cancers, the genes most frequently harboring point mutations are *SPOP*, *TP53*, and *PTEN*, whereas more advanced and metastatic tumors treated with androgen-targeting regimes show amplifications and point mutations of the *AR* gene, which may suggest that AR is not involved in PCa pathogenesis but appears with resistance to AR treatment. However, proteins regulating the AR function have been shown to be affected by mutations or otherwise dysregulated in prostate tumors, for example, the product of the *FOX1A* gene which is hyperactivated by point mutations in about 5% of prostate cancers.

SPOP mutations are mutually exclusive with the *TMPRSS-ERG* fusion and other *ETS* rearrangements as well as with *TP53* alterations. Moreover, *SPOP* mutations are usually associated with lack of abnormalities in the *PI3K/PTEN/Akt* pathway and a higher prevalence of *CHD1* deletions. Dysregulation of *PTEN* is the most common way of inactivating the *PI3K/PTEN/Akt* and is consistently associated with a poor prognosis (**Table 2**).

A model of progressive accumulation of genetic alterations in the prostate carcinogenesis is shown in **Fig. 2**.

Biomarkers

Prostate-Specific Antigen (PSA) Serum Levels

PSA is a glycoprotein secreted by prostatic epithelial cells. Its protease activity lyses clotted ejaculate to increase sperm motility. PSA enters the circulation through unknown mechanisms and anti-PSA antibodies can be measured in blood serum using commercially available assays. As there is no single standard for comparing the results of these tests, repeat testing to monitor changes in serum PSA levels is recommended. PSA testing is a widely accepted method of population screening for prostate cancer (see section "**Presentation and Diagnosis**") A number of assays that combine PSA testing with measurements of other biomarkers are

Table 2 Gene loci that are most frequently altered in prostate carcinoma

Pathway	*Gene (locus)*	*Alteration type*	*Estimate*
MAPK/ERK signaling	Members of the *ETS* family	Recurrent gene fusions (most common: *TMMPRSS2-ERG* fusion)	Approximately 50% of tumors detected by PSA screening
P13K/PTEN/Akt signaling (control of cell metabolism)	*PTEN* (10q23)	Deletions and inactivating point mutations	40% and 45%–50% of prostate cancers, respectively; more common in advanced tumors
	PIK3CA (3q26)	Amplifications and point mutations	25% and 5%, respectively
Androgen signaling/*MAPK/ERK* signaling	*AR* (Xq12)	Amplifications and point mutations	40% and 50% of treated metastatic tumors respectively; alterations absent in localized disease
p53 signaling	*TP53* (17p13.1)	Deletions and point mutations	Deletions in 25%–40% and point mutations in 5%–40% of tumors; more common in metastatic disease but 25%–30% localized tumors harbor some *TP53* alterations
Hedgehog signaling	*SPOP* (17q21.33)	Point mutations	6% to 15% of primary prostate cancers
Chromatin remodeling and transcription control	*CHD1* (5q15–q21)	Deletions, rearrangements and point mutations	Deletions in 10%–25% of prostate tumors (both primary and metastatic); restricted to *ETS*-negative tumors

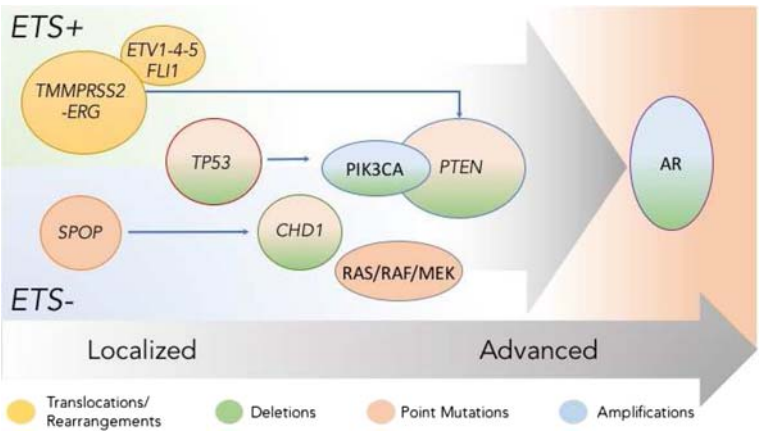

Fig. 2 Temporal sequence of genetic events in the progression of prostate cancer (PCa). Genes with frequent somatic alterations are shown. Progression is represented by a *gray arrow*. Conversion to androgen resistance in advanced lesions is shown by a change in the background color. This model distinguishes tumors with rearrangements in *ETS* gene family (*ETS*+) and without such rearrangements (*ETS*−). Somatic inactivations of *TP53* and/or *PTEN* are common events in both pathways. Modified from Barbieri et al. (2015). *Urologic Oncology: Seminars and Original Investigations* **33**, 95–102.

commercially available (Table 3). Serum PSA measurement is also used for monitoring PCa patients for recurrence following primary treatment as well as to evaluate response to treatment and the probability of distant metastases.

PCA3 Levels and the *ERG* Gene Rearrangements

Prostate cancer gene 3 (PCA3) is a prostate-specific, non-coding mRNA biomarker which is detectable in urine sediments obtained during DRE and can be measured by real-time PCR. It is highly overexpressed in prostatic adenocarcinoma as compared to normal prostate and benign prostatic hyperplasia. However, due to difficulties in determining the cut-off values, and to the complexity and cost of the test, it cannot replace PSA testing as an initial screening or a diagnostic test. As PCA3 score increases with PCa volume, it has been suggested it could be used as a monitoring marker but there are conflicting data on whether it independently predicts Gleason score and so its usefulness for active disease surveillance has not yet been confirmed.

ERG is a member of the family of the ETS transcription factors and is affected by gene rearrangements in about a half of prostate cancers. Currently, two commercially available assays (ExoDx Prostate and Mi-Prostate Score) aim to improve prostate cancer detection and risk stratification based on measuring the expression of the *ERG* gene in combination with that of PCA3.

Table 3 Biomarkers and tissue-based tests for the detection and management of prostate carcinoma

Biomarker	*Characteristics and clinical utility*
Early detection (screening) biomarkers	
Prostate-specific antigen (PSA) serum levels	Changes in serum PSA levels upon repeated testing, in particular in conjunction with abnormal digital rectal examination, indicate a suspicion of prostate cancer (FDA-approved)
PCA3 in urine	Useful in determining the need for a repeat biopsy (FDA-approved); clinical usefulness of commercial kits measuring the PCA3 expression in combination with testing for *ERG* rearrangements [ExoDx *Prostate(IntelliScore)* (EPI) and *Mi-Prostate Score* (MiPS)] is still under study
Prostate Health Index (PHI)	A combination of tPSA, fPSA, and proPSA tests; discriminates between high-grade and low-grade tumors/negative biopsies; predicts aggressive cancers (FDA-approved)
ConfirmMDx (MDxHealth, USA)	A multiplex epigenetic assay measuring hypermethylation of *GSTP1*, *APC*, and *RASSF1* promoter regions in core biopsy tissue samples; aims to improve stratification of men considered for repeat prostate biopsy
DLX1 and *HOXC6* expression in urine	The *SelectMDx* commercial kit (MDxHealth) used to perform this measurement on post-DRE urine; aims to identify men with clinically significant (more aggressive) prostate cancer prior to biopsy; still at an investigational stage
Prognostic biomarkers: tissue-based prognostic tests	
OncotypeDX genomic prostate score (Genomic Health, USA)	Testing the expression of 12 prostate cancer-related genes and 5 housekeeping genes; a potential tool for predicting recurrence and time-to-recurrence following a biopsy in very low and low risk PCa patients considered for active surveillance versus definitive therapy
Prolaris (Myriad Genetics Inc., USA)	Measures the expression of 31 genes related to cell-cycle and 15 housekeeping genes; a potential tool for predicting mortality, recurrence, and metastasis following a biopsy in very low and low risk PCa patients considered for active surveillance versus definitive therapy
Promark® (Metamark, USA)	Quantitates the expression of eight protein biomarkers correlated with disease aggressiveness; a potential tool for predicting mortality, recurrence, and metastasis following a biopsy in very low and low risk PCa patients considered for active surveillance versus definitive therapy
Decipher (GenomeDX, Canada)	Measures the expression of 22 genes involved in multiple biological pathways and associated with aggressive prostate cancer; predicts metastasis following radical prostatectomy

DRE, digital rectal examination; *FDA*, US Food and Drug Administration.

Germline Mutations in *BRCA1* and *BRCA2*, and in Other DNA Repair Genes

Recent reports suggest that men suffering from inherited syndromes associated with germline mutations in DNA repair genes are at an increased risk of prostate cancer. In particular, carriers of *BRCA1* and *BRCA2* germline mutations have a higher risk of earlier-onset prostate cancer and cancers in these patients have a more aggressive phenotype associated with reduced survival.

Gene Expression Profiles as Prognostic Biomarkers

The progression from benign prostate lesions to aggressive prostate cancers involves alterations in the expression of multiple genes involved in different cellular pathways. A number of commercial assays aiming to predict recurrence-free survival, the probability of recurrence and metastases, the aggressiveness of the tumor, and/or disease-related mortality based on cancer gene signatures are available (Table 3).

Predictive Biomarkers

Given a plethora of evidence-based therapeutic sequences for treatment of prostate cancer, the precision medicine approach seems to be the choice of the future. However, no effective predictive biomarkers have been validated up to date. Current search for these biomarkers turns to the detection of circulating tumor cells and cell-free DNA in the blood stream ("liquid biopsies") rather than tissue-based assays.

Management and Therapy

The management of all stages of prostate cancer is controversial. Given that the disease occurs in older men, deferred treatment is often recommended, in particular for lower stages localized carcinoma. A life expectancy of at least 10 years is considered mandatory for any benefit of local treatment. For elderly patients with limited life expectancy, watchful waiting with symptom-guided treatments to maintain life quality despite progressing disease is usually considered the most appropriate, regardless of the disease stage. For younger patients with longer life expectancy and low-risk localized disease, active surveillance (sometimes also called expectant management) is the first-choice treatment. In contrast to watchful waiting which aims to delay palliative treatments, active surveillance has a curative intent: it aims to achieve correct timing for curative treatment considering individual life expectancy of each patient. However, precise risk assessment for patients with longer life expectancy (and so potentially candidates for active surveillance) remains a challenge. A number of biomarkers exist (see section "**Biomarkers**") and numerous models, including nomograms which take into account different patient and tumor parameters, have been used in clinical practice but none of them has been clearly shown to be optimal or superior to others.

The decision to switch to active treatment is taken based on changes in biopsy results or T stage progression, or at the patient's request. Surgery and radiotherapy (RT) are usually the first treatment choices under consideration.

Radical prostatectomy is a common choice for primary treatment. It is appropriate for any patient whose cancer clinically appears as localized to the prostate. The goal of radical prostatectomy is to eradicate the disease, while preserving continence and potency whenever possible. Open prostatectomy can be performed with retropubic or perineal approach. The two approaches seem to be equivalent in terms of long-term cancer control outcomes. Laparoscopic and robot-assisted radical prostatectomy (RARP) are commonly used alternatives to open prostatectomy. If performed by experiences surgeons, they are usually considered to be comparable, even though some recent data suggest that the robot-assisted approach may be associated with perioperative benefits and a quicker recovery after surgery. Return of urinary continence after radical prostatectomy may be improved by preserving the urethra beyond the prostatic apex and by avoiding damage to the distal sphincter mechanism, while preserving bladder neck may allow quicker recovery of urinary control. Pelvic lymph node dissection is simultaneously performed in case of high-risk disease and/or advanced disease with nodal involvement.

External beam RT (EBRT) as primary treatment is an alternative to surgery, giving the same oncological outcomes at 10 years follow-up as radical prostatectomy. Intensity-modulated radiation therapy (IMRT), the second-generation 3D radiotherapy technique, is increasingly used in clinical practice because of reduced risk of gastrointestinal toxicities. Brachytherapy, that is placing sources of radioactivity in the prostate tissue, is traditionally used in patients with low-risk disease. Some earlier studies found it less effective than EBRT for high-risk disease. However, increasing evidence suggests that technical advancements in the contemporary brachytherapy may make it appropriate also for treatment of high-risk PCa patients. Brachytherapy can also be considered in men with biochemical recurrence (i.e. increasing PSA levels but no clinical evidence of recurrence; sometimes also called PSA recurrence) after EBRT.

Other options for primary treatment of localized PCa include cryosurgery, high-intensity focused ultrasound (HIFU) and focal therapy (brachytherapy or CyberKnife Robotic Radiosurgery). Cryosurgery (also called cryotherapy or cryoablation) is a minimally invasive treatment that damages tumor tissue through local freezing. Focal therapy of any sort appears promising but remains investigational. Therefore, the European Association of Urology recommends to offer focal therapy, as well as cryotherapy and HIFU only in clinical trial settings.

In advanced disease, androgen deprivation therapy (ADT) is administered as primary systemic treatment. It remains the gold standard for initial treatment of patients presenting with metastatic disease. ADT is also used as neoadjuvant or adjuvant treatment combined with radiation in localized or locally advanced PCa.

Androgen deprivation can be achieved either by suppressing the secretion of testicular androgens or by inhibiting the action of circulating androgens at the level of their receptors. Suppressing androgen secretion can be obtained by surgical castration (bilateral orchiectomy) or by using agonists of the luteinizing hormone-releasing hormone (LHRH; also called gonadotropin-releasing hormone; "medical castration"). The two methods are equally effective, however some recent data suggest that surgical castration may be safer. These two methods can also be combined to achieve complete androgen blockade (also called combined androgen blockade, CAB). The use of LHRH antagonists (e.g. degarelix) has the advantage of avoiding the phenomenon of testosterone surge ("flare-up", the initial release of LH and FSH) which is associated with the use of LHRH agonists. Blocking androgen receptors can be obtained by using synthetic antiandrogens. These can be either steroidal (e.g. cyproterone acetate, CPA) or nonsteroidal compounds (e.g. flutamide or bicalutamide).

ADT is associated with substantial side effects, like decreased bone mineral density and increased risk of cardiovascular disease, which usually increase with the duration of the treatment. Moreover, developing castration resistance renders the treatment ineffective with time. Actually most patients with advanced disease eventually become castration-resistant (also called castration-recurrent), that is they stop responding to ADT. One of the approaches suggested to partially overcome this problem is intermittent ADT, that is administering cycles of androgen deprivation followed by reexposure to androgens, with the assumption that this may delay "androgen independence" and also reduce treatment-related morbidity. Furthermore, a number of novel hormonal therapeutics have also been developed with the aim to replace ADT in treatment of advanced and/or metastatic castration-resistant PCa. Out of those, the following two agents have been approved by the US Food and Drug Administration: abiraterone acetate (abiraterone; Zytiga®) and enzalutamide (Xtandi®). Abiraterone blocks androgen synthesis by inhibiting CYP17 and has been approved for treatment of metastatic castration-resistant PCa in combination with low-dose prednisone in patients who had received chemotherapy

with docetaxel. Enzalutamide is a novel antiandrogen with a higher affinity for the androgen receptor than bicalutamide. While nonsteroidal antiandrogens still allow transfer of androgens to the nucleus, enzalutamide also blocks androgen transfer and therefore suppresses any possible agonist-like activity. Enzalutamide has been approved for treatment of metastatic castration-resistant PCa in patients who had received chemotherapy with docetaxel and also for treatment of hormone-sensitive PCa in combination with ADT. Moreover, recent studies suggest that it may be a reasonable treatment option also in pre-docetaxel setting or in patients who are not candidates for chemotherapy.

Chemotherapy is also one of the treatment options in the management of PCa. Patients with progressive androgen-stimulated PCa and distant metastases are usually treated with docetaxel. Moreover, the guidelines of the US National Comprehensive Cancer Network (NCCN) recommend using docetaxel in combination with ADT and EBRT in fit patients with high-risk localized disease. Cabazitaxel, a semi-synthetic taxane derivative is approved by FDA for treatment of metastatic castration-resistant PCa in patients who had undergone a docetaxel-based therapy. The results of recent trials suggest that it may also be an alternative for patients who are not candidates for docetaxel treatment, in particular those who are intolerant of docetaxel or who have a preexisting mild peripheral neuropathy (cabazitaxel treatment is associated with lower rates of peripheral neuropathy than docetaxel regimens).

With the advent of new, targeted therapies, immunotherapy may also have a role in the treatment of PCa. Autologous dendritic cell vaccine sipuleucel-T (PROVENGE®) has been shown to extend overall survival in asymptomatic or minimally symptomatic metastatic castrate-resistant (hormone refractory) PCa patients and is approved by FDA for treatment of the disease meeting these criteria. Using checkpoint inhibitors for treatment of advanced PCa is currently being tested. Also some targeted molecular therapies currently tested in clinical trials seem promising. In particular, PARP1 inhibitors (like olaparib) have given encouraging results for treatment of metastatic castration-resistant PCa in patients with *BRCA1* and/or *BRCA2* mutations in phase II trials.

See also: Prostate Cancer: Pathology and Genetics.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.

European Association of Urology Guidelines on prostate Cancer, 2017: http://uroweb.org/guideline/prostate-cancer/ (accessed on January 12, 2018).

International Agency for Research on Cancer, 2016. In: Moch, H., Humphrey, P.A., Ulbright, T.M., Reuter, V.E. (Eds.), WHO classification of Tumours of the urinary system and male genital organs, 4th edn, 8. IARC Press, Lyon, France.

Kretschmer, A., Tilki, D., 2017. Biomarkers in prostate cancer - current clinical utility and future perspectives. Critical Reviews in Oncology/Hematology 120, 180–193.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Prostate Cancer. Version 2.2017, February 21, 2017a: https://www.nccn.org/professionals/physician_gls/default.aspx#site.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Prostate Cancer. Early Detection. Version 2. 2017September 15, 2017. https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Relevant Websites

http://www.auanet.org/—American Urological Association.

www.euroweb.org—European Association of Urology.

Prostate Cancer: Pathology and Genetics

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Nomenclature

Aka Also known as
ADT Androgen deprivation therapy
AR Androgen receptor
BPH Benign prostate hyperplasia
CD Cluster of differentiation
CRPC Castration resistant prostate carcinoma
IDC Intraductal carcinoma
ISUP International Society of Urological Pathology
mpMRI Multimodal magnetic resonance imaging
NE Neuroendocrine
PIN Prostate intraepithelial neoplasia

The Prostate Gland

Introduction

The goal of this article is to provide the current state of knowledge of the pathology of prostate cancer and of the genetic features of prostate cancer, primary and metastatic, and of the men who have prostate cancer. Starting with the anatomy of the prostate gland, we will provide basic information on the histology, immunophenotype, and molecular features of different cell types in the prostate and of prostate carcinoma cells. We will briefly discuss clinical implications, while focusing on the current standard of practice in diagnosing and pathologically characterizing prostate cancer. We will also present our thoughts on limitations of what is known currently and we will speculate on future directions. Recognizing the importance of standardizing terms across disciplines we will use Human Gene Ontology (HUGO) terms for specific molecules, acknowledging that some terms are not intuitive. For example, the HUGO term for "Prostate Specific Antigen" is KLK3.

Anatomy of the Prostate Gland

The prostate gland, which is positioned between the urinary bladder and penis, is a truncated, cone-shaped organ through which the urethra and the ejaculatory ducts pass. The prostate produces, stores, and, upon demand, secretes proteins which, combined with the proteins expressed in the seminal vesicle, forms the ejaculate. Enzymes in the ejaculate, including kallikreins, such as KLK3 (prostate specific antigen or "PSA"), liquefy the ejaculate to enable sperm motility. Histologically, the prostate is composed of three zones—peripheral, transition, and central zones. These are based on the branching pattern of the ducts and subtle histological differences of both the glands and the stroma of the zones. The zones differ in the frequency of being the site of prostate cancer or of benign prostatic hyperplasia (BPH), and in the frequency of histological variants of prostate carcinoma. BPH consists of non-neoplastic, hyperplastic nodules of parenchyma which enlarge the transition and central zones of the prostate. As a consequence, the increased mass of prostate parenchyma (termed BPH) can compress the urethra and bladder neck, leading to lower urinary tract syndrome (LUTS), or obstructive uropathy. Conversely, the peripheral zone, which is juxtaposed to the outer wall of the rectum and is the region of the prostate most accessible to biopsy, is the most frequent site of prostate carcinoma. Finally, the foamy gland histological variant of prostate cancer is found more frequently in the transition zone than in the peripheral zone.

Cell Types

The glands (aka acini and ducts) of the prostate are embedded in a fibromuscular stroma which is innervated by alpha adrenergic neurons. Two predominant cell types comprise the pseudostratified epithelium of the glands—luminal cells, which synthesize and secrete kallikreins and other proteins, and basal cells, the apparent precursor cells to luminal epithelial cells. Within the basal epithelium is a third cell type—the neuroendocrine cell. These cells comprise less than 1% of the epithelial cells of the prostate. The stromal cell population is composed predominantly of admixed fibroblasts, myofibroblasts, and smooth muscle cells. Less frequent are functionally specialized cells—nerve sheath cells, perineural cells, vessels (endothelial and smooth muscle cells), and cells of the hematopoietic/lymphoreticular system.

Genome of Normal Prostate

Cell phenotype

Cells of the prostate have distinct protein profiles, which are readily characterized immunohistochemically. By cell type the proteins are:

- *Luminal epithelial cells*: Intermediate filaments, low molecular keratins weight KRT 8, 18; enzymes KLK3 (aka PSA), ACPP (aka prostatic acid phosphatase); cell membrane proteins CD24, MME (aka CD10), B3GAT1 (aka CD57); transcription factors AR (androgen receptor), NKX 3.1.
- *Basal epithelial cells*: Intermediate filaments, high molecular weight keratins KRT 5/6, 7, 10, 20; cell membrane proteins CD44, 49b, 49c, 104; transcription factor TP63. Note that basal cells, which do not express contractile proteins smooth muscle actin, desmin or myosin, are not categorized as myoepithelial cells.
- *Neuroendocrine cells*: Membrane-bound organelles CHGA (chromogranin A) of endocrine granules and SYP (synaptophysin) of neuron vesicles; cell membrane protein NCAM1 (aka CD56), which is also expressed by prostate fibromuscular stromal cells, thus not being specific for neuroendocrine cells.
- *Smooth muscle stromal cells*: Intermediate filaments Des (desmin), VIM (vimentin), and DES (desmin); myogenic filaments ACTA2 (smooth muscle actin); cell membrane proteins ITGA5 (aka CD49e), NCAM1 (aka CD56), ITGB3 (aka CD61).
- *Fibroblasts*: Intermediate filament VIM (vimentin). Since vimentin is expressed by many cells types it is not a fibroblastic-specific marker.
- *Cells of the hematopoietic system*: Cell membrane proteins (primarily cluster of differentiation/CD biomarkers) CD45 (all lineages), CD's specific to basic hematopoietic cell types: granulocytes CD15, B lymphocytes CD19, T lymphocytes CD3 (including PD-1 of activated T cells), monocytes CD14 (including PDL1 of antigen presenting cells) and natural killer cells CD56, thrombocytes CD61. Of note the number and distribution of hematopoietic cell types in disease-free prostate tissue has not been determined. Thus, the number of any hematopoietic cell type that is regarded as abnormal and associated with disease is not known. Accordingly the term "chronic prostatitis" is not strictly defined as a tissue-based abnormality.

The protein phenotype of cells provides a basis for diagnosing prostate lesions using immunohistochemistry. For example, absence of gland-based cells expressing basal cell proteins KRT 5/6 or TP63 is evidence that the glands are carcinoma. Other proteins are ubiquitous, including cell cycle gene products (cyclin D1, E, MKI67), and DNA repair gene products, including mismatch repair gene proteins (MSH2, MSH6, MLH1, PMS1), and homologous recombination helicases and exonucleases. Of note, many markers are not specific for these nonneoplastic prostate cell types. For example, NCAM1 (CD56) is expressed by neuroendocrine, natural killer cells, and fibromuscular stromal cells.

Investigators have identified additional epithelial cells based on distinct keratin profiles, that is, intermediate epithelial cells—KRT 15, 17, 19; and transit amplifying cells—KRT 8, 19. No role for these subtypes of epithelial cells has been reported in prostate carcinoma.

Several potential considerations confound using immunohistochemical stains to identify cell types. Preanalytical variables include loss of analyte and consequent false negativity if fixation is inadequate to stop protein degradation. Conversely overfixation may impair immunoreactivity, that is, prolonged (weeks) fixation in formalin decreases keratin immunoreactivity. Incomplete characterization of the range of expression of a given analyte can result in inaccurate interpretations of immunostains. Likewise incomplete characterization of the range of immunoreactivity of a given antibody can also lead to false positivity. Use of monoclonal antibodies addresses some questions of specificity.

The Host (A Man With Prostate Carcinoma)

Demography

Prostate carcinoma is primarily a disease of old age, rarely diagnosed below the age of 40. Incidence and mortality are higher in African-Americans and lower in Japanese. Incidence is higher among family members—both of men who have prostate cancer and of women who have breast or ovarian cancer.

Environmental Factors

Numerous factors have been associated with patients who have prostate carcinoma, such as obesity, a diet that is high in carbohydrates, in total and saturated fat, and/or includes overcooked meat. In addition, the frequency of prostate carcinoma is increased in family members who immigrate to the United States from Japan. Whether this change in incidence is a consequence of exposure to a non-Japanese diet, to a change in lifestyle, or to use of more sensitive detection methods is unknown. Associating possible external sources of tumorigenesis is confounded by the high prevalence of undiagnosed prostate carcinoma in men, which makes unraveling of prostate cancer etiology a real challenge. Based on several autopsy studies, the proportion of males affected by prostate carcinoma is equal to their age. Another challenge in these studies is controlling for diet and environment in a disease which has such a long natural history.

Host Genomics

There is clinical evidence that a notable number of patients have heritable prostate carcinoma, evidenced by male family members having prostate carcinoma and female family members having breast and/or ovarian carcinoma.

- Linkage studies of family members have been undertaken to identify germ-line gene features. Genes that have been identified as possible prostate carcinoma susceptibility genes include HPC1, PCAP, HPCX, 17q21-22 (HOXB13). Case control-based studies identified other genes including androgen receptor gene variants, AMACR, CHEK2, EMSY, as susceptibility genes. However, results have been mixed. For example, the length of the polymorphic trinucleotide CAG and GGN microsatellite repeats in exon 1 of the AR gene, which is located on the X chromosome, have been associated with the risk of developing prostate carcinoma in some, but not other, studies. Based on genome wide association studies (GWAS) in population-based cohorts, more common low-penetrance genetic polymorphisms have been identified, including 3q26.2, 6q25.3, 8q24.21, 10q11.23, 11q13.3, and 17q12. Of note, SNP rs138042437 ($P = 1.7e\ (-8)$) at 8q24.21 achieved a large estimated effect size in this cohort (odds ratio = 13.3).
- Recent sequencing studies provide evidence that mutations in some DNA repair genes correlate with prostate carcinoma that carries a high risk of progression. More than 12% of patients with metastatic androgen deprivation resistant prostate carcinoma (also termed castration resistant prostate carcinoma or CRPC) have mutation(s) in a mismatch repair gene, that is, BRCA2, ATM, CHEK2, BRCA1, RAD51D or PALB2, or in a mismatch repair gene, that is, MSH2. The combined BRCA1/2 and ATM mutation carrier rate was significantly higher in patients who died of CRPC (6%) than in patients with localized prostate carcinoma (1.4%). Clinics to screen for carriers of DNA repair gene mutations have opened. The goal is to treat these patients earlier in the course of a possibly high risk prostate adenocarcinoma. In addition, patients with CRPC who have germ-line pathogenic variants in BRCA2 and other DNA repair genes have higher response rates to PARP inhibitors and platinum compounds than to taxanes.

Prostate Carcinoma

Primary Prostate Carcinoma

Clinical presentation

In 2014, the number of men presenting with newly diagnosed prostate carcinoma in North America was 230,000, of which 95% of cancers were localized to the prostate. At initial diagnosis 5% were already metastatic; 20,000 men died of prostate carcinoma. The number of patients presenting with clinically localized disease was equal to the number of patients with metastatic disease at presentation.

Screening

Historically, the primary modality used to screen for prostate carcinoma was a digital rectal examination. Firm prostate nodules were biopsied. However, most nodules were benign—firm regions of nodular hyperplasia of the prostate in BPH. There is no evidence that these nodules of BPH have malignant potential. Prostate carcinoma in many patients manifested clinically as painful metastases to bones. Approximately three decades ago clinicians began using levels of serum KLK3 (PSA) to screen for prostate carcinoma. A serum value of 4 ng/mL of PSA best distinguished populations of men with clinically manifest prostate carcinoma from men without clinical evidence of prostate carcinoma. Widespread use serum PSA to screen men resulted in increased discovery of predominantly clinically occult prostate carcinomas and a decrease in the proportion of men with metastatic prostate carcinoma. Modifications of the serum PSA assay, such as PSA velocity (rate of change of serum PSA over months), PSA density (PSA concentration normalized to the size of the prostate), free:bound PSA ratios and the 4-kallikrein panel have not significantly improved the precision of detecting high-risk prostate carcinoma. Based on a series of 1200 patients treated by radical prostatectomy, serum PSA was a better measurement of the size of the prostate than it is a detector of high risk prostate carcinoma. Recognition that many prostate carcinomas were small, of low Gleason grade, and were limited to the prostate and, thus, of low-risk of progression, has led to ever more widespread implementation of active surveillance programs. The goal of these programs is to minimize complications of treating men whose cancer would not progress if not treated and optimize treatment of men whose cancer would progress if not treated. The typical design of an active surveillance program is to examine the patient on a regular schedule, during which biopsies are taken and serum PSA is assayed. Intent-to-cure treatment is considered if the number of biopsies with cancer or the grade of the cancer is greater than that of the study entry biopsy.

The current state of PSA screening is summarized in two reports—in the Cochrane Database of Systematic Reviews and by the US Preventive Services Task Force. The latter organization estimates that "of 1000 men (55–69 years old) offered PSA-based screening, no more than two will avoid death from prostate cancer" if they receive intent-to-cure therapy. We expect a consequent decline in per capita incidences of PSA testing, prostate biopsy, and definitive therapy (radical prostatectomy and radiation therapy). It should not be surprising that the majority of prostate carcinomas do not lead to death, since the prevalence of prostate carcinoma in a large forensic study is equal to the age of the man (e.g., 80% of 80 years old men has prostate cancer). And most men die of cardiovascular disease.

Biopsy

An elevated serum PSA level, abnormal DRE exam and/or family history of prostate or of breast cancer are the most common indications for a prostate biopsy. The current procedure is to obtain 12 needle core biopsies (2.2 cm long, 0.9 mm diameter) which systematically sample the prostate gland using a probe with a built-in ultrasound imaging camera to help the urologist position the biopsy instrument. Since the biopsy is directed transrectally, the anterior region (which is predominantly transition zone) is undersampled. Consequently the volume and grade of some anterior cancers are underestimated. More thorough sampling of the prostate by needle biopsy can be done by saturation biopsies, that is, more than 50 symmetrically distributed biopsies, done under general anesthesia in patients from whom multiple previous biopsies failed to detect carcinoma and the clinical suspicion that there is carcinoma is high.

Recently multiparametric proton-based magnetic resonance imaging (mpMRI) in conjunction with ultrasound imaging (MRI/US fusion) has been used to target biopsies to hypothetically cancer enriched areas of the prostate and potentially increase the rate of detection of clinically significant, high risk of progression cancer. The rationale that proton MRI will detect cancer is based on the observation that tumor cell lines have a higher water content than normal tissues. The performance of this expensive imaging modality in distinguishing carcinoma, particularly high grade carcinoma, from benign prostate parenchyma has not been rigorously tested. One limitation of mpMRI is that spatial resolution is no greater than 3 mm, whereas individual cancer cells have a maximum dimension of 0.03 mm. Consequently a cancer consisting only of Gleason pattern 5 cells (the highest risk cancer) may not be detected. Furthermore, histologically distinguishing some benign entities, such as adenosis, from low-grade cancer can be virtually impossible without immunohistochemically determining whether the glands have a basal cell population.

Needle biopsies are the most common way tissue for diagnosing prostate cancer is obtained. Transurethral resections of prostate tissue, typically done to alleviate the clinical symptoms of lower urinary tract obstruction, can also reveal prostate cancer. These cancers are generally of low grade, of transition zone origin and are incidental, without clinical significance.

Histological architecture

The large majority ($>95\%$) of carcinomas of the prostate are acinar adenocarcinomas. Subtypes of prostate carcinomas are neuroendocrine carcinoma, ductal adenocarcinoma, and the sarcomatoid component of some acinar adenocarcinomas. Less frequent are other cancers which involve the prostate–urothelial carcinoma, which originates in the urothelium of prostatic urethra or of the urinary bladder, sarcomas (most common are leiomyosarcoma in adults and rhabdomyosarcoma in infants), and lymphoma (virtually all originate in lymph nodes and secondarily involve the prostate). We shall focus on primary carcinomas of the prostate-acinar adenocarcinoma, ductal adenocarcinoma, and neuroendocrine carcinoma.

Acinar Adenocarcinoma

The histological architecture of prostate acinar adenocarcinoma is variable. Adenocarcinoma is histologically distinguished from benign prostate glands by histological features viewed at low ($20\times$–$100\times$) and high ($200\times$) magnification. At low magnification, the architecture is disrupted, with loss of the normal lobular distribution of profiles of prostate glands and an infiltrative growth pattern between and around benign glands. At high magnification, large nuclei, prominent nucleoli, and a more basophilic cytoplasm characterize carcinoma. Other histologic features help make the diagnosis—intraluminal corpora amylacea (round aggregates of proteins, predominantly lactoferrin from neutrophils), characterizes benign glands; conversely, intraluminal crystalloids (of unknown chemical composition) and mucin are often seen in the lumens of cancer glands. The pathognomonic feature of prostate adenocarcinoma is absence of a basal cell population, which is a specific component of benign glands. Since identifying basal cells by light microscopy is sometimes problematic, immunohistochemical staining of tissue sections for basal cell markers KRT 5/6 or TP63 can improve diagnostic precision. Two other useful immunohistochemical markers are the enzyme AMACR (alpha-methylacyl-coA-racemase) and the transcription factor erg (overexpressed, consequent to the translocation of the TMPRSS2 gene promoter to the erg gene). These proteins are expressed by more than 90% and 45% of primary and metastatic prostate carcinomas, respectively. Notable is nonspecificity of these markers. Some benign glands express AMACR at a level of staining intensity similar to that of prostate carcinoma cells. And erg is expressed by endothelial cells and by some sarcomas.

Additional architectural features of adenocarcinoma provide the basis for grading. The current system for grading, formulated in the 1980s, is the Gleason system. The system is based solely on the architecture of prostate carcinoma in prostates which have not been subjected to systemic or radiation therapy. Cytologic features of carcinoma cells, that is, nuclear size, nuclear pleomorphism, nucleolar size, are not relevant for grading. By convention small cell/neuroendocrine carcinoma is not graded. The cells of prostate carcinoma can form many different histological patterns. These have been categorized into five patterns, termed Gleason patterns 1–5. The grade (or "score") of a carcinoma is the sum of the most frequent pattern and second most frequent pattern. Though the patterns are numbered, they are not continuous variables. Thus, there is no such entity as an average Gleason score. And analyses of a population of carcinomas of different Gleason scores are most appropriately done using a nonparametric statistical test. As currently defined, patterns 1–3 are "well-formed" glands consisting of a cluster of cells. Pattern 3 carcinoma infiltrates between benign glands; this architecture contrasts with patterns 1 and 2 glands, which form circumscribed aggregates of glands (pattern 1) from which foci of glands extend (pattern 2). There are different histologic forms of both patterns 4 and 5. Included in pattern 4 are four forms—poorly formed ("ill-formed") glands, fused glands, cribriform glands, and glomeruloid structures. Pattern 5 can appear as three forms—single cells, sheets of cells without histological evidence of gland formation and comedocarcinoma (**Figs. 1 and 2**).

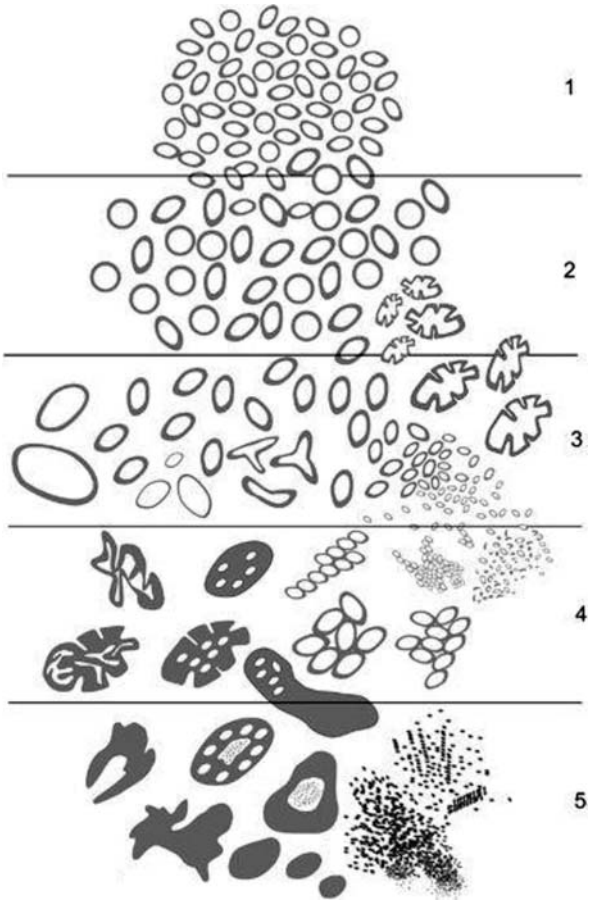

Fig. 1 Current modified schematic Gleason diagram (courtesy of David Grignon, Indiana University Medical Center). Schematic of grade patterns of prostate adenocarcinoma, modified from the original Gleason schematic of grade patterns. From Epstein, J. I. (2018). Prostate cancer grading: A decade after the 2005 modified system. *Modern Pathology* **31**, S47–S63, with permission.

At consensus meetings sponsored by the International Society of Urological Pathology (ISUP), the grading system has been refined. One refinement that had a potentially profound effect on both population studies and on the clinical management of individual patients is virtual exclusion of patterns 1 and 2 from the Gleason score of carcinoma in needle biopsies. The rationale is that extension of carcinoma glands between benign glands excludes categorizing a carcinoma as pattern 1 or 2 since the diameter of the current needle biopsy (less than 0.1 cm in diameter) is less than most clusters of prostate carcinoma glands. Furthermore, many cancers graded as pattern 1 or 2 were probably "adenosis" (a benign proliferation of prostate glands) since immunohistochemical stains, used to more accurately distinguish prostate carcinoma from its mimics, were not used when the Gleason system was developed. Adoption of this principle has resulted in grade shifting (colloquially termed the Will Rogers phenomenon).

A rule of thumb for investigators studying a population of men with prostate carcinoma is to consider upgrading to pattern 3 all carcinomas which have been assigned patterns 1 or 2. An exception to this rule would be specimens of a size sufficient for the pathologist to assess the periphery of a cluster of carcinoma glands. Such specimens are typically the tissue ("chips") of transurethral resections and carcinoma within the transition zone of prostatectomy specimens. Another refinement is changing the definition of the secondary pattern in a biopsy sample from the second most frequent pattern to the pattern that has the highest numerical value. Thus a cancer which is a Gleason $3 + 4$ carcinoma with a minor (or, tertiary) pattern 5 is graded as a Gleason $3 + 5 = 8$ carcinoma. A refinement which has less effect on population studies, though equivalent effect on the care of individual patients, is categorizing all carcinoma glands with a cribriform architecture as Gleason pattern 4. In a previous iteration of the Gleason system uniformly round or ellipsoidal cribriform glands were regarded as pattern 3.

Ductal Adenocarcinoma

The cells of ductal adenocarcinoma, a variant of prostate carcinoma, have a defined histology—ellipsoidal cells with nuclear atypia form a pseudostratified. Ductal adenocarcinoma glands can have different architecture. Most common are papillary and cribriform. Less common are solid and gland-like patterns. While this histological variant of prostate carcinoma is rare, occurring in up to 3% of cancers, it is most frequently a component of an acinar adenocarcinoma. Pure ductal adenocarcinoma represents no more than

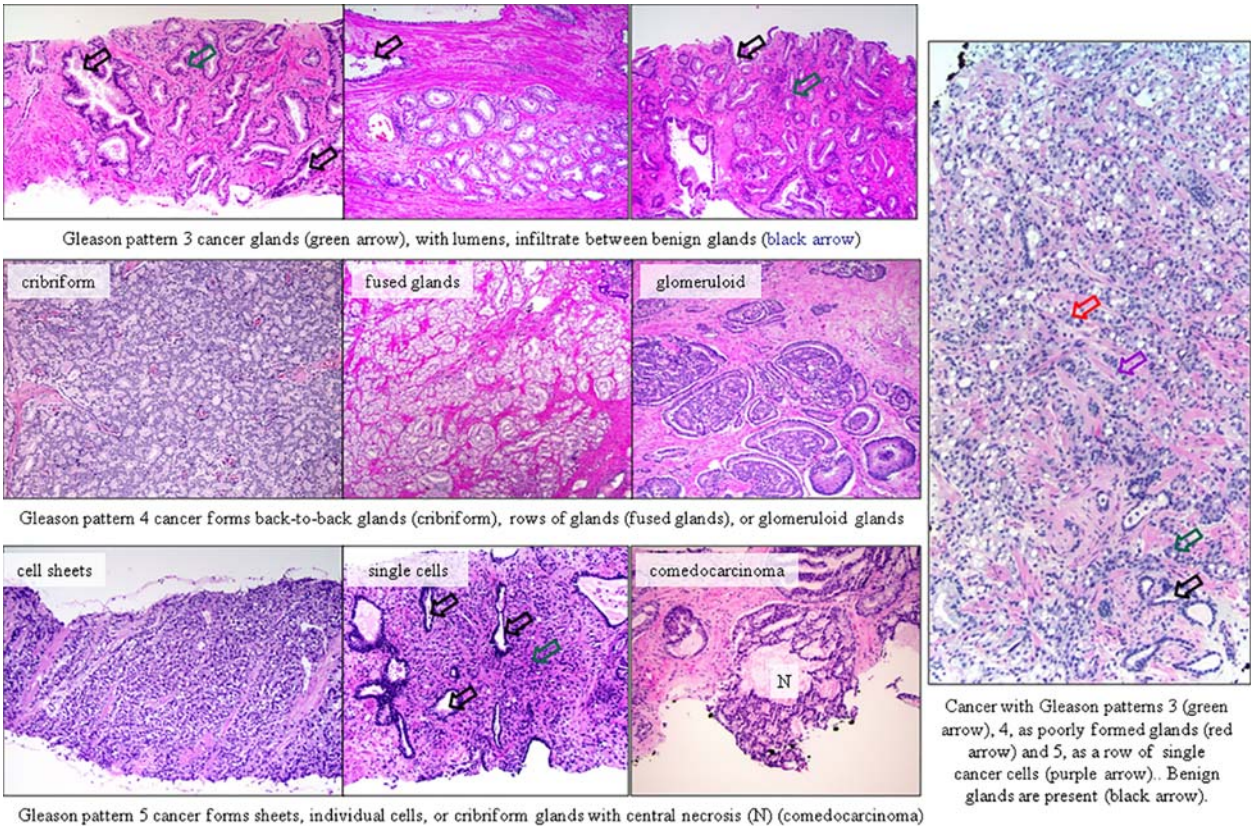

Fig. 2 Gleason patterns. Montage of photographs of different histological architectures of prostate adenocarcinoma, categorized by grade pattern.

0.2% of prostate cancers. Since the molecular phenotype of ductal adenocarcinoma extensively overlaps with acinar adenocarcinoma, we might question whether it should be categorized as a separate histological entity.

Neuroendocrine Carcinoma

Based on a consensus meeting sponsored by the Prostate Cancer Foundation, and subsequently accepted by the ISUP, neuroendocrine (NE) carcinoma has five histological phenotypes—mixed neuroendocrine-acinar adenocarcinoma, small cell carcinoma, and three very rare phenotypes—carcinoid, large cell NE carcinoma, and adenocarcinoma with Paneth cell-like NE differentiation. Tumor cells with a NE phenotype have a distinct protein profile—expressing immunoreactive SYP and/or CHGA and lacking androgen pathway gene products AR, NKX3.1, PSA, and ACPP. Most clinically distinctive is pure small cell carcinoma. Since this highly proliferative tumor (the MKI67 positive tumor cell fraction is at least 80%) does not express androgen pathway gene biomarkers, the organ of origin of metastases of pure small cell carcinoma of prostate cannot be pathologically distinguished from small cell NE carcinoma of more common sites of origin, such as the lung. One exception to this observation is those NE carcinomas which have the TMPRSS2-erg rearrangement. Expression of erg immunoreactivity, by immunohistochemistry or by FISH, is strong evidence that a neuroendocrine carcinoma is of prostate origin, once erg-expressing tumors, such as some sarcomas, have been ruled out. A clinically significant consequence of AR deficiency is that these tumors are not sensitive to androgen deprivation therapy (ADT).

Intraductal Carcinoma

Intraductal carcinoma (IDC) is a proliferation of cells that have the cytological features of acinar adenocarcinoma but which are confined to an acinus. Typically IDC expands the acinus. The most common architecture of IDC is cribriform. The presence of IDC correlates with adenocarcinoma that is high volume and high grade. Mechanistically, IDC is thought to be extension of invasive adenocarcinoma into an acinus of a benign gland. IDC is a microscopic lesion—a truly minor component of adenocarcinoma. IDC is histologically distinctive, to be distinguishable from both PIN and ductal adenocarcinoma.

Prostate Intraepithelial Neoplasia (PIN)

PIN is thought to be the preinvasive state of acinar adenocarcinoma due to similar molecular and cytological features. PIN is characterized by a proliferation of cells that have the cytological of acinar adenocarcinoma but that are confined to an acinus. PIN cells form a pseudostratified epithelium that usually has a papillary architecture. The presence of PIN in a prostate biopsy is evidence that there is a prostate adenocarcinoma, of any grade or volume, within the prostate.

Histological Mimics

Knowing the histological mimics of prostate carcinoma is important so that a patient is treated appropriately. We briefly summarize the histological mimics, to make the reader aware of the challenge a pathologist has in correctly classifying a histological lesion of the prostate parenchyma.

Differential diagnosis

A wide range of cell and tissue entities histologically mimic prostate carcinoma. Correct identification of these mimics relies upon knowledge of these entities and, often, use of appropriate immunohistochemical stains.

Small glands

- Simple atrophy versus acinar adenocarcinoma (Gleason pattern 3) or acinar adenocarcinoma (poorly formed gland variant of Gleason pattern 4)
- Adenosis versus acinar adenocarcinoma (Gleason pattern 3)

Large glands

- Cribriform hyperplasia versus acinar adenocarcinoma (cribriform variant of Gleason pattern 4)
- Hyperplasia versus PIN or pseudohyperplastic acinar adenocarcinoma (Gleason pattern 3)
- Seminal vesicle and ejaculatory duct versus acinar adenocarcinoma (Gleason pattern 3)

Diffuse cell infiltrates

- Acinar adenocarcinoma, Gleason pattern 5 versus hematopoietic cell infiltrate (lymphocytic inflammation, granulomatous prostatitis or lymphoma)
- Acinar adenocarcinoma, Gleason pattern 5 versus small cell and large cell neuroendocrine carcinoma

Spindle cell infiltrate

- Sarcomatoid component of prostate adenocarcinoma or of urothelial carcinoma versus benign stromal cell nodule, inflammatory myofibroblastic pseudotumor, leiomyoma or malignant sarcoma

Nuclear structure

The typical microscopic image of nuclei of prostate cancer cells is that of enlarged, round nuclei with prominent, round nucleoli. This iconic image, which is neither specific for carcinoma nor associated with all prostate carcinomas, contributes to the basis of the microscopic diagnosis of prostate cancer. Confounding the specificity of large nucleoli for carcinoma are histological variants of prostate carcinoma, including the foamy gland variant of primary prostate carcinoma and androgen deprived prostate carcinoma, both of which have small nuclei and nucleoli, and the large nucleoli of some benign hyperplastic basal cells.

Genomics of Primary Prostate Carcinoma

- *Acinar adenocarcinoma*. Prostate adenocarcinoma cells have a phenotype which, in general, recapitulates that of the nonneoplastic luminal epithelial cells: Intermediate filaments, low molecular keratins weight KRT 8, 18; enzymes KLK3 (aka PSA), ACPP (aka prostatic acid phosphatase), cell membrane proteins CD24, MME (aka CD10), B3GAT1 (aka CD57), transcription factors AR (androgen receptor), NKX 3.1. Of note, the protein of some genes is expressed at higher levels in carcinoma, that is, AMACR (alpha-methylacyl-coA-racemase), CD24, and HPN (hepsin). Of these, a subset are expressed at higher levels in higher grade carcinoma than in lower grade carcinoma, that is, MAOA (aka monoamine oxidase a) and DAD1. In addition to transcript features, there are DNA abnormalities in many prostate carcinomas. Changes in DNA involve smaller regions of the genome than can be detected by flow cytometry. Most primary prostate carcinomas are DNA diploid. A small number of carcinomas are DNA tetraploid. DNA aneuploidy is rare. There is hemizygous loss or mutation of PTEN in approximately 40% of acinar adenocarcinomas. Androgen receptor amplification and selection of AR variants, that is, ARv7 and ARv567, increase in frequency in androgen deprivation. The NCI Cancer Genome Anatomy Project (TCGA) characterized the molecular features of more than

300 primary prostate acinar adenocarcinomas. The majority of tumors could be categorized into seven molecular subtypes. Approximately half involved fusion of a promoter to an ets family gene (erg, etv1, or etv4). The other categories were fusion to fli1 and mutations of SPOP, FOXA1, and IDH1. Twenty-five percentage of these carcinomas had a potentially actionable abnormality in the PI3K and/or MAP kinase signaling pathway. DNA repair genes were mutated in 20% of cases. In contrast to metastatic CRPC, there is little evidence of AR variants in primary prostate adenocarcinoma. A subsequent small study reported mutations in DNA repair genes—mismatch repair and homologous recombination genes—in ductal adenocarcinomas of the prostate. Confirmation of this latter study awaits a larger study.

- *Neuroendocrine carcinoma*. The genotype is, in large part, distinctive. Neuroendocrine carcinomas are characterized by loss of RB and of TP53 and virtually all genes and gene products in the androgen pathway—AR, PSA, NKX3.1, hepsin, APCC—and increase expression of AURKA and myc. One exception is that the TMPRSS2-erg rearrangement, which characterizes 45% of acinar adenocarcinomas. This rearrangement is also present in a large number of NE carcinomas.

Prognosis and Prediction

There are two categories of tissue biomarkers—predictive and prognostic. Predictive biomarkers predict response to a given therapy. Prognostic predict clinical outcome, that is, time to biochemical failure (evidenced by posttreatment rising serum PSA), time to metastasis, time to death.

Prognostic parameters

Histological grade

Assigning a grade to a primary acinar (gland forming) adenocarcinoma of the prostate is one of the most clinically important activities of pathologists. The three basic parameters—tumor grade, clinical stage, and serum PSA—stratify patients for prognosis, clinical outcome, and treatment. To generalize, patients with Grade Group 1 carcinoma are candidates for active surveillance. Patients with Grade Group 2 or 3 carcinoma are candidates for intent-to-cure therapy (radical prostatectomy, external beam radiotherapy, or implantation of radiation-emitting "seeds"/brachytherapy). Patients with Grade Group 4 or 5 carcinoma may be offered neoadjuvant therapy prior to intent-to-cure therapy, or may be treated with androgen deprivation. Nomograms of the three basic parameters, supplemented with additional parameters, such as patient age, location of needle biopsies with cancer, amount of cancer in each biopsy, have been developed by different groups, that is, Kattan nomogram, Partin tables, UCSF-Capra score. These prognostic tables are applicable to both biopsies and to prostatectomies.

Grade groups

A recent development is reporting the Gleason score as a Grade Group, of which there are five. Grade Groups correlate as well with clinical outcome as do Gleason scores. The rationale for reporting a carcinoma as a Grade Group is a simpler value and clearer communication of pathologic grade. For example, Grade Group 4 includes Gleason score 4 + 4, score 3 + 5, and score 5 + 3 carcinomas. The consequences of using grade groups are, in general, beneficial. The Grade Group of the carcinoma which has the lowest frequency of progression is 1. These carcinoma are Gleason score 6 carcinomas. Telling a patient with a prostate carcinoma that his carcinoma is Grade Group 1 instead of Gleason score 6 conveys a more "benign" message.

Composite grade

A standard practice of many pathologists has been to grade each core. The clinician then has used the highest grade of a core as the basis for managing the patient. An alternative is for the pathologist to report a single composite grade, which is based on the relative amount of cancer of each grade in each biopsy, in addition to reporting the grade of cancer in each core. The composite grade correlates better with the grade of carcinoma in the whole prostate at prostatectomy than does the highest grade of a core. The composite grade seems more intuitive since, with few exceptions, the cores sample different parts of the same cancer in a prostate.

Pathologic staging

As one of the prognostic parameters, pathologic staging provides information about the extent of the disease—whether the tumor is confined to the prostate or it extends into periprostatic fibrofatty tissue, seminal vesicles, or lymph nodes. This information, also known as TNM staging, is reflected in the American Joint Committee on Cancer staging system, which was updated in 2016. As an example, a prostatic adenocarcinoma in a prostatectomy specimen which invades the seminal vesicle but has not metastasized to lymph nodes or distant sites is classified as pT3bN0M0. The stage predicts outcome after radical prostatectomy.

Predictive models and nomograms incorporate clinical stage, grade, and PSA to stratify patients into risk groups—low-risk or high-risk of progression. Risk categories are based on the grade and volume of cancer in the biopsies. Clinical stage is based on the findings in the biopsies, physical examination, and imaging studies, including pelvic CT, MRI, and bone scans. Although clinical stage is used to aid the clinician in the treatment modality she/he recommends to the patient, pathologic stage, assessed only after prostatectomy, provides more accurate information about the extent of the disease. A carcinoma that is localized to the prostate at prostatectomy but that extends into extraprostatic fibroadipose tissue or into the seminal vesicle, or that involves the radial surgical margin, predicts a shorter time to PSA recurrence. These findings provide a basis for treating a patient with adjuvant radiation therapy.

Tumor volume

The goal of assessing tumor volume in biopsies and in prostatectomies differs. The total volume of tumor in all biopsies in a biopsy procedure is a parameter for recommending active surveillance to a patient. A typical volume threshold value for active surveillance is carcinoma in fewer than four biopsies. The volume of carcinoma in a prostatectomy correlates in some studies with tumor progression and response to neoadjuvant therapy. However, there are discrepant studies. A major source of discrepancy in these studies is the lack of a standard approach to determining tumor volume and of a reference volume. That said, the two most frequent methods used to assess volume are based on visual assessment of the fraction of cells in a region of parenchyma that contains cancer. One method reports volume as a percentage of prostate cells that are carcinoma cells. The second reports volume as the volume of cancer containing parenchyma.

Shortcomings of current grading

The current grading system has shortcomings. One is that variants of different patterns are assumed to have an identical biology. This assumption has been contravened by recent studies which provide evidence that carcinomas with any component of the cribriform variant of pattern 4 have a clinical outcome similar to that of Gleason pattern 5 carcinoma. A future development might be categorizing the cribriform variant as pattern 5 instead of pattern 4. A second shortcoming is inter- and intrapathologist variance in grading carcinomas. For example, pathologists are challenged when distinguishing tangential sections of well-formed carcinoma glands (pattern 3) from poorly formed glands (pattern 4). A possible solution to this problem is substituting transcript profiles of cancer cells for grading. However, basing prognosis on transcript profiles as presently practiced seems unlikely to succeed since the profiles of Gleason grades reported by different investigators differ. Explanations for these differences include inadequate sampling (typically only a few thousand cells are analyzed from a tumor composed of 10^9 cells), contamination (most studies have not isolated tumor cells from stromal cells and inflammatory cells), and different molecular technologies.

Candidate tissue-based prognostic biomarkers

Numerous biomarkers of different categories of analytes (protein, RNA's, DNA) have been assessed in tissue, urine, cells in blood, and cell free samples of blood. We will limit our discussion to tissue-based analytes, of which hundreds have been reported. In univariate analyses many have prognostic value, that is, the fraction of carcinoma cells in cell cycle (the MKI67 fraction), increased expression of MME (CD10), increased fraction of SPINK1 expressing cells, decreased fraction of AZGP1 expressing cells, loss of tumor suppressor gene PTEN and methylation of tumor promoters of CD01, GST-pi, RASSF1, PITX2. Another type of biomarker is a transcript panel from homogenates of tumor containing tissue. Several have been commercialized—ConfirmMDx, Prolaris gene panel, OncotypeDX Genomic Prostate score, and Decipher classifier. However, to date, no biomarker has been shown to improve prognosis stratification in a multivariate analysis which includes carcinoma grade, stage, and diagnostic level of serum PSA. The reasons are multifold. There are shortcomings with preanalytical and analytical study designs and with statistical analyses. A general explanation for the failure of these biomarkers may be that the investigators who discover the biomarkers have not allied the clinical laboratorians who have the tools and experience to validate clinical use.

Predictive biomarker

Currently, there are two predictive tissue-based biomarkers—one histological, the second molecular. Prostate carcinoma that has an exclusively neuroendocrine histological phenotype is predictive to be insensitive to androgen deprivation. Metastatic small cell neuroendocrine carcinoma of the prostate is responsive, somewhat, to cytotoxic chemotherapy. Acinar adenocarcinomas which have a neuroendocrine component are not predictably responsive to chemotherapy. These carcinomas are initially treated with androgen deprivation therapy (ADT). Many patients with metastatic CRPC that expresses AR variant 7 do not respond to even the latest generation of androgen deprivation therapy, that is, enzalutamide and abiraterone. Nonresponse to ADT of ARv7 carcinomas is attributed to absence of the C terminal AR ligand binding domain. Accordingly, these CRPC cancers are treated, forthwith, with cytotoxic chemotherapy. A second histological phenotype which hypothetically predicts response to therapy is ductal adenocarcinoma of the prostate. This type of carcinoma is predicted to not respond to ADT. Extending the possible predictive power of tumor histology further, we have evidence that the cribriform pattern of ductal adenocarcinoma, which tends to have mutations in homologous recombination genes, responds to PARP inhibitors or cytotoxic platinum therapy. Conversely, the papillary pattern of ductal adenocarcinoma, which tends to have mismatch DNA repair gene defects, responds to immune checkpoint blockade therapy.

Metastatic Prostate Carcinoma

Clinical presentation

Most frequently metastatic prostate carcinoma presents in bone, causing pain, and producing fractures and resulting in an often markedly elevated serum PSA. Obtaining biopsies of bone, which can be challenging due to the typically osteoblastic nature of bone metastases, is important, both to confirm the clinical diagnosis and to obtain tissue for sequencing.

Histological architecture

Metastatic prostate carcinoma can be categorized as either subjected, or not, to systemic therapy.

- Metastatic prostate carcinoma not subjected to systemic therapy. The histology of carcinoma not subjected to androgen deprivation therapy is similar to that of the primary carcinoma. However, since neither architecture nor tumor cell cytology is specific for prostate adenocarcinoma, immunohistochemical stains for androgen regulated genes, that is, AR, KLK3, NKX3.1, can often, though not always, be confirmatory. One exception is neuroendocrine carcinoma, as discussed previously.
- Metastatic prostate carcinoma subjected to systemic therapy. The nonsurgical treatment modalities of prostate carcinoma, that is, hormonal ablation and radiation therapy, alter the histology of both the carcinoma and of the nonneoplastic glandular tissue. Both therapies result in decrease of tumor cell and nuclear size and cytoplasmic vacuolization. In general, tumor cells become inconspicuous and appear to be of higher grade. Changes in benign glands are also distinctive. Radiated benign glands are atrophic with a multilayered epithelium enriched in basal cells, including a subset of large, pleomorphic basal cells. In contrast, androgen-deprived benign gland are small and round with a prominent rim of hyperplastic basal cells. Both treatment modalities result in two potentially misleading types of histological features. Since there is a decrease in the number of tumor cells in a region of cancer containing parenchyma, the volume of actual cancer cell mass may be overestimated. And since cancer cells shrink, many appearing as single cells, tumor may be over-graded. General practice is to not grade cancer which has been radiated or androgen deprived, unless there is no histological evidence of treatment effect in either the benign or malignant glands. Of note, the neuroendocrine variant of prostate carcinoma is more frequent in patients with CRPC than in patients with androgen deprivation sensitive metastatic prostate adenocarcinoma. A subcategory of CRPC is prostate carcinoma which has neither an acinar nor neuroendocrine phenotype. Whether this phenotype has a unique immunophenotype or whether it is expresses biologically significant, but immunohistochemically undetectable, levels of either a neuroendocrine or acinar phenotype awaits further studies.

Genomics of metastatic prostate carcinoma

The only detailed studies of large numbers of patients with multiple metastases have been studies of CRPC in patients treated with androgen deprivation. Compared with primary untreated acinar adenocarcinoma, molecular changes in DNA repair genes BRCA2, BRCA1, and ATM were more frequent, occurring in 19% of cases. The large majority of patients had a potentially actionable molecular change. Most frequent were changes of AR (63% of cases). AR changes included single base pair changes, amplification, and an increase in AR variants, most frequently ARv7. Changes in PTEN and in TP53 were also more frequent than in primary prostate carcinoma. The question of heterogeneity of metastases has been addressed in a study of 176 metastases from 63 men in a rapid autopsy program. Interpatient heterogeneity of the molecular phenotype of metastases was great, based on whole exome sequencing, array comparative genomic hybridization, and RNA transcript profiles. In contrast, the molecular profiles of cell cycle and AR activity genes in separate metastases in an individual were similar. Consequently a biopsy of any one metastasis is likely to represent the molecular phenotype of all metastasis.

Prospective Vision

More accurate grading

Although grade is of proven prognostic value in populations of patients and of clinical value in categorizing patients for treatment, there is multiinstitutional evidence that individual patients are often not accurately graded. Interpathologist, and intrapathologist, variance is high. Kappa values (a metric of observer variance where kappa < 0 is no agreement and kappa $= 1$ is perfect agreement) range between 0.3 and 0.8. The following approaches may improve the accuracy of grading:

- Regarding any carcinoma that has a cribriform component as being of highest grade, or as a Grade Group 5 carcinoma. At present carcinomas with a cribriform component can have a Grade Group score from 2 (Gleason score $3 + 4 = 7$) to 5 (Gleason score $5 + 4 = 9$). More than six studies have correlated the presence of any cribriform component with worse outcome, which, in one study, is comparable to the outcome of Grade Group 5 cancer.
- Providing a web-based set of reference images vetted unanimously by a large group of internationally recognized experts in grading prostate adenocarcinoma. Given the present state of interobserver variance, this goal seems to be more aspirational than achievable.
- Histologically assessing prostate carcinomas in 3-D instead of basing grading on 2 dimensional sections of 5 μm thick sections on glass slides. Hypothetically many or all sections of the poorly formed gland variant of Gleason pattern 4 carcinoma are tangential sections of well-formed Gleason pattern 3 carcinomas. An open table top light sheet microscope can assess the architecture of tissue measuring up to 10×10 cm in the x, y axes and at least 0.3 cm in the z axis at a resolution sufficient to distinguish carcinoma from benign glands. The goal of more accurately grading cancer by the Gleason system may be succeeded by a novel system based on 3-D architecture. Confirmation that grading by 3-D microscopy improves prognostic accuracy and decreases pathologist variance would likely involve analyzing thousands of primary prostate carcinomas for which the 10 year outcome is known.
- Replacement of histological grading by a molecular assay. Investigators have reported transcript profiles which distinguish carcinomas of different Gleason grade. To date, transcript profiling has not led to more accurate prognosis by multivariate analysis. And transcript profiles differ by investigator, which raises questions about the accuracy of any profile. Reasons for these differences include the effect of preanalytical variables, that is, warm and cold ischemia time, hypoxia, and pH, degree of

contamination of the tissues by such noncarcinoma cells as stromal and hematopoietic cells and by benign prostate glands, and the molecular heterogeneity of a given tumor. Samples which are profiled typically represent a small component of a tumor. For example, a laser microdissected sample contains only hundreds of cells from a tumor composed of a million tumor cells as well as stromal and inflammatory cells. Analyzing larger samples may not detect minor biologically important subclones, despite use of such a PCR artifact correcting methods as duplex sequencing. Genomic profiling of each tumor can be done more accurately by controlling for preanalytical and analytical variables and by obtaining additional, sequential samples until the marginal data from the next sequential sample leads to no significant change in the data.

- Spatial-based molecular sequencing. As the ability to obtain accurate transcript profiles of single cells improves, the prospect of locating the most malignant subclones in a population of tumor cells increases. Using a combination of 3-D based localization of cells with "driver" genes and in vivo imaging techniques to guide the biopsy hand of the urologist would be a laudable goal, and a formidable challenge.

New histological variants

Different histological variants of carcinoma which cannot be categorized by the system developed for neuroendocrine differentiation have been identified.

- Novel forms of NE-type carcinomas include amphicrine (or, adenoneuroendocrine) carcinoma and metastases of pure NE carcinoma. The tumor cells of amphicrine carcinoma coexpress biomarkers of both neuroendocrine differentiation and of acinar differentiation. The response of this mixed phenotype tumor has not been characterized. A second histological phenotype is pure NE carcinoma. This carcinoma lacks features of both small cell carcinoma (sheets of mitotically active small cells with crush artifact, overlapping nuclei, and frequent apoptotic bodies) and of large cell NE carcinoma (large, pleomorphic cells forming a pseudostratified epithelium around areas of necrosis). Since these tumor cells have more abundant cytoplasm than the cells of small cell carcinoma and are less cytologically atypical than large cell NE cells, we can consider naming them "Intermediate cell neuroendocrine carcinoma."
- A clinically distinctive and pathologically nondistinct form of metastatic CRPC has been termed "anaplastic" carcinoma. What is distinctive about these carcinomas is a serum PSA which is disproportionately low for the mass of tumor metastases, a higher ratio of visceral to bone metastases than is seen in a typical metastatic CRPC, absence of tissue markers of acinar and neuroendocrine differentiation, and insensitivity to ADT. Since the term anaplastic is conventionally used for cytologically pleomorphic cells, a better term might be "null" prostate carcinoma, in lieu of naming it for a specific marker(s). Another curious phenomenon is the variability in expression of the two biomarkers of neuroendocrine differentiation—CHGA and SYP. What is curious is that these proteins, associated with neurosecretory granules of endocrine cells and with synaptic vesicles of neurons, respectively, are expressed by the same tumor cells. This observation raises a question—Is the biology of CHGA-expressing NE tumor cells different than the biology of SYP-expressing NE tumor cells?

More accurate staging

More accurate prognosis may be provided for individual patients if we include in the margin-positive stage pT2 cancers (cancer limited to the prostate), those cancers which are within 0.1 mm of, but not at, the surgical margin. Conventionally such tumors are classified as margin-negative. At least two studies report that the hazard ratio of tumor progression in these patients is the same as that of patients with positive margins.

Nuclear structure

The iconic prostate cancer nucleus is large, round, with a prominent, round nucleolus. This phenomenon, which is clinically so important, raises the question—Why are prostate cancer nuclei histologically different from the nuclei of nonmalignant prostate epithelial cells? Understanding genome organization might provide a basis for both understanding why translocations are characteristic of specific cancers and how genome structure can be diagnostically useful. There is pilot data that nuclear matrix protein SP100, which inhibits expression of some matrix metalloproteinases, is underexpressed in prostate cancer nuclei compared with paired benign nuclei, and that the gene is repositioned in cancer cell nuclei. Hypothetically, carcinomas expressing SP100 are less likely to progress than carcinomas which do not express SP100.

Imaging sciences

We anticipate that spatial resolution of both conventional ex vivo imaging modalities (mpMRI and CT) and of functional modalities (PET scans) will improve in both resolution and in the range of molecules that can be imaged so that cancer can be detected and, perhaps, even graded. Staging, thus prognosis, will become more accurate as the sensitivity and specificity of imaging modalities increases. In about one third of patients whose prostate carcinoma is clinically and pathologically confined to the prostate at radical prostatectomy the carcinoma will recur as metastases. Better imaging promises to detect many of these carcinomas prior to radical prostatectomy. The question then will be how to treat patients with clinically occult, minimal volume metastatic carcinoma. A second possibility is that all prostate carcinoma is metastatic at time of radical prostatectomy, and the cancer remains dormant for many years, even until the individual dies of a nonprostate carcinoma-associated disease. It is also conceivable that a prognosis and

predictive grading system, that is not based on the structure of cancer cells but on functional features which can be imaged, might be developed.

Communication with patients

The vocabulary of pathology reports grows in complexity as new methods of analysis of tissue evolve and are used in clinical practice. Consequently, clearly and successfully communicating pathology and genetic findings to patients, and, even, to clinicians, is a challenge. Without clear communication, patients and their families do not fully comprehend the consequences and risks of treatments. And they risk feeling dis-empowered. Several multidisciplinary teams are developing patient-centered pathology reports. Using graphics and written in a manner that is understandable by patients of common literacy level, these reports promise to improve communication between pathologists, other health care providers, and patients.

See also: Prostate Cancer: Diagnosis and Treatment.

Further Reading

Cancer Genome Atlas Research Network, 2015. The molecular taxonomy of primary prostate cancer. Cell 163 (4), 1011–1025.
Epstein, J.I., 2018. Prostate cancer grading: A decade after the 2005 modified system. Modern Pathology 31 (S1), S47–63.
Glaser, A.K., Reder, N.P., Chen, Y., McCarty, E.F., Yin, C., Wei, L., Wang, Y., True, L.D., Liu, J.T.C., 2017. Light-sheet microscopy for slide-free non-destructive pathology of large clinical specimens. Nature Biomedical Engineering 1 (7), 0084.
Humphrey, P.A., 2018. Variants of acinar adenocarcinoma of the prostate mimicking benign conditions. Modern Pathology 31 (S1), S64–70.
Ilic, D., Neuberger, M.M., Djulbegovic, M., Dahm, P., 2013. Screening for prostate cancer. Cochrane Database of Systematic Reviews 31 (1), CD004720.
Kumar, A., Coleman, I., Morrissey, C., et al., 2016. Substantial interindividual and limited intraindividual genomic diversity among tumors from men with metastatic prostate cancer. Nature Medicine 22 (4), 369–378.
Kweldam, C.F., Wildhagen, M.F., Steyerberg, E.W., et al., 2015. Cribriform growth is highly predictive for postoperative metastasis and disease-specific death in Gleason score 7 prostate cancer. Modern Pathology 28 (3), 457–464.
Magi-Galluzzi, C., 2018. Prostate cancer: Diagnostic criteria and role of immunohistochemistry. Modern Pathology 31 (S1), S12–21.
Newcomb, L.F., Brooks, J.D., Carroll, P.R., et al., 2010. Canary Prostate Active Surveillance Study: Design of a multi-institutional active surveillance cohort and biorepository. Urology 75 (2), 407–413.
Pritchard, C.C., Mateo, J., Walsh, M.F., et al., 2016. Inherited DNA-repair gene mutations in men with metastatic prostate cancer. The New England Journal of Medicine 375 (5), 443–453.
Robinson, D., Van Allen, E.M., Wu, Y.M., et al., 2015. Integrative clinical genomics of advanced prostate cancer. Cell 161 (5), 1215–1228.
Rubin, M.A., Demichelis, F., 2018. The genomics of prostate cancer: Emerging understanding with technologic advances. Modern Pathology 31 (S1), S1–11.
Sakr, W.A., Grignon, D.J., Haas, G.P., Heilbrun, L.K., Pontes, J.E., Crissman, J.D., 1999. Age and racial distribution of prostatic intraepithelial neoplasia. European Urology 30 (2), 138–144.
U.S. Preventive Services Task Force, 2008. Screening for prostate cancer: U.S. Preventive Services Task Force recommendation statement. Annals of Internal Medicine 149 (3), 185–191.
Walsh, P.C., 2017. The search for the missing heritability of prostate Cancer. European Urology 72 (5), 657–659.

Pyruvate Kinase

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Glossary

Aerobic glycolysis (Warburg effect) A phenomenon where unlike normal cells, cancer cells take-up large amounts of glucose and break it primarily into lactate regardless of the presence of molecular oxygen (O_2), a tendency exploited in clinical detection of cancer by fluorodeoxyglucose positron emission tomography (FDG-PET) scan.

Protein moonlighting A phenomenon that elucidates the ability of a protein to perform more than one function. In common the primary function of these proteins are catalysis of a biochemical reaction, but through their secondary moonlighting property they can involve in signal transduction, transcriptional regulation, replication, translation, scaffolding, motility, and apoptosis. Researchers believe that proteins would have acquired their moonlighting function through evolution, further they believe that the ancestral protein would have possessed only single function.

Substrate-level phosphorylation An enzyme-catalyzed biochemical reaction that yields a molecule of ATP or GTP by directly transferring a phosphate group to ADP or GDP (energy) from high-energy rich phosphate containing metabolic intermediates, for example, phosphoenol pyruvic acid. Unlike mitochondrial oxidative phosphorylation, the energy (ATP) produced through substrate-level phosphorylation is independent of oxygen.

Introduction

Characterization of PK Isoforms

Enzyme pyruvate kinase (PK; ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40) catalyzes an irreversible, decisive step of the glycolytic pathway. PK catalyzes a transphosphorylation reaction between phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) to yield a molecule of pyruvate and ATP. Energy (ATP) generated from the above biochemical reaction is through substrate-level phosphorylation which is oxygen independent, substantially contributing to the net glycolytic ATP. PK is ubiquitously expressed across all living beings, originating from simple unicellular organism to complex multicellular organism, where the presence of at least one isoform of PK is essential to drive glycolysis. Mammals possess four isoforms of pyruvate kinase, named as PKR, PKL, PKM1 and PKM2; and these PK isoforms primarily differ from one another by their differential kinetic properties. The expression of PK isoforms is strictly regulated to demonstrate tissue specificity, which is essential to meet the different metabolic demands of diverse cell types. For instance, the expression of PKL is predominant in hepatocytes, to support gluconeogenesis. Similarly, expression of PKM2 is ideal for cells with high anabolic biosynthesis rate, such as embryonic cells. Among four isoforms of PK, M2-PK has gained enormous interest; and has been extensively studied. Since PKM2 has been shown to preferentially express in cancer cells, it is considered as one of the metabolic hallmark features of such cells. Cancer cells in addition to expressing PKM2, alter its quatnery state into an enzymatically less active dimeric form. This in-active form of PKM2 is indispensable for cancer cells to rewire its metabolism to exhibit aerobic glycolysis, a mechanism through which cancer enhances glycolysis and replenishes its biosynthetic and energetic demand to sustain rapid growth and proliferation. Moreover, PKM2 aids cancer cells with manifold nonmetabolic benefits, such as protein kinase activity, cotranscriptional activation of gene expression, maintaining redox balance, chromosomal segregation and regulation of apoptosis.

Allosteric Regulation of PK Isoforms

Although, the biochemical reaction (pyruvate kinase activity) catalyzed by PK isoforms are identical in nature, however, they markedly vary among one another for their kinetic properties, in relation to varied affinity to its substrate PEP and allosteric regulation by metabolic intermediates. Moreover, PK isoforms show almost similar affinity for its substrate ADP. PKM1, a nonallosteric, constitutively more active form, in comparison to the other PK isoforms has the highest affinity to PEP. Likewise, PKR and PKL have the least affinity to their substrate PEP and both isoforms can be allosterically activated by fructose 1, 6-bisphosphate (FBP) and -inhibited by the concentration of ATP. Intriguingly, PKM2 affinity for its substrate PEP varies based on its quaternary structure (dimer or tetramer). The kinetic characterization of two oligomeric forms of PKM2 has revealed that the tetramer of PKM2 has high affinity to its substrate PEP and it is enzymatically active; whereas, the dimeric form of PKM2 has a relatively very weak affinity for PEP and is enzymatically inactive at physiological conditions.

PK Isoforms and Tissue Distribution

The genome of *Homo sapiens* comprises of two distinct PK genes, termed as *PKLR* and *PKM* located on chromosome 1q22 and 15q23, which encode for four PK isoforms, PKR, PKL, PKM1 and PKM2. *PKLR* gene codes for PKR exclusively in erythrocytes

(red blood cells, RBCs), and expresses PKL predominantly in liver and sparsely in kidney, and intestine. The expression of PKL and PKR in the selective tissue types is achieved by the activation of two alternate promoters located in *PKLR* gene by the tissue-specific transcription factors. The upstream promoter present in the *PKLR* gene drives the expression of a full-length PKR transcript, exclusively in RBC, possessing an extra exon in its 5′ terminal end, in comparison to a shorter variant PKL (**Fig. 1**), thus coding for a polypeptide chain of 574 amino acids. Whereas, the PKL transcript codes for 543 amino acids (58.4 kDa) containing polypeptide chain.

The *PKM* gene, located on chromosome 15q23, is 32 kb long with 12 exons and 11 introns; and codes for two alternative splice variants, PKM1 and PKM2. Out of 12 exons' that *PKM* gene codes for, the mature PKM mRNA that acquires exon 9 and omits exon 10 is termed as PKM1 (**Fig. 1**). On the other hand, the transcript that includes exon 10 and excludes exon 9 is designated as PKM2. The mature mRNAs of PKM1 and PKM2, harbor coding sequences with identical length (1593 base pairs), however, they differ by 160 nucleotide sequences from 1143 to 1303 bases, due to their alternative selection between exon 9 and 10. The polypeptide chain of 531 amino acids (aa) translated from theses mature mRNAs of PKM1 and PKM2 are of identical size. The amino acid sequence of PKM2, based on its characteristic biochemical properties, is categorized into three functional domains (A-, B- and C). The interlinking region between the A-domain (aa 44–116 to aa 219–389) and B-domain (aa 117–218) forms the catalytic active site, which binds to the enzyme substrates PEP and ADP. The C-domain (aa 390–531) situated at the C-terminal end of PKM2 harbors the binding site for allosteric activator—fructose 1, 6 bisphosphate (FBP), nuclear localization signal sequence and an intersubunit contact domain (ISCD) (**Fig. 2**A). The ISCD sequence is unique to PKM2 and provides it with some exclusive privilege over other PK isoforms to exist in two distinct oligomeric states with different enzyme kinetic properties that is, enzymatically more active tetramer and an enzymatically inactive dimer. All other PK isoforms (M1, -L and -R) exist only in a tetrameric quaternary state to act as active enzymes. Two monomers of PKM2 interact at their A-domain to give rise to a dimer; further two dimers associate at their C-domain-ISCD-interface in a dimer-of-dimers arrangement to form an enzymatically active tetrameric oligomeric state. Unlike PKM2, PKM1 is a constitutively active form and lacks ISCD, as it differs from PKM2 due to alternative splicing. The amino acid sequences coded by exon 9 and exon 10, corresponding to amino acids sequence between 378 and 434 in the C-terminal of PKM isoforms, differs by 23- out of 56-amino acids. Hence, PKM1 exists only in tetrameric form and its enzyme activity cannot be activated by allosteric activators, such as FBP.

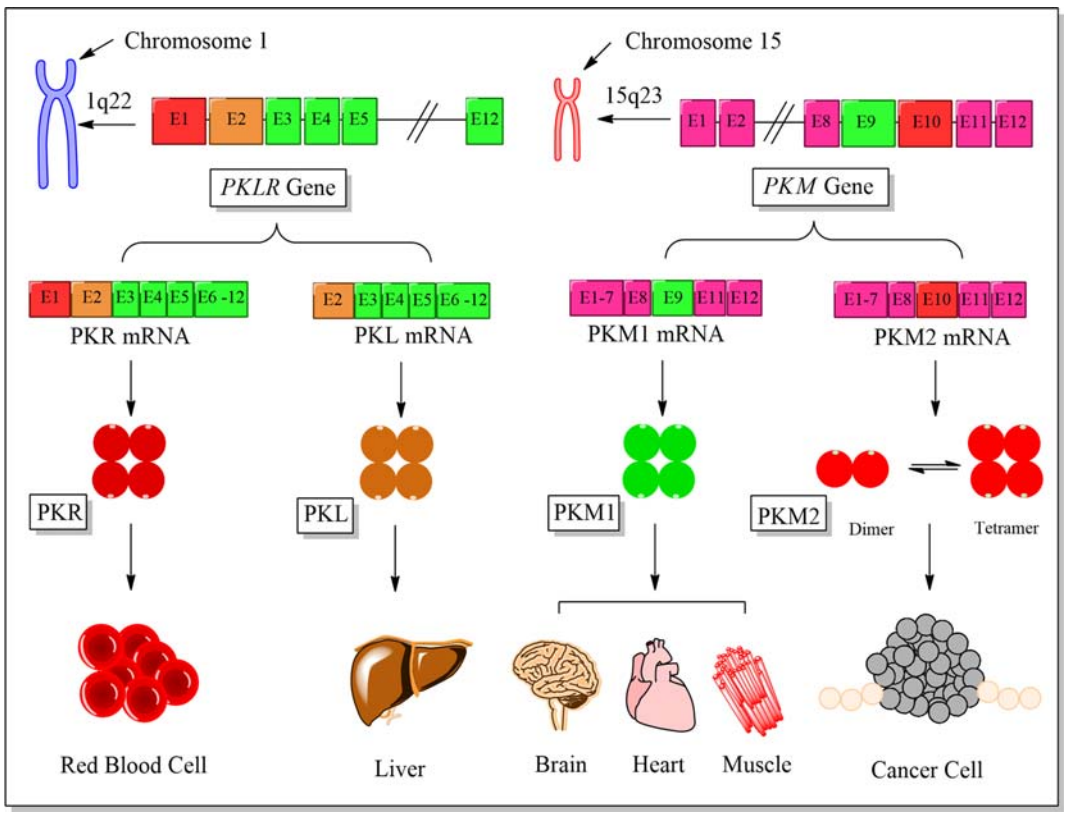

Fig. 1 Pyruvate kinase isoforms and tissue distribution. *PKLR* gene located on chromosome 1q22 region codes for PKR and PKL isoforms by activating tissue-specific promoters and restricts their expression to particular tissues, such as PKR in RBC and PKL in Liver. Likewise, *PKM* gene on chromosome 15q23 encodes for two more isoforms, termed PKM1 and PKM2, through alternative splicing of mutually exclusive exon 9 and 10. The expression of PKM1 is confined to differentiated cells, such as skeletal muscle, brain and heart. On the contrary, the expression of PKM2 is restricted to proliferating cells, for example, malignant cancer cells.

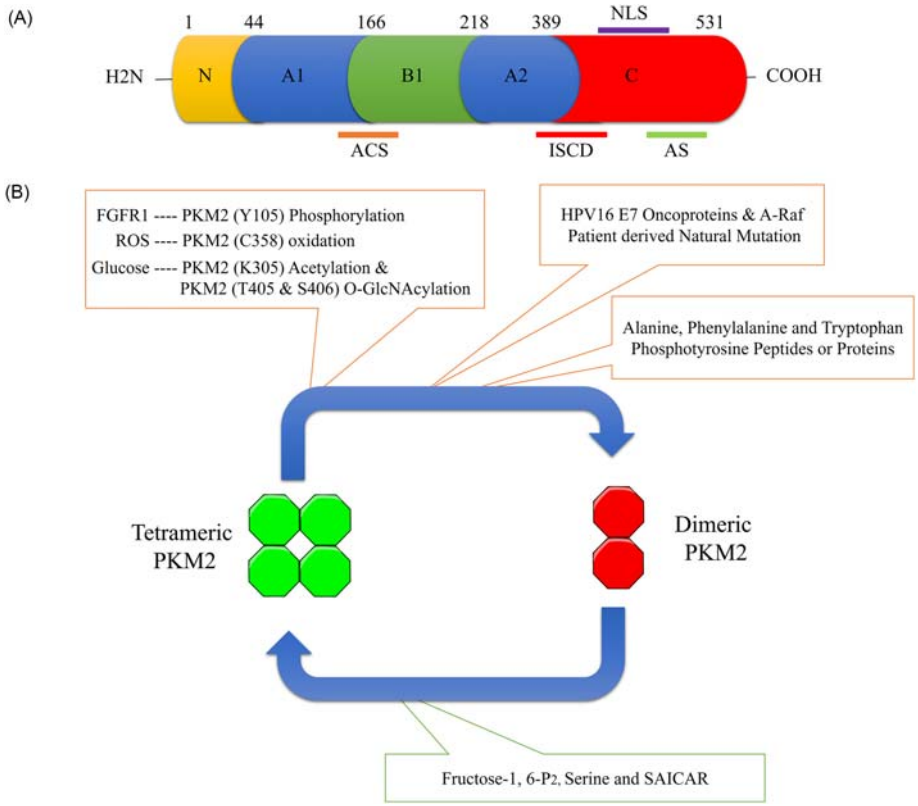

Fig. 2 A schematic diagram portraying the domain structures of PKM2 and the cellular molecular cues that influence PKM2 enzyme activity. (A) The adjoining region between the PKM2 A1 and B-domain forms a catalytic active site (ACS) and the interface between A2 and C-domain forms an intersubunit contact domain (ISCD), the latter enables the two dimers of PKM2 to interact and form tetrameric oligomer. Further, C-domain harbors a nuclear localization signal sequence (NLS) and an allosteric activator (FBP) binding site. (B) Illustration depicts the metabolic intermediates, oncogenes and posttranslational modification that activates and inhibits PKM2 enzyme activity and its oligomeric state. PKM2 exists as an enzymatically active tetramer and relatively inactive dimer and the allosteric activation of PKM2 involves stabilization of its enzymatically active tetrameric state. PKM2 is allosterically activated by glycolytic intermediate FBP, amino acid serine and SAICAR, a metabolic intermediate from purine biosynthesis pathway. Likewise, PKM2 activity is inhibited (i.e., dissociation of PKM2 into dimers) by metabolites (alanine, phenylalanine and tryptophan), binding of oncoproteins (HPV16 E7 oncoprotein and A-Raf), natural mutations, phospho-tyrosine-mediated release of FBP and PTMs, such as phosphorylation, acetylation, oxidation and O-GlcNAcylation.

Tissues that comprise of highly differentiated cells with high basal metabolism rate (i.e., catabolism) and high-energy (ATP) turnover to support normal physiology, such as skeletal muscle, heart and brain, ideally express the constitutively active M1 isoform of PK (PKM1). PKM2 expression is abundant in cells that undergo rapid growth and frequent cell division, such as embryonic cells and the transformed neoplastic cells (Fig. 1). Embryonic cells during their differentiation, gradually replace the expression of prototype PKM2 isoform with other tissue-specific isoforms. However, malignant transformation of adult differentiated cells results in reexpression of PKM2. For instance, PKL to PKM2 shift was reported in the cases of hepatocellular carcinoma and PK-M1 to -M2 in a variety of cancer types, such as glioblastoma, lungs and so on. Studies have cited the expression of PKM2 in normal differentiated cells, such as intestinal epithelium, pancreatic islets, lung, retina, distal renal tubules and brown adipose tissue; and the exclusive presence of PKM2 in cancer cells has also been questioned. Interestingly, the coexpression of PKM2 and PKM1 isoforms has been noticed; and reported in sporadic breast cancer tissue as well as in multiple cancer cell lines from different tissue origin. The latter suggests that besides PKM2 supporting cancer cell growth and division, there is a unique role played by PKM1 in cancer cells as well. Besides occurring as homo-tetramers, PKM2 can exist as hetero-oligomers. Hybrids between PKM2 and PKL have been reported in liver, colon and rectum. Similarly, PKM1 and PKM2 hetero-tetramer hybrids have been described in stomach, esophagus and recently in lung cancer cell lines.

Regulation of PK-Isoform Expression and Enzyme Activity

Expression Regulation of PKL/R Isoforms

PKL expression is linked with the tissue (Hepatocytes) that principally supports gluconeogenesis, an adaptive metabolism during fasting or nutrient starvation. Intriguingly nutritional status is one of the essential features that impact the expression of PKL in the

liver. Carbohydrate-rich diet stimulates the activation of carbohydrate-response element-binding protein (ChREBP) to induce the expression of PKL. On the other hand, nutrient starvation suppresses the PKL expression. Hormone, glucagon, phosphorylates- and inhibits-PKL enzyme activity through a mechanism apparently involving cyclin-AMP activated protein kinase. Similarly, hormone epinephrine blocks the activity of PKL to promote gluconeogenesis. However, hormone insulin reverses the effects of glucagon and epinephrine on PKL activity, thus promoting glycolysis and inhibiting gluconeogenesis. There are insufficient reports to elucidate the expression regulation of PKR.

Expression Regulation of PKM Isoforms

The molecular mechanism behind the alternative splicing of PKM isoforms, as well as the mechanistic link by which cancer cells reexpress PKM2 has been elucidated recently. In this article, authors propose an apparent role of c-Myc (frequently deregulated oncogene) driven activation of heterogeneous nuclear ribonucleoproteins (hnRNPs; hnRNPA1, hnRNPA2 and polypyrimidine tract binding protein (PTB)), in dictating alternative splicing of PKM isoforms. The hnRNPs directly bind the sequences flanking exon 9 of the PKM precursor RNA and repress its inclusion into the mature PKM transcript, thus indirectly facilitating the inclusion of exon 10 and resulting in increased copy number of PKM2 transcripts in cancer cells. Moreover, serine/arginine-rich splicing factor 3 (SRF3) binds- and facilitates-exon 10 inclusion to mature PKM mRNA; thus, increasing the proportion of PKM2 mRNA in the cancer cells through one another distinct mechanism. Since numerous splice factors exist to check (repress) the inclusion of exon 9 and prevent the expression of PKM1, one can assume that PKM1 could be the default protein to express in mammalian cells, however, more studies are required to conclude anything further.

The expression of PKM2 in normal proliferating as well as in malignant tumor cells is broadly influenced by diverse signaling pathways, stimulated by growth factors, hormones, aberrantly mutated oncoproteins and the elements from tumor microenvironment (hypoxia and nutrient status). These molecular cues facilitate the expression of PKM2, by stimulating the expression of numerous transcription factors that bind their consensus DNA binding site in *PKM* gene (promoter or enhancers). Hypoxia-inducible factor 1α (HIF-1α), peroxisome proliferator-activated receptor gamma (PPARγ), nuclear factor kappa (NFκB), specificity protein (Sp1 and Sp3) are few of the transcription factors known to directly regulate PKM2 expression. HIF-1 transcription factor, which is canonically stabilized under hypoxic conditions, induces the expression of PKM2 isoform by binding hypoxic response element (HRE; HIF-1α consensus DNA binding sequence) located at the first intron of *PKM* gene. The PKM2 that is overexpressed under hypoxic stress, localizes into the nucleus and interacts with HIF-1 transcription complex to coregulate the transcription of glycolytic enzymes including PKM2, through a positive feedback loop. The resultant changes in expression of glycolytic enzymes, including PKM2 is necessary to support aerobic glycolysis (an adaptive metabolic reprogramming) in cancer cells, which is essential to support tumor progression and to survive the recurrent hypoxic stress that ascends from tumor microenvironment. In addition to hypoxia, insulin and aberrant oncogenic signaling networks, such as receptor tyrosine kinase, PI3K and AKT, which are frequently mutated and deregulated in cancer, constitutively trigger the downstream effector mammalian target of rapamycin (mTOR) and facilitate the normoxic stabilization of HIF-1 and induce the expression of c-Myc transcription factor. HIF-1 and c-Myc transcription factors together activate the expression of PKM2 and other essential glycolytic enzymes to rewire cancer-metabolism. Sp1 transcription factor stimulates the transcription of PKM2 by binding GC-boxes (Sp1 consensus DNA binding site) located in *PKM* gene promoter. Sp3 transcription factor has been shown to synergize this Sp1 function by enhancing the expression of PKM2. In addition, under hyperglycemic conditions protein phosphatase 1 has been shown to dephosphorylate- and stimulate-Sp1 to bind PKM gene at GC-box to facilitate the expression of PKM2. Hyperactivated AKT2 in PTEN null mouse liver cells has been shown to induce the expression of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), also a nuclear hormone receptor. PPARγ by specifically binding PKM and HK (Hexokinase) gene promoters was shown to induce the expression of PKM2 and HK2, resulting in liver metabolism rewiring, which paves the way for liver pathophysiology. EGFR stimulated NFκB (p65) recruits HIF-1α transcription factor to PKM gene promoter to coregulate the expression of PKM2 and rewire the metabolism of cancer cells. Hormones, including, glucocorticoid, triiodothyronine-T3 and insulin have been shown to regulate the transcription of PKM2. Collectively, the above evidences suggest that cancer cells exploit the flexibility in the expression regulation of PKM2 and reap the benefit of reprogramming metabolism to meet the demands of biomass and energy to support their persistent growth and proliferation.

PKM2 at Crossroads of the Metabolic Rewiring of Cancer Cells

Cancer cells are facilitated by the concomitant expression of PKM2 and the alteration of its quaternary state by promoting subunit dissociation into an enzymatically less active dimeric form. The accumulation of dimeric PKM2 obstructs the terminal step of glycolysis. This, as a result stockpiles the high energy-rich glycolytic metabolites above the pyruvate kinase reaction, which is then channeled into anabolic metabolism to support the biosynthesis of amino acid, nucleotides and lipids, offering a metabolic advantage for cancer cell growth and proliferation. The tetramer:dimer ratio of PKM2 and its enzyme activity, in normal proliferating cells and notably in cancer cells, is controlled by multiple cellular intrinsic parameters, such as binding metabolic intermediates (that act as an allosteric regulators), physically interacting oncoproteins, dominant negative natural mutations, competitively binding phosphotyrosine peptides and most importantly posttranslation modifications (PTMs). Glycolytic intermediate, fructose 1, 6-P2 (FBP), and amino acid serine, bind- and enhances-PKM2 activity by stimulating the subunit association of dimeric PKM2. However,

a reduction in the concentration of FBP results in reversible disassociation and inactivation of PKM2 activity. Likewise, succinylaminoimidazolecarboxamide ribose-50-phosphate (SAICAR), a metabolic intermediate from the purine biosynthetic pathway, which accumulates under nutrient-deprived conditions, specifically binds M2-PK to enhance its activity. Whereas, amino acids alanine, phenylalanine and tryptophan serve as an allosteric inhibitors of PKM2.

Apart from the metabolic intermediates, numerous oncogenic protein interacting partners of PKM2 have been shown to modulate its oligomeric state and enzyme activity. Remarkably, human papillomavirus 16 (HPV16; high-risk type papillomavirus) encoded E7 oncoprotein, physically interacts with tetrameric PKM2 to stimulate its subunit disassociation into enzymatically inactive dimers. FBP fails to reverse the above PKM2 oligomeric shift mediated by HPV16 E7 oncoprotein. Through a similar mode of mechanism, A-Raf promotes the dimerization and inactivation of PKM2 in A-Raf transformed NIH3T3 cells. Besides, enzyme activity of PKM2 has been shown to affect its interaction with EGF stimulated Jumonji C-domain containing dioxygenase (JMJD5), cytosolic promyelocytic leukemia (cPML) or Tyr-46 phosphorylated MUC1-C (Mucin 1). JMJD5 reduces PKM2 activity by obstructing the association of PKM2 dimers to form enzymatically active tetrameric form. cPML decreases PKM2 activity by selectively binding tetrameric form of PKM2, without altering its quatnery state. In case of EGF stimulated Tyr-46 phosphorylated MUC1-C, the molecular mechanism by which it impedes the activity of PKM2 is not known. Moreover, recently the interaction between PKM2 and a serine/threonine kinase, death-associated protein kinase (DAPK), has been shown to increase PKM2 activity. Differing from the above molecular mechanism, PARP-14 has been shown to suppress PKM2 activity by inhibiting JNK-1 (proapoptotic factor) mediated Thr365 phosphorylation- and activation of -PKM2 enzyme, and therefore PARP-14 indirectly supports aerobic glycolysis. Further, natural mutations in PKM2, especially within the sequence that codes for inter-subunit-contact-domain located at the C-terminus of PKM2 were shown to decrease its enzyme activity by manifold. The reason being, mutations in ISCD obstruct the association of two dimers to form an enzymatically active PKM2 tetramer, accumulating more dimers, which has been validated using both computational and experimental techniques.

The amino acid sequence of PKM2 retains many conserved PTM sites, frequently modified by phosphorylation, acetylation, oxidation, hydroxylation, ubiquitination, glycosylation, methylation and so on. These wide variety of PTMs can be categorized into two types based upon their impact on PKM2 and resultant metabolic and nonmetabolic sustenance to cancer cells. The modifications that specifically affect PKM2 structural and functional features and provide metabolic advantage to cancer cells include, PKM2 tyrosine (Y) 105 phosphorylation, lysine (K) 305 acetylation, cysteine (C) 358 oxidation, threonine (T) 405/serine (S) 406 O-GlcNAcylation and Arginine (A) 445/447/455 methylation of PKM2. PKM2 Y105 phosphorylation (mediated by the deregulated Fibroblast growth factor receptor 1 (FGFR1), BCR-ABL and JAK2) decreases PK enzyme activity by stimulating the enzymatically active tetramer form of PKM2 to release the bound FBP (allosteric activator), and to undergo subunit disassociation. The above metabolic alteration channels the precursors within the glycolytic pathway toward the biosynthesis of macromolecules, supporting the metabolic necessity of cancer cells. Similar to the above mechanism, in response to high glucose, acetyltransferase P300/CBP-associated factor (PCAF), acetylates K305 residue of PKM2 and reduces its enzyme activity; and also directs acetylated PKM2 for lysosomal degradation. The relative poor stability of PKM2 and resultant reduced activity eventually contributes to the proproliferative metabolic phenotype of cancer cells. An upsurge of reactive oxygen species (ROS) has been shown to oxidize PKM2 at C358 residue, prompting PKM2 dissociation into a less active dimeric form. The accumulated glycolytic intermediates due to less PKM2 activity are directed toward PPP shunt for the production of reducing potential ($NADPH + H^+$) to detoxify ROS in cancer cells. Multiple factors have been shown to trigger ROS production in cancer cells such as growth factors, hormone (insulin), aberrant growth factor signaling as well as defective mitochondria that harbor mutant subunits of oxidative phosphorylation complexes, for example, ND5, ND3 and COI. Nutrient status of cancer cells has been shown to dynamically regulate O-GlcNAc transferase (OGT) to interact and O-GlcNAcylate PKM2 at T405 and S406 residues. O-GlcNAcylation reduces PKM2 activity by destabilizing the PKM2 tetramer into less active dimer, remodeling the metabolism of cancer cells to acquire aerobic glycolysis. In addition, O-GlcNAcylated PKM2 localizes inside the nucleus, for which it requires additional S37 phosphorylation, mediated by EGF stimulated ERK1/2 protein kinase. Nuclear PKM2 (i.e., T405 and S406 O-GlcNAcylated and S37 phosphorylated PKM2) coregulates the transcription of c-Myc target genes GLUT1 and LDHA and enhances procancerous metabolism and tumor progression, a unique and pioneering study to emphasize the crosstalk between the PTMs of PKM2, where it turns out both PKM2 O-GlcNAcylation and phosphorylation are mutually exclusive and essential to translocate PKM2 in nucleus and to exhibit nonglycolytic nuclear function in cancer cells. Methylation of PKM2 at Arg445/447/455 residues, by the coactivator associated arginine methyltransferase 1 (CARM1), promotes aerobic glycolysis to support tumor cells proliferation, invasion and metastasis. This study is unique to elucidate a novel molecular mechanism through which PKM2 methylation influences the aerobic glycolysis without altering its activity and the quaternary state. The methylated PKM2 is translocated into mitochondrial associated—endoplasmic reticulum membrane to interact—and reduce the stability of—$InsP_3Rs$ (inositol 1,4,5-trisphosphate receptors), which significantly decreases the calcium influx from endoplasmic reticulum to mitochondria, thus decreasing the mitochondrial membrane potential and paving the way for a shift from aerobic glycolysis to oxidative phosphorylation. Knockdown of PKM2 or the introduction of methylation ablative PKM2 mutant ectopically or by delivering nanoparticles harboring competitive nonmethylated PKM2 peptide in cancer cells have been shown to abrogate tumor favoring features of PKM2.

Concomitantly, a few PTMs of PKM2, varying marginally from the above defined molecular mechanism, can direct nuclear translocation of PKM2 to execute several nonglycolytic moonlighting functions. These collectively orchestrates metabolic rewiring in cancer cells to acquire procancerous aerobic glycolytic metabolic trait. EGF stimulated ERK1/2, mediates PKM2 S37 phosphorylation in cancer cell and offers PKM2 an advantage to selectively bind PIN and importin α5 (Nuclear exporter) and localize inside the nucleus. Nuclear PKM2 act as a transcriptional coactivator of β-catenin to promote the expression of its target gene, c-Myc. The

transcriptional activation of c-Myc influences the expression of glycolytic enzymes, GLUT1, LDHA and remarkably PKM2, through a positive feedback loop, to enhance the metabolic capabilities of cancer cells. Similarly, a secretory glycoprotein, extracellular matrix protein 1 (ECM1), also shown to stimulate PKM2 S37 phosphorylation, directs nuclear localization and transcription activation of glycolytic enzymes in tumor cells. Besides posttranslational modification, PKM2 stands unique in comparison to all other PK isoforms, by its atypical characteristic of binding the tyrosine-phosphorylated peptides or proteins. The latter prompts PKM2 to release its allosteric activator FBP, resulting in the inhibition of PKM2 activity and its subunit disassociation, which in turn supports the procancerous anabolic metabolism. The report of PKM2 profound affinity for phosphotyrosine protein is further substantiated by a recent study, which has revealed that PKM2 interacts specifically with Y333 phosphorylated β-catenin, in EGF stimulated cells. PKM2- β-catenin complex in nucleus interacts with T-cell factor (TCF) and then binds to the CCDN1 gene promoter for the transcription activation of cyclin D1 expression to promote brain tumor cell division. To sum up, PKM2 acts as a metabolic tuner, which decides the fate of glycolytic intermediates (used for anabolic biosynthesis or energy generation) by modulation of its activity and structural configuration by above-stated mechanisms.

The Nonmetabolic Attributes of PKM2 and the Hallmark Features of Cancer

Malignant cancer cells markedly differ from nontransformed normal cells by six cardinal hallmark features, such as self-sufficiency in proliferating signal, resisting growth inhibitory signals, immortality, infinite replicative competence, angiogenesis and metastasis. In addition, aerobic glycolysis, one of the key biochemical hallmark feature of the cancer cells, has been added to the above list in the recent past. A large body of evidence has corroborated the indispensable role of PKM2 in aerobic glycolysis. Moreover, emerging studies have elucidated a wide range of remarkable nonglycolytic functions of PKM2 that collectively support the stated hallmark features of cancer cells, such as growth, proliferation, evasion of cell death and spreading. The diverse nonglycolytic attributes of PKM2 associated with tumor progression are primarily by virtue of its moonlighting-protein kinase, transcriptional coactivator or co-repressor-functions. In this section of the article, we bring in all the new developments and landmark discoveries that highlight the nonmetabolic attributes of PKM2 and their link with other cardinal-hallmark features of malignant tumors (Fig. 3).

Self-Sufficiency in Growth Factor Signaling

Unlike normal cells, tumor cells are least dependent on the extracellular mitogenic cues for their proliferation. Rather, they acquire the ability to proliferate with the sustenance of oncogenes. Cancer cells harbor a variety of such aberrant oncogenes, which are key

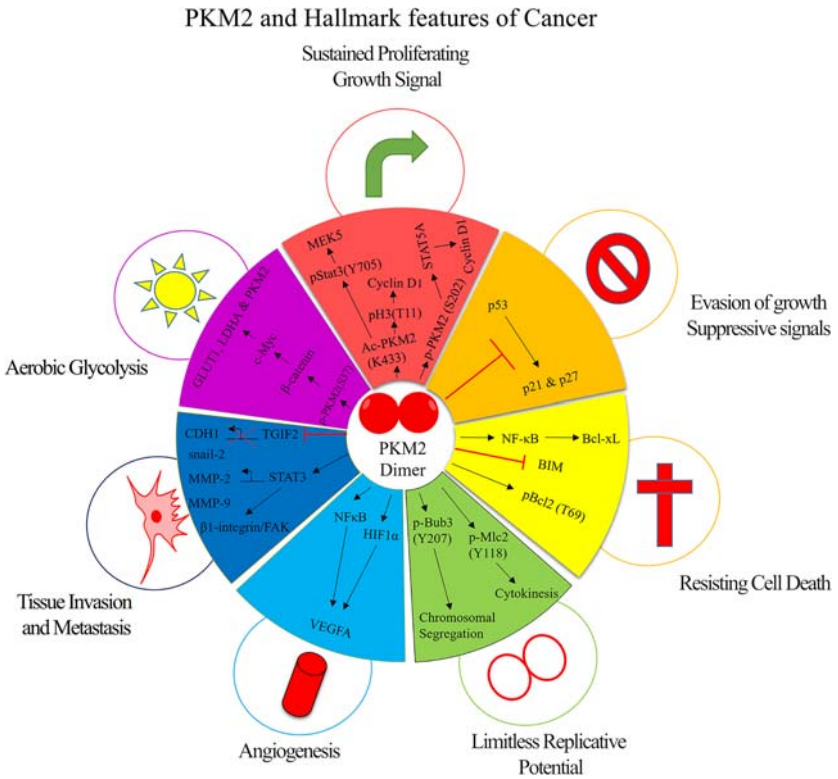

Fig. 3 PKM2 and the hallmark feature of cancer. Illustration highlights the association of PKM2 with established key hallmark features of cancer, suggesting an apparent role of PKM2 in cancer progression.

components of constitutively active growth factor signal transduction pathway due to gain of function mutations or aberrant post-translational modifications. Emerging studies have emphasized prooncogenic properties of PKM2 through its nonglycolytic virtues, such as protein kinase and transcription coactivator functions. Growing body of evidence suggests that the tetramer of PKM2 is an active enzyme, pyruvate kinase; and the dimeric form of PKM2 is relatively an inactive enzyme and a protein kinase. In difference to a typical protein kinase, the dimeric PKM2 utilizes PEP (phosphoenolpyruvate) as the phosphate donor for its protein kinase activity. It has been suggested that the dimeric PKM2 is a dual-specificity protein kinase (i.e., it can act as both tyrosine kinase and serine/threonine kinase), which is highly specific in binding and phosphorylating its protein substrates. However, there has not been characterization of any specific consensus binding site of PKM2 protein kinase on its protein substrates.

In a recent study, PKM2 has been shown to phosphorylate Y705 residue of Stat3 transcription factor (mechanism which is independent of the canonical Janus kinase (JAK)) and enhance its transcriptional activity to express its target gene MEK5, which in turn promotes the cell growth and proliferation. EGF stimulated cancer cells have been shown to facilitate PKM2 dimerization and its nuclear localization to phosphorylate histone (H3) at T11 residue, the latter stimulates the expression of two master transcription factors, c-Myc and cyclin D1, which transactivates the expression of genes that directly dictate cell proliferation. SAICAR, a metabolic intermediate from purine biosynthesis pathway that accumulates vastly in the proliferating cells, has been shown to specifically bind dimeric PKM2 and enhance its protein kinase activity. The PKM2–SAICAR complex has been shown to phosphorylate several novel protein substrates (typically protein kinase) including Erk1/2 which in turn sustains the mitogen-induced cell proliferation. Acetyltransferase-p300 stimulated by the spectrum of mitogenic signals and oncogenes were shown to acetylate K433 residue of PKM2. K433 acetylation enables PKM2 to localize into the nucleus and act as a protein kinase to phosphorylate Stat3 at Y705 and H3 at T11 residues, which in turn transcriptionally activate the expression of key factors that promote cell proliferation and tumorogenesis. Marginally differing from the above molecular mechanisms, insulin-like growth factor (IGF-1) stimulated cancer cells trigger AKT to interact and phosphorylate PKM2 at S202 residue to facilitate its nuclear entry and to transcriptionally activate the Stat5A target gene—cyclin D1 expression. PKM2 mediated cyclin D1 expression allows the cells to enter into cell division. This unique observation highlights how downstream to IGF/PI3K/AKT axis PKM2 functions as a transcriptional coactivator of Stat5A, which promotes tumor growth (**Fig. 3**). PKM2 can also serve as a transcriptional coactivator of transcription factors such as β-catenin and signal transducer and activator of transcription (Stat-3) and regulate the expression of their target genes that directly regulate cell proliferation or reprogramming of the metabolism of cancer cells.

Insensitivity to Growth Inhibitory Signals and the Evasion of Cell Death

In addition to the role of PKM2 in stimulating progrowth signaling in cancer cells, emerging evidences support the immense significance of PKM2 in stimulating antiapoptotic factors and in repressing proapoptotic factors (i.e., regulating apoptosis). The studies that have corroborated with this line of thought are: silencing of PKM2 expression in multiple cancer cells by introducing small interference RNA, resulting in the proliferation inhibition and apoptosis. Likewise, knockdown of PKM2 in non-small cell lung carcinoma (NSCLC), both in vitro and in vivo has demonstrated an increased sensitivity for radiation therapy; and also resulted in apoptosis. The molecular mechanism to elucidate the significance of PKM2 in apoptosis regulation continued with some uncertainty until recently, where emerging studies proposed multiple molecular mechanisms through which PKM2 could regulate programmed cell death. The expression of PKM2 in gastric cancer (GC) cells was shown to enhance the protein stability of NFκB (p65) transcription factor and in doing so, PKM2 promoted the transcription of NFκB target gene Bcl-xL (an antiapoptotic protein). PKM2 silencing in GC cells was shown to disrupt the stability of NFκB and thus decreasing the expression of Bcl-xL, resulting in apoptotic cell death. Thus, the axis of PKM2/NFκB/Bcl-xL was proposed as a potential survival signaling in gastric cancer cells. This correlation between the expression of PKM2 and Bcl-xL was also recognized in hepatocellular carcinoma. On the contrary, the high level of PKM2 expression in HCC tissue samples was inversely correlated with the expression of a proapoptotic factor, Bcl-2-like protein 11 (BIM). PKM2 knockdown in HCC cell line stabilized BIM expression and the latter stimulated apoptotic cell death, which could be prevented by prior silencing of BIM. In this article, it was proposed that the negative correlation with the expression of BIM and PKM2 could be exploited for its therapeutic benefits. More recently, PKM2 was shown to localize in the mitochondria in response to the oxidative stress (induced by treating the cells with H_2O_2) to stabilize the antiapoptotic factor Bcl2. Mitochondria localized PKM2 phosphorylated Bcl2 at T69 residue to prevent its interaction with Cul3-based E3 ubiquitin ligase and its subsequent proteasomal degradation, suggesting that PKM2 prevented oxidative stress-induced apoptosis in glioblastoma cells. PKM2 mediated T69 phosphorylation and stabilization of Bcl2 protein have been suggested to provide endurance in tumor cells against oxidative stress and promote gliomagenesis. The study sheds light on the mechanistic link between PKM2 regulation and apoptosis, resulting in the tolerance in cancer cells against oxidative stress (**Fig. 3**). A novel transcription corepressor role of PKM2 has also been shown to provide the cancer cells with tolerance against DNA damaging agents. PKM2 selectively interacts- and represses-p53 to activate the transcript of cell-cycle inhibitors (p21 and p27) in cancer cells that are treated with DNA damaging agents, providing a unique molecular mechanism in cancer cells to acquire resistance against DNA damaging agents involving PKM2 (**Fig. 3**).

Limitless Replicative Competency

The primary objective of the dividing cell is to inherit genetic material equally in the daughter cells with high fidelity. In addition to the availability of enough nutrient supply and exogenous growth factor signals to support cell division, a proliferating cell should gain license to advance further through multiple cell-cycle checkpoints. Cancer cells surpass these well-orchestrated cell-cycle

checkpoints with the help of aberrant oncogenic signal pathways. A few emerging studies have emphasized the crucial role of PKM2 in cell division. Apart from controlling the G1-S transition by regulating the expression of cyclin D1, PKM2 also regulates mitosis. In particular, the protein kinase activity of PKM2 is considered instrumental to achieve transition across mitotic checkpoints, such as chromosomal segregation and cytokinesis, eventually to establish cancerous state with unlimited replicative competency. For instance, PKM2 physically interacts- and phosphorylates-Bub3 (a spindle checkpoint protein) at Y207 residue. Phosphorylated Bub3 interacts with Bub1 and resultant Bub3-1 complex is recruited to kinetochores, where it interacts with Blinkin to enable a proper kinetochore-microtubule attachment and to advance through spindle-assembly checkpoint for proper chromosome segregation and eventually cell division of cancer cells (brain tumorogenesis) (Fig. 3). Subsequent to this observation, an independent investigation carried out by the same group has revealed the significance of PKM2 in cytokinesis. Where, PKM2 phosphorylated at T45 residue by Aurora B kinase has been shown to localize near the contractile ring of dividing cells to interact and phosphorylate myosin light chain 2 (MLC2) at Y118 residue. The phosphorylated Y118 residue of MLC2 (mediated by PKM2) serves as a priming site for ROCK2 protein kinase binding, which in addition to PKM2, phosphorylates MLC2 at S15 residue to commence cytokinesis (Fig. 3). Together, this study highlights the pivotal role of PKM2-guided MLC2 phosphorylation in cytokinesis, cell proliferation and brain tumor development. Moreover, the above role of PKM2 could also be enhanced by EGF stimulation or ectopic expression of EGFRvIII, K-Ras G12 V and B-Raf V600E mutants.

PKM2 and Tumor Angiogenesis

PKM2 contributes to tumor angiogenesis by regulating the expression of proangiogenesis factor, vascular endothelial growth factor (VEGF); and by promoting endothelial cell division. PKM2 through its nonglycolytic function of cotranscriptional activation regulates the expression of VEGF. For instance, studies carried out using endothelial cells infected with Kaposi's sarcoma-associated herpesvirus to examine pathological angiogenesis have revealed the mechanistic link involving PKM2 dependent transcriptional coactivation of HIF-1α to express proangiogenic factor, VEGF. Similarly, human cytomegalovirus-encoded US28-chemokine binding receptor has been shown to promote angiogenesis in glioblastoma cells, by stimulating the HIF1-PKM2 axis to express VEGF. Moreover, in solid tumors under hypoxic condition, enzyme prolyl hydroxylase 3 (PHD3) hydroxylates PKM2 at proline (P)-403 and -408 residues, which is essential for PKM2 to localize inside nucleus to interact with HIF1 transcription factor and eventually to express HIF1 target genes. This includes most of the glycolytic pathway enzymes, including PKM2, through a positive feedback loop that reprograms the glycolytic metabolism to resist hypoxic condition. However, the role of PKM2 in facilitating the transcriptional activation of HIF1 to express proangiogenic factor, VEGF, under hypoxic condition is still elusive. In addition to HIF-1α, PKM2 can act as a transcriptional co-activator of NFκB and governs the expression of VEGF. A recent investigation using pancreatic adenocarcinoma cells have revealed that PKM2 activates both NFκB/p65 and HIF-1α to enhance the transcription of VEGF-A, the latter triggers tumor angiogenesis and alleviates tumor from recurrent hypoxic stress. Immuno-histological studies using sporadic breast tumor specimens with their matched adjacent tissue controls has revealed an overexpression of PKM2 and VEGF-C in breast cancer, associated with poor prognosis (Fig. 3).

It has long been known that patients with gastrointestinal-, colorectal-, pancreatic-, lung- and ovarian-cancer were shown to release dimeric PKM2 in plasma and stool. This was exploited as a diagnostic and prognostic marker to identify early-stage cancer development, and in the follow-up studies, respectively, to examine the treatment response of antineoplastic drugs. However, the mechanism through which cancer cells secrete (or release) PKM2 in blood circulation and the potential benefits that cancer cells harness by releasing PKM2 in circulation remains ambiguous. Investigations of the objectives have shed light on this long-standing puzzle and revealed the direct involvement of dimeric PKM2 (i.e., released by the cancer cells in blood circulation) in tumor angiogenesis. This study revealed the vital role played by the circulating dimeric PKM2 in triggering angiogenic endothelial cell proliferation, migration and extracellular matrix (ECM) adhesion. In addition, PKM2 isotype expressed preferentially in ontogeny, poses a provocative objective of examining the involvement of PKM2 in governing angiogenesis during fetal organ development and maturation.

PKM2 in Cancer Cell Invasion, Migration and Metastasis

For a cancer cell to invade and metastasize to distant organs, it requires to undergo morphological changes, such as cell–cell junction dissolution, loss of cell polarity (apical and basolateral) and expression of mesenchymal markers, a phenomenon collectively termed as epithelial–mesenchymal transition (EMT). Growing body of evidence has revealed an association between deregulated PKM2 expression and EMT in cancer cells. Evidence from a recent study have suggested a novel role for PKM2 in colorectal cancer (CRC) migration, where the study used both in vitro cell culture system and immuno-histological study to validate the association between the deregulated expression of PKM2 and the advanced stage lymph metastasis of tumors cells. However, the study failed to characterize the molecular mechanism. Following this preliminary observation, several studies have attempted to examine the molecular mechanism through which PKM2 regulates the tumor cell- EMT and metastasis. Recently, Hamabe et al. revealed that CRC cells that are cultured with EMT stimulating factors, facilitated PKM2 to localize inside the nucleus to interact with TGF-beta induced factor homeobox 2 (TGIF2) and repress the expression of the TGIF2 target gene CDH1 (E-cadherin).

PKM2 impedes the TGIF2 dependent transactivation of E-cadherin by recruiting HDAC (histone deacetylase) to CDH1 gene and deacetylate its promoter, eventually repressing epithelial phenotype, and facilitating the expression of the mesenchymal trait. In a similar kind of study, EMT stimulation in colorectal cancer cells was shown to enhance the nuclear translocation of dimeric

PKM2 to activate STAT3 transcription activation. Activated STAT3 is known to enhance the transcription of snail-2, matrix metallopeptidase-2 (MMP-2), matrix metallopeptidase-9 (MMP-9) and also induce the β1-integrin/FAK signaling. Furthermore, snail-2 activation has been shown to modulate the expression of N-cadherin and repress E-cadherin to demonstrate epithelial to mesenchymal transition (Fig. 3). The molecular mechanism involving PKM2, thus, has been shown to regulate EMT in hepatocellular carcinoma cells (HCC). EGF stimulated HCC in an ERK1/2 dependent manner influences PKM2 to localize inside the nucleus and transactivate β-catenin-TCF/LEF-1 transcription complex to code for EMT markers to exhibit mesenchymal phenotype. In breast cancer cells that are stimulated with leptin (an adipokine), it has been shown to activate PI3K/AKT pathway to stimulate EMT in a PKM2 dependent manner. Either abolishment of PI3K/AKT activation using LY294002 or silencing of PKM2 expression attenuated the leptin-induced expression of EMT markers in breast cancer cells, inhibiting the invasion and migration of cancer. An aberrant high level of PKM2 expression was reported in cases of hilar cholangiocarcinoma (HC). Notably, the expression of PKM2 was shown to directly correlate with the expression of syndecan 2 (SDC2), the latter involved in the neuronal invasion. Silencing of PKM2 expression in HC downregulated SDC2 expression and attenuated the neural invasion of HC in vitro and in vivo.

PK Isoforms in Noncancer Human Pathologies and in Cell Physiology

Pyruvate Kinase Deficiency (PKD)

Inherent reasons, such as aberrant mutations in *PKLR* gene that affect the expression of PKR isoform and its biochemical function are referred to as pyruvate kinase deficiency (PKD). PKD is inherited as an autosomal recessive trait and until recent 195 distinct mutations have been identified in *PKLR* gene. Such aberrant mutation in PKR gene directly affect the glycolytic metabolism of RBCs and alter these in unhealthy cells that fail to meet the demand for the oxygen supply of body tissues, causing a severe pathological condition, chronic nonspherocytic hemolytic anemia. The unhealthy RBCs with hemolytic anemia differ from the normal cells with respect to their lifespan, which is lesser than 120 days. The severity of disease differs from case to case, where PKD results in a mild chronic neonatal anemia that may turn worse in childhood and in rare cases results in neonatal death. Until now, the practice of bone-marrow transplantation is one of the prominent ways to cure PKD, but with huge limitations. Moreover, advancement in the practices of gene therapy is providing new hopes and in future may cure PKD.

PKM2 and Nonmalignant Pathologies

Evidences accumulated in the past decade have substantiated the indispensable role of PKM2 in the pathophysiology of cancer, keeping in view the glycolytic and nonglycolytic attributes of PKM2. In addition to cancer, the aberrant PKM2-expression, -enzyme activity and consequential metabolic rewiring (i.e., aerobic glycolysis) is now well-recognized to be associated with numerous human pathologies, such as myotonic dystrophy, coronary artery disease (CAD), Bloom syndrome (cases with natural mutations in PKM2), severe aplastic anemia (SAA), preclampsia (PE), Crohn's disease and rheumatoid arthritis.

Nonmetabolic Role of PKM2 in Cell Physiology

Besides, what is now known about PKM2 expressing in adult progenitor and differentiated cells, only a few studies in the recent past have revealed the multifunctional role of PKM2 in governing physiology at cellular and whole-body level, such as regulation of inflammatory response against invading pathogens and in the processes of wound healing. Macrophages that were stimulated with LPS or *S. typhimurium* both in vitro and in vivo, promoted the accumulation of the dimeric form of PKM2 and its nuclear localization to form complex with HIF1α. PKM2-HIF1α complex enhanced the transcription of a proinflammatory cytokine IL-1β (a HIF1 target gene). Notably, either knockdown of PKM2 expression or stimulation of PKM2 tetramerization in LPS-induced macrophages abrogated PKM2 and HIF1 interaction; as well as IL-1β expression and secretion. The study proposed a unique and novel nonglycolytic physiological role for PKM2 in inflammatory responses. In addition, a recent study has revealed, that in activated macrophages, PKM2 complex with HIF1α and regulated the expression of HIF target gene, high mobility group box-1 (HMGB1; a secretory proinflammatory cytokine). Likewise, PKM2 in LPS-induced colorectal cancer cells stimulated Stat3 transactivation to express its target genes, IL-1β and TNF- α. A recent study has demonstrated the role of PKM2 in the early inflammatory response and wound healing, where activated neutrophils infiltrate the site of injury and secrete PKM2, the latter promoting angiogenesis at the wound site; and as a consequence, healing the wound. Parkin, an E3 ubiquitin ligase (an autosomal recessive mutation in Parkinson disease) has been shown to monoubiquitinate PKM2 and PKM1 at lysine (K) -186 and 206 residues and reduce its enzyme activity, however without altering protein stability, depicting the apparent role of Parkin in energy metabolism and offering a novel understanding of Parkinson's disease with possible novel therapeutic strategies.

Potential of PKM2 in Onco-Therapy

Considering the quantum of metabolic and nonmetabolic functions of PKM2 that support the cancer cell growth, proliferation, and spreading, PKM2 has emerged as a potential target with immense anticancer therapeutic opportunities. Several studies have proposed a variety of therapeutic strategies to target PKM2 expression and thus curtail cancer cell progression. This includes

RNA interference, natural compounds (phytochemicals), small molecule inhibitors and -activators. In brief, knockdown of PKM2, using small interfering RNA (siRNA), has been shown to significantly affect the growth and proliferation of tumor cells, both in vitro and in vivo, the latter corroborated with the observation of increased caspase-dependent apoptosis. It is also reported that the cancer cells silenced for PKM2 are highly sensitive to radiation therapy as well as for anticancer drugs that are potential DNA damaging agents, suggesting the synergetic effect of PKM2 siRNA with other approaches of oncotherapy to formulate a tailored therapeutic strategy with more efficacy. Further, natural compounds that are derived from the phytochemicals, such as shikonin, resveratrol and gemcitabine have also been shown to inhibit tumor growth by perturbing the expression of PKM2. High throughput chemical screen has revealed several novel small molecule inhibitors of PKM2. Compounds, such as Arava and *N*-(3-carboxy-4-hydroxy) phenyl 1-2, 5,-dimethylpyrole were shown to specifically bind PKM2 and inhibit its enzyme activity. In addition to these drugs or therapeutic strategies, recently cell permeable structural analogs of the allosteric activator FBP, such as TEPP-46 (ML265; thieno-[3,2-*b*]pyrrole[3,2-*d*]pyridazinone) and DASA-58 (ML203; substituted *N*,*N*′-diarylsulfonamide) have been shown to promote PKM2-tetramerization and -enzyme activity. Lung cancer cells treated with TEPP-46 and DASA enhanced PKM2 enzyme activity and eventually inhibited lung cancer cell progression in in vitro conditions and in xenograft mouse models. The plausible molecular mechanism through which compounds that stimulated the tetramerization of PKM2 and inhibited cancer cell proliferation is by limiting the biosynthesis of macromolecules, which was actually supported by dimeric form of PKM2. Collectively, results from a multitude of studies have emphasized that PKM2 could be a potential target to design anticancer therapy with improved efficacy. However, one of our recent publication has shed light on the molecular mechanism involving cell survival signaling pathways that could develop resistance and may diminish the efficacy of therapeutic strategy, involving inhibition or silencing of PKM2, proposing a potential therapeutic strategy that may improve the efficacy of PKM2 silencing to curtail tumor propagation. The multifaceted nonglycolytic role of PKM2 in cancer and in a few physiological processes is due to its PTMs, however, more studies are required to identify that the PTMs of PKM2 that are unique to cancer cells and not shared by the other adult differentiated cells, may open up new therapeutic opportunities with fewer side effects.

Concluding Remarks

PKM2 is evolutionarily conserved prototypic glycolytic enzyme. Its expression, the quaternary structure and a physiological role are controlled by a variety of extrinsic and intrinsic cellular signals. Considering the key position that PKM2 occupies in the glycolytic pathway, it senses a variety of signals and integrates these with the metabolic status to modulate the glucose metabolism to meet the bioenergetic and biosynthetic expenses of cells that either grow, proliferate, differentiate, rest or resist the stresses from the microenvironment (i.e., hypoxia and hypoglycemia). For instance, differentiated cells that are under quiescent state retain an enzymatically active tetrameric form of PKM2, which primarily supports energy synthesis with limited biosynthesis of macromolecules. On the contrary, embryonic cells or malignant cancer cells that often divide, necessitate a hefty demand for macromolecules with balanced energy supply, thus preferring to overexpress PKM2 and simultaneously alter it into enzymatically inactive dimeric form.

A variety of growth factors and survival signals regulate PKM2 expression and function (i.e., glycolytic and nonglycolytic attributes) to achieve vital physiological functions and to preserve cellular and whole-body level homeostasis. Aberration in these signaling pathways (e.g., oncogenes that constitutively stimulate signaling) result in deregulated PKM2-expression and -enzyme activity, leading to malignancy and numerous pathologies, such as myotonic dystrophy, coronary artery disease, inflammatory bowel disease and neuronal disorders. Thus, the therapeutic strategies that would target PKM2 to curtail tumor growth, may hold a promise to treat other pathologies as well. However, more studies are desired to understand the respective roles of the two isoforms of pyruvate kinase (PKM1 and PKM2) under aberrant and normal cellular genetic backgrounds.

Further Reading

Chaneton, B., Gottlieb, E., 2012. Rocking cell metabolism: Revised functions of the key glycolytic regulator PKM2 in cancer. Trends in Biochemical Sciences 37 (8), 309–316.
Gupta, V., Bamezai, R.N., 2010. Human pyruvate kinase M2: A multifunctional protein. Protein Science 19 (11), 2031–2044.
Gupta, V., Wellen, K.E., Mazurek, S., Bamezai, R.N., 2014. Pyruvate kinase M2: Regulatory circuits and potential for therapeutic intervention. Current Pharmaceutical Design 20 (15), 2595–2606.
Iqbal, M.A., Gupta, V., Gopinath, P., Mazurek, S., Bamezai, R.N., 2014. Pyruvate kinase M2 and cancer: An updated assessment. FEBS Letters 588 (16), 2685–2692.
Luo, W., Semenza, G.L., 2012. Emerging roles of PKM2 in cell metabolism and cancer progression. Trends in Endocrinology & Metabolism 23 (11), 560–566.
Mazurek, S., 2011. Pyruvate kinase type M2: A key regulator of the metabolic budget system in tumor cells. The International Journal of Biochemistry & Cell Biology 43 (7), 969–980.
Mazurek, S., Boschek, C.B., Hugo, F., Eigenbrodt, E., 2005. Pyruvate kinase type M2 and its role in tumor growth and spreading. Seminars in Cancer Biology 15 (4), 300–308.
Prakasam, G., Iqbal, M.A., Bamezai, R.N.K., Mazurek, S., 2018. Posttranslational modifications of pyruvate kinase M2: Tweaks that benefit cancer. Frontiers in Oncology 8 (22).
Prakasam, G., Iqbal, M.A., Gupta, V., Kumar, B., Bamezai, R.N.K., 2017. Pyruvate kinase M2. In: Choi, S. (Ed.), Encyclopedia of signaling molecules. Springer, New York, NY, pp. 1–11.
Vander Heiden, M.G., Cantley, L.C., Thompson, C.B., 2009. Understanding the Warburg effect: The metabolic requirements of cell proliferation. Science 324 (5930), 1029–1033.
Wong, N., De Melo, J., Tang, D., 2013. PKM2, a central point of regulation in cancer metabolism. International Journal of Cell Biology 242,513.

Radiation Oncology

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Glossary

Image-guided radiotherapy (IGRT) General term describing the process of integrating various imaging modalities into the treatment workflow of radiotherapy to improve precision and accuracy of radiotherapy. IGRT is used to support and verify patient positioning and alignment, to compensate for motion of the patient or target (mainly used for respiratory motion) and to monitor anatomical changes during the course of radiotherapy that potentially influence the treatment application and therapy outcome.

Intensity-modulated radiotherapy (IMRT) Advanced technique of external beam radiotherapy applying irradiation fields with specifically inhomogeneous dose distributions mostly using high energy photons.

Linear energy transfer (LET) Term used in dosimetry describing the energy transfer of ionizing radiation to the tissue traversed per unit distance. A distinction can be made between sparsely ionizing radiation such as photons, electrons and protons exhibiting a low LET, and densely ionizing radiation such as alpha particles and ions exhibiting a high LET.

Oxygen enhancement factor (OER) Term used in radiobiology describing the ratio of absorbed dose between hypoxic and oxic conditions within a specific biological system resulting in the same defined effect or experimental endpoint.

Particle therapy Type of external beam radiotherapy using high energy particle beams such as protons or ions (e.g., C-12, O-15) generated by dedicated medical circular accelerators.

Photon therapy Type of external beam radiotherapy using high energy photon beams generated by medical linear accelerators.

Relative biological effectiveness (RBE) Term used in radiobiology describing the ratio of absorbed dose between different fields of ionizing radiation (i.e., type, energy) resulting in the same defined effect or experimental endpoint within a specific biological system. Thereby, an investigated radiation field is typically compared to a reference radiation field, which is traditionally 200 kV X-rays or Co-60 and today often megavoltage photons from medical linear accelerator.

Introduction

Radiotherapy together with surgery, chemotherapy, and emerging, immunotherapy is one of the four main treatment modalities for cancer patients. The value of radiotherapy for cancer treatment was established more than 100 years ago and up today the role of this therapeutic option is continuously gaining importance. At present, more than 50% of all cancer patients receive radiotherapy during their course of disease. Rapid advances in the field of radiation oncology with respect to biology (e.g., tumor environment, normal tissue reactions, and radiation effects) and treatment technology lead to a more personalized cancer treatment. For this purpose, individual biological and tumor-specific factors are considered for the therapy decision besides anatomical and clinical information.

The main aim of radiotherapy is to achieve local or locoregional control of tumor disease without major damage to the surrounding normal tissue. Radiotherapy can also decrease distant metastases by preventing metastatic spread from locally uncontrolled tumors. Radiotherapy is often applied in combination with systemic treatments. Today, mostly classical chemotherapeutics like Cisplatin are used, but in recent years the combination with small molecules and immunotherapy has gained increasing importance. For many tumor entities, radiotherapy is also combined in a pre- or postoperative setting with surgery. Radiotherapy can be applied with curative intention but also plays an important role in palliative care of patients with very locally advanced tumors or distant metastases. In this situation, radiotherapy can efficiently reduce pain, for example by bone metastasis, prevent paraplegia caused by metastases to the vertebral column and dissolve life-threatening symptoms of vena-cava-superior syndrome. For this palliative intention, reduction of symptoms and preservation of quality of life are primary therapy goals, which usually can be achieved rapidly and with relatively low doses. A special situation is so called oligometastasis, in which radiotherapy can be applied with curative intention to a limited number of distant metastases.

Radiobiology

Radiobiology describes the biological effects of irradiation on molecules, cells, tissues, organs, and organisms. The interactions of ionizing radiation with tissues are in clinical practice broadly divided into effects on tumors and early- or late-responding normal tissues.

The main interactions of radiation with target molecules are ionization and excitation. The intensity of these processes and the resulting biological effect are beam-quality-dependent. To characterize a beam and its effects, two parameters are used namely the linear energy transfer (LET) and the relative biological effectiveness (RBE). The LET describes the energy transfer of ionizing radiation to the tissue dE traversed per unit distance dx, which represents the ionization density along the beam (Eq. 1). A distinction is made between sparsely (low LET, e.g., photons, electrons, protons) and densely (high LET, e.g., ions) ionizing radiation. The spatial density of damage caused by high-LET radiation is high, meaning that damage to target molecules is clustered and often difficult or even impossible to repair. Therefore, LET correlates with the biological effect (high LET leads to a large effect) and is dependent from the radiation energy.

$$LET = {dE}/{dx} \tag{1}$$

Beside of the physical characteristics, the biological effect of ionizing radiation is dependent on many other factors, for example the irradiated tissue, the evaluated effect and dose fractionation scheme. The relative biological effectiveness (RBE) describes the biological effect of a specific beam and dose on a specific biological system using a specific endpoint (Eq. 2). A RBE above 1 means, the biological effect of the investigated beam is greater than the reference beam (traditionally usually 200 kV X-rays or Co-60, today often megavoltage photons), and vice versa for a value lower than 1.

$$RBE = \frac{\text{Dose reference beam}}{\text{Dose investigated beam}} | \text{isoeffect} \tag{2}$$

When radiation travels through matter, secondary electrons are produced through interactions. These secondary electrons deposit the energy to the matter and thereby are the mediator of biological radiation effects. Interactions between secondary electrons and target molecules can be direct or indirect. Direct interaction leads to ionization or excitation and as a consequence to damage of the target molecule. Indirect effects of radiation generate radicals, which then interact with target molecules thereby causing damage. Most important in this context is radiolysis of water, which constitutes the largest component of molecules within cells, leading to hydrogen and hydroxyl radicals as well as hydrogen and hydrogen peroxide. These radicals are highly reactive, interact with the target molecules and damage them. Whether a beam leads to indirect, direct or both interactions, is dependent on the radiation quality and energy, and correlates with LET. With increasing LET, the impact of direct interactions increases and of indirect interaction decreases.

The most important radiation target determining the fate of irradiated cells is the deoxyribonucleic acid (DNA), which contains the genetic information of the individual cell. Important radiation-induced types of DNA damages include single- and double-strand breaks, as well as clusters of these damages. Only a small proportion of the DNA damage leads to cell death, because cells during evolution have developed very efficient DNA repair mechanisms. Important examples include base excision repair, homologous recombination, and nonhomologous end joining. Only incorrectly repaired or unrepaired DNA damage, most prominently unrepaired DNA double-strand breaks, lead to cell death. Radiation-induced cell death can occur by several mechanisms including apoptosis, senescence and, most importantly and quite distinct from other anticancer agents, mitotic catastrophe.

Beside of DNA repair capacity, additional factors, most importantly reassortment, reoxygenation, intrinsic radiosensitivity and repopulation, have an influence on cell survival and tissue damage after irradiation. These factors are usually referred to as the five R's of fractionated radiotherapy.

Cycling cells run through the cell cycle, from which mitosis is the most sensitive and the late S phase the most radioresistant. After irradiation of a cell population, the most sensitive cells are killed while the more resistant cells have a higher chance to survive. In addition, cells build dose-dependently a G2 phase arrest allowing improved DNA repair of radiation damage before entering mitosis. All these mechanisms lead to a partial and temporary phase synchronization of surviving cells after irradiation, with selection of more resistant cells. With increasing time after irradiation, the cells distribute again over the cell cycle, which is called reassortment or redistribution. This phenomenon is associated with increasing radiosensitivity of cell populations. Cells can also temporarily leave the cell cycle and enter the dormant G0 phase. Particularly stem cells are preferentially dormant, which allows better protecting their DNA from damage. Dormant stem cells in tumors may therefore be the cause of radioresistance. Recruitment of stem cells into the cell cycle may occur as a consequence of sensing depletion of more differentiated cells during radiotherapy, which would be expected to be associated with an increasing radiosensitivity on a cellular level. However, at the same time the radiosensitivity of cells with stem cell characteristic may increase. The effect of high-LET radiation is less dependent on the cell differences and different radiosensitivity of the cell cycle phases. This is maybe one of the reasons why high-LET radiation is on average more efficient in very slowly proliferating tumors.

Cellular radiation sensitivity importantly depends on the oxygen concentration during irradiation. Comparing the dose leading to the same cell surviving fraction under anoxic and oxic conditions (photon irradiation), an oxygen enhancement ratio (OER) of about 2–3 has been derived in many cell lines and tissues:

$$OER = \frac{\text{Dose hypoxic conditions}}{\text{Dose oxic conditions}} | \text{isoeffect} \tag{3}$$

Oxygen plays an important role for the fixation of the DNA damage caused by free radicals. The indirect interaction of radiation in tissue builds unstable radicals, which interact with the DNA. In the presence of oxygen, changes in the DNA are stabilized and thereby the damage is fixed. In contrast, radiation with high LET produces more direct damage resulting in a lower OER. Thus, high-LET radiation is expected to be particularly effective in hypoxic tumors. After irradiation with low-LET radiation, preferentially

radioresistant hypoxic cells survive in tumors. Over time following irradiation, these cells may reoxygenate, for example (1) by reperfusion of vessels, which were not perfused at the time of treatment, (2) by shrinkage of the tumor, which may lead to a higher ratio of vessels over tumor cells, (3) by renormalization of the vasculature or (4) by decreased oxygen consumption of surrounding tumor cells with radiation damage.

Intrinsic radiosensitivity describes the genetically determined sensitivity of cells to radiation. Intrinsic radiosensitivity may differ significantly between different cell lines. On average radioresistant tumor entities and normal tissues are also characterized by low cellular radiosensitivity.

Repopulation refers to an increase in the number of stem cells in tumors or normal tissues during the course of radiation. Repopulation can be caused either by proliferation during treatment or by decreased cell loss. In any case, repopulation leads to increasing radioresistance with increasing overall time of treatment. This has also been coined as time factor of fractionated radiotherapy. For example, it is well recognized from preclinical experiments and clinical studies that, because of repopulation, many tumors, most prominently squamous cell carcinomas, have a lesser chance to be controlled after long compared to short treatments when applying comparable doses. This accounts on average for 0.5–1.0 Gy for each day of treatment prolongation, which is highly relevant for clinical practice.

In clinical practice, effects on tumors have to be differentiated from effects on so called early- and late-responding normal tissues. The tumors are not simply a bag of homogenous tumor cells, but a complex tissue of many different cell populations interacting with each other. Description of these interactions is beyond this article. However, for radiobiological considerations it is important to differentiate between stroma, containing a large number of nonmalignant cell populations, on the one side and tumor cells on the other side. Tumor cells again can be divided into cancer stem cells (CSCs), which usually constitute a minority of the tumor cells, and noncancer stem cells, which usually constitute the majority of the tumor burden. CSCs and non-CSCs are not exclusive to each other, as CSCs can lose their stemness while non-CSCs can gain stemness in the course of disease or during treatment. However, only CSCs can cause recurrence of the primary tumor after radiotherapy or metastases. If not eliminated by the host using other mechanisms including immune responses, a single surviving CSC can lead to a recurrence. Therefore, the aim of the curative radiotherapy is to kill all CSCs. The probability of local tumor control increases with increasing dose according to an S-shaped curve, with a steep dose response relationship after a threshold dose. In principle, all tumors could be eradicated by radiotherapy if high enough doses can be applied. However, this is limited by the tolerance of the tumor surrounding normal tissues. Thus, the clinical challenge is to prescribe and deliver the dose leading to the optimum of uncomplicated local control, resulting in a high chance for local tumor control at an acceptable risk of serious normal tissue damage (**Fig. 1**). This concept was first described by Holthusen in 1936.

Side effects of radiation are classified into stochastic and deterministic according their dose-effect relationship. The probability of stochastic radiation damage, for example mutations and secondary cancer induction, increases with dose without a threshold value. In contrast, deterministic radiation damage occurs only above a threshold dose, the likelihood and severity are dose-dependent. Deterministic radiation damage is further subdivided by the time point of occurrence, with regard to treatment, into early and late side effects. Early (acute) side effects occur during radiotherapy up to 90 days after start of treatment and usually recover. The duration and severity of early side effects are dose-dependent. Mucositis and dermatitis are typical examples. These tissues are hierarchically structured and have a high turnover. Irradiation leads to death of stem and precursor cells with subsequent reduced cell replacement, which leads to tissue depletion. The mechanism of repopulation allows the tissue to recover. In contrast,

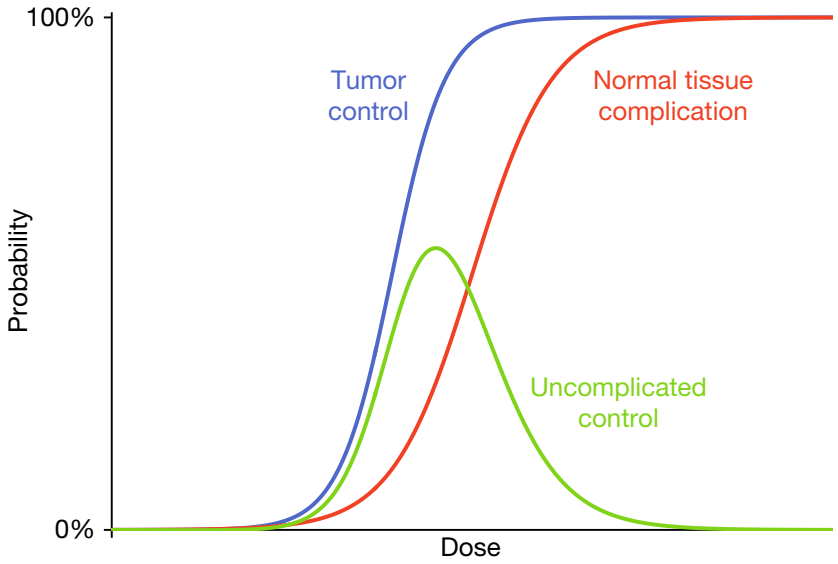

Fig. 1 Dose response relationship for tumor control and normal tissue complications. The closer the dose response curves for tumor and normal tissues are located to each other, the smaller is the therapeutic window for uncomplicated local control and vice versa.

late effects do generally not recover, latency is inversely dose-dependent and effects can occur from day 90 after start of treatment up to months or even many years. Fibrosis of the lung, bladder, subcutaneous tissues or neurons of the central nervous system are typical examples. The radiobiological mechanism is more complex than for early side effects and depends on the interplay of different cell types and tissues, including the immune system. Subclinical damage induced by irradiation may lead to late damage over time, which is often progressive. Early and late side effects usually occur independent of each other, with the exception of consequential late effects. These occur as a result of a particularly severe acute reaction with underlying radiobiological mechanisms combining early and late reactions. Early side effects increase with total dose and shortened overall treatment times. Late effects are mainly dependent on total dose, high dose per fraction and short intervals between fractions.

Radiotherapy is usually applied over several weeks with daily fractions of 1.8–2 Gy. This so called conventionally fractionated schedule has been developed empirically over decades and exploits the radiobiological mechanism underlined by the R's of fractionated radiotherapy. Several modified fractionation schedules have been developed on a radiobiological basis. Accelerated radiotherapy means shortening the overall treatment time by increasing the weekly dose above 10 Gy. It has been shown that shortening the overall treatment time counteracts the repopulation of CSCs during radiotherapy and increases local tumor control, but also increases early side effects. In contrast, a prolongation of the planned treatment time results in worsening tumor control and should therefore be avoided or compensated using radiobiological isoeffect calculations. Another concept is hyperfractionated radiotherapy, for which the total dose is divided into a larger number of fractions with smaller dose per fraction (< 1.8 Gy) maintaining the overall treatment time. Because of the dependence of late normal tissue damage on dose per fraction, hyperfractionation can be used to decrease late normal tissue toxicity or, at an isotoxic level, to increase the dose to the tumor. In clinical practice, hyperfractionation is typically used in combination with slight acceleration, which potentially allows a reduction of late side effects and improved local tumor control. Treatments with doses higher than 2 Gy per fraction are called hypofractionated radiotherapy. Doses up to approximately 3 Gy per fraction are often called moderate hypofractionation. Hypofractionation is frequently applied for palliative treatments. In this case, limited total doses are given, for example 10×3 Gy, 5×4 Gy or 1×8 Gy. In recent years, the application of moderate hypofractionated radiotherapy with only slightly reduced total dose has gained in importance also for curative treatments, for example for breast and prostate cancer. Usually tumor treatments are also moderately accelerated. Compared to conventional fractionated radiotherapy moderately hypofractionated and accelerated radiotherapy to lower total dose may achieve a comparable rate of tumor control and late normal tissue damage. As less fractions need to be given, the treatments may be more convenient for patients and may allow treatment of more patients at a given number of machines. The fact that, despite higher doses per fraction, late normal tissue damage was not increased in a number of clinical trials testing this approach can be explained by reduced total doses and high precision treatments of small volumes with modern technologies. This is also the reason for successful application of very high doses per fraction using stereotactic radiotherapy approaches, which allow precisely treating small target volumes with a very steep dose gradient. For example, stereotactic radiotherapy is applied with so called ablative doses (e.g., 3×18 Gy) to small lung tumors or metastases in bone, lung, liver and brain. Hypofractionation appears also to be a promising option for curative treatments with particle therapy and should be tested in clinical trials.

The linear-quadratic (LQ) model describes the biological effect of irradiation to tumor and normal tissues and allows the comparison of different fractionation protocols. The LQ model is based on the observation that a typical cell survival curve after irradiation, when plotted in the usual semilogarithmic way, can be described by a linear component proportional to dose and a quadratic component proportional to dose squared. The constants α and β characterize the slopes of these components and vary between different tumors and normal tissues. The α/β ratio can be used to evaluate normal tissue reactions and convert between fractionation protocols. Thereby, a high α/β ratio goes along with a low fractionation sensitivity, which is typical for most tumors and early-responding normal tissues. Inversely, a low α/β ratio indicates high fractionation sensitivity and is typical for late-responding normal tissues but also for some tumors (e.g., breast cancer, prostate cancer melanoma, chondrosarcoma).

Beside the reported radiotherapy parameters (e.g., dose per fraction, total dose) the irradiated volume of normal tissues has significant influence on damage and associated function loss. For example, a large part of the liver can be damaged by irradiation without severe function deficits of the whole organ if a sufficient proportion of liver can be spared. In contrast, radiation damage of just a small volume of the spinal cord may lead to paralysis. These different volume effects depend on the respective organ architecture. The organs at risk (OAR) can be classified broadly according to their functional tissue organization as serial and parallel. Serially organized organs consist of a continuous chain of functional units (FUs) and damage to only one unit causes complete organ failure, which applies, for example, for the spinal cord and esophagus. In contrast, parallelly organized organs consist of individual-acting functional units and damage is compensated to a certain extend. Typical examples are the lung and liver, where organ function is maintained until an organ-specific threshold of damaged FUs is exceeded. The volume effects in different tissues are taken into account during the radiotherapy treatment planning by dose-volume constraints for the individual OARs. However, the recommended constraints are empirical data without considering additional influencing factors for organ damage requiring individual evaluation for every patient (e.g., simultaneous or previous systemic therapy). For parallelly organized organs, function preservation is essentially determined by the remaining FUs. Is the function already impaired before radiotherapy, for example due to exogenous noxae, illness or age, the remaining FUs and therewith the dose-volume tolerance are different compared to healthy individuals. Therefore, additional diagnostics like lung or kidney function tests are crucial before radiotherapy.

Tumor response and normal tissue complications vary greatly between patients with, according to clinical and histopathological parameters, apparently similar disease. With clinical and pathological parameters it is not possible to define, for example, the number of CSCs or their radiosensitivity, which importantly influences the treatment result. Therefore their predictive power for treatment outcome is limited. Genetic variations and a host of other radiobiologically important factors influence the reaction

of tumors and normal tissues to radiotherapy. It is therefore necessary to develop clinically suitable radiotherapy specific biomarkers to predict treatment response regarding tumor and normal tissues. Such biomarkers can be assessed using medical imaging, tissue samples or liquid biopsies. A predictive biomarker is usually evaluated before treatment and indicates a different outcome for a specific treatment in biomarker-positive versus-negative patients. An example is the methylation status of O^6-methylguanine-DNA methyltransferase (MGMT) in glioblastoma. Patients with MGMT-methylated tumors exhibit a better response to radiochemotherapy with Temozolomide compared to MGMT-unmethylated tumors. A prognostic biomarker can be evaluated before or during treatment and indicates the likely course of the disease without providing information on advantages or disadvantages of specific treatments. In clinical practice, it is sometimes difficult to distinguish between predictive and prognostic biomarkers. Additionally, the treatment itself may have an influence on the prognostic or predictive power, such that the value of a given biomarker has always to be referred to a specific clinical situation. The predictive and prognostic value of biomarkers may also change during treatment necessitating repeated evaluation of the biomarker's dynamic. A typical example for a prognostic marker is the human papillomavirus (HPV) status of oropharyngeal carcinoma, of which HPV-positive tumors are more radiosensitive compared to HPV-negative ones. Note that, if future interventional studies should show that HPV-positive tumors can be treated equally effective with reduced doses or without chemotherapy, HPV status would change from a prognostic to a predictive biomarker. Radiomics, that is, the evaluation of profiles derived from quantitative texture analysis of imaging data, is an emerging approach to generate spatially resolved biomarkers switches for radiotherapy treatment planning and monitoring. A great advantage of this approach lies in the fact, that imaging data are available for all radiotherapy patients from treatment planning and increasingly from monitoring tests during treatment. So far, radiomics in mostly retrospective clinical studies has shown similar potential for prognostic stratification as tissue-based markers.

Further investigations and identifications of biomarkers are needed to classify patients and their disease not only according to anatomical and histological information but also to biomarker status to decide for the optimal therapy and avoid under- or over-treatment. The biomarkers for tumor and patient characteristics as well as the radiomics approach support the development toward a personalized radiotherapy.

Hardware and Technique

Due to the enormous development in radiotherapy, a variety of modern equipment and techniques are currently available for treatment. Application in patients requires modern information technology consisting of (1) high performance computers for fast device control, calculations and visualization, (2) high capacity servers for data storage and management using large databases (e.g., for images, backup in case of crash, long term storage), (3) fast network connection between the devices including import/export options, and (4) dedicated software with clear and intuitive graphical user interfaces facilitating simple operation and assistance of all processes.

Four main categories of radiotherapy can be distinguished: external beam therapy, brachytherapy, intraoperative radiotherapy (IORT) and systemic radioisotope therapy. Differences of these categories result from the position of the radiation source and therewith the distance between source and target. With external beam radiotherapy, irradiation is delivered from outside the patient's body without direct contact between source and target. To obtain adequate penetration of the tissue, typically high energy electrons, photons or particles, produced by clinical accelerators, are used but also high energy gamma rays from Co-60 are still of relevance. For brachytherapy, sealed radioactive sources are temporarily or permanently placed in direct vicinity of the target area. Irradiation is applied using low and medium energy beta and gamma rays from radioisotopes such as Ir-192 or I-125. IORT is a special type of radiotherapy applied during surgery. Dedicated applicators are brought in direct contact with the tumor bed after tumor resection and irradiation is delivered using low or medium energy photons or high energy electrons. Systemic radioisotope therapy is a category of targeted therapy usually applied by a nuclear medicine specialist. Targeting is achieved due to the chemical properties of the radioisotope (e.g., iodine absorption of thyroid gland) or attaching the radioisotopes to a transporting molecule (e.g., a hormone) or antibody specifically binding to the target tissue. The resulting therapeutic agent is then delivered to the patient through infusion or ingestion.

Selection of therapy options for cancer treatment is mainly determined by evidence summarized in guidelines according to tumor characteristics (e.g., stage, volume, spread, and position), patient condition and wishes, treatment intention and expertise and infrastructures of the treatment center. The central goal of physical planning and application of radiotherapy is to maximize the dose to the target volume while minimizing the exposure to normal tissues. Hence, delivered dose distribution should be as conformal as possible, which is attained by using design features of the treatment devices as well as physical radiation properties differing between the types of radiotherapy.

External Beam Therapy Using High Energy Photons and Electrons

External beam therapy using high energy photons and electrons is the current standard of radiotherapy. Radiation is usually produced by a clinical linear accelerator (LINAC), which consists of three main components: (1) a static structure holding (2) an isocentric gantry that can vertically rotate 360 degree around (3) a patient couch. High-frequency electromagnetic waves are used to accelerate electrons to high energies through a linear tube of approximately 1 m length inside the gantry. The generated pulsed electron pencil beam is bent toward the patient by means of magnets inside the gantry head. For photon irradiation, the

beam hits a target to produce bremsstrahlung as well as a flattening filter to homogenize the beam intensity. For electron irradiation, the beam passes a scattering foil to widen it. Following these, ionization chambers are used to monitor dose, dose rate and beam characteristics (e.g., flatness, symmetry) and therewith to control the machine output. At the beam exit at the gantry head, several steps of collimators are integrated for field formation and radiation protection purpose. The LINAC thus provides electron and photon cone beams of typically 5–25 MeV energy with a focus-isocenter-distance of normally 1 m, a maximum field length of approximately 40 cm and a dose rate of 3–6 Gy min^{-1}.

Beam shaping of photon beams today is mainly achieved by a multileaf collimator (MLC). MLCs consist of opposing sets of tungsten alloy leaves with a height of about 7–12 cm and, projected to the plane of isocenter, a width of about mostly 3–10 mm. Resulting transmission through the leafs is few percent of the open field dose rate. Each individual leaf is moveable by a computer-controlled motor allowing for stepped beam shaping. MLCs are mostly supported by preceding or succeeding motor-driven jaws, which are used for field shaping and reduction of scattered or leakage radiation. These collimation features have a typical distance of about 40 cm to the isocenter. Due to an increased scattering in air, beam shaping of electron beams is achieved by additional applicators comprising metal scrapers to absorb leakage radiation as well as an insert to hold apertures casted from Rose's metal. Alternatively, an electron MLC (eMLC) is available as add-on component exhibiting similar characteristics as integrated MLC. These applicators or eMLC can be mounted to the gantry and patients are typically positioned with a small air gap of few centimeters to it.

Fig. 2 exemplarily shows lateral and depth dose profile distributions of photon and electron beams. Lateral dose profiles are characterized by a flat, homogenous plateau and a penumbra of several millimeters. Depth dose profiles differ between electrons and photons, both showing dose built-up when entering tissue. Electron beams are stopped at a certain range defined by their energy (within several centimeter depth) and are therefore suitable for superficial targets whereby tissue in greater depth is protected. In contrast, photon beams exhibit an energy-dependent, exponential dose fall-off in the depth. Sparing of normal tissue is attained by delivering photon beams from different directions and with field shapes adapted to the target shape resulting in a superposition of applied dose distributions.

Irradiation with electrons is usually applied to the patient by a single fixed beam from a defined gantry angle. For each beam, an individual aperture has to be casted according the target shape before and needs to be inserted to the applicator, or a specific eMLC needs to be utilized. Irradiation with photons can be delivered by fixed beams from various gantry angles. For simple cases (e.g., with palliative intent), single or opposing beams are used but typically multiple beams are applied to achieve higher dose conformity. Each photon beam is shaped by the MLC resulting in a homogeneous dose distribution but also inhomogeneity can be useful, for example to compensate for varying materials (i.e., density, thickness, atomic composition) traversed by the beams. In addition, some advanced high precision techniques explicitly require areas of higher dose inside the target volume (i.e., simultaneous integrated boost) to increase treatment intensity in these areas while keeping limited doses in other areas of the target volume and in healthy tissues. Such intended dose inhomogeneity's can be generated by leaf motion and are therewith an important characteristic of an advanced technique called intensity-modulated radiotherapy (IMRT). Two delivery modes of IMRT are distinguished (Fig. 3). For step-and-shoot IMRT, radiation and leaf motion are executed sequentially (leaves cannot be moved when the beam is on). For sliding-window IMRT, radiation and leaf motion are executed simultaneously (leaves can be moved when the beam is on). Beside fixed beams, another way of delivering photons to the patient is arc therapy. Thereby, the gantry rotates during irradiation in combination with either static MLC shape or leaf motion (e.g., dynamic conformal arc therapy, intensity-modulated arc therapy). Arc therapy is provided by LINACs equipped with gantry and MLC generating a cone beam, but also by TomoTherapy® devices. These devices consist of a LINAC that is mounted on a CT ring gantry for continuous 360 degree rotation around a patient couch. A pneumatic MLC is used for field shaping to produce a 6 MeV photon fan beam, which is applied to the patient as a helical arc through continuous couch translation.

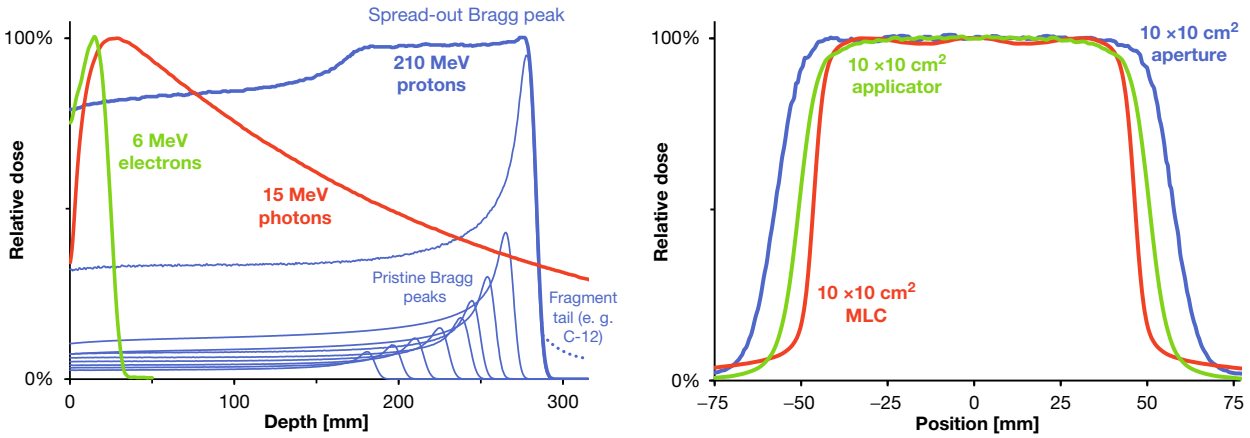

Fig. 2 Schematic depth dose (*left*) and lateral dose profiles (*right*) of 6 MeV electrons, 15 MeV photons, and 210 MeV protons (passive scattering) used for external beam radiotherapy.

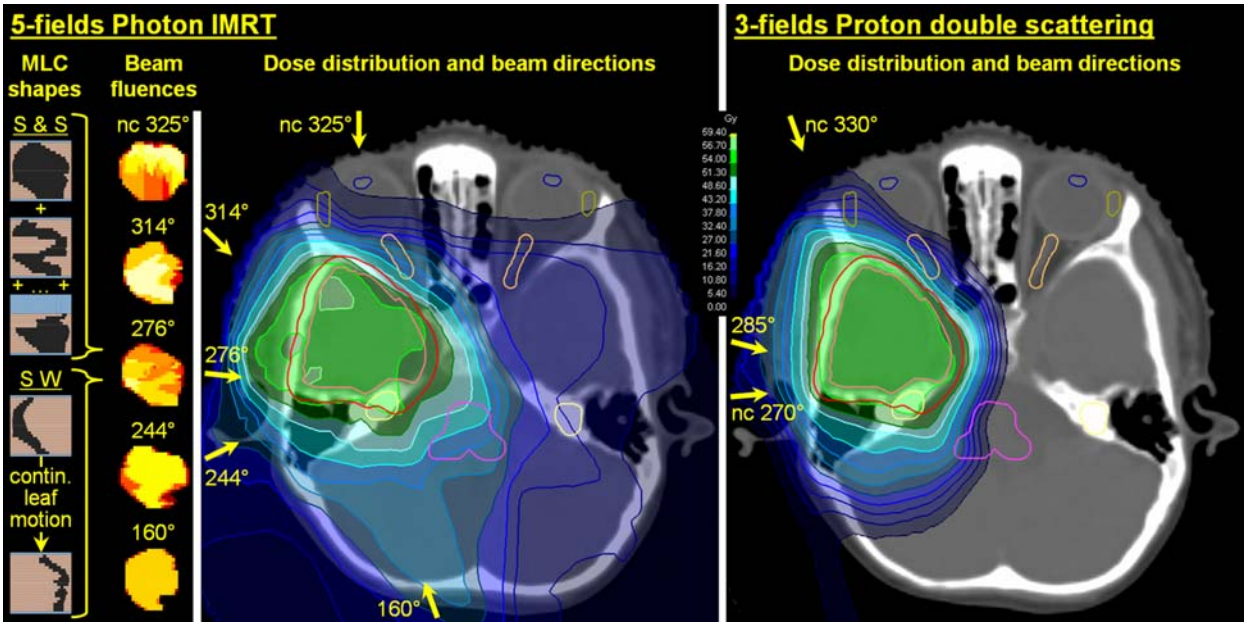

Fig. 3 Comparison of dose distributions for external beam radiotherapy using 5-fields photon IMRT (left) and 3-field proton double scattering irradiation (right). For IMRT, beam fluences as well as exemplary MLC shapes for one of these fluences using the two different IMRT delivery modes (*S&S*, step-and-shoot; *SW*, sliding-window) are included. Beam directions are indicated as gantry rotation angles, in some cases with additional patient couch rotation marked as noncoplanar (nc).

A specific type of external beam radiotherapy with photons is the stereotactic radiotherapy (SRT). The particularity of SRT is the high accuracy and precision of beam application resulting in high dose conformity as well as steep dose fall-off outside the treatment volume. This allows application of very high doses to a small target volume (<3 cm diameter) in a single treatment or a limited number of fractions. For that, multiple fixed beams or several arcs with largest possible spatial separation are needed to focus the high dose to the target volume while minimizing exposure of surrounding tissues by widely distributing entrance and exit doses. SRT can be performed with a LINAC equipped with an MLC with particular thin leaves or cylindrical cone collimators for beam shaping. To shorten the irradiation time for the patient and therefore increase the dose rate (up to factor four), the flattening filter inside gantry head of dedicated LINACs was removed. This leads to a nonuniform, radially decreasing lateral dose distribution and a reduction of the scattered radiation. Another device for intracranial SRT is the Gamma Knife®, which consists of about 200 Co-60 sources arranged in a hemispherical or cylindrical configuration as well as a helmet or a ring array collimator focusing circular field shapes (4–18 mm diameter) to the isocenter. During the treatment with both LINAC and Gamma Knife®, the patient position needs to be very stable and motion needs to be controlled. For extracranial targets, patients are ideally positioned inside a vacuum cushion, whereas for intracranial targets, rigid fixation of the head is feasible, especially used at the Gamma Knife®. The CyberKnife® allows for SRT without fixation. This system consists of a LINAC and a patient table both mounted on computer-controlled robots enabling near-complete freedom to position source and target. Beam shaping is alternatively achieved by exchangeable fixed cone collimators, a motorized Iris™ variable aperture collimator or an MLC. An integrated stereoscopic radiography system continuously evaluates the target position by means of anatomical structures (e.g., bones) or fiducial markers during the treatment enabling online adaption of beam and target position.

External Beam Radiotherapy Using Particles

Particle therapy with protons or heavier ions like C-12 is an emerging development in radiotherapy with various options for treatment optimization. Particles are accelerated by means of high-frequency electromagnetic waves using circular accelerators. The large mass of particles necessitates a much larger therapy devices compared to LINACs, resulting in higher costs and efforts for operation. The particle devices consist of four main components: (1) circular accelerator, (2) energy selection system, (3) beam line, and (4) patient application system (nozzle). For protons, a cyclotron of 2–5 m diameter and about 100–200 t weight and, for ions, a synchrotron of 20–40 m diameter with upstream LINACs for preacceleration are used to generate a mono-energetic beam. The following energy selection system is comprised of a degrader in form of a graphite wedge or wheel. The beam line is an evacuated tube of several 10 m length, surrounded by magnetic quadrupole lenses for beam focusing and dipole magnets for bending in case of curves or junctions. The patient application device differs between protons and ions. Protons are usually delivered by an isocentric 360 degree rotating gantry with about 10 m diameter and about 100 t weight. For ions, the worldwide only one 360 degree gantry is installed in the Heidelberg Ion-Beam Therapy Center with 13 m diameter, 25 m length and about 600 t rotating weight.

Other institutes using ions operate static beam lines guided into the treatment room, which is equipped with a respective couch or chair system for patient positioning.

For particle therapy, two different kinds of field shaping can be differentiated namely passive scattering and active beam scanning (also termed as pencil-beam or spot scanning). Beam shaping with passive scattering is achieved by guiding the mono-energetic pencil beam through a first scatterer to widen the beam. A range modulator or ridge filter forms the spread-out Bragg peak (SOBP) by partially decelerating particles to adapt the energies to the required ranges in tissue according to the tumor width in the beam direction. Afterwards, a second scatterer for further beam widening can be necessary. The lateral shaping of the homogenous beam is performed with a collimator milled from brass or poured from Rose's metal. Distal beam shaping is achieved by a compensator from acrylic or wax of varying thickness. These hardware devices have to be manufactured for each patient and irradiation field, and are mounted close to the patient at the beam nozzle before irradiation. Due to the fact that the proximal target contour cannot be fully shaped with passive scattering, normal tissue is to some extend unnecessarily exposed to higher doses. This can be overcome by active beam scanning, which allows also shaping of the proximal contour of the target without patient-specific hardware. By varying the beam energy through the degrader, a change from one range to another within the target is possible. The spatial deflection of the pencil beam is ensured by two vertical and horizontal magnet couples, such that single spots can be irradiated one after another scanning over the treatment area. Active beam scanning can be used to apply homogenous dose distributions, but also a highly complex dose distribution can be achieved by modulating the number of particles and thus the applied dose at each spot. Analogues to IMRT using photons, the latter is termed as intensity-modulated particle therapy (IMPT).

Treatment with particles has several advantages compared to photons and electrons. While passing through matter particles are continuously stopped mainly by collisional but also radiative processes having the highest energy transfer at the end of their track. Therefore, the depth dose profile exhibits a plateau region with a sharp increase of dose in a well-defined, energy-dependent depth (Bragg peak) and steep dose fall-off beyond the maximum (**Fig. 2**). For ions, additional nuclear fragmentation processes lead to a fragment tail in the depth dose profile. Lateral and range straggling decreases with particle mass such that both the width of the Bragg peak and the penumbra of the lateral dose profile are larger for the light protons as compared with the heavier ions like C-12. The physical characteristics of particle beams allow sparing of normal tissue by reducing the irradiated volume, which, based on radiobiological principals and clinical evidence gained from photon therapy, is expected to lead to a reduction of risk and severity of side effects as well as secondary cancer. However, on the other hand, the accurate and precise dose deposition of particles makes the treatment very sensitive to anatomical changes (e.g., changed body weight, edema) as well as organ motion and filling. Already minor changes can cause underdosage of the target and overdosage of the surrounding normal tissue. To prevent this, continuous dose and patient monitoring as well as knowledge of organ motion are even more important for particles than for photons. Beside the physical benefits, the increased RBE of particles may also be advantageous in some clinical situations. Differences between RBE of particles and photons are less pronounced for protons (RBE 1.1) than for ions (e.g., RBE 2–3 for C-12). The benefit of particle therapy using either protons or ions is still under investigation for each tumor entity and not generally accepted as a clinical standard. At present, the treatment of chondrosarcoma near the spinal cord or skull base, chordoma, ocular melanoma, pediatric tumors of the central nervous system, brain tumors with long life expectancy in adults as well as curative treatments in preirradiated tissues are among the accepted indications for particle therapy. To obtain a better database for evidence-based treatment allocation guidelines, it is necessary to perform comparative treatment planning with best practice photon therapy and treatment of patients in prospective trials.

Brachytherapy

Brachytherapy (BT) is a form of radiotherapy to treat cancer by placing sealed radioactive sources within the tumor volume. Through the direct contact, high dose can be locally applied whereas the dose to distant tissues decreases rapidly following the inverse square law. Therefore, BT is a highly efficient and conformal treatment, but with a limited applicability for several tumor entities and therapy schemes due to the required accessibility to the target area and its maximum size. BT can be used as monotherapy but it is typically delivered as additional local dose (boost) in combination with external beam radiotherapy.

Different types of BT can be defined according to the placement and dose rate of the radioactive sources as well as the way and duration of treatment delivery. The placement of sources can be interstitial using hollow needles or plastic catheters that are brought directly into the tissue by an invasive procedure. Another possibility is contact BT using the space adjacent to the target area for source placement, which might be the patient external, body cavities, lumens or vessels. Interstitial BT can be used for breast and prostate cancer, contact BT can be applied for gynecological, lung, esophageal and skin cancer. Regarding the dose rate of sources, BT is clinically applied with high dose rate (HDR) and low dose rate (LDR). Using HDR sources, irradiation time is in the range of several minutes and usually several fractions are applied to allow for repair of DNA damage in normal tissues thereby reducing morbidity. Conversely using LDR sources, the irradiation time is in the range of days to weeks. As repair in normal tissues takes place during treatment, fractionation is not necessary. To combine the radiobiological advantages of LDR BT with regard to repair of DNA damage and the physical advantages of HDR BT (e.g., flexibility in treatment planning, adaption and optimization), a third option termed as pulsed dose rate (PDR) BT was developed. Fractions of HDR BT with 0.4–1.2 Gy are given in 1–2 h interval over several days up to the prescribed dose (mostly boost).

Two main techniques of treatment delivery for BT can be distinguished: afterloading and seed implantation. Afterloading refers to a technique of first preparing the target area for irradiation by positioning nonradioactive applicators, needles or catheters that are subsequently connected to a remote-controlled afterloader for temporary placement of a radioactive source. Such afterloader

contains a shielded HDR point source (e.g., Ir-192 or Co-60) attached to a flexible wire enabling fast, motor-driven placement inside connected applicators. Treatment is applied according to calculated dwell times and positions taking into account exponential decay of the source activity. Seed implantation refers to a technique of implanting LDR point sources (so called "seeds," e.g., I-125 or Pd-103) in a planned pattern into the target volume by interstitial BT. This implantation is mostly permanent, for example, to treat prostate and brain tumors, but also temporary implantation with removal after few weeks has been performed. Radionuclides like I-125 and Pd-103 emit photons with lower than 30 keV energy, for which an increased RBE (I-125: 1.3–1.5; Pd-103: 1.9) has been reported. Radioactive seeds are also used for the treatment of ocular tumors. For this, dedicated ophthalmic applicators are sutured to the sclera for local irradiation over several days. These applicators can alternatively be equipped with seeds or a foil coated with a beta emitter (Ru-106) both enclosed by a thin gold or silver shielding for sterilization.

Intraoperative Radiotherapy (IORT)

Intraoperative radiotherapy (IORT) is usually applied after surgical resection directly in the operating room. A suitable applicator is placed in direct contact with the tumor bed or residual tumor and aligned for connection to the irradiation device. Deeper normal tissue can thereby be protected from exposure by inserting additional shielding material. IORT is typically given in a single fraction with doses of 10–20 Gy. In this ranges, lower doses are applied when IORT is followed by external beam radiotherapy whereas higher doses are prescribed when IORT is applied alone. IORT has been found effective for different tumor entities that are treated by surgery, for example breast, skin, pancreas, rectum, soft tissue sarcoma. The advantages include application of high local doses with direct visualization of the target area and very good preservation of normal tissues and OARs from irradiation.

There are two main types of irradiation devices for delivering IORT. Electron-beam IORT is applied by compact LINACs generating 3–12 MeV electron beams that are guided to the target area through circular applicators. The LINACs are mounted to a motorized stand or C-arm structure enabling flexible positioning for delivering beams directed from above onto the surgical table. For radiation protection purposes, a beamstop opposite the electron beam is integrated. To improve beam uniformity and flatness, scattering foils can be incorporated, however, requiring additional shielding to reduce stray radiation. The source-surface distance ranges from 50 to 100 cm such that electron beam IORT is subsumed under external beam radiotherapy. In contrast, IORT using low energy photons is also referred as electronic brachytherapy. Respective devices are equipped with low kilovoltage X-ray sources (≤ 50 keV), either point sources or collimated tubes, which are connected to applicators for intraoperative treatment with 1–4 cm focus-surface distance. For flexible applicator positioning, the X-ray source is carried by an articulated arm or, in case of the point source, a connecting tube. Due to the low energy of the X-ray source, radiation protection is comparatively simple. The radiant units of the described IORT devices are mobile due to integrated transportation systems and are therefore very flexible.

Systemic Radioisotope Therapy

Radionuclides, usually beta or alpha emitters, can be used for therapeutic purposes. This approach gains importance in the context of developing targeted molecules including antibodies that specifically bind to the surface of cancer cells. If these are labeled with therapeutic radioisotopes, the radiation can be guided directly to the tumor. As the range of the emitted electrons is in the order of several cell layers, also neighboring cells are irradiated besides the actual target cells to which the antibody is bound. An example is radioimmunotherapy of CD20-positive non-Hodgkin lymphoma with the antibody Ibritumomab-Tiuxetan labeled with Y-90. Particularly promising seems to be the combination of internal irradiation using targeted radionuclides and external beam radiotherapy. Preclinical studies using radionuclide-labeled Cetuximab in with combination external beam radiotherapy showed a significant improvement of local tumor control for some squamous cell carcinoma. Theranostic approaches refer to the use of targeted drugs, which can be labeled with radionuclides suitable for imaging (e.g., PET) and, if accumulating in the tumor, can be applied with therapeutic radionuclides for treatment. Such approaches promise personalized application of systemic radioisotope therapy.

Radiotherapy Workflow

Clinical Decision Making

Today, radiation oncologists are increasingly working as member of a multidisciplinary and multiprofessional team. They cooperate closely with medical oncologists, radiologists, surgeons, pathologists, additional disciplines depending on the tumor entity (e.g., dermatologists in case of melanoma) as well as other health professionals including psycho-oncologists, dieticians and physical therapists. This team discusses the overall disease situation of the patient based on relevant information, such as general condition, tumor staging, histology and images, and recommends the treatment according to guidelines or, increasingly, personalized treatment concepts. Before starting radiotherapy, the patient has to be informed in detail about the treatment intention (curative or palliative) and chances of success, treatment planning and application, possible early and late side effects, alternative treatment options, and wherever possible on potential use of data for clinical cancer research and quality control of treatment. After the patient consent, radiotherapy planning starts with treatment prescription including detailed information for treatment planning and application (e.g., required imaging, target volume, total dose, fractionation, beam quality and technique).

Imaging for Treatment Planning

Radiotherapy requires a defined, reproducible, stable and as convenient as possible patient position with free access to target area as basis for treatment planning. Therefore, imaging for radiotherapy, outside of the context of basic staging, has specific requirements. Patient position is determined during imaging using different aids, such as thermoplastic masks for head and neck region, skin marking stain or tattoo and immobilization devices such as vacuum cushion, knee rolls and arm rests. In recent years, additional procedures for increased reproducibility of organ fillings and for movement reduction have been developed. For example, for prostate cancer patients, bladder filling can be reproduced by a daily drinking protocol and ultrasound monitoring, and the rectum can be stabilized by a special rectal balloon. A spacer can be implanted between prostate and rectum, thereby reducing dose to the anterior rectum wall. For brachytherapy planning, CT imaging of the treated area has to include applicators or catheters to determine source positions and dwell times. For seed implantation, an ultrasound examination and, if necessary, an MRI is performed to determine the target volume (e.g., postage volume) in order to plan probable seed numbers and positions.

A CT scan of the target region typically builds the basis for treatment planning. Scans are usually performed by a modern multislice scanner with high spatial resolution in the range of submillimeters and a short acquisition time due to fast rotation. These CT scanners also have a large field-of-view due to the built-in detector array and options for low dose imaging by modulating the X-ray tube current during rotation. CT reconstruction is performed using filtered backprojection algorithm or additional iterative reconstruction algorithms for low dose protocols and artifact reduction. To enhance visibility and differentiation between anatomical and tumor structures, contrast agent may be applied or fiducial marker may be implanted. For periodically moving targets, which can typically be found in the thoracic or abdominal region due to heartbeat and breathing, 4D CT imaging is advantageous. The breathing cycle can be monitored, for example, by a pressure sensor, surface monitoring or spirometry. The respective signals are synchronized with the CT image acquisition such that defined phases of the 4D CT dataset can be reconstructed (e.g., inspiration) or further image processing using algebraic algorithms (e.g., average CT, maximum or minimum intensity projection) can be carried out. Dual energy CT, which describes the acquisition of two CT scans with different X-ray tube potential, has a growing importance in clinical practice for diagnostics and treatment planning. These scans can be performed with a single source scanner allowing subsequent acquisition or also synchronous acquisition using a fast alternation between the tube voltages. With this technique, artifacts by implants and also application of contrast agent can be reduced. Furthermore, assessment of physical tissue properties (e.g., density) is more precise, which improves precision of dose calculation for radiotherapy using photons, electrons and even more particles. Finally, acquired images are transferred to the treatment planning system (TPS) to start the process of contouring and thereafter treatment planning.

For dose calculation, in particular for external beam radiotherapy, the Hounsfield unit of each voxel from the CT dataset is converted by the TPS to the physical quantity relevant for radiation interaction (e.g., electron density, stopping power). This requires calibration for defined CT scanners and imaging protocols to determine respective relation. Since the turn of the millennium and the progress in the availability of MRI scanners, the usage of MRI in radiotherapy is of growing interest. Due to its superior soft-tissue contrast and radiation-free acquisition compared with CT, MRI has become increasingly relevant for contouring, treatment planning and verification besides the traditional immense importance for diagnostics, However, many challenges have to be overcome regarding the compatibility to radiotherapy equipment (e.g., for patient positioning, treatment delivery, and dosimetry), the geometrical accuracy (e.g., system- and patient-related distortions, patient, and organ motion) and the conversion of MRI signal to physical quantities for dose calculation. Continuous progress in research and development has provided innovative solutions and enables versatile clinical application by now.

Contouring

In the reports of the International Commissions on Radiation Units and Measurements (ICRU), recommendations for target volume definition for radiotherapy are given. Extent and location of the malignant growth including primary tumor as well as lymph node and other targets, such as (distant) metastasis, needs to be delineated as the gross tumor volume (GTV). This target volume is determined by clinical information (e.g., inspection, palpation) and various imaging modalities (e.g., MRI, PET-CT, and endoscopy). As the GTV does not include the subclinical microscopic extension, it needs to be enlarged by empirical margins in three dimensions to generate the clinical target volume (CTV). For this, tumor characteristics like local invasion capacity, potential spread to different regions and results from recurrences patterns need to be considered. To compensate for expected physiologic movement as well as variation in size, shape and position of the CTV, a so called internal margin is added to the CTV resulting in the internal target volume (ITV). These variations arise from respiration, heartbeat, swallowing, different fillings and movement of hollow organs (stomach, intestine, and bladder) and extent of margin can be assessed, for example, by time-resolved imaging (4D, consecutive). A further margin is added to account for uncertainties in patient positioning and beam alignment resulting in the planning target volume (PTV). This setup margin may differ substantially depending on factors like variations of patient position, influenced by helping aids and image-guidance techniques, mechanical uncertainties of the equipment as well as dosimetry-based uncertainties. The latter applies in particular for external beam therapy using particles since the beam characteristics necessitates consideration of range uncertainties because the location of the Bragg peak in depth depends importantly on the amount of tissue in the entrance channel (vide supra).

In the proximity to target volumes, normal tissues may significantly influence the treatment planning and prescribed dose due to their radiation sensitivity. For serial-like organs, the dose at or close to the maximum is the best predictor of loss of function. In

contrast, for parallel-like organs the mean dose or excess of dose given to a defined volume have been used as the best predictors of loss of function. Actually, tissue organization of many organs is a mixture of both with more or less pronounced characteristics. Analogous to the margins between CTV and PTV, uncertainties and variations of the OAR must be considered to avoid complications generating the planning organ at risk volumes (PRV).

For delineating target volumes and organs at risk (OAR), TPS provide versatile tools for manual contouring, for example freehand, polygon and brush tools as well as options for local deformations of the resulting region of interest (ROI). Simplification tools, inter- and extrapolation as well as the possibility of 3D contouring ease the completion of manually drawn ROIs and Boolean algebra for margining and combining ROIs (e.g., union, difference, and intersection) can be used. In addition, TPS include sophisticated (semi-)automatic tools for contouring such as thresholding (global, local), edge detection and region-growing methods. Model-based segmentation makes use of statistical models of shape and appearance, for example, of organs that is combined with image information (gray value, gradients) for delineating ROIs. In atlas-based segmentation, segmented structures from multiple image sets in a clinical database are taken to find the best matching for contouring the current image set. The given methods can also be found in combination, which additionally can be supported by machine-learning approaches. Especially automatic tools enable simultaneous contouring of multiple ROIs and even patients increasing efficiency and throughput. Furthermore, accuracy and reproducibility may be improved since manual contouring may vary with experience and training of the observer as well as image modality (and quality) and treatment protocol. To reduce this variability and increase the reproducibility, guidelines for contouring of tumor entities according to stage and situation as well as OARs are available.

Frequently, different CT datasets complemented by other image modalities are required for comprehensive contouring. This applies for time-resolved imaging to examine target or organ movement or monitor the course of treatment for possible adaptions. Another reason might be the low soft-tissue contrast of CT impeding differentiation of normal and abnormal tissue as well as organ boundaries. Furthermore, functional imaging modalities such as PET or functional MRI add physiological information such as metabolism, oxygenation (e.g., hypoxia), perfusion, diffusion and substance distribution (e.g., proteins) to already existing anatomical information. TPS typically provide import and export functionality for various image modalities based on the DICOM format (Digital Imaging and Communications in Medicine). For image registration, rigid image transformations are integrated using manual operations (e.g., translation, rotation, scaling) as well as (semi)-automatic algorithms based on comparison of landmarks or gray values (e.g., mutual information). Moreover, several TPS enable nonrigid, deformable registration, which is particularly important for therapy adaption due to volume changes of the patient (e.g., edema, swelling) or treatment planning within a previously irradiated area (e.g., in case of recurrence, metastasis, and secondary tumor), possibly even with long time interval.

In addition to target volumes and OARs, contouring of helping aids as well as applicators and catheters for brachytherapy is relevant for consideration during treatment planning, for example to avoid collision with the irradiation device or to calculate radiation interaction or source dwell times. For inverse treatment planning used for IMRT, help contours are typically required to control the optimization mostly drawn by the physicists by means of manual contouring tools and Boolean algebra. An important feature of TPS is the density override of a ROI needed, for example, to cope with image artifacts caused by metal implants or to correct the Hounsfield unit of tissue modified by contrast agent. Without this function, dose calculation might be wrong due to incorrect gray values. In this context, generation of a so-called bolus at the patient surface to compensate for the dose built-up of high energy photons and electrons is also a necessary feature. Such bolus can usually be drawn with desired thickness based on the external contour using a semi-automatic routine. For treatment application, this bolus is placed onto the patient at the intended area. Besides delineation of ROIs, TPS also enable definition of points of interest facilitating patient alignment, plan modeling and evaluation as well as dose verification and quality assurance.

Treatment Planning

TPS provide models for treatment devices and sources used for the different types of radiotherapy. On the one hand, these models comprise all design features (e.g., gantry) and beam shaping components (e.g., MLC, electron applicators) relevant for treatment application as well as their characteristics regarding material, motion and geometry. On the other hand, dosimetric properties of radiation source and field are included. For modeling, extensive measurements of absolute dose as well as relative depth and lateral dose profiles are required using appropriate detectors (e.g., ionization chamber, diode detector, radiochromic film) and phantoms (e.g., motorized water phantom, slab phantom) conforming to national standards. These measurements are mostly performed during the commissioning phase of a treatment device and, for monitoring purpose, during the regular quality assurance procedures.

The aim of designing a treatment plan is to achieve an optimal dose distribution complying with prescribed dose objectives for the target volume and the tolerance doses of surrounding normal tissues. The traditional way is termed as forward planning, which is an iterative trial-and-error approach of the planner by manually specifying and modifying a set of plan parameters to accomplish this goal. The result depends largely on the experience, effort and decisions of the planner. During the last decades, computing power has strongly improved allowing for an alternative way of plan design termed as inverse planning. Here, the planner defines dose constraints for target volume and OARs as well as some framework conditions (e.g., beam directions, number of MLC shapes). Then, an optimization algorithm determines the plan parameters that best satisfy the constraints using an objective function. The planner may influence the optimization by modifying the penalty weight of each constraint to indicate relative importance. In a powerful IT environment, inverse planning can potentially be very time-efficient because much of the trial-and-error time is removed. Furthermore, plan quality with regard to dose conformity or OAR sparing can be superior to those resulting from forward planning.

Many TPS provide both ways of plan design for the individual types of radiotherapy. The resulting treatment plan consists of a set of parameters differing between the respective treatment devices. For external beam radiotherapy, these parameters comprise beam positions, energies, directions, shapes, and weights (i.e., monitor unit). Inverse planning is mostly associated with IMRT using photons for external beam radiotherapy as the optimization algorithm results in inhomogeneous dose distributions for each fixed beam or arc, which is realized by the two IMRT delivery modes (i.e., step-and-shoot or sliding-window). IMRT is also feasible with electrons using the eMLC and with particles using IMPT. In addition, there are applications for inverse planning in connection with brachytherapy to spare an OAR by optimized dwell times. Two interesting approaches of inverse planning have recently been developed possessing a great potential for future treatment planning of external beam radiotherapy but requiring high computing power to be performed with reasonable time requirement (i.e., within several minutes). The first one is termed as automated planning and describes the automatic generation of numerous treatment plans for defined clinical goals and combinations of treatment techniques (e.g., IMRT with varying beam number, types of arc therapy) and devices (e.g., different LINACs, TomoTherapy®). The decision for a certain plan might be taken based on criteria fulfillment and treatment efficiency considerations. A variant of automated planning is the so called fallback planning, which can be very valuable in contingency situation (e.g., device breakdown), is the so called fallback planning. Here, additional plans are automatically created, possibly with a different treatment technique and/or device, by replicating the dose distribution of a given plan. The second one is termed as multicriteria optimization and describes an alternative workflow for the optimization. Instead of the iterative process of modifying dose constraints and penalty weights by the planner, TPS automatically generates Pareto optimal plans, meaning that all constraints are respected and no criterion can be improved without impairing another one. The planner may select a certain plan through continuous interpolation between these precalculated alternatives.

An important step of plan design is the dose calculation based on the treatment device model and patient anatomy. Several algorithms have proven suitable for calculating high accurate and realistic dose distributions. For external beam radiotherapy and brachytherapy using photons, dose calculation is typically performed by a collapsed cone convolution superposition algorithm or an approach based on solving the linear Boltzmann transport equation. Alternatively, a Monte-Carlo simulation is implemented in TPS, which is furthermore suitable for dose computation from electron beams. In contrast, the formerly widespread pencil-beam algorithm is hardly used as it does not accurately account for inclined beam directions as well as disturbed secondary electron equilibrium and lateral photon scattering from patient heterogeneities. For particles therapy, two central aspects have to be considered for dose calculation. Physical dose distribution can be computed by a pencil-beam algorithm for protons as well as analytical algorithms or Monte-Carlo simulation for ions. In addition, the increased relative biological effectiveness requires a conversion of physical absorbed dose to RBE-weighted dose. For protons, today usually a RBE of 1.1 is assumed although radiobiological data indicate some variability. RBE dependencies for ions are more complex involving parameters such as ion type, energy, depth, LET and cell type, conversion of physical absorbed dose is carried out using a fixed (HIMAC approach) or variable (local effect model) RBE scheme.

Plan Evaluation

Plan evaluation is an important step to finally select a treatment plan for application. This decision is usually taken by the radiooncologist, optimally as a team effort together with medical physicists. For plan evaluation, criteria for both target volumes and OARs are recommended in reports and guidelines of professional organizations (e.g., ICRU, RTOG, and EORTC) for the different types of radiotherapy. For example, for external beam radiotherapy the ICRU report 50 specifies that in the best technical and clinical conditions the dose heterogeneity in the PTV should be kept within 95%–107% of the prescribed dose. As these minimum and maximum criteria are difficult to be maintained for IMRT plans, ICRU report 83 recommends replacement of these values by a near-minimum $D_{98\%}$ and near-maximum $D_{2\%}$ (D_V means dose D [% or Gy] received by volume V [% or mL] of a target volume or OAR). Criteria for radiation tolerance of OARs can also be found from various literature expressed as maximum doses or dose-volume-parameters (e.g., D_V or analogous V_D). These criteria are typically related to relevant side effects of the OARs (i.e., toxicity endpoint and rate, severity grade, and time of occurrence) allowing the radiooncologist a risk-benefit assessment taking into account the patient situation. In addition to physical dose, also biological dose, that is dose taking into account the dose per fraction, overall treatment time and time interval between fractions, needs to be considered during treatment plan evaluation, for example by converting accepted tolerance doses according to the LQ model.

The gold standard of plan evaluation is a dose-based assessment combining qualitative and quantitative methods. The calculated 3D dose distribution is superimposed on the treatment planning image dataset by means of isodose contours or color wash using absolute or percentage dose scaling. This allows a direct, qualitative slice-by-slice review of dose in relation to target volumes and OARs. However, the amount of information is huge and the visual evaluation of target volume dose coverage as well as OAR tolerance dose compliance is difficult. Therefore, various quantitative surrogates serve for simplification of the 3D dose information within a specific ROI. Dose statistics such as minimum, maximum or mean and median values are easy to calculate. A dose-volume histogram (DVH) is a 2D graph showing the frequency distribution of dose values within a certain ROI ignoring spatial information. There are two variants of DVH, namely the differential and the commonly used cumulative (integral) DVH. Dose statistics and DVH are important tools for plan evaluation as both facilitate comparison to recommended criteria. These features should always be used in conjunction with each other and the visual analysis of the 3D dose distribution. For example, separate DVH interpretation can cause serious mistakes as the DVH is only calculated for delineated ROIs, it is insensitive to small spots of low or high doses and the graph shape can be misleading (e.g., in case of ROIs containing high density differences).

An additional aspect of plan evaluation is the comparison of different treatment plans with each other to find an optimum for the current patient situation with the best possible clinical outcome. Plan scoring can be based solely on dose information (i.e., 3D distribution, statistics, and DVHs). For this, various indices can be calculated as a measure of, for example, dose homogeneity inside the target volume or conformity of the dose distribution with respect to the target volume and prescription also considering the extent of sparing relevant OARs. However, these indices mostly lack clinical relevance. Therefore parameters such as the tumor control probability (TCP), the normal tissue complication probability (NTCP) or the equivalent uniform dose (EUD) can be incorporated for plan scoring. These parameters are interesting for research purpose and future clinical practice, however for current clinical decisions, these tools should be used with caution as the validity of underlying models and their input parameters are often yet uncertain.

The practicalities of treatment delivery pose further important issues for plan evaluation, in particular for external beam radiotherapy using intensity-modulated beams. Complexity of a treatment plan can be assessed considering the limits of the irradiation devices, the treatment time for the patient as well as clinical aspects such as the patient's general condition and treatment intention. Safety of delivery can be evaluated in terms of sensitivity of a treatment plan to patient positioning errors or anatomical changes (e.g., due to breathing or differing organ fillings) by recalculating the dose distribution in spatially-shifted CT datasets or different phases of a 4D CT. In this context, patient-specific quality assurance is often conducted. Thereby, dosimetric measurements are performed for the comparison with the planned dose distribution typically using a gamma-index analysis. In addition, the treatment plan can be checked for collisions (e.g., between irradiation device and patient, positioning aids or couch) and necessary patient-specific equipment can be tested prior to the treatment delivery.

Fig. 3 provides an example for dose plan assessment, comparing a proton dose distribution in comparison with photons in the same patient suffering from brain tumor. Very significant differences occur with regard to the dose in normal tissue. While large portions of normal brain receive low to intermediate doses when the photon plan is applied ("dose bath"), the portion of irradiated normal brain is much less for proton treatment. Differences between the plans are much less for target volume coverage, target volume conformity and high dose regions in healthy tissues. Overall, the proton plan is from a physical and radiobiological perspective superior for this patient and was selected for clinical treatment. However, it needs to be noted that clinical benefit cannot be directly be determined from treatment plan comparison at present time and requires further data to be validated in predictive models.

Treatment Application and Verification

The course of radiotherapy often lasts several weeks, during which inter- and intra-fractional changes can occur due to shifts in patient positioning as well as target- and patient-related alterations or motion. Nevertheless, precise and accurate application of the irradiation according to the treatment plan needs to be ensured over the entire radiotherapy period. Therefore, integration of various imaging techniques into the workflow for treatment verification is advisable for three main purposes. Correct positioning of patient, target volume and OARs can be supported by suitable imaging, which is typically carried out for all fractions before irradiation delivery, but also position verification by imaging should be performed in regular intervals. Moreover, anatomical changes such as swelling, tumor shrinkage or patient weight loss should be monitored with the help of imaging, for example on a weekly basis or on demand of the radiooncologist, possibly leading to a necessary treatment adaption. The third purpose is the management of patient motion during the irradiation, mainly used for respiratory motion. Two methods for compensating the detected motion have been established. Gating refers to restricted irradiation delivery to a defined breathing phase (e.g., inspiration). Tracking refers to a nonrestricted irradiation delivery during the breathing cycle while continuously adjusting the patient or beam position. Both methods require a correlation between motion signal and target position as well as an approved connection between the motion sensing device and the irradiation device, which is sometimes problematic due to medical products regulations. All the above mentioned utilization of proper imaging techniques for treatment verification and application are subsumed under the term image-guided radiotherapy (IGRT).

Several imaging modalities are used for IGRT, of which X-ray imaging is currently still of highest importance. Modern devices for external beam radiotherapy are equipped with on-board imaging systems. LINACs include an electronic portal imaging device (EPID) opposite the gantry head using the megavoltage photon beam for imaging. As the tissue contrast for such high energy is low and the exposure can be high compared with kilovoltage X-ray imaging, an additional X-ray tube and corresponding flat-panel detector are integrated into several LINACs arranged parallel or perpendicular to the therapy beam. Both EPID and X-ray imaging systems allow for radiography and, with additional gantry rotation, for cone-beam CT (CBCT). For particle therapy, even a pair of orthogonal X-ray imaging systems can be installed within the gantry enabling radiography. Alternatively, a ceiling-mounted robotic C-arm X-ray imager or a table-mounted X-ray imaging ring allows for radiography and CBCT. Beside these on-board systems, additional in-room X-ray imaging systems are used. Such systems are, for example, permanently-installed X-ray systems for (stereoscopic) radiography, mobile C-arms mostly used for brachytherapy or in-room CT scanners. The latter can also allow for CT imaging with a fixed position of the patient couch, as is the typical case for external beam radiotherapy, when a CT sliding gantry is mounted on rails. The acquired helical CT scans are of diagnostic image quality, which is advantageous over CBCT scans, however a patient relocation from the treatment position through couch rotation is required.

As radiation-based imaging always involves additional dose exposure of the patient, various nonradiation-based methods for IGRT are available. Different systems enable monitoring of the patient's surface and are valuable for superficial target (e.g., breast) and respiratory motion detection. These systems are ceiling-mounted and use methods like infrared-light reflection of respective

markers, speckled-light projection or laser triangulation. Ultrasound is a simple, fast method primarily applied to treatment sites like prostate, lung or breast. However, ultrasound requires comprehensive user experience as well as a direct patient contact associated with an inherent sensitivity to conduction and pressure of the probe to the tissue. Electromagnetic tracking is another method using seed-like transponders that are implanted subcutaneously or into the target area (e.g., prostate), or placed on the patient's skin. During recent years, MRI has been increasingly used for IGRT. Due to the special requirements with regard to the magnetic fields and the lack of compatibility to irradiation devices, off-room MRI scanners in combination with a dedicated patient couch transfer system have been implemented. In addition, research and development was pursued to directly integrate MRI into external beam radiotherapy devices and tackle the resulting technological challenges such as attaining material compatibility and considering magnetic fields for dosimetry and dose calculation (charged primary and secondary particles are affected by Lorentz force). A solution has been found by equipping an MRI scanner with a rotating-ring gantry, which holds three highly radioactive Co-60 sources and corresponding MLCs mounted with 120 degree separation. The newest, most innovative solution is the hybrid MR-LINAC, which combines complete functionalities of latest MRI scanners and LINACs. Furthermore, there is on-going effort of integrating MRI into particle therapy, for example using open MRI systems.

Different image modalities are differently suited for the variable purposes of IGRT and need to be assessed accordingly by the radiooncologist for each patient. Factors like additional dose exposure, image quality as well as requirements of time, effort and costs have to be individually balanced. All image modalities are suitable to support and verify patient positioning with varying effort and resulting precision and accuracy. Volumetric imaging like (CB)CT or MRI is usually needed for monitoring anatomical changes, however these modalities are inappropriate for motion management due to low temporal resolution. For this purpose, fast IGRT methods such as (stereoscopic) planar imaging (e.g., X-ray, MRI), surface monitoring, ultrasound, electromagnetic tracking are used. The acquired images are subjected to manual or (semi-)automatic evaluation, which is normally a comparison to former images acquired for treatment planning or during previous fractions. For example, radiographs from on-board or in-room X-ray devices are compared to digitally reconstructed radiographs (DRR) calculated from the treatment planning CT. Similarly, surface monitoring systems can make use of the delineated patient external contour for comparison whereas (CB)CT and MRI scans can be directly co-registered to the treatment planning dataset. Image evaluation is thereby performed by means of anatomical structures (e.g., bones) or fiducial markers, which have been placed on the surface or implanted into body of the patient.

An additional issue is the verification of dosimetric properties of the irradiation field during treatment application using external systems. A method named portal dosimetry has been specifically developed for monitoring IMRT delivery of a LINAC using the EPID for dose measurement. Alternatively, an independent transmission ionization chamber array is available, which is attached to the gantry head allowing online monitoring. For brachytherapy, in vivo dose measurement can be performed during irradiation using small-size dosimeters (e.g., thermoluminescent dosimeters, ionization chambers) that are placed in the vicinity of the target volume. For particle therapy, range verification is of particular interest due to the sensitivity to changes of the anatomical conditions in relation to the treatment plan. PET has been extensively used to measure the induced activity distribution of positron-emitting target (and projectile) residuals and subsequently compare them with Monte-Carlo simulations. Three options namely in-beam, in-room, and off-line PET have been implemented associated with different advantages and disadvantages. For example, the benefit of in-beam PET is the short, synchronous data acquisition and treatment application at the highest nuclide activity level, but implementation is complex, expensive and technically demanding. In contrast, off-line PET uses commercial PET scanners outside the treatment room, however necessitating a patient transport system to reduce repositioning uncertainties and longer acquisition times due to the rapid decay and biological washout of radionuclides, the latter is additionally impairing the image quality. In recent years, approaches for detecting prompt gammas, emitted as a result of nuclear reactions, have been explored. The underlying interactions happen within nanoseconds and are therefore a more direct indicator for the delivered particle distribution compared with the activity of positron emitters. Furthermore, prompt gamma emission exhibits a broad energy spectrum in wide range of 1–20 MeV including characteristic lines from specific elements of irradiated tissues. Different systems comprising a slit or Compton camera have been investigated and first clinical application for proton range verification has been demonstrated. Further promising systems are under development using time- and energy-resolved prompt gamma measurements instead.

Follow-Up

After the end of radiotherapy, long term follow-up is necessary to evaluate the treatment result and normal tissue toxicity. In this context, quality of life and patient-reported outcome should be evaluated possibly through standardized questionnaires. According to the tumor entity, the follow-up is usually combined with diagnostic imaging (e.g., CT, MRI), laboratory tests as well as medical examination by the radiooncologist and responsible medical specialists (e.g., gynecologist, surgeons). The treatment and follow-up data are required for early detection of potentially treatable recurrence, side effects and secondary tumors as well as for scientific evaluation and quality assurance.

Combination of Radiotherapy and Systemic Therapy

Radiotherapy in combination with chemotherapy is currently the standard treatment for a number of different tumor entities (e.g., head and neck, lung, rectal, and cervical cancer). By adding systemic agents to local irradiation, two potential effects namely increased local tumor control and/or elimination of occult metastasis can be expected. Chemotherapy can be applied

neoadjuvantly, simultaneously or adjuvantly to radiotherapy and also mixed forms are possible. The schedule depends on the aim (i.e., simultaneous to increase local control, (neo-)adjuvant to reduce distant metastasis) and evidence of treatment for the tumor entity. The main mechanisms for increased therapeutic efficacy are independent tumor cell killing (reducing CSC population) or superadded local effects by interaction with radiation-induced damages (e.g., inhibition of DNA repair, synchronization of cells in sensitive cell cycle phases). Combined therapy is also associated with additional and more severe toxicity due to a relatively unspecific cell killing by cytostatic drugs. With increasing knowledge of tumor biology and behavior, it has become possible to develop substances, antibodies and small molecules that specifically intervene in signal transduction pathways and modulate the reaction of the tumor cell to radiation. A well-known example is the epidermal growth factor receptor (EGFR) antibody Cetuximab. Studies showed an improved overall survival of the combination of Cetuximab and radiotherapy when compared with radiotherapy alone, but also uncommon side effects such as acne-like skin reaction. Conversely, other substances interacting in the EGFR pathway like tyrosine kinase inhibitors did not show the same effects in combination with radiotherapy. It is assumed that, in contrast to the small molecules, antibodies trigger an additional immune effect.

Another interesting approach is the combination of DNA-repair inhibitors with radiotherapy. Irradiation induces approximately 30–40 double-strand breaks per cell per Gy, of which almost all can be repaired by the tumor cells. The inhibition of this DNA-repair mechanism, for double-strand breaks especially nonhomologous end joining (NHEJ) and homologous recombination, leads to a strongly increased radiosensitivity and may furthermore improve the clinical outcome. Currently, a number of inhibitors of the DNA-dependent protein kinase, which is the key enzyme for NHEJ, are under investigations and the first are in clinical phase I testing. Beside the promising improvement of clinical outcome and tumor control, the side effects on normal tissue have to be considered and the therapeutic ratio for new combination treatments have to be evaluated.

A further quite new approach is the combination of radiotherapy with immunotherapy. The immune system has a complex structure with a plethora of parallel and consecutive, cellular and humoral processes to protect living beings against foreign substances, microorganisms or diseased tissues including malignant cells. The immune system is usually able to distinguish between own and foreign structures. Unfortunately, cancer cells develop mechanisms to escape from immune surveillance. The aim of immunotherapy is to stimulate the immune system to attack cancer cells. The currently most prominent example is the use of checkpoint inhibitors for CTLA-4 (e.g., Ipilimumab) or PD-1/PD-L1 (e.g., Pembrolizumab), which lead to a significant benefit in overall survival for patients with metastases in a number of tumor entities. Case reports and increasingly also clinical studies show that radiotherapy may lead to effects on metastases located outside the target area after or during Ipilimumab treatment. These so called abscopal effects are in line with data showing that radiotherapy can enhance the immune system by increasing vulnerability for T-cell-depending cell death, recruitment of T-cells to the treated tumor and induction of immunogenic cell death. To translate combinations of radio- and immunotherapy to clinical practice, a number of issues including dose fractionation, treatment sequence and the impact of irradiating adjacent lymphatic tissues on the immune response need to be clarified. To utilize the abscopal effects of radiotherapy in metastasized disease, another important question is how many and which lesions need to be irradiated. Beside of or in addition to combinations of radiotherapy with checkpoint inhibitors, other approaches such as addition of IL-2, usage of chimeric antigen receptor (CAR) cells and peptide or ribonucleic acid (RNA) vaccination, as well as the development of biomarkers are of current research interest.

Future

The advances in radiotherapy over the last years have led to an anatomical personalized and tumor-entity-specific treatment (e.g., fractionation, total dose, and chemotherapy). Further investigations are necessary according predictive and prognostic biomarkers for personalized treatment decisions and applications. These developments will lead to increased treatment efficiency and will avoid over- or undertreatment. Another important research field/aim is the identification and development of new selective and tumor-specific drugs for combination with radiotherapy to improve treatment outcome and reduce severe side effects and late tissue complications. Especially immunotherapy provides a range of potential combinations but also selective intervention in the DNA-repair and survival pathways seems promising. The focus of technical developments will be on clinical studies using particle therapy and on the establishment of fully image-guided particle therapy. Furthermore, individualized concepts for regular adaption during radiotherapy in a quick and uncomplicated way will gain in importance to cope with patient- and target-specific alterations using the entire range of technical possibilities of modern radiation oncology.

Further Reading

Baumann, M., Krause, M., Overgaard, J., et al., 2016. Radiation oncology in the era of precision medicine. Nature Reviews Cancer 16, 234–249.

Baumann, M., Kurth, I., Cordes, N., Krause, M., Linge, A., 2018. Molecular cancer and radiation biology. In: Halperin, E.C., Wazer, D.E., Perez, C.A., Brady, L.W. (Eds.), Perez and Brady's principals and practice of radiation oncology, 7th edn. Lippincott Williams & Wilkins, Philadelphia (PA).

De Ruysscher, D., Sterpin, E., Haustermans, K., Depuydt, T., 2015. Tumour movement in proton therapy: Solutions and remaining questions: A review. Cancers (Basel) 7, 1143–1153.

Goyal, S., Kataria, T., 2014. Image guidance in radiation therapy: Techniques and applications. Radiology Research and Practice 2014, 705604.

Joiner, M.C., Van der Kogel, A. (Eds.), 2009. Basic clinical radiobiology, 4th edn. Tayler & Francis Ltd, London.

Kraan, A.C., 2015. Range verification methods in particle therapy: Underlying physics and Monte Carlo modeling. Frontiers in Oncology 5, 150.

Parodi, K., Paganetti, H., Shih, H.A., et al., 2007. Patient study of in vivo verification of beam delivery and range, using positron emission tomography and computed tomography imaging after proton therapy. International Journal of Radiation Oncology, Biology, Physics 68, 920–934.

Seidlitz, A., Combs, S.E., Debus, J., Baumann, M., 2016. Practice points for radiation oncology. In: Kerr, D.J., Haller, G., van de Velde, C.J.H., Baumann, M. (Eds.), Oxford textbook of oncology, 3rd edn. Oxford University Press, Oxford, pp. 173–184.

Van Limbergen, E.J., De Ruysscher, D.K., Olivo Pimentel, V., et al., 2017. Combining radiotherapy with immunotherapy: The past, the present and the future. The British Journal of Radiology 90, 20170157.

Radiation Therapy-Induced Metastasis and Secondary Malignancy

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Introduction

Radiation therapy (RT) is a popular treatment modality for many cancer patients. Recent advances in RT technology, along with surgical procedures and chemotherapy, have significantly increased the lifespan of cancer patients (DeSantis et al., 2014). However, many patients suffer adverse effects from RT, one of them being the development of secondary malignant neoplasms, or SMNs (Suit et al., 2007). This is especially apparent in survivors of prostate, breast, and pediatric cancers (Sountoulides et al., 2010; Ning et al., 2011; Choi et al., 2014). It is estimated that 1 in 12 cancer survivors develop a secondary malignancy and greater than half will succumb to the occurrence of secondary cancer (Donin et al., 2016). Additionally, time from exposure plays a major role, as the relative risk (RR) of survivors of childhood cancers for developing any SMN after RT was 2.7, and RT was the highest independent risk factor for SMN development (Choi et al., 2014). However, it should be noted that the incidence of RT-induced SMN is difficult to calculate, as many patients will develop SMNs due to other genetic abnormalities and lifestyle choices (e.g., tobacco and alcohol use) (Vaughan et al., 1995). Whether SMNs arise from metastatic cohorts of the primary tumor or develop as completely new tumors that are unrelated to the primary is a controversial subject. This review will specifically discuss how radiation-induced metastasis contributes to the development of SMN (**Fig. 1**), the mechanisms by which this occurs, and future perspectives to overcome metastatic-induced SMN development.

Radiobiology

The main goal of radiation therapy is to damage as many tumor cells as possible while not impacting normal cells. Radiation can target tumor cells both directly and indirectly. Direct damage by RT occurs when energy is directly absorbed by nuclear DNA and induces DNA damage that is hard to repair, especially in tumor cells that lack specific DNA repair mechanisms (Altmeyer and Lukas, 2013; Thompson, 2012; Hunt et al., 2013; Lavelle and Foray, 2014). Indirect DNA damage occurs when molecules within the tumor microenvironment, such as oxygen, absorb energy emitted from RT and become molecules that are highly reactive (ROS, reactive oxygen species) and damaging to DNA (Widel et al., 2014; Cadet and Wagner, 2013; Alan Mitteer et al., 2015). Hence, tumor cells are more susceptible to damage by RT than normal cells as they continuously copy their DNA in order to proliferate. When tumor cells are unable to repair genomic DNA, they initiate apoptosis or cellular suicide (Alan Mitteer et al., 2015). On the other hand, normal cells have appropriate DNA repair mechanisms in place and are able to survive fractionated RT. However, some cancerous cells survive RT because of their microenvironment or mutation status, primarily loss-of-function mutations in the DNA damage response pathway or gain-of-function mutations in DNA repair pathways. It is quite likely that RT-induced DNA damage results in faulty repair, which introduces further mutations in tumor cell DNA and may confer selective advantage to those cells or emergence of RT-resistant cells (Van Houten et al., 2018; Weeden and Asselin-Labat, 2018; Chang et al., 2015; Cao et al., 2002). These RT-resistant cancer cells are highly aggressive and confer metastatic behavior leading to the progression of disease.

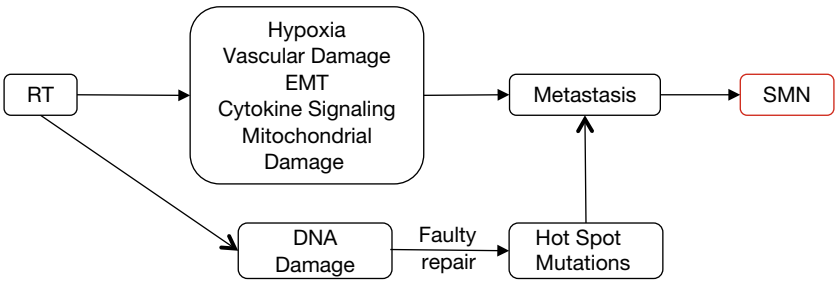

Fig. 1 RT induces metastasis to facilitate SMN development. A graphical representation depicting the mechanisms by which RT potentiates metastasis, ultimately leading to SMN development.

Mechanisms of Radiation-Induced Metastasis

Epithelial-Mesenchymal Transition

Epithelial cells are round in shape and typically resemble a cobblestone phenotype (Gonzalez and Medici, 2014). They express markers such as E-cadherin and maintain cell polarity with distinct apical and basal membranes (Wodarz and Nathke, 2007). Epithelial cells form adherent and tight junctions with other neighboring cells and prefer to stay in one place. Meanwhile, mesenchymal cells express markers such as vimentin, are more elongated than epithelial cells and display spindle-like profusions and lose polarity with respect to their apical and basal membranes (Gonzalez and Medici, 2014). Because of their genetic characteristics and phenotype, mesenchymal cells readily migrate. Although migratory potential plays a critical role in tumor cell dissemination, cells must also be able to invade the surrounding tumor microenvironment (TME) (Quail and Joyce, 2013). Mesenchymal cells typically express and secrete proteins such as matrix metalloproteinases (MMPs) that degrade TME (Gilkes et al., 2013). Thus, epithelial-mesenchymal transition (EMT) is the first step in tumor cell dissemination, as epithelial tumor cells must acquire migratory and invasive characteristics before they can disseminate from the primary tumor.

Several studies have already investigated the link between RT and EMT. In an orthotopic model of hepatocellular carcinoma, RT induces greater dissemination of primary tumor cells and development of distant metastatic lesions compared to the non-irradiated tumor cells (Li et al., 2011). Tumors receiving RT show increased protein expression of mesenchymal markers such as N-cadherin and vimentin along with concomitant loss of E-cadherin (Li et al., 2011). Interestingly, the transmembrane protease, serine 4 (TMPRSS4) seems to control RT-induced EMT in vivo (Li et al., 2011). This is an important observation because TMPRSS4 is overexpressed or mutated in various cancers and is associated with malignant phenotypes, most notably invasion and remodeling of the extracellular matrix, which are considered a pre-requisite for metastasis (de Aberasturi and Calvo, 2015). A similar study was performed using rat glioma cells, whereby irradiation of tumor cells induced EMT, invasion, and development of metastatic lesions in an in vivo model (Park et al., 2012). Human specimens have also been tested for the role of RT in metastasis. In this regard, a recent study profiled human prostate cancer samples for EMT markers pre- and post-RT (Stark et al., 2017). Indeed, EMT occurred after RT in those prostate cancer patients as evidenced by an increase in vimentin, a decrease in E-cadherin and a decrease in the cytoskeleton remodeling protein cofilin (Stark et al., 2017). As mentioned previously, cytoskeleton remodeling is an essential step in EMT, as cells must become de-polarized in order to migrate. Cytoskeleton remodeling changes the biomechanical makeup of single cells as observed via atomic force microscopy in tongue squamous cell carcinoma (TSCC) cells after X-ray irradiation (Zheng et al., 2015). Specifically, TSCC cells decreased in height, became more elongated, and actin fibers were disorganized after X-ray radiation, which allows the cells to de-polarize and for migration to occur (Zheng et al., 2015).

Vascular Damage

RT damages the TME in several ways, including demolishing vasculature of TME (Juntermanns et al., 2014; Sabatasso et al., 2011; Grabham et al., 2012; Zywietz, 1990; Kinoshita et al., 2014). Tumors require vasculature for oxygen and nutrient supply. If the primary tumor becomes too large, tumor cells will have no access to oxygenated blood vessels and become hypoxic and nutrient-deprived. In this scenario, many tumors will create their own blood vessels to connect to the main vasculature system, a process known as angiogenesis. High dose RT has been shown to severely damage tumor vasculature, which also leads to hypoxic conditions in tumors post-RT (Juntermanns et al., 2014; Sabatasso et al., 2011; Grabham et al., 2012; Zywietz, 1990; Kinoshita et al., 2014). High dose RT-induces activation of HIFs (hypoxia-inducible factors), most importantly HIF-1α and 2α leading to the activation of several signaling cascades including stress response pathways and secretion of various pro-survival cytokines and chemokines, which are discussed later in this article. However, low dose RT activates several pathways involved in angiogenesis and metastasis (He et al., 2013; Sofia Vala et al., 2010). One such study demonstrated that in hepatoma cells, low dose RT induces vascular endothelial growth factor (VEGF) secretion and MMP2 activation, both of which seem to rely on p53 activation by low dose RT (He et al., 2013). VEGF is a family of cytokines that initiate angiogenesis after binding to their receptor, VEGFR, on endothelial cells and as previously mentioned, MMP's degrade the TME to allow for cell dissemination (Vempati et al., 2014). In another study, low dose RT initiated endothelial cell migration and angiogenesis in the zebrafish fin regeneration model, as well as increased invasion in a murine matrigel plug assay—all of which seem to rely on VEGF activation (Sofia Vala et al., 2010). In addition, RT has been reported to alter the expression of cell adhesion proteins like ICAM-1, VCAM-1 and selectins on tumor-associated endothelial cells, thus further contributing towards alteration of the TME (Gaugler et al., 1997; Hallahan and Virudachalam, 1999).

Hypoxia

It has long been established that low oxygen levels within the tumor microenvironment, or hypoxia, promote the development of aggressive cancer phenotypes including metastatic potential (Gilkes et al., 2014). Hypoxia is also a contributing factor of resistance to RT. As previously mentioned, molecular oxygen is one of the main molecules that absorb energy from radiation to transform into damaging reactive oxygen species (ROS). Therefore, low oxygen levels inhibit DNA damage produced by indirect RT damage. Chronic hypoxia potentiates the development of a dysfunctional TME, so there are underlying mechanisms that tumor cells activate in order to escape the harsh environment (Hemmerlein et al., 2001; Ji, 2014; Mimeault and Batra, 2013). Low oxygen levels activate the transcription factor hypoxia inducible factor 1 (HIF-1α). HIF-1α is responsible for inducing transcription of genes involved with metastasis, such as VEGF, MMP-2, TGF-A, vimentin, and c-Met (Krishnamachary et al., 2003; Pennacchietti et al., 2003; Semenza,

2003). Activation of these genes promotes migration and invasion of tumor cells and in this way allow tumor cells to escape the hypoxic conditions. HIF-1α not only promotes the activation of migratory genes, but also induces angiogenic genes. For example, a recent study demonstrated that HIF-1α levels increase in response to RT, but also modulate VEGF expression levels (Fu et al., 2015). Interestingly, HIF-1α can be activated by RT under both normoxic and hypoxic conditions in glioma cells (Kim et al., 2014). Thus, HIF-1α and the hypoxic tumor microenvironment promote both radio resistance and metastasis.

Mitochondrial Damage

Mitochondria contain their own DNA, referred to as mitochondrial DNA or mtDNA. Unlike nuclear DNA, mtDNA is not wrapped around protective histones and lacks DNA repair proteins and is therefore more susceptible to damage than nuclear DNA (Croteau and Bohr, 1997). Thus, mtDNA is more sensitive to DNA-damaging agents including RT and RT-induced ROS. The mitochondrial proteome consists of a delicate balance between nuclear- and mitochondrial-encoded genes. Specifically, the mitochondrial genome contains 13 structural genes that encode subunits of the electron transport chain. The stoichiometry between nuclear- and mitochondrial-encoded genes must be kept constant or a "mitochondrial (mito)-nuclear imbalance" will occur (Karpac and Jasper, 2013; Houtkooper et al., 2013). A mito-nuclear imbalance can occur whenever mtDNA is damaged, mutated, amplified or depleted (Karpac and Jasper, 2013; Houtkooper et al., 2013). Mito-nuclear imbalances lead to defective oxidative phosphorylation, the generation of ROS and ultimately cell death in normal cells if the proper repair mechanisms are not set in place (Karpac and Jasper, 2013; Houtkooper et al., 2013). However, tumor cells have adapted the ability to hijack and prolong the mitochondrial unfolded protein response (mt^{UPR}), a stress response system that alleviates mitochondrial damage through activation of mitochondrial-specific proteases, chaperones and antioxidants (Kalen et al., 2017; Fiorese et al., 2016; Jovaisaite et al., 2014; Shpilka and Haynes, 2017; Pellegrino et al., 2013; Mouchiroud et al., 2013). In this way, the mt^{UPR} increases the apoptotic threshold and promotes cell survival even in the presence of mitochondrial stressors. Importantly, the mt^{UPR} was shown to be activated in response to RT in *Caenorhabditis elegans* (Li et al., 2017). Additionally, irradiation was shown to change mtDNA copy number, damage mtDNA and alter mtDNA structure, all of which are known activators of mt^{UPR} (Zhang and Okunieff, 2014; Zhou et al., 2011, 2012). Other studies have demonstrated that mtDNA-depleted cells increase cell survival in response to RT and employ different mtDNA replication modalities in response to low dose RT, which ultimately preserves mitochondrial maintenance (Torregrosa-Munumer et al., 2015; Nieri et al., 2013). Thus, cancer cell mitochondria can be preserved, despite being damaged by RT, due to mt^{UPR} activation (**Fig. 2**).

Several studies have explored the relationship between mitochondrial dysfunction and metastatic potential, thus establishing a concrete link between mitochondria and metastasis. For example, mtDNA-depleted cells are reported to have elevated metastatic potential (Moro et al., 2008, 2009). Additionally, mutated mtDNA associates with increased migration and invasion of cancer cells (Arnold et al., 2015; Imanishi et al., 2011; Ishikawa et al., 2008). One of the best methods to study the influence of mutant mtDNA on metastasis is through cybrid technology, whereby mtDNA is transferred from one cell to another (Ishikawa et al., 2008). In this way, the direct effect of mtDNA mutations on different phenotypes can be assessed. Indeed, when mutated mtDNA was transferred into a non-invasive cell line, the non-invasive cell line became highly invasive (Ishikawa et al., 2008). On the contrary, the replacement of mtDNA from a highly metastatic cell line, MDA-MB-231, with mtDNA derived from normal human cells suppressed metastatic potential (Imanishi et al., 2011). Overall, it is clear that RT induces mitochondrial damage, and damaged mitochondria contribute to an invasive phenotype.

Cytokine/Chemokine Signaling

Tumor cells develop within a TME where they communicate with stromal cells to sustain their growth and survival (Quail and Joyce, 2013). This communication between these cells may be through either direct cell-to-cell interaction or via cytokines and chemokines. Similar to RT, disruption of this TME can produce both beneficial and undesirable outcomes. Although RT is known to be directly cytotoxic towards tumor cells, radiation can also cause the release of various pro-inflammatory cytokines and growth factors like FGF (fibroblast growth factors), EGF (epidermal growth factors) and TGF (transforming growth factors) within the TME to promote cell migration, invasion, and metastatic progression.

For example, in response to RT, non-small cell lung cancer (NSCLC) cells experience an increase in the stability of β-catenin, subsequent activation of the PI3K/Akt pathway, followed by release of granulocyte-colony-stimulating factor (G-CSF) (Cui et al., 2015). Acting in an autocrine manner, G-CSF triggers the G-CSFR/JAK1/STAT3 signaling pathway to induce EMT of NSCLC cells (Cui et al., 2015). In addition, RT promotes HIF-1α signaling in NSCLC to enhance CXCR4 expression. CXCR4 expression increases sensitivity to CXCL12 and this CXCL12/CXCR4 interaction activates the Akt and ERK1/2 pathways to enhance MMP-2 and MMP-9

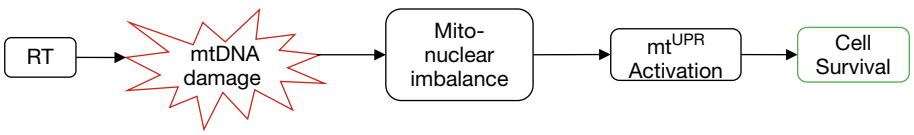

Fig. 2 Mt^{UPR} is activated by RT and potentiates cell survival. A graphical representation that describes how RT induces mitochondrial damage and promotes cell survival via the unfolded protein response.

expression, which in turn increase the invasiveness of NSCLC cells (Gu et al., 2015). RT also stimulates the release of IL-6, which acts in an autocrine manner to enhance CCL2 and CCL5 secretions and attracts macrophages to the tumor site (Wang et al., 2017) (Fig. 3A).

Similarly, RT of breast cancer cells causes the release of granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes tumor cell invasion as well as recruitment of circulating tumor cells at distant metastatic sites (Vilalta et al., 2014) (Fig. 3B). Additionally, both breast cancer and renal cell carcinoma cells release macrophage migration inhibitory factor (MIF) due to RT (Gupta et al., 2016) (Fig. 3C), a factor known to promote metastasis (Simpson et al., 2012; Lv et al., 2016). Notably, during breast cancer RT the lung experiences radiation as well. This causes the release of CXCL12 by lung epithelial cells, which enhances the migratory activity of breast cancer cells and promotes lung-specific metastasis (Feys et al., 2015) (Fig. 3B).

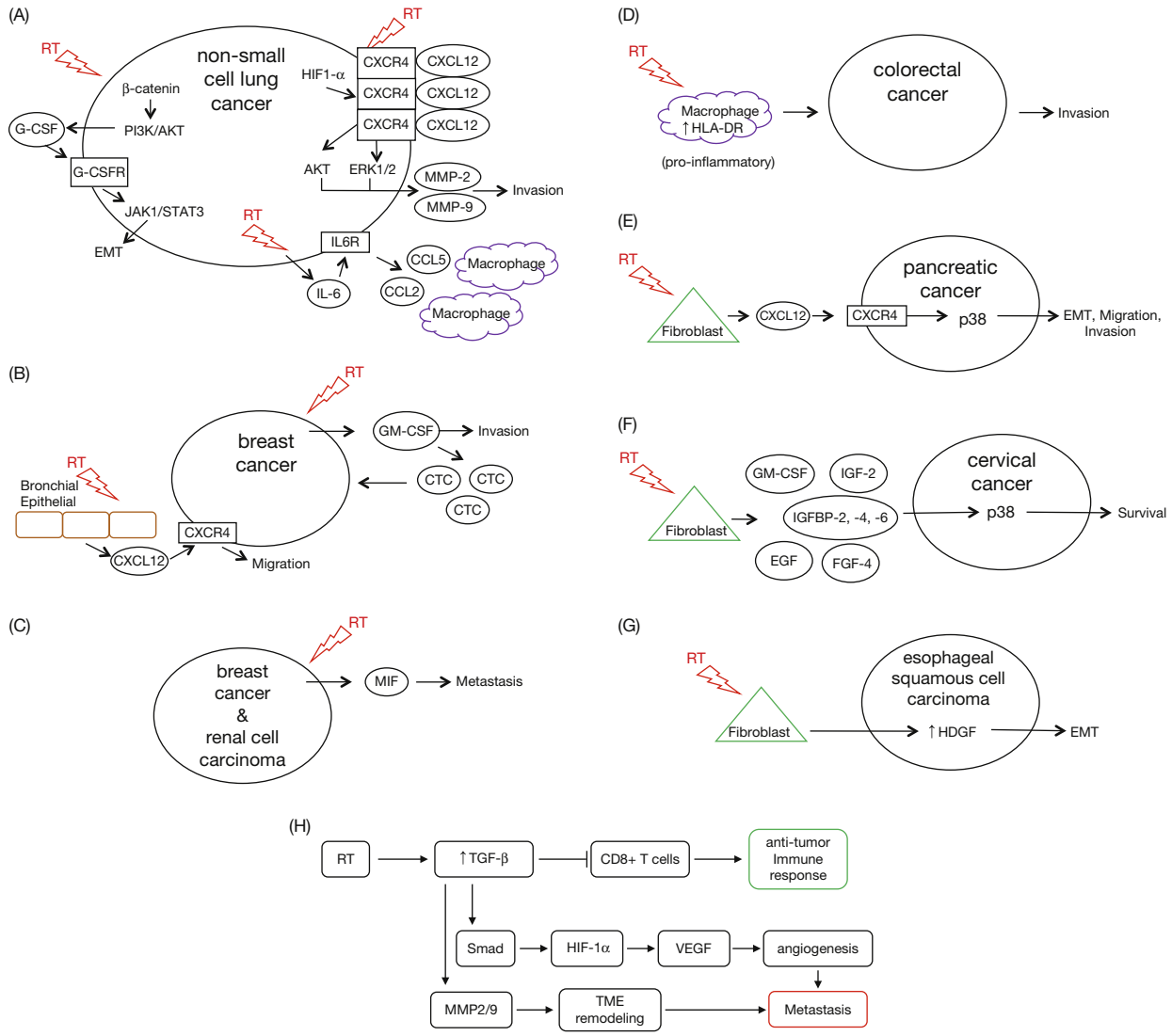

Fig. 3 RT induces the release of cytokines, chemokines, and growth factors within the tumor microenvironment to promote metastasis. (A) In non-small cell lung cancer, RT causes the release of G-CSF that acts in an autocrine manner to induce EMT. RT upregulates CXCR4 expression to enhance MMP-2 and MMP-9 secretion and promote invasion. RT induces the release of IL-6, which acts through autocrine signaling to promote the release of CCL2 and CCL5 which attract macrophages. (B) In breast cancer, RT causes the release of GM-CSF, which promotes invasion and attracts circulating tumor cells (CTCs) at metastatic sites. Also, RT induces the release of CXCL12 by bronchial epithelial cells of the lung, which in turn enhances the migration of breast cancer cells. (C) RT causes the release of MIF by breast cancer and renal cell carcinoma cells to promote metastasis. (D) RT induces macrophages towards a pro-inflammatory phenotype that act upon colorectal cancer cells to promote invasion. (E) RT induces the release of CXCL12 by fibroblasts that acts upon pancreatic cancer cells to promote EMT, migration, and invasion. (F) RT prompts the secretion of GM-CSF, IGF2, IGFBP-2, -4, -6, EGF, and FGF-4 by fibroblasts to enhance survival of cervical cancer cells. (G) During RT, fibroblasts act on esophageal squamous cell carcinoma cells to upregulate HDGF and promote EMT. (H) RT induces expression of TGF-β, which inhibits the anti-tumor immune response and promotes metastasis.

Stromal cells such as macrophages and fibroblasts influence the metastatic potential of tumor cells. Although RT inhibits the invasive ability of colorectal cancer cells, RT-treated macrophages are able to restore and even enhance their invasive properties due to a shift towards a pro-inflammatory phenotype, which includes increased expression of HLA-DR (Teresa Pinto et al., 2016) (**Fig. 3**D). Fibroblasts from pancreatic tumors secrete CXCL12 in response to RT, and in turn this CXCL12 acts through CXCR4 to activate the p38 MAPK pathway and promote epithelial-mesenchymal transition (EMT), migration, and invasion of pancreatic cancer cells (**Fig. 3**E). In vivo, this RT-induced CXCL12/CXCR4 interaction accelerates the growth of lung metastases (Li et al., 2016). Similarly, cervical cancer-associated fibroblasts activate p38 in HeLa cells to increase their survival after RT. The release of granulocyte-macrophage colony-stimulating factor (GM-CSF), insulin-like growth factor 2 (IGF2), insulin-like growth factor binding proteins (IGFBP)- 2, -4, -6, epidermal growth factor (EGF), and fibroblast growth factor 4 (FGF-4), by fibroblasts may contribute to cancer cell survival (Chu et al., 2014) (**Fig. 3**F). RT-treated fibroblasts from esophageal squamous cell carcinoma (ESCC) tissue enhance the migratory and invasive properties of ESCC cells by induction of epithelial-mesenchymal transition (EMT). These effects are initiated by the upregulation of hepatoma-derived growth factor (HDGF) in ESCC due to interactions with RT-treated fibroblasts (Bao et al., 2015) (**Fig. 3**G).

RT also modulates anti-tumor immunity and promotes the elusion of tumor cells against anti-tumor immune responses (**Fig. 3**H). The major factor modulating anti-tumor immune response is TGF-β, which acts by inhibiting the differentiation of $CD8^+$ cytotoxic T cells, as well as inhibiting the expression of cytolytic proteins in these cells, which include granzymes, perforin, fas ligand, IFN-γ, etc. Without these factors, cytotoxic T cells fail to impart its cell death-inducing function on its target tumor cells (Thomas and Massague, 2005). In addition, TGF-β also modulates the function of other immune cells in the TME like dendritic cells, a subtype of antigen presenting cells, which lose their ability to activate T cells (Geissmann et al., 1999). Furthermore, TGF-β induces angiogenesis, TME remodeling, EMT, and therefore, promotes metastasis in solid tumors (Padua and Massague, 2009). The pro-metastatic ability of TGF-β has been attributed to its ability to activate HIF-1α and Smad signaling pathways, which in turn induce the expression of VEGF and MMPs (MMP2 and MMP9). TGF-β is known to promote EMT by suppressing the expression of E-cadherin and other junction proteins by activation of Snail and Slug transcription factors and MAPK pathway molecules (Oft et al., 1996). Taken together, enhanced expression of TGF-β expression in TME post-RT has been a significant problem and may be one of the main reasons behind emergence of SMNs.

Therapeutic Approaches to Overcome RT-Induced Metastasis

Several studies have investigated how to surmount this negative side effect of RT (**Fig. 4**). For example, HIF-1α is a frontrunner for therapeutic development since it is activated both by hypoxia and RT, regulates EMT and angiogenesis and participates in cross-talk with the TME (Hu et al., 2013). Several HIF-1α inhibitors have shown promising results and may have potential for clinical use, such as silibinin and everolimus, each of which work by blocking mTOR and thus inhibit HIF-1α translation (Garcia-Maceira and Mateo, 2009; Cejka et al., 2008). The FDA-approved HDAC inhibitor SAHA (vorinostat) also inhibits HIF-1α transcription and activity and was shown to decrease metastatic spread in several cancer types (Zhang et al., 2017; Shankar et al., 2009). Furthermore, there are many HIF-1α inhibitors being tested in a preclinical setting.

The angiogenic factor VEGF has been targeted as well, but with mixed outcomes (Heist et al., 2015; Papadimitriou et al., 2015; Rahbari et al., 2016; Das et al., 2014). For example, bevacizumab is an FDA approved monoclonal antibody that targets VEGF and inhibits angiogenesis (Panoilia et al., 2015). However, the success of this agent is short-lived and the cancer typically recurs in a few months (Carmeliet and Jain, 2011). Recently, Rahbari et al. have demonstrated that bevacizumab alters the extracellular matrix and induces stiffness via hyaluronic acid aggregation of liver metastatic sites in metastatic colorectal mouse models, and that targeting hyaluronic acid build-up sensitizes these models to treatment with bevacizumab (Rahbari et al., 2016). Thus, anti-VEGF therapy may still be beneficial as long as the proper agents are targeted in combination.

Recently, radio immunotherapy (RIT) (Behr et al., 2002) also gained much consideration as targeted therapy against tumor cells. Tumor-specific antibodies conjugated with chemotherapeutic agents or radioisotopes have been shown as a targeted

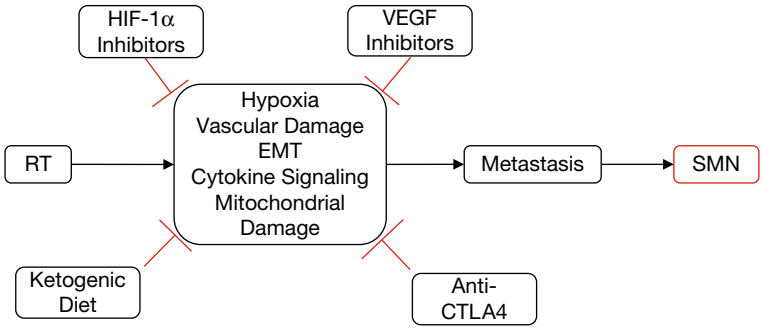

Fig. 4 Potential therapeutic approaches to overcome RT-induced SMN. A flowchart describing advances in therapeutic approaches to inhibit metastatic-induced SMN development.

delivery method to tumor cells with minimal exposure to adjacent normal cells. In this regard, CD20 targeting antibodies ibritumomab tiuxetan (Zevalin) conjugated with yttrium-90 (Y-90) and tositumomab (Bexxar) conjugated with iodine-131 (I-131) were used for treatment of non-Hodgkin's lymphoma (Ghobrial and Witzig, 2004). RIT has also shown promise for treatment of metastatic cancers such as colorectal (Behr et al., 2002) and prostate (O'Donnell et al., 2001). More research needs to be done to analyze the effect of radioisotope-conjugated antibodies on tumor stroma and other immune cells in the TME.

Lifestyle choices can also impact response to RT. Most notably, a calorie restricted or ketogenic diet (KD) has shown promising results. Specifically, KD combined with RT was demonstrated to have a greater anti-tumor affect than RT alone (Abdelwahab et al., 2012; Allen et al., 2013). Since KDs are low in carbohydrates, and tumor cells require carbohydrates for glycolysis, KDs selectively starve out the cancer cells (Lv et al., 2014). Additionally, the lack of carbohydrates pushes the cancer cell to use oxidative phosphorylation over glycolysis, which promotes the generation of mitochondrial ROS and induces damage.

Ironically, RT itself has been implicated to act as a type of anti-cancer vaccine by activating tumor-specific T-cells, and is reviewed extensively in (Demaria et al., 2016). To summarize, the common problem in immunotherapy is the concept of sensitizing tumor cells to immunotherapy, or turning "cold" tumors into "hot" ones (Gajewski, 2015). The basic premise is that dendritic cells must activate T-cells, and these activated T-cells must be able to successfully infiltrate the tumor microenvironment in order for immunotherapy to be effective. However, refractive tumor microenvironments have poor T-cell infiltration. Several approaches have been put forth to overcome this barrier. An interesting approach, the combination of RT with the anti-Cytotoxic T-lymphocyte-Associated Protein 4 (CTLA-4, an immunosuppressive protein receptor), has shown to decrease growth of the primary tumor and metastatic spread in several mouse models of breast cancer (Demaria et al., 2005; Dewan et al., 2009). The combination of RT with anti-CTLA-4 has also demonstrated clinical benefit, as metastatic lesions and tumor burden were both decreased in patients with metastatic non-small cell lung cancer and metastatic melanoma after the combination therapy (Golden et al., 2013; Hiniker et al., 2012). These results are both promising and surprising, as RT was previously thought to be strictly immunosuppressive. However, these results demonstrate that the immunogenicity of RT may be circumstantial with regards to cancer biology. In addition, TGF-β also causes anti-tumor immune suppression in the TME by inhibiting $CD8^+$ cytotoxic T cells as well as promotes metastasis by inducing HIFs and Smad signaling. Therefore, TGF-β inhibitors in the form of small molecules and neutralizing antibodies have been extensively studied and it is safe to assume that in combination with RT, TGF-β inhibition will be helpful in inhibiting SMN development post-RT by activating the immune response and inhibiting metastasis.

Concluding Remarks

RT is a useful anti-cancer tool. Its long-term use in the clinical setting is evidence of its efficacy. The risk of developing SMNs after RT is well described but it is seen as a necessary risk to cancer patients that have no other options. In this modern era, we have made impressive advances in understanding how SMNs develop with respect to RT-induced metastasis. Because of these recent advances, new therapeutic targets are currently being characterized and validated in order to prevent RT-induced metastasis. Nonetheless, all of these advances have helped to better our understanding of tumor biology and will pave the way for new therapeutic ideas and approaches to combat RT-induced metastases with the ultimate goal of eliminating the risk of SMN development in cancer survivors.

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See also: Cell Responses to DNA Damage. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics.

References

Abdelwahab MG, Fenton KE, Preul MC, Rho JM, Lynch A, Stafford P, Scheck AC. The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. PLoS One. 2012;7(5):e36197. Epub 2012/05/09. https://doi.org/10.1371/journal.pone.0036197. PubMed PMID: 22563484; PMCID: PMC3341352.

Alan Mitteer R, Wang Y, Shah J, Gordon S, Fager M, Butter PP, Jun Kim H, Guardiola-Salmeron C, Carabe-Fernandez A, Fan Y. Proton beam radiation induces DNA damage and cell apoptosis in glioma stem cells through reactive oxygen species. Science Reports 2015; 5: 13961. Epub 2015/09/12. https://doi.org/10.1038/srep13961. PubMed PMID: 26354413; PMCID: PMC4564801.

Allen BG, Bhatia SK, Buatti JM, Brandt KE, Lindholm KE, Button AM, Szweda LI, Smith BJ, Spitz DR, Fath MA. Ketogenic diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts. Clinical Cancer Research. 2013; 19(14): 3905–13. Epub 2013/06/08. https://doi.org/10.1158/1078-0432.CCR-12-0287. PubMed PMID: 23743570; PMCID: PMC3954599.

Altmeyer M, Lukas J. To spread or not to spread—Chromatin modifications in response to DNA damage. Current Opinion in Genetics & Development 2013;23(2):156–65. Epub 2013/01/15. https://doi.org/10.1016/j.gde.2012.11.001. PubMed PMID: 23312207.

Arnold RS, Fedewa SA, Goodman M, Osunkoya AO, Kissick HT, Morrissey C, True LD, Petros JA. Bone metastasis in prostate cancer: Recurring mitochondrial DNA mutation reveals selective pressure exerted by the bone microenvironment. Bone. 2015; 78:81–6. Epub 2015/05/09. https://doi.org/10.1016/j.bone.2015.04.046. PubMed PMID: 25952970; PMCID: PMC4466124.

Bao CH, Wang XT, Ma W, Wang NN, Un Nesa E, Wang JB, Wang C, Jia YB, Wang K, Tian H, Cheng YF. Irradiated fibroblasts promote epithelial-mesenchymal transition and HDGF expression of esophageal squamous cell carcinoma. Biochemical and Biophysical Research Communications 2015;458(2):441–7. Epub 2015/02/14. https://doi.org/10.1016/j.bbrc.2015.02.001. PubMed PMID: 25677618.

Behr TM, Liersch T, Greiner-Bechert L, Griesinger F, Behe M, Markus PM, Gratz S, Angerstein C, Brittinger G, Becker H, Goldenberg DM, Becker W. Radioimmunotherapy of small-volume disease of metastatic colorectal cancer. Cancer 2002;94(4 Suppl):1373–81. Epub 2002/03/06. PubMed PMID: 11877768.

Cadet J, Wagner JR. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harbor Perspectives in Biology 2013; 5(2). a012559 Epub 2013/02/05. https://doi.org/10.1101/cshperspect.a012559. PubMed PMID: 23378590; PMCID: PMC3552502.

Cao J, Liu Y, Sun H, Cheng G, Pang X, Zhou Z. Chromosomal aberrations, DNA strand breaks and gene mutations in nasopharyngeal cancer patients undergoing radiation therapy. Mutation Research 2002;504(1–2):85–90. Epub 2002/07/11. PubMed PMID: 12106649.

Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011; 473(7347): 298–307. Epub 2011/05/20. https://doi.org/10.1038/nature10144. PubMed PMID: 21593862; PMCID: PMC4049445.

Cejka D, Preusser M, Woehrer A, Sieghart W, Strommer S, Werzowa J, Fuereder T, Wacheck V. Everolimus (RAD001) and anti-angiogenic cyclophosphamide show long-term control of gastric cancer growth in vivo. Cancer Biology & Therapy 2008;7(9):1377–85. Epub 2008/08/19. PubMed PMID: 18708754.

Chang CH, Zhang M, Rajapakshe K, Coarfa C, Edwards D, Huang S, Rosen JM. Mammary stem cells and tumor-initiating cells are more resistant to apoptosis and exhibit increased DNA repair activity in response to DNA damage. Stem Cell Reports. 2015; 5(3): 378–91. Epub 2015/08/25. https://doi.org/10.1016/j.stemcr.2015.07.009. PubMed PMID: 26300228; PMCID: PMC4618454.

Choi DK, Helenowski I, Hijiya N. Secondary malignancies in pediatric cancer survivors: Perspectives and review of the literature. International Journal of Cancer 2014;135(8):1764–73. Epub 2014/06/20. https://doi.org/10.1002/ijc.28991. PubMed PMID: 24945137.

Chu TY, Yang JT, Huang TH, Liu HW. Crosstalk with cancer-associated fibroblasts increases the growth and radiation survival of cervical cancer cells. Radiation Research 2014;181(5):540–7. Epub 2014/05/03. https://doi.org/10.1667/RR13583.1. PubMed PMID: 24785588.

Croteau DL, Bohr VA. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. The Journal of Biological Chemistry 1997;272(41):25409–12. Epub 1997/11/05. PubMed PMID: 9325246.

Cui YH, Suh Y, Lee HJ, Yoo KC, Uddin N, Jeong YJ, Lee JS, Hwang SG, Nam SY, Kim MJ, Lee SJ. Radiation promotes invasiveness of non-small-cell lung cancer cells through granulocyte-colony-stimulating factor. Oncogene 2015;34(42):5372–82. Epub 2015/02/03. https://doi.org/10.1038/onc.2014.466. PubMed PMID: 25639867.

Das P, Eng C, Rodriguez-Bigas MA, Chang GJ, Skibber JM, You YN, Maru DM, Munsell MF, Clemons MV, Kopetz SE, Garrett CR, Shureiqi I, Delclos ME, Krishnan S, Crane CH. Preoperative radiation therapy with concurrent capecitabine, bevacizumab, and erlotinib for rectal cancer: A phase 1 trial. International Journal of Radiation Oncology, Biology, Physics. 2014; 88(2): 301–5. Epub 2013/12/10. https://doi.org/10.1016/j.ijrobp.2013.10.034. PubMed PMID: 24315563; PMCID: PMC5592790.

de Aberasturi AL, Calvo A. TMPRSS4: An emerging potential therapeutic target in cancer. British Journal of Cancer. 2015; 112(1): 4–8. Epub 2014/09/10. https://doi.org/10.1038/bjc.2014.403. PubMed PMID: 25203520; PMCID: PMC4453593.

Demaria S, Kawashima N, Yang AM, Devitt ML, Babb JS, Allison JP, Formenti SC. Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. Clinical Cancer Research 2005;11(2 Pt 1):728–34. Epub 2005/02/11. PubMed PMID: 15701862.

Demaria S, Coleman CN, Formenti SC. Radiotherapy: Changing the game in immunotherapy. Trends Cancer. 2016;2(6):286–94. Epub 2016/10/25. https://doi.org/10.1016/j.trecan.2016.05.002. PubMed PMID: 27774519; PMCID: PMC5070800.

DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS, Jemal A. Cancer treatment and survivorship statistics, 2014. CA: A Cancer Journal for Clinicians 2014;64(4):252–71. Epub 2014/06/04. https://doi.org/10.3322/caac.21235. PubMed PMID: 24890451.

Dewan MZ, Galloway AE, Kawashima N, Dewyngaert JK, Babb JS, Formenti SC, Demaria S. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. Clinical Cancer Research 2009; 15(17): 5379–88. Epub 2009/08/27. https://doi.org/10.1158/1078-0432.CCR-09-0265. PubMed PMID: 19706802; PMCID: PMC2746048.

Donin N, Filson C, Drakaki A, Tan HJ, Castillo A, Kwan L, Litwin M, Chamie K. Risk of second primary malignancies among cancer survivors in the United States, 1992 through 2008. Cancer 2016;122(19):3075–86. Epub 2016/07/06. https://doi.org/10.1002/cncr.30164. PubMed PMID: 27377470.

Feys L, Descamps B, Vanhove C, Vral A, Veldeman L, Vermeulen S, De Wagter C, Bracke M, De Wever O. Radiation-induced lung damage promotes breast cancer lung-metastasis through CXCR4 signaling. Oncotarget. 2015; 6(29): 26615–32. Epub 2015/09/24. https://doi.org/10.18632/oncotarget.5666. PubMed PMID: 26396176; PMCID: PMC4694940.

Fiorese CJ, Schulz AM, Lin YF, Rosin N, Pellegrino MW, Haynes CM. The Transcription Factor ATF5 Mediates a Mammalian Mitochondrial UPR. Current Biology 2016; 26(15): 2037–43. Epub 2016/07/19. https://doi.org/10.1016/j.cub.2016.06.002. PubMed PMID: 27426517; PMCID: PMC4980197.

Fu Z, Chen D, Cheng H, Wang F. Hypoxia-inducible factor-1alpha protects cervical carcinoma cells from apoptosis induced by radiation via modulation of vascular endothelial growth factor and p53 under hypoxia. Medical Science Monitor 2015; 21: 318–25. Epub 2015/01/28. https://doi.org/10.12659/MSM.893265. PubMed PMID: 25623525; PMCID: PMC4316866.

Gajewski TF. The next hurdle in cancer immunotherapy: Overcoming the non-T-cell-inflamed tumor microenvironment. Seminars in Oncology 2015; 42(4): 663–71. Epub 2015/09/01. https://doi.org/10.1053/j.seminoncol.2015.05.011. PubMed PMID: 26320069; PMCID: PMC4555998.

Garcia-Maceira P, Mateo J. Silibinin inhibits hypoxia-inducible factor-1alpha and mTOR/p70S6K/4E-BP1 signalling pathway in human cervical and hepatoma cancer cells: Implications for anticancer therapy. Oncogene 2009;28(3):313–24. Epub 2008/11/04. https://doi.org/10.1038/onc.2008.398. PubMed PMID: 18978810.

Gaugler MH, Squiban C, van der Meeren A, Bertho JM, Vandamme M, Mouthon MA. Late and persistent up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by ionizing radiation in human endothelial cells in vitro. International Journal of Radiation Biology 1997;72(2):201–9. Epub 1997/08/01. PubMed PMID: 9269313.

Geissmann F, Revy P, Regnault A, Lepelletier Y, Dy M, Brousse N, Amigorena S, Hermine O, Durandy A. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. Journal of Immunology 1999;162(8):4567–75. Epub 1999/04/14. PubMed PMID: 10201996.

Ghobrial I, Witzig T. Radioimmunotherapy: A new treatment modality for B-cell non-Hodgkin's lymphoma. Oncology (Williston Park). 2004;18(5): 623–30; discussion 33–4, 37–8, 40. Epub 2004/06/24. PubMed PMID: 15209190.

Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. The Journal of Biological Chemistry. 2013; 288(15): 10819–29. Epub 2013/02/21. https://doi.org/10.1074/jbc.M112.442939. PubMed PMID: 23423382; PMCID: PMC3624462.

Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: Drivers of tumour metastasis. Nature Reviews. Cancer. 2014; 14(6): 430–9. Epub 2014/05/16. https://doi.org/10.1038/nrc3726. PubMed PMID: 24827502; PMCID: PMC4283800.

Golden EB, Demaria S, Schiff PB, Chachoua A, Formenti SC. An abscopal response to radiation and ipilimumab in a patient with metastatic non-small cell lung cancer. Cancer Immunology Research 2013; 1(6): 365–72. Epub 2014/02/25. https://doi.org/10.1158/2326-6066.CIR-13-0115. PubMed PMID: 24563870; PMCID: PMC3930458.

Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Science Signaling, 2014; 7(344): re8. Epub 2014/09/25. https://doi.org/10.1126/scisignal.2005189. PubMed PMID: 25249658; PMCID: PMC4372086.

Grabham P, Bigelow A, Geard C. DNA damage foci formation and decline in two-dimensional monolayers and in three-dimensional human vessel models: Differential effects according to radiation quality. International Journal of Radiation Biology 2012;88(6):493–500. Epub 2012/03/28. https://doi.org/10.3109/09553002.2012.679382. PubMed PMID: 22449005.

Gu Q, He Y, Ji J, Yao Y, Shen W, Luo J, Zhu W, Cao H, Geng Y, Xu J, Zhang S, Cao J, Ding WQ. Hypoxia-inducible factor 1alpha (HIF-1alpha) and reactive oxygen species (ROS) mediates radiation-induced invasiveness through the SDF-1alpha/CXCR4 pathway in non-small cell lung carcinoma cells. Oncotarget. 2015;6(13): 10893–907. Epub 2015/04/07. https://doi.org/10.18632/oncotarget.3535. PubMed PMID: 25843954; PMCID: PMC4484427.

Gupta Y, Pasupuleti V, Du W, Welford SM. Macrophage migration inhibitory factor secretion is induced by ionizing radiation and oxidative stress in cancer cells. PLoS One. 2016; 11(1): e0146482. Epub 2016/01/08. https://doi.org/10.1371/journal.pone.0146482. PubMed PMID: 26741693; PMCID: PMC4704778.

Hallahan DE, Virudachalam S. Accumulation of P-selectin in the lumen of irradiated blood vessels. Radiation Research 1999;152(1):6–13. Epub 1999/06/25. PubMed PMID: 10381836.

He M, Dong C, Ren R, Yuan D, Xie Y, Pan Y, Shao C. Radiation enhances the invasiveness of irradiated and nonirradiated bystander hepatoma cells through a VEGF-MMP2 pathway initiated by p53. Radiation Research 2013;180(4):389–97. Epub 2013/09/26. https://doi.org/10.1667/RR3355.1. PubMed PMID: 24059678.

Heist RS, Duda DG, Sahani DV, Ancukiewicz M, Fidias P, Sequist LV, Temel JS, Shaw AT, Pennell NA, Neal JW, Gandhi L, Lynch TJ, Engelman JA, Jain RK. Improved tumor vascularization after anti-VEGF therapy with carboplatin and nab-paclitaxel associates with survival in lung cancer. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(5):1547–52. Epub 2015/01/22. https://doi.org/10.1073/pnas.1424024112. PubMed PMID: 25605928; PMCID: PMC4321320.

Hemmerlein B, Kugler A, Ozisik R, Ringert RH, Radzun HJ, Thelen P. Vascular endothelial growth factor expression, angiogenesis, and necrosis in renal cell carcinomas. Virchows Archiv 2001;439(5):645–52. Epub 2002/01/05. PubMed PMID: 11764385.

Hiniker SM, Chen DS, Reddy S, Chang DT, Jones JC, Mollick JA, Swetter SM, Knox SJ. A systemic complete response of metastatic melanoma to local radiation and immunotherapy. Translational Oncology 2012;5(6):404–7. Epub 2013/01/17. PubMed PMID: 23323154; PMCID: PMC3542835.

Houtkooper RH, Mouchiroud L, Ryu D, Moullan N, Katsyuba E, Knott G, Williams RW, Auwerx J. Mitonuclear protein imbalance as a conserved longevity mechanism. Nature. 2013; 497(7450): 451–7. Epub 2013/05/24. https://doi.org/10.1038/nature12188. PubMed PMID: 23698443; PMCID: PMC3663447.

Hu Y, Liu J, Huang H. Recent agents targeting HIF-1alpha for cancer therapy. Journal of Cellular Biochemistry 2013;114(3):498–509. Epub 2012/09/11. https://doi.org/10.1002/jcb.24390. PubMed PMID: 22961911.

Hunt CR, Ramnarain D, Horikoshi N, Iyengar P, Pandita RK, Shay JW, Pandita TK. Histone modifications and DNA double-strand break repair after exposure to ionizing radiations. Radiation Research 2013; 179(4): 383–92. Epub 2013/02/05. https://doi.org/10.1667/RR3308.2. PubMed PMID: 23373901; PMCID: PMC4133051.

Imanishi H, Hattori K, Wada R, Ishikawa K, Fukuda S, Takenaga K, Nakada K, Hayashi J. Mitochondrial DNA mutations regulate metastasis of human breast cancer cells. PLoS One. 2011; 6(8): e23401. Epub 2011/08/20. https://doi.org/10.1371/journal.pone.0023401. PubMed PMID: 21853128; PMCID: PMC3154938.

Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, Nakada K, Honma Y, Hayashi J. ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science 2008;320(5876):661–4. Epub 2008/04/05. https://doi.org/10.1126/science.1156906. PubMed PMID: 18388260.

Ji RC. Hypoxia and lymphangiogenesis in tumor microenvironment and metastasis. Cancer Letters 2014;346(1):6–16. Epub 2013/12/18. https://doi.org/10.1016/j.canlet.2013.12.001. PubMed PMID: 24333723.

Jovaisaite V, Mouchiroud L, Auwerx J. The mitochondrial unfolded protein response, a conserved stress response pathway with implications in health and disease. The Journal of Experimental Biology. 2014; 217(Pt 1): 137–43. Epub 2013/12/20. https://doi.org/10.1242/jeb.090738. PubMed PMID: 24353213; PMCID: PMC3867496.

Juntermanns B, Grabellus F, Zhang H, Radunz S, Bernheim J, Fingas CD, Sauerwein W, Paul A, Kaiser GM. Vascular and nerval damage after intraoperative radiation therapy of the liver hilum in a large animal model. Journal of Investigative Surgery 2014;27(3):163–8. Epub 2014/01/01. https://doi.org/10.3109/08941939.2013.868961. PubMed PMID: 24377883.

Kalen AL, Ahmad IM, Abdalla MY, O'Malley YQ, Goswami PC, Sarsour EH. MnSOD and cyclin B1 coordinate a mito-checkpoint during cell cycle response to oxidative stress. Antioxidants (Basel). 2017; 6(4). Epub 2017/11/18. https://doi.org/10.3390/antiox6040092. PubMed PMID: 29149089.

Karpac J, Jasper H. Aging: Seeking mitonuclear balance. Cell 2013;154(2):271–3. Epub 2013/07/23. https://doi.org/10.1016/j.cell.2013.06.046. PubMed PMID: 23870118.

Kim YH, Yoo KC, Cui YH, Uddin N, Lim EJ, Kim MJ, Nam SY, Kim IG, Suh Y, Lee SJ. Radiation promotes malignant progression of glioma cells through HIF-1alpha stabilization. Cancer Letters 2014;354(1):132–41. Epub 2014/08/12. https://doi.org/10.1016/j.canlet.2014.07.048. PubMed PMID: 25109450.

Kinoshita K, Ishimine H, Shiraishi K, Kato H, Doi K, Kuno S, Kanayama K, Mineda K, Mashiko T, Feng J, Nakagawa K, Kurisaki A, Itami S, Yoshimura K. Cell and tissue damage after skin exposure to ionizing radiation: Short- and long-term effects after a single and fractional doses. Cells, Tissues, Organs 2014;200(3–4):240–52. Epub 2015/09/12. https://doi.org/10.1159/000435809. PubMed PMID: 26359658.

Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P, Semenza GL. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Research 2003;63(5):1138–43. Epub 2003/03/05. PubMed PMID: 12615733.

Lavelle C, Foray N. Chromatin structure and radiation-induced DNA damage: From structural biology to radiobiology. The International Journal of Biochemistry & Cell Biology 2014;49:84–97. Epub 2014/02/04. https://doi.org/10.1016/j.biocel.2014.01.012. PubMed PMID: 24486235.

Li T, Zeng ZC, Wang L, Qiu SJ, Zhou JW, Zhi XT, Yu HH, Tang ZY. Radiation enhances long-term metastasis potential of residual hepatocellular carcinoma in nude mice through TMPRSS4-induced epithelial-mesenchymal transition. Cancer Gene Therapy 2011;18(9):617–26. Epub 2011/06/04. https://doi.org/10.1038/cgt.2011.29. PubMed PMID: 21637307.

Li D, Qu C, Ning Z, Wang H, Zang K, Zhuang L, Chen L, Wang P, Meng Z. Radiation promotes epithelial-to-mesenchymal transition and invasion of pancreatic cancer cell by activating carcinoma-associated fibroblasts. Am J Cancer Res. 2016; 6(10): 2192–206. Epub 2016/11/09. PubMed PMID: 27822411; PMCID: PMC5088285.

Li Q, Shi J, Chen L, Zhan F, Yuan H, Wang J, Xu A, Wu L. Spatial function of the oxidative DNA damage response in radiation induced bystander effects in intra- and inter-system of *Caenorhabditis elegans*. Oncotarget. 2017; 8(31): 51253–63. Epub 2017/09/09. https://doi.org/10.18632/oncotarget.17229. PubMed PMID: 28881645; PMCID: PMC5584246.

Lv M, Zhu X, Wang H, Wang F, Guan W. Roles of caloric restriction, ketogenic diet and intermittent fasting during initiation, progression and metastasis of cancer in animal models: A systematic review and meta-analysis. PLoS One. 2014; 9(12): e115147. Epub 2014/12/17. https://doi.org/10.1371/journal.pone.0115147. PubMed PMID: 25502434; PMCID: PMC4263749.

Lv W, Chen N, Lin Y, Ma H, Ruan Y, Li Z, Li X, Pan X, Tian X. Macrophage migration inhibitory factor promotes breast cancer metastasis via activation of HMGB1/TLR4/NF kappa B axis. Cancer Letters 2016;375(2):245–55. Epub 2016/03/10, https://doi.org/10.1016/j.canlet.2016.02.005. PubMed PMID: 26952810.

Mimeault M, Batra SK. Hypoxia-inducing factors as master regulators of stemness properties and altered metabolism of cancer- and metastasis-initiating cells. Journal of Cellular and Molecular Medicine 2013; 17(1): 30–54. Epub 2013/01/11. https://doi.org/10.1111/jcmm.12004. PubMed PMID: 23301832; PMCID: PMC3560853.

Moro L, Arbini AA, Marra E, Greco M. Mitochondrial DNA depletion reduces PARP-1 levels and promotes progression of the neoplastic phenotype in prostate carcinoma. Cellular Oncology 2008; 30(4): 307–22. Epub 2008/07/09. PubMed PMID: 18607066; PMCID: PMC4618976.

Moro L, Arbini AA, Yao JL, di Sant'Agnese PA, Marra E, Greco M. Mitochondrial DNA depletion in prostate epithelial cells promotes anoikis resistance and invasion through activation of PI3K/Akt2. Cell Death and Differentiation 2009;16(4):571–83. Epub 2008/12/17. https://doi.org/10.1038/cdd.2008.178. PubMed PMID: 19079138.

Mouchiroud L, Houtkooper RH, Moullan N, Katsyuba E, Ryu D, Canto C, Mottis A, Jo YS, Viswanathan M, Schoonjans K, Guarente L, Auwerx J. The NAD(+)/Sirtuin pathway modulates longevity through activation of mitochondrial UPR and FOXO signaling. Cell. 2013; 154(2): 430–41. Epub 2013/07/23. https://doi.org/10.1016/j.cell.2013.06.016. PubMed PMID: 23870130; PMCID: PMC3753670.

Nieri D, Fioramonti M, Berardinelli F, Leone S, Cherubini R, De Nadal V, Gerardi S, Moreno S, Nardacci R, Tanzarella C, Antoccia A. Radiation response of chemically derived mitochondrial DNA-deficient AG01522 human primary fibroblasts. Mutation Research 2013;756(1–2):86–94. Epub 2013/06/01. https://doi.org/10.1016/j.mrgentox.2013.05.010. PubMed PMID: 23721903.

Ning Y, Manegold PC, Hong YK, Zhang W, Pohl A, Lurje G, Winder T, Yang D, LaBonte MJ, Wilson PM, Ladner RD, Lenz HJ. Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. International Journal of Cancer. 2011; 128(9): 2038–49. Epub 2010/07/22. https://doi.org/10.1002/ijc.25562. PubMed PMID: 20648559; PMCID: PMC3039715.

O'Donnell RT, DeNardo SJ, Yuan A, Shen S, Richman CM, Lara PN, Griffith IJ, Goldstein DS, Kukis DL, Martinez GS, Mirick GR, DeNardo GL, Meyers FJ. Radioimmunotherapy with (111)in/(90)Y-2IT-BAD-m170 for metastatic prostate cancer. Clinical Cancer Research 2001;7(6):1561–8. Epub 2001/06/19. PubMed PMID: 11410491.

Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes & Development 1996;10(19):2462–77. Epub 1996/10/01. PubMed PMID: 8843198.

Padua D, Massague J. Roles of TGFbeta in metastasis. Cell Research 2009;19(1):89–102. Epub 2008/12/04. https://doi.org/10.1038/cr.2008.316. PubMed PMID: 19050696.

Panoilia E, Schindler E, Samantas E, Aravantinos G, Kalofonos HP, Christodoulou C, Patrinos GP, Friberg LE, Sivolapenko G. A pharmacokinetic binding model for bevacizumab and VEGF165 in colorectal cancer patients. Cancer Chemotherapy and Pharmacology. 2015;75(4): 791–803. Epub 2015/02/18. https://doi.org/10.1007/s00280-015-2701-3. PubMed PMID: 25687989; PMCID: PMC4365273.

Papadimitriou K, Rolfo C, Dewaele E, Van De Wiel M, Van den Brande J, Altintas S, Huizing M, Specenier P, Peeters M. Incorporating anti-VEGF pathway therapy as a continuum of care in metastatic colorectal cancer. Current Treatment Options in Oncology 2015;16(4):18. Epub 2015/03/31. https://doi.org/10.1007/s11864-015-0333-9. PubMed PMID: 25813037.

Park JK, Jang SJ, Kang SW, Park S, Hwang SG, Kim WJ, Kang JH, Um HD. Establishment of animal model for the analysis of cancer cell metastasis during radiotherapy. Radiation Oncology 2012;7: 153. Epub 2012/09/12. https://doi.org/10.1186/1748-717X-7-153. PubMed PMID: 22963683; PMCID: PMC3493326.

Pellegrino MW, Nargund AM, Haynes CM. Signaling the mitochondrial unfolded protein response. Biochimica et Biophysica Acta. 2013; 1833(2): 410–6. Epub 2012/03/27. https://doi.org/10.1016/j.bbamcr.2012.02.019. PubMed PMID: 22445420; PMCID: PMC3393825.

Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 2003;3(4):347–61. Epub 2003/05/03. PubMed PMID: 12726861.

Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nature Medicine 2013;19(11): 1423–37. Epub 2013/11/10. https://doi.org/10.1038/nm.3394. PubMed PMID: 24202395; PMCID: PMC3954707.

Rahbari NN, Kedrin D, Incio J, Liu H, Ho WW, Nia HT, Edrich CM, Jung K, Daubriac J, Chen I, Heishi T, Martin JD, Huang Y, Maimon N, Reissfelder C, Weitz J, Boucher Y, Clark JW, Grodzinsky AJ, Duda DG, Jain RK, Fukumura D. Anti-VEGF therapy induces ECM remodeling and mechanical barriers to therapy in colorectal cancer liver metastases. Science Translational Medicine. 2016; 8(360): 360ra135. Epub 2016/10/14. https://doi.org/10.1126/scitranslmed.aaf5219. PubMed PMID: 27733559; PMCID: PMC5457741.

Sabatasso S, Laissue JA, Hlushchuk R, Graber W, Bravin A, Brauer-Krisch E, Corde S, Blattmann H, Gruber G, Djonov V. Microbeam radiation-induced tissue damage depends on the stage of vascular maturation. International Journal of Radiation Oncology, Biology, Physics 2011;80(5):1522–32. Epub 2011/07/12. https://doi.org/10.1016/j.ijrobp.2011.03.018. PubMed PMID: 21740994.

Semenza GL. Targeting HIF-1 for cancer therapy. Nature Reviews. Cancer 2003;3(10):721–32. Epub 2003/09/18. https://doi.org/10.1038/nrc1187. PubMed PMID: 13130303.

Shankar S, Davis R, Singh KP, Kurzrock R, Ross DD, Srivastava RK. Suberoylanilide hydroxamic acid (Zolinza/vorinostat) sensitizes TRAIL-resistant breast cancer cells orthotopically implanted in BALB/c nude mice. Molecular Cancer Therapeutics 2009;8(6):1596–605. Epub 2009/06/11. https://doi.org/10.1158/1535-7163.MCT-08-1004. PubMed PMID: 19509267.

Shpilka T, Haynes CM. The mitochondrial UPR: Mechanisms, physiological functions and implications in ageing. Nature Reviews. Molecular Cell Biology 2017. Epub 2017/11/23. https://doi.org/10.1038/nrm.2017.110. PubMed PMID: 29165426.

Simpson KD, Templeton DJ, Cross JV. Macrophage migration inhibitory factor promotes tumor growth and metastasis by inducing myeloid-derived suppressor cells in the tumor microenvironment. Journal of Immunology. 2012; 189(12): 5533–40. Epub 2012/11/06. https://doi.org/10.4049/jimmunol.1201161. PubMed PMID: 23125418; PMCID: PMC3518629.

Sofia Vala I, Martins LR, Imaizumi N, Nunes RJ, Rino J, Kuonen F, Carvalho LM, Ruegg C, Grillo IM, Barata JT, Mareel M, Santos SC. Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis. PLoS One. 2010; 5(6): e11222. Epub 2010/06/25. https://doi.org/10.1371/journal.pone.0011222. PubMed PMID: 20574535; PMCID: PMC2888592.

Sountoulides P, Koletsas N, Kikidakis D, Paschalidis K, Sofikitis N. Secondary malignancies following radiotherapy for prostate cancer. Therapeutic Advances in Urology 2010; 2(3): 119–25. Epub 2011/07/27. https://doi.org/10.1177/1756287210374462. PubMed PMID: 21789089; PMCID: PMC3126090.

Stark TW, Hensley PJ, Spear A, Pu H, Strup SS, Kyprianou N. Predictive value of epithelial-mesenchymal-transition (EMT) signature and PARP-1 in prostate cancer radioresistance. The Prostate 2017;77(16):1583–91. Epub 2017/10/25. https://doi.org/10.1002/pros.23435. PubMed PMID: 29063620.

Suit H, Goldberg S, Niemierko A, Ancukiewicz M, Hall E, Goitein M, Wong W, Paganetti H. Secondary carcinogenesis in patients treated with radiation: A review of data on radiation-induced cancers in human, non-human primate, canine and rodent subjects. Radiation Research 2007;167(1):12–42. Epub 2007/01/12. https://doi.org/10.1667/RR0527.1. PubMed PMID: 17214511.

Teresa Pinto A, Laranjeiro Pinto M, Patricia Cardoso A, Monteiro C, Teixeira Pinto M, Filipe Maia A, Castro P, Figueira R, Monteiro A, Marques M, Mareel M, Dos Santos SG, Seruca R, Adolfo Barbosa M, Rocha S, Jose Oliveira M. Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities. Science Reports. 2016; 6: 18765. Epub 2016/01/07. https://doi.org/10.1038/srep18765. PubMed PMID: 26735768; PMCID: PMC4702523.

Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 2005;8(5):369–80. Epub 2005/11/16. https://doi.org/10.1016/j.ccr.2005.10.012. PubMed PMID: 16286245.

Thompson LH. Recognition, signaling, and repair of DNA double-strand breaks produced by ionizing radiation in mammalian cells: The molecular choreography. Mutation Research 2012;751(2):158–246. Epub 2012/06/30. https://doi.org/10.1016/j.mrrev.2012.06.002. PubMed PMID: 22743550.

Torregrosa-Munumer R, Goffart S, Haikonen JA, Pohjoismaki JL. Low doses of ultraviolet radiation and oxidative damage induce dramatic accumulation of mitochondrial DNA replication intermediates, fork regression, and replication initiation shift. Molecular Biology of the Cell 2015; 26(23): 4197–208. Epub 2015/09/25. https://doi.org/10.1091/mbc.E15-06-0390. PubMed PMID: 26399294; PMCID: PMC4642854.

Van Houten B, Santa-Gonzalez GA, Camargo M. DNA repair after oxidative stress: current challenges. Current Opinion in Toxicology. 2018; 7: 9–16. Epub 2017/11/22. https://doi.org/10.1016/j.cotox.2017.10.009. PubMed PMID: 29159324; PMCID: PMC5693256.

Vaughan TL, Davis S, Kristal A, Thomas DB. Obesity, alcohol, and tobacco as risk factors for cancers of the esophagus and gastric cardia: Adenocarcinoma versus squamous cell carcinoma. Cancer Epidemiology, Biomarkers & Prevention 1995;4(2):85–92. Epub 1995/03/01. PubMed PMID: 7742727.

Vempati P, Popel AS, Mac Gabhann F. Extracellular regulation of VEGF: Isoforms, proteolysis, and vascular patterning. Cytokine and Growth Factor Reviews. 2014; 25(1):1–19. Epub 2013/12/18. https://doi.org/10.1016/j.cytogfr.2013.11.002. PubMed PMID: 24332926; PMCID: PMC3977708.

Vilalta M, Rafat M, Giaccia AJ, Graves EE. Recruitment of circulating breast cancer cells is stimulated by radiotherapy. Cell Reports 2014; 8(2): 402–9. Epub 2014/07/16. https://doi.org/10.1016/j.celrep.2014.06.011. PubMed PMID: 25017065; PMCID: PMC4121080.

Wang X, Yang X, Tsai Y, Yang L, Chuang KH, Keng PC, Lee SO, Chen Y. IL-6 mediates macrophage infiltration after irradiation via up-regulation of CCL2/CCL5 in non-small cell lung cancer. Radiation Research 2017;187(1):50–9. Epub 2017/01/06. https://doi.org/10.1667/RR14503.1. PubMed PMID: 28054838.

Weeden CE, Asselin-Labat ML. Mechanisms of DNA damage repair in adult stem cells and implications for cancer formation. Biochimica et Biophysica Acta 2018;1864(1):89–101. Epub 2017/10/19. https://doi.org/10.1016/j.bbadis.2017.10.015. PubMed PMID: 29038050.

Widel M, Krzywon A, Gajda K, Skonieczna M, Rzeszowska-Wolny J. Induction of bystander effects by UVA, UVB, and UVC radiation in human fibroblasts and the implication of reactive oxygen species. Free Radical Biology & Medicine 2014;68:278–87. Epub 2014/01/01. https://doi.org/10.1016/j.freeradbiomed.2013.12.021. PubMed PMID: 24373962.

Wodarz A, Nathke I. Cell polarity in development and cancer. Nature Cell Biology 2007;9(9):1016–24. Epub 2007/09/01. https://doi.org/10.1038/ncb433. PubMed PMID: 17762893.

Zhang SB, Okunieff P. Mitochondrial genetic abnormalities after radiation exposure. Advances in Experimental Medicine and Biology 2014;812:1–7. Epub 2014/04/15. https://doi.org/10.1007/978-1-4939-0620-8_1. PubMed PMID: 24729208.

Zhang C, Yang C, Feldman MJ, Wang H, Pang Y, Maggio DM, Zhu D, Nesvick CL, Dmitriev P, Bullova P, Chittiboina P, Brady RO, Pacak K, Zhuang Z. Vorinostat suppresses hypoxia signaling by modulating nuclear translocation of hypoxia inducible factor 1 alpha. Oncotarget. 2017; 8(34): 56110–25. Epub 2017/09/17. https://doi.org/10.18632/oncotarget.18125. PubMed PMID: 28915577; PMCID: PMC5593548.

Zheng Q, Liu Y, Zhou HJ, Du YT, Zhang BP, Zhang J, Miao GY, Liu B, Zhang H. X-ray radiation promotes the metastatic potential of tongue squamous cell carcinoma cells via modulation of biomechanical and cytoskeletal properties. Human & Experimental Toxicology 2015;34(9):894–903. Epub 2015/01/15. https://doi.org/10.1177/0960327114561664. PubMed PMID: 25586002.

Zhou X, Li N, Wang Y, Wang Y, Zhang X, Zhang H. Effects of X-irradiation on mitochondrial DNA damage and its supercoiling formation change. Mitochondrion 2011;11(6):886–92. Epub 2011/08/13. https://doi.org/10.1016/j.mito.2011.07.005. PubMed PMID: 21835270.

Zhou X, Liu X, Zhang X, Zhou R, He Y, Li Q, Wang Z, Zhang H. Non-randomized mtDNA damage after ionizing radiation via charge transport. Science Reports. 2012; 2:780. Epub 2012/10/31. https://doi.org/10.1038/srep00780. PubMed PMID: 23110249; PMCID: PMC3483021.

Zywietz F. Vascular and cellular damage in a murine tumour during fractionated treatment with radiation and hyperthermia. Strahlentherapie und Onkologie 1990;166(7):493–501. Epub 1990/07/01. PubMed PMID: 2382213.

Renal Cell Cancer: Pathology and Genetics

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Glossary

Cytogenetics The study of chromosomal structure and function.
Histology Microscopic study of tissues.
Immunohistochemistry Detection of proteins in tissue samples using antibody-based staining techniques.
Nephron The microscopic functional and structural unit of the kidney, composed of the glomerulus and renal tubule.
Renal cell carcinoma Cancer of the kidney that is thought to recapitulate the phenotype of the renal tubules.
Renal tubules Structures that carry urine and modify its salt and water composition after filtration by the kidney.

Introduction

In adults, most cancers of the kidney are renal cell carcinomas, tumors that are largely thought to recapitulate the phenotype of cells of the tubules of the nephron. A smaller subset of kidney cancers are urothelial carcinomas originating from the urothelial lining of the renal pelvis or ureter, and rarer are sarcomas and mesenchymal tumors of the kidney. This article summarizes the pathology and genetics of the most common form of adult kidney cancer, renal cell carcinoma. This cancer type is somewhat unique compared to those of many other organs, in that tumors often form predominantly spherical masses, a growth pattern that is prototypically associated with benign tumors, and they do not necessarily exhibit the characteristic pattern of infiltrative, crab-like growth for which cancer is named. Nonetheless, renal cancer is known for sometimes surprising behavior, including metastasis decades after the first diagnosis, and sometimes unusual sites of metastases, such as to the pancreas or visceral organs. With increasing sophistication of radiologic imaging and more widespread use of imaging, however, there is also a shift toward detecting renal cancers at an earlier stage, in which case the treatment paradigm has begun to shift toward less aggressive and nonsurgical management of early-stage tumors.

Epidemiology

Kidney cancer is approximately the eighth most common site of cancer diagnosis, with 60,000 cases estimated to be diagnosed in the United States in 2017. Renal cell carcinoma is more common in men, in countries with higher levels of socioeconomic development, particularly northern and eastern Europe, North America, and Australia, and in particular the Czech Republic has the highest incidence, for incompletely understood reasons. Risk factors include obesity, smoking, hypertension, acquired cystic kidney disease (cystic changes of the kidney from long-term renal failure, usually associated with dialysis), and occupational exposure to certain chemicals. A subset of renal cancers is found in the setting of hereditary susceptibility, estimated to make up about 2%–4%, with each syndrome tending to associate with a specific tumor histology. These are discussed in more detail with each type of renal cancer in this article.

Clinical Management

Usual clinical management for localized (non-metastatic) renal cancer includes local treatment of the primary tumor, most typically in the form of radical nephrectomy (removal of the entire kidney) or partial nephrectomy (surgical removal of only the tumor with a small rim of normal kidney tissue). For the most part, adjuvant therapy is not employed in addition to surgical resection in the setting of localized kidney cancer; however, this remains an area of exploration with ongoing clinical trials evaluating possible roles for adjuvant therapies in high-risk patients. Alternate therapies to surgery are also increasingly considered, especially in patients whose health status would make surgery riskier, such as cryoablation or radiofrequency ablation of the tumor. Metastatic kidney cancer, in contrast, is typically managed with one or more chemotherapeutic treatment pathways, including interleukin-2 (IL-2) or interferon therapy, molecularly-targeted agents, and more recently, anti-PD-1/PD-L1 therapies that modulate immune response. Nonetheless, metastatic kidney cancer is typically a progressive disease that is rarely, if ever, completely cured.

Renal Mass Biopsy

Renal cell cancer also differs somewhat from other cancers in that a diagnostic tissue sample or biopsy is not as a rule undertaken prior to proceeding with definitive surgery, since a solid and/or vascular renal mass identified by radiographic imaging is usually

considered presumptive malignancy until proven otherwise. A relatively small number of renal masses are proven benign, predominantly angiomyolipomas (which can often be diagnosed by the presence of fat on imaging studies and therefore biopsy or surgery avoided) and oncocytomas (approximately 5% or less of renal masses), which remains difficult to distinguish from renal cell carcinoma by imaging. This, combined with historical fears of "seeding," or causing tumor cells to implant into the soft tissue of the biopsy site, among other factors, had resulted in limited use of renal mass biopsies in the past.

However, more recently, the paradigm for kidney cancer management has begun to shift, with historical fears of safety and efficacy and renal mass biopsy largely mitigated. Now, renal mass biopsies are increasingly used, especially in patients for whom a small renal mass is identified, especially in the setting of other medical comorbidities, such that surgery or other treatment may be risky. In this context, various strategies, such as surveillance, ablation, and surgery are now more widely tailored to the patient, based on biopsy information and patient factors. Likewise, in the setting of metastatic renal cancer, a biopsy may be the only confirmation of tumor histology, used to select tumor-specific treatment options or to employ molecular studies in search of targetable treatment.

Clear Cell Renal Cell Carcinoma

Clear cell renal cell carcinoma is by far the most common subtype of renal cell cancer, making up at least 60%–75% of adult tumors, although novel entities continue to be extracted from this category based on refined understanding of genetics and subtle differences in histology. Clinical presentation has become more variable for these tumors in current practice, as many are now recognized incidentally when radiographic imaging is done for other reasons, in contrast to historically larger tumors that presented with clinical symptoms, such as hematuria or flank pain or mass. However, generally, clear cell renal cell carcinomas form spherical masses that can be endophytic, entirely within the kidney, or can bulge well beyond the normal contour of the kidney and substantially distort its shape. This often-spherical configuration differs from many other cancers that prototypically have an infiltrative growth pattern with stromal reaction, or desmoplasia. In contrast, invasion by renal cell carcinoma often manifests as subtler, tongue-like, rounded protrusions into veins or soft tissue.

The characteristic gross pathologic appearance of clear cell renal cell carcinoma includes a bright, golden-yellow cut surface, although this may vary with the presence of hemorrhage or cystic areas, imparting red-brown areas or fluid-filled spaces (Fig. 1). Microscopically, clear cell renal cell carcinoma prototypically is composed, as its name suggests, of cells with empty-appearing (clear cytoplasm), resulting from cytoplasmic lipid and glycogen (Fig. 2). However, the histologic appearance can also be quite variable, especially in tumors of higher grade, which can have eosinophilic cytoplasm (historically often known as "granular cell renal cell carcinoma") and a variety of unusual patterns.

Immunohistochemistry

Due to alterations in the von Hippel-Lindau gene (*VHL*) pathway, these tumors characteristically demonstrate upregulation of multiple downstream targets of the *VHL* gene, which has led to several targeted therapies against these pathway components. In pathologic practice, diagnostic immunohistochemical staining can demonstrate diffuse membrane positivity for carbonic anhydrase IX (Fig. 3), a downstream element of the hypoxia pathway, which has been found to be helpful for diagnosis of clear cell renal cell carcinoma and debatably in some settings possibly a prognostic marker. Radiographic imaging techniques directed against carbonic anhydrase IX have also been tested in some centers as a possible mechanism to support the diagnosis prior to tissue sampling. In contrast to adenocarcinomas of many other organs, clear cell renal cell carcinomas are usually negative for cytokeratin 7, and therefore they are usually among the group of adenocarcinomas that are positive for neither cytokeratin 7 nor 20, two

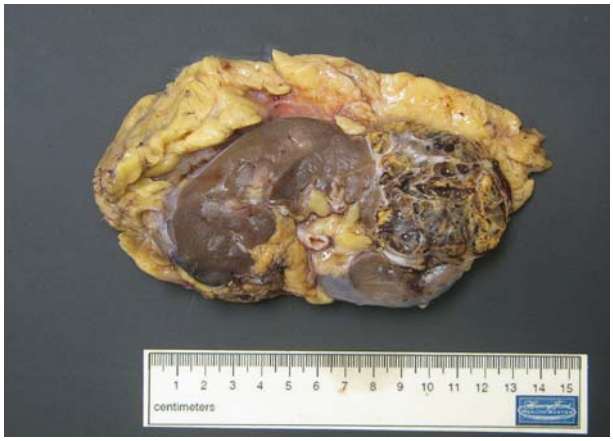

Fig. 1 The characteristic gross appearance of clear cell renal cell carcinoma is often *bright yellow*. This tumor includes a mixture of *yellow* and *red-brown* areas, as well as cystic spaces.

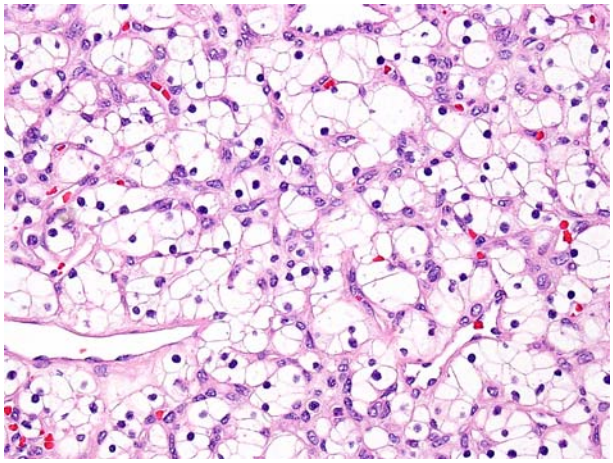

Fig. 2 The classic microscopic appearance of clear cell renal cell carcinoma includes a nested arrangement of cells with empty-appearing (clear) cytoplasm, although a wide variety of patterns can occur, especially in higher grade tumors.

markers which are often used in assessment of metastatic carcinoma from an unknown origin. Immunohistochemistry for paired box gene 8 (PAX8) has emerged in pathologic practice as a lineage-specific marker for renal cell tumors (among other tumors, such as gynecologic and thyroid tumors). Although not specific for clear cell renal cell carcinoma, labeling for this antigen can be used to support an origin from renal cancer in the appropriate context. Additionally, many renal cell carcinomas, including clear cell renal cell carcinoma, share the feature of reactivity for both epithelial markers and vimentin. Although the latter is nonspecific, it is generally considered a marker of mesenchymal lineage and dual reactivity for both epithelial markers and vimentin is somewhat more restricted to specific cancer types, especially renal cell and endometrial cancer. Interestingly, renal oncocytoma, a benign renal cell neoplasm, and chromophobe renal cell carcinoma, discussed additionally later, essentially always lack reactivity for vimentin. In general, it is thought that the cells of clear cell renal cell carcinoma, and most renal cancers, have a phenotype or "cell of origin" resembling the proximal tubules of the nephron.

Genetics

Clear cell renal cell carcinoma, as the most common form of adult kidney cancer, has paved the road for understanding of renal cancer genetics, based on work identifying the *VHL* gene and chromosome 3p25 (the region where the *VHL* gene is located) as critical in the development of these tumors. Patients with the syndrome VHL disease have germline mutations of the *VHL* gene and develop multiple tumors, including clear cell renal cell carcinoma of the kidney, pheochromocytomas of the adrenal gland, neuroendocrine tumors of the pancreas, pancreatic cysts, renal cysts, and hemangioblastomas of the central nervous system and retina, among others. Knowledge of the *VHL* gene can also be extrapolated to the sporadic setting (patients without germline mutation), in which patients likely progress through a two-hit mechanism to tumor development, whereas in VHL patients already harbor one

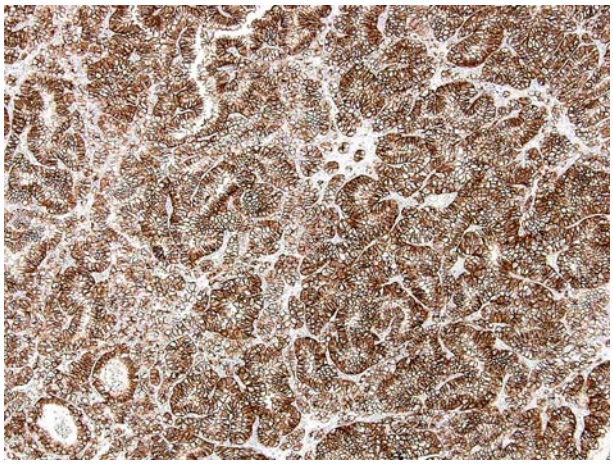

Fig. 3 Carbonic anhydrase IX, a component of the hypoxia-inducible pathway, is typically markedly upregulated in clear cell renal cell carcinoma and can be detected by immunohistochemistry with diffuse membranous labeling (*brown*).

VHL gene alteration from birth, accelerating this process. *VHL* is a tumor suppressor gene, and usually one copy is inactivated via mutation and the other is lost via sizable deletion of large regions of chromosome 3p or the entire 3p arm. Therefore, fluorescence in situ hybridization (FISH) or other molecular techniques have been used as one approach to support a diagnosis of clear cell renal cell carcinoma via detection of chromosome 3p deletion. More recent work as part of large-scale sequencing studies has revealed that several other key genes, many of which are also located on chromosome 3p, are also frequently altered in clear cell renal cell carcinoma, especially *PBRM1* (and other members of the switching defective/sucrose nonfermenting or SWI/SNF family), as well as *SETD2*, and *BAP1*. Deregulation of the mammalian target of rapamycin complex 1 (mTORC1) pathway is also found in a subset of tumors, resulting from mutations in *MTOR*, *TSC1*, *PIK3CA*, and *PTEN*. From this knowledge of clear cell renal cancer genetics, recent decades have yielded multiple additional treatment options targeting these pathways, especially the vascular endothelial growth factor (VEGF) pathway and the MTOR pathway.

Multilocular Cystic Renal Cell Neoplasm of Low Malignant Potential

Multilocular cystic renal cell carcinoma was considered a distinct tumor subtype in the 2004 World Health Organization (WHO) Classification of Tumors, strictly defined as an entirely cystic renal cell neoplasm with thin fibrovascular septa containing only aggregates of cells, resembling clear cell renal cell carcinoma cells, but not forming a gross mass (Fig. 4). When defined according to these criteria, no example has been published that exhibited aggressive behavior, resulting in the 2016 WHO recategorization of this entity as multilocular cystic renal cell neoplasm of low malignant potential. Despite the highly favorable prognosis of this entity, there is nonetheless some evidence for genetic overlap with clear cell renal cell carcinoma, including chromosome 3p25 loss and a subset with mutation of *VHL* (although the rate of these alterations may be lower than those of usual clear cell renal cell carcinoma). Though the WHO definition of this entity requires an entirely cystic tumor with no solid growth, other studies have also argued that a cystic component in clear cell renal cell carcinoma may impart a good prognosis, even for tumors that are not entirely cystic, and sometimes with even a relatively minor cystic component.

Papillary Renal Cell Carcinoma

Papillary renal cell carcinoma is the second most common form of adult renal cancer but considerably less common, at approximately 15% of renal cell carcinoma. Presentation of papillary renal cell carcinoma can be highly variable, ranging from small renal nodules (single or multiple) to large masses that bulge beyond the normal shape of the kidney. Patients with end-stage renal disease tend to develop papillary renal cell carcinoma, among other tumor histologies, sometimes multifocally; however, multifocal papillary renal cell carcinoma can also occur in non-end-stage kidneys for unknown reasons, and as part of hereditary syndromes, especially the hereditary papillary renal cell carcinoma syndrome, characterized by germline mutation of *MET*.

The gross appearance of papillary renal cell carcinoma can also be highly variable, depending on factors such as intratumoral hemorrhage, which can impart a red-brown appearance, or abundant foamy macrophages, which can impart a yellow appearance like clear cell renal cell carcinoma. Histologically, papillary tumors have been subdivided into type I and type II tumors. Type I tumors are more common, composed of cuboidal cells (Fig. 5) with a generally basophilic appearance. In contrast, type II tumors have eosinophilic cytoplasm, more elongated and pseudostratified nuclei. Prognosis is generally considered to be better for type I tumors; however, some authors have found distinguishing these two tumor types difficult in practice due to overlapping or mixed patterns. This classification may also be confounded by the fact that certain aggressive tumor types, especially hereditary

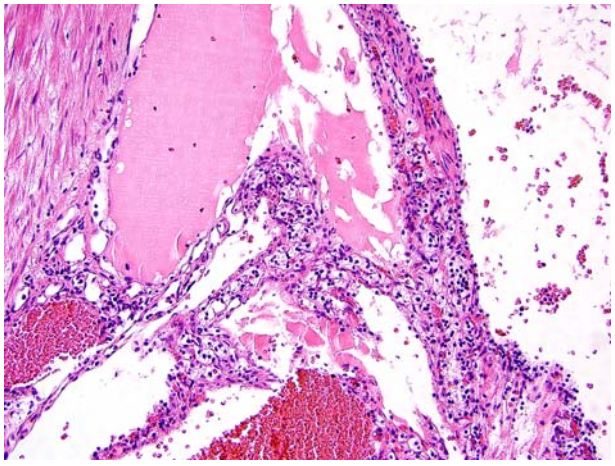

Fig. 4 Multilocular cystic renal neoplasms of low malignant potential are composed of cells that are similar to those of clear cell renal cell carcinoma; however, by definition these tumors are entirely cystic and associated with an excellent prognosis.

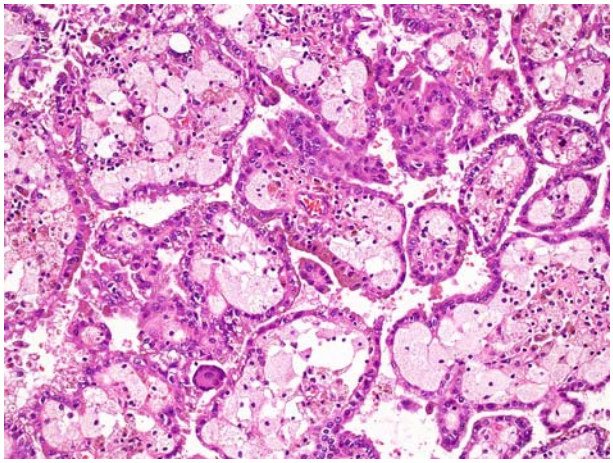

Fig. 5 Papillary renal cell carcinoma is characteristically composed of cuboidal-shaped cells lining papillary structures. Foamy macrophages within the papillae are a common feature, as present in this example.

leiomyomatosis and renal cell carcinoma syndrome (HLRCC)-associated tumors, have been historically considered type II papillary renal cell carcinoma, but are currently thought better classified as a distinct tumor type.

Immunohistochemistry

Using immunohistochemistry, papillary renal cell carcinoma is generally considered to be cytokeratin 7-positive, in contrast to clear cell renal cell carcinoma; however, this holds true primarily for classic type I tumors with basophilic cells. Staining for cytokeratin 7, in contrast, is often negative or limited in examples with eosinophilic cytoplasm. Alpha-methylacyl-CoA racemase (AMACR) reliably demonstrates strong, diffuse reactivity independent of cytology in almost all papillary tumors, and like clear cell renal cell carcinoma, labeling of epithelial cells for the mesenchymal marker vimentin is common. Carbonic anhydrase IX labeling is usually limited compared to clear cell renal cell carcinoma, although as a marker of the hypoxia pathway, carbonic anhydrase IX labeling can be present in other tumors or non-tumor tissues, especially in areas of tissue ischemia or necrosis.

Genetics

In the setting of the hereditary papillary renal cell carcinoma syndrome, patients have germline mutations of *MET* and develop numerous bilateral, multifocal papillary renal cell carcinomas (type I). In contrast to the *VHL* gene, however, this association is not as clearly translated to the sporadic setting, in which only a relatively small subset of papillary tumors is *MET* mutant. Characteristic chromosomal alterations in papillary renal cell carcinoma include trisomy of chromosome 7 and/or 17 and loss of the Y chromosome in male patients. Detection of these changes by conventional karyotype, FISH, or other techniques has been used as supportive of papillary renal cell carcinoma classification; however, the specificity of these alterations is debatable, as copy number changes for these chromosomes can also be found in other renal tumors and diseases of other organs. Multiple other chromosomal copy changes have also been recognized, including amplification of 8q with overexpression of *MYC*. Recent large-scale genomic characterization studies have also supported the interpretation of type I and type II tumors as different entities, with distinct genomic alterations in each category; however, this classification undoubtedly remains to be further refined. For example, *TFE3* gene fusions have been reported as one pattern of molecular alteration in type II tumors, a finding which likely in reality should justify classification as not papillary renal cell carcinoma but translocation-associated renal cell carcinoma, discussed additionally later. Some papillary renal cell carcinomas also harbor alterations in chromatin-modifying genes such as *SETD2*, *BAP1*, and *PBRM1*, which as noted previously are also now recognized to have important roles in clear cell renal cell carcinoma. *CDKN2A* silencing has also been recognized in some papillary renal cell carcinomas.

Chromophobe Renal Cell Carcinoma

Chromophobe renal cell carcinoma is typically considered the third most common subtype of renal cell carcinoma, usually making up approximately 5% of tumors. Overall, chromophobe renal cell carcinoma is usually regarded as a nonaggressive or less aggressive renal cancer type; however, there are some notable exceptions this rule, especially in the case of sarcomatoid dedifferentiation. The gross appearance of chromophobe renal cell carcinoma can resemble that of renal oncocytoma (a benign renal cell tumor), often demonstrating a tan or brown cut surface, either similar in color to normal renal parenchyma or paler in color. Although central fibrosis or "central scar" is considered a characteristic feature of oncocytoma, this can also be found in chromophobe renal cell carcinoma and other tumors, making it not entirely specific.

The prototypical histologic appearance of chromophobe renal cell carcinoma includes cells with variable amounts of pale-staining cytoplasm (hence the name "chromo" meaning color and "phobe" meaning fearing, or color-fearing, Fig. 6). Tumor cells are sometimes described as resembling plant cells or vegetable cells, since their histologic appearance includes very prominent cell borders, mimicking the cell walls that are found in plant cells. Cell volume is characteristically highly variable, with preservation of the nuclear-cytoplasmic ratio, so that some cells are very large and may appear to have no nucleus due to histologic sectioning through the large cell that entirely misses the nucleus. In contrast, other cells are much smaller and more crowded, yet still with a similar nuclear-cytoplasmic ratio.

Although this tumor type is named for its characteristic pale cytoplasm, it was not long after its recognition that the eosinophilic variant of chromophobe renal cell carcinoma was recognized, which has dense eosinophilic cytoplasm (making the chromophobe designation a misnomer in this setting). This appearance can lead to considerable overlap with the benign renal neoplasm oncocytoma, such that discriminating these two entities continues to be a challenge in diagnostic practice even today. As such, it is also a topic of debate whether oncocytoma and chromophobe renal cell carcinoma represent a spectrum of disease, with the benign counterpart (oncocytoma) at one end and classic (pale-staining) chromophobe renal cell carcinoma at the other end, separated by a gray zone of borderline tumors. A progressive model in which tumors evolve from oncocytoma to chromophobe renal cell carcinoma is not entirely intuitive, however, as many chromophobe renal cell carcinomas have a pure pale cell morphology, leading to no significant overlap with oncocytoma. Therefore, an alternative hypothesis is that chromophobe renal cell carcinoma and oncocytoma are unrelated entities, for which the former may occasionally mimic the latter.

Both oncocytoma and chromophobe renal cell carcinoma have been subject to study at the electron microscopic level (ultrastructural examination), in which oncocytoma exhibits numerous, densely-packed mitochondria, and chromophobe renal cell carcinoma contains numerous cytoplasmic microvesicles, structures usually 140–300 nm in size that may be related to defective mitochondrial development. Colloidal iron histochemical staining (historically known as Hale colloidal iron, or in modern practice modified Mowry or Muller–Mowry colloidal iron staining) has been exploited as a technique to highlight the differences in cytoplasmic constituents between oncocytoma and chromophobe carcinoma, which is thought to reflect disrupted microvesicles (positive staining in chromophobe and negative staining in oncocytoma). However, like many other diagnostic parameters, there remains overlap in both the presence of microvesicles ultrastructurally and staining for colloidal iron histochemically, especially in tumors that are equivocal for diagnosis of oncocytoma and chromophobe renal cell carcinoma. Therefore, due to lack of availability in many diagnostic pathology laboratories and challenges and subjectivity in interpretation, electron microscopy and colloidal iron staining are often not employed in current practice. Nonetheless, the balance of features at the morphologic, immunohistochemical, and ultrastructural level has led to the theory that oncocytoma and chromophobe renal cell carcinoma are tumors that arise from, or recapitulate the phenotype of, intercalated cells of the collecting ducts.

Although chromophobe renal cell carcinoma is generally considered a less aggressive renal cancer type, aggressive behavior sometimes occurs, particularly in tumors with sarcomatoid dedifferentiation, tumor necrosis, and vascular invasion. Interestingly, although clear cell renal cell carcinoma is the most common renal cell carcinoma subtype and likely is the origin for the largest *number* of sarcomatoid renal cancers, chromophobe carcinoma has been found in some studies to have a paradoxically high rate of sarcomatoid change, despite its much lower incidence and usual nonaggressive behavior, perhaps related to its tendency for *TP53* mutation, which is discussed later.

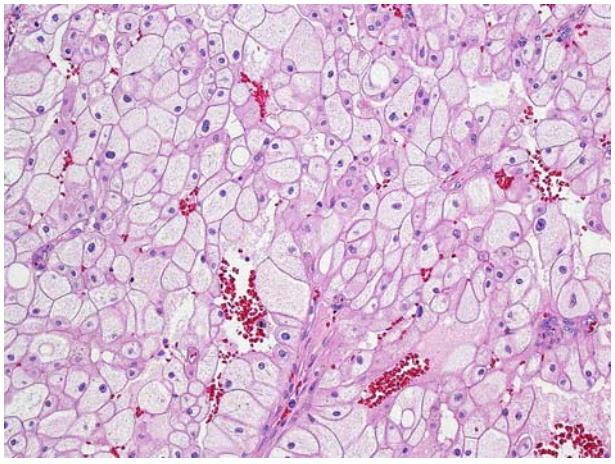

Fig. 6 Chromophobe renal cell carcinoma is composed of cells with pale-staining cytoplasm and prominent cell borders, resembling the cell wall of plant tissue as observed microscopically. In this example, some cells are so voluminous that they appear to have no nucleus, due to the plane of tissue sectioning entirely missing the nucleus.

Immunohistochemistry

Using immunohistochemistry, chromophobe renal cell carcinoma characteristically differs from clear cell and papillary renal cell carcinoma, in that it uniformly does not label for mesenchymal marker vimentin. Staining for KIT (CD117) often reveals a membranous pattern of labeling, which can be used to distinguish chromophobe from clear cell renal cell carcinoma (although this feature is shared with oncocytoma and therefore not helpful in discriminating these two entities). Classic chromophobe renal cell carcinoma (with pale cells) usually exhibits diffuse staining for cytokeratin 7, which differs from clear cell renal cell carcinoma and oncocytoma; however, this becomes less helpful in tumors with eosinophilic cells and the eosinophilic variant of chromophobe carcinoma, in which staining for cytokeratin 7 can be quite limited and minimally different from that of oncocytoma, if at all. A precise threshold of amount of cytokeratin 7 staining that precludes a diagnosis of oncocytoma remains to be fully agreed upon, although in general oncocytomas exhibit only rare single cells and clusters of cells that label for cytokeratin 7, making up typically 5% of cells or less. Other immunohistochemical markers that are used by some pathologists and researchers to discriminate chromophobe carcinoma from oncocytoma include S100A1, kidney-specific cadherin, among a wide variety of others that have been investigated in many studies; however, usage in diagnostic practice remains limited for most of these.

Genetics

At the chromosomal level, chromophobe renal cell carcinoma typically exhibits abnormal copy numbers for multiple chromosomes, often loss, particularly for chromosomes Y, 1, 2, 6, 10, 13, 17, and 21. However, there are conflicting data as to whether the eosinophilic variant exhibits a similar chromosomal profile. Some studies using FISH have found similar chromosomal losses in the eosinophilic variant when defined according to strict criteria; however, others, such as the Cancer Genome Atlas cohort, have found that the eosinophilic variant may have a diploid (normal) number of chromosomes, further blurring the distinction from oncocytoma. For the most part, oncocytomas exhibit a normal karyotype, loss of chromosome 1, loss of chromosome Y, or rearrangement of 11q13. The latter likely represents *CCND1* (cyclin D1) gene rearrangement, which has recently emerged as a molecular finding in some oncocytomas, which some authors suggest may define a molecular subtype of oncocytoma.

Regarding chromophobe renal cell carcinoma in general, other studies have also found that some tumors have chromosomal gains as well as losses. Other genetic alterations that have been recognized in chromophobe renal cell carcinoma include somatic mutations in mitochondrial DNA, such as NADH dehydrogenase, and alterations of *TP53*, *PTEN*, and rearrangements involving the *TERT* promoter. Although mutations of *TP53* are not entirely specific and can be found in clear cell renal cell carcinoma, these mutations appear to be enriched in chromophobe renal cell carcinoma, which might account for its predilection for sarcomatoid dedifferentiation, as *TP53* mutations are associated with a wide variety of cancers, including sarcomas.

"Hybrid" Oncocytic Tumors

An incompletely defined term in the renal cancer literature is the so-called "hybrid" oncocytoma-chromophobe tumor (HOCT or hybrid oncoytic tumor/HOT), which generally has some features of oncocytoma and some features of chromophobe renal cell carcinoma (Fig. 7). The context in which this term is used can be somewhat variable, with some authors using it for any oncocytic tumor with borderline diagnostic features, others using it only when the tumor has a mosaic morphology (some areas identical to oncocytoma and others to chromophobe carcinoma), and others using it particularly for tumors that occur in the context of an apparent

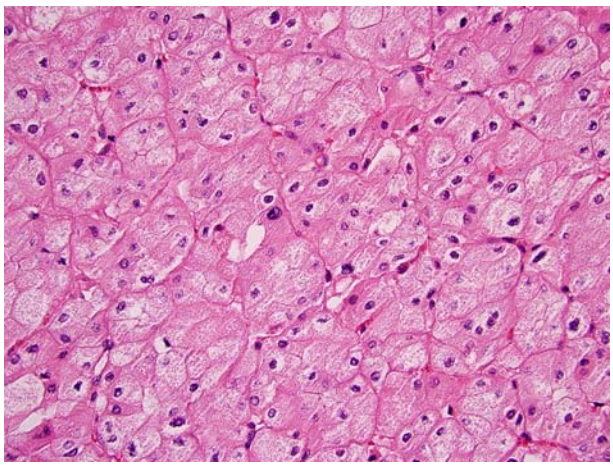

Fig. 7 The term hybrid tumor is used in some contexts for tumors that have mixed features of oncocytoma and chromophobe renal cell carcinoma. This tumor contained a mixture of morphology and exhibited multiple chromosomal gains using fluorescence in situ hybridization, making it somewhat atypical for either oncocytoma or chromophobe renal cell carcinoma.

syndrome (multiple oncocytic renal tumors). One context in which patients have multiple oncocytic renal tumors is Birt–Hogg–Dubé syndrome (germline mutation of the folliculin gene, *FLCN*), in which patients develop multiple skin tumors, fibrofolliculomas, as well as lung cysts. In this context, tumors have been reported as oncocytomas, chromophobe renal cell carcinomas, and hybrid tumors; however, it remains to be better understood whether these are the same entities as their sporadic counterparts or whether Birt–Hogg–Dubé-associated tumors are a distinct tumor type. In the absence of this syndrome, the constellation of multiple oncocytic renal tumors is otherwise known as renal oncocytosis, and again, it is debated whether these tumors are true oncocytomas or a distinct tumor type. For now, the WHO Classification has designated "hybrid" tumors as a subcategory of chromophobe renal cell carcinoma, principally due to a lack of consensus on this topic.

Clear Cell Papillary Renal Cell Carcinoma

Clear cell papillary renal cell carcinoma has only been recognized as a type of renal neoplasm since approximately 2006, first distinguished from conventional clear cell renal cell carcinoma in patients with end-stage renal disease. Since this recognition, however, it is now known that most cases occur in non-end-stage kidneys, and this tumor may make up as much as 4% of renal cell carcinoma, making it probably the fourth most common renal cell carcinoma subtype, close in incidence to that of chromophobe renal cell carcinoma. Due to close resemblance in many cases to clear cell renal cell carcinoma, it is not surprising that this entity eluded recognition for decades; however, several of its features are distinctive, including aligned nuclei in branched glandular structures (Fig. 8) and small papillae protruding into cysts. Grossly, these tumors are usually white or tan, and often not golden-yellow as seen in clear cell renal cell carcinoma. A cystic component is common. Microscopically, areas of solid growth are not unusual, and may be morphologically indistinguishable from clear cell renal cell carcinoma; however, histologic clues to this diagnosis include prominent alignment of the nuclei at the same height within the cytoplasm, a branched configuration of the glandular structures (rather than solid, round nests), and variable papillae protruding into cystic spaces (ranging from small stubby papillae to more complex branching).

Although this tumor is currently classified as a subtype of renal cell carcinoma, mostly based on the relatively recent discrimination from clear cell renal cell carcinoma and close resemblance, there is no convincing example with metastasis to date, suggesting that these may be reclassified as benign neoplasms or low malignant potential neoplasms in the future, as this entity accumulates more widespread recognition and longer-term follow-up. Interestingly, the morphology and immunohistochemistry of these tumors is quite similar to several other tumor entities, including clear cell papillary cystadenoma of the epididymis and broad ligament and serous cystadenoma of the pancreas, which curiously are both VHL disease-associated tumors, yet as discussed later, clear cell papillary renal cell carcinoma of the kidney is characteristically not *VHL*-mutant or associated with VHL disease, for unknown reasons.

Immunohistochemistry

One piece of the puzzle that has led to recognition of clear cell papillary renal cell carcinoma as a distinct entity is its overlapping but different immunohistochemical staining pattern. They share with clear cell renal cell carcinoma the finding of diffuse labeling for carbonic anhydrase IX; however, in contrast to the usual findings of clear cell renal cell carcinoma, these tumors are consistently diffusely positive for cytokeratin 7 and have only focal labeling for CD10, if any. In contrast to papillary renal cell

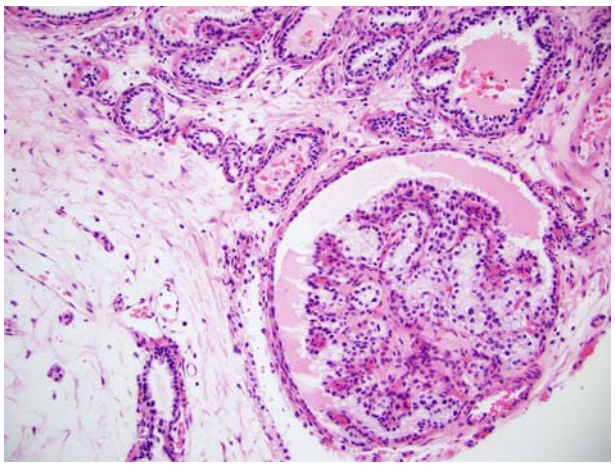

Fig. 8 Clear cell papillary renal cell carcinoma has been recently recognized to be the fourth most common renal cell carcinoma, often having substantial overlap in appearance with clear cell renal cell carcinoma. However, subtle diagnostic clues include alignment of nuclei at the same height within the cells and branched shape of the glandular structures.

carcinoma, these tumors have minimal to no labeling for AMACR. Recent studies have found these tumors to often label for GATA3 and high molecular weight cytokeratin, suggesting that the originate from, or recapitulate the phenotype of, the distal nephron.

Genetics

After the recognition of this entity based on unique morphology, studies using immunohistochemistry and genetic techniques have supported its consideration as distinct from clear cell and papillary renal cell carcinomas, the two most common entities and its two closest mimics. In general, almost all have been found to lack *VHL* mutations and chromosome 3p25 loss, in contrast to clear cell renal cell carcinoma, with perhaps rare exception. However, the VHL pathway likely appears to be upregulated nonetheless, as evidenced by their consistent labeling for markers such as hypoxia-inducible factor (HIF) and carbonic anhydrase IX. Although these tumors are typically not *VHL* mutant, some clear cell renal cell carcinomas in patients with VHL disease have been noted to have a morphology closely mimicking this entity, for unknown reasons. In this setting, however, the immunohistochemical phenotype generally does not match that of sporadic clear cell papillary renal cell carcinoma. In contrast to papillary renal cell carcinoma, the clear majority of clear cell papillary tumors have been lacking chromosome 7 or 17 gains, although this has been reported in a minority of cases and may not be entirely specific for papillary renal tumors. Otherwise, a distinctive genetic profile has yet to be recognized for this entity, largely with absence of recurrent copy number changes reported to date.

Translocation Renal Cell Carcinoma

Renal cell carcinomas with translocations involving the microphthalmia transcription factor (MITF) family of genes, including *TFE3* and *TFEB*, and recently perhaps *MITF* itself, are now well recognized as a distinct category of renal neoplasia. These tumors were first recognized in children and young adults, and if a renal cell carcinoma occurs in a child or young adult, the likelihood of translocation renal cell carcinoma is substantially increased. However, since renal cell carcinoma is rare in children, there is likely an overall greater *number* of translocation-associated renal cancer cases in older adults, and indeed these tumors can be found in patients of all ages. Interestingly, the gene fusion between *ASPSCR1* and *TFE3* involves the same two genes as in alveolar soft part sarcoma, a rare soft tissue tumor that shares epithelioid cytologic features with renal cell carcinoma but differs in several other ways.

Morphologically, translocation-associated renal cancers typically exhibit a mixture of morphologic features that may make them difficult to classify as a single specific subtype of renal cell carcinoma, such as mixed solid and papillary architecture, or mixed clear and eosinophilic cytologic features (Fig. 9). In general, these tumors often most closely resemble clear cell renal cell carcinoma, although the spectrum of morphology continues to widen with increasing awareness of these entities and more accessible molecular testing, and therefore tumors mimicking a wide spectrum of neoplasms have been reported. Specific morphologic associations are also known, such as more voluminous cytoplasm, papillary architecture, and psammomatous calcifications in tumors with *ASPSCR1-TFE3* fusion, compared to more compact architecture and less calcifications in tumors with *PRCC-TFE3* fusions. Tumors with *NONO-TFE3* fusion as well as those with *SFPQ* fusion may have a pattern of clear cell-like morphology with nuclear alignment, resembling that pattern of clear cell papillary renal cell carcinoma.

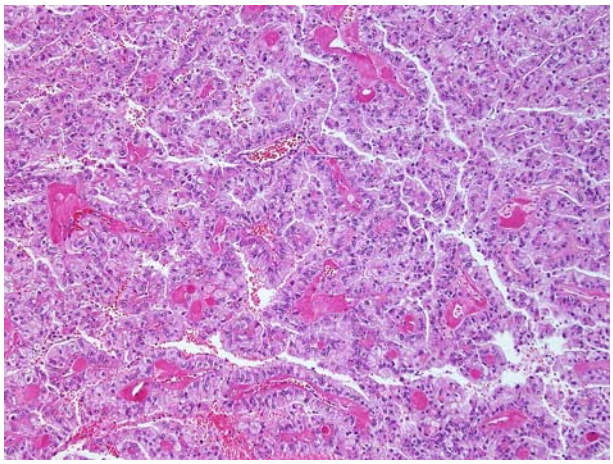

Fig. 9 Translocation-associated renal cell carcinomas often have a mixture of features of clear cell and papillary renal cell carcinoma and sometimes occur in younger patients. This tumor, occurring in a child, demonstrates papillary architecture with cells that have clear to eosinophilic cytoplasm.

Immunohistochemistry

Translocation-associated renal cell carcinomas have some unique immunohistochemical features compared to conventional renal cell carcinomas, including positivity for markers of melanocytic differentiation (HMB45, melan-A), and decreased labeling for epithelial markers (cytokeratins and epithelial membrane antigen). TFE3 and TFEB proteins can often be detected with immunohistochemical antibodies against them, and if there is strong positivity, this generally correlates well with gene fusion. However, molecular techniques are usually regarded as more sensitive and specific for confirmation of the gene fusions, particularly FISH, which is one of the most common diagnostic techniques in current practice. Cathepsin K, a papain-like cysteine protease, has also been recognized to have positive immunohistochemical labeling in many translocation-associated renal cell carcinoma (*TFE3* rearrangement tumors, depending on the fusion partner, and most *TFEB* rearrangement tumors).

Genetics

The *TFE3* gene is located at Xp11.2, and therefore translocation renal cell carcinomas are variably also referred to as Xp11 translocation-associated tumors; however, gene fusions involving the related family member *TFEB* (located at chromosome 6p21) also occur. The typical fusion partner for *TFEB* is *MALAT1* (also known as Alpha), resulting in a *MALAT1-TFEB* gene fusion, or t(6;11). In contrast to alveolar soft part sarcoma, multiple other fusion combinations in renal cell carcinoma have been found, including *PRCC-TFE3*, *SFPQ-TFE3*, *NONO-TFE3*, *CLTC-TFE3*, and unknown partners.

Very recently, an additional group of tumors with amplification of the *TFEB* and *VEGFA* gene region located at 6p21 has also been reported (usually without rearrangement). These tumors have some features overlapping with those of translocation carcinoma, including positivity for melanocytic markers (especially melan-A) and cathepsin K. Morphologically, these have been noted to have a tendency for an eosinophilic tubulopapillary appearance, although like translocation carcinomas, they have heterogeneous morphology with other areas resembling clear cell or chromophobe renal cell carcinoma and some showing collecting duct carcinoma-like features. Although data on these tumors so far are limited, they appear to have aggressive behavior.

Sarcomatoid Renal Cell Carcinoma

Sarcomatoid renal cell carcinoma is not per se a distinct entity, but a form of aggressive transformation or dedifferentiation that can occur in multiple renal cancer types. In most cases, conventional morphology of one of the other described renal cell carcinoma types can be appreciated in some areas, and therefore current recommendations are that these be diagnosed as sarcomatoid carcinomas arising from a specific renal cell carcinoma subtype, if possible. However, examples in which the sarcomatoid pattern is predominant and no definite low-grade component can be appreciated do occur, which may be diagnosed as pure sarcomatoid renal cell carcinomas or unclassified sarcomatoid renal cell carcinoma. The principle diagnostic feature for sarcomatoid dedifferentiation is that areas of the tumor lack apparent epithelial differentiation and could be mistaken for a mesenchymal neoplasm (sarcoma) if viewed in isolation (**Fig. 10**). However, occasional cases of clear cell renal cell carcinoma occur in which the cells take on a spindle-shaped appearance but are still largely recognizable as epithelial. In this context, it remains debatable whether this should be considered an early form of sarcomatoid change or a transitional pattern, not yet reaching sarcomatoid features. Rhabdoid cytologic features, formed by large cells with voluminous cytoplasm containing a globule of eosinophilic material, are so named for their resemblance to rhabdomyoblastic cells of rhabdomyosarcoma, yet without true muscle differentiation as

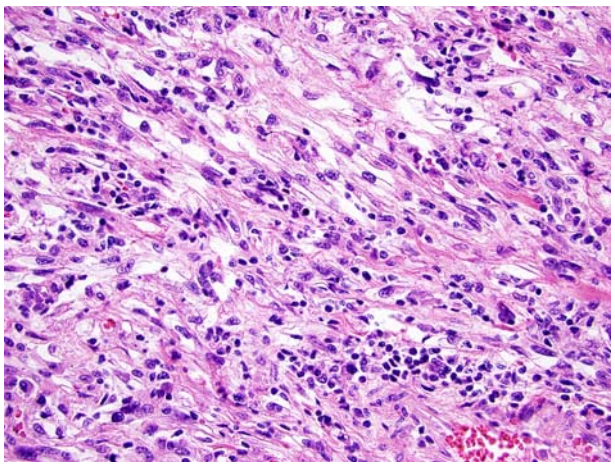

Fig. 10 Sarcomatoid renal cell carcinoma is defined as a transition of renal cell carcinoma to areas resembling sarcoma (cancer of mesenchymal or connective tissue). In this example, the tumor cells take on a spindle-shaped rather than rounded appearance.

detectable immunohistochemically. In current thinking, this is largely considered a pattern of sarcomatoid dedifferentiation, although there are conflicting data on whether this feature carries an equally adverse prognosis to that of sarcomatoid carcinoma formed by sarcoma-like morphology.

Immunohistochemistry

Sarcomatoid renal cancers may be difficult to recognize as of renal cell origin, if the sarcomatoid component is predominant. In general, these tumors have much more limited evidence of epithelial origin, and therefore immunohistochemistry for multiple markers of epithelial origin, such as cytokeratin, epithelial membrane antigen, and PAX8 (a transcription factor involved in renal organogenesis), may be used in combination to garner some support of epithelial origin, combined with careful search for areas with epithelial morphology. Carbonic anhydrase IX typically exhibits diffuse labeling in clear cell renal cell carcinoma; however, this may be decreased in sarcomatoid tumors.

Genetics

There is some evidence that sarcomatoid renal cell carcinomas share major molecular events with the conventional renal cancers from which they originated, such as *VHL* mutation and 3p25 deletion when originating from clear cell renal cell carcinoma. Alterations in other clear cell renal cell carcinoma-associated genes, such as the chromatin remodeling genes discussed previously, have also been documented, and there is also some evidence that additional distinct genetic alterations may be enriched in sarcomatoid renal cancers, including alterations of *NF2*, *TP53*, *CDKN2A*, among others. Other data suggest that some sarcomatoid renal cell carcinomas may progress to the sarcomatoid phenotype through different molecular pathways that may bypass some of the presumed early steps in carcinogenesis.

Other Renal Cell Carcinoma Subtypes

Collecting Duct Carcinoma

Renal collecting duct carcinoma is a rare, aggressive subtype of renal cancer that is more like a prototypical cancer, differing from the previously discussed, more common renal cell carcinoma types. In contrast to usual renal cell carcinoma, which usually forms a spherical, circumscribed mass, collecting duct carcinoma typically manifests as a diffusely infiltrative cancer with desmoplastic reaction, composed of tubular structures. This diagnosis is largely one of exclusion, requiring the pathologist to discern these tumors from urothelial carcinoma arising from the renal pelvis invading the kidney, renal medullary carcinoma (a largely similar histology that occurs characteristically in patients with sickle cell trait), and metastatic cancer from another origin. A medullary location is also considered one of the diagnostic features for this entity. In general, this diagnosis can be supported by immunohistochemical labeling for renal-specific markers such as PAX8 and usually negative labeling for markers of urothelial carcinoma, such as p63 and GATA3 (although these patterns may overlap in some cases, likely due to overlapping phenotypes of distal renal tubules and urothelium), and negative markers of other cancer origins. Genetically, recent studies have found alterations of *NF2*, *SETD2*, *SMARCB1*, and *CDKN2A* in collecting duct carcinomas, although a defining set of molecular alterations is not currently recognized. The *SMARCB1* gene (also known as INI1) has gained recent recognition as a key molecular alteration in renal medullary carcinoma, a tumor almost by definition occurring in patients with sickle cell trait. A novel terminology of "renal cell carcinoma unclassified with medullary phenotype" has been proposed for tumors with this morphology, combined with loss of INI1 protein labeling by immunohistochemistry or *SMARCB1* alteration, yet with absence of known sickle cell trait. Therefore, the cases of collecting duct carcinoma with *SMARCB1* alteration may now fall into this category, which has only recently been proposed.

Renal Medullary Carcinoma

As discussed in relation to collecting duct carcinoma, renal medullary carcinoma has substantial overlap in morphologic features with collecting duct carcinoma, requiring evaluation of a similar differential diagnosis; however, unique features of this diagnostic entity include occurrence at a relatively young age (often 2nd or 3rd decade of life) in patients with sickle cell trait and related hemoglobinopathies. As noted previously, loss of *SMARCB1* via translocation and deletion appears to be a key molecular feature of all or almost all tumors, which can be detected with abnormal absence of immunohistochemical staining (for INI1/SMARCB1 protein). Behavior of these tumors is highly aggressive, with survival in months rather than years.

Mucinous Tubular and Spindle Cell Carcinoma

Mucinous tubular and spindle cell carcinoma is considered a separate entity in the WHO Classification of renal tumors; however, it has debatably several features that overlap with papillary renal cell carcinoma, leading some authors to speculate whether it might be a morphologic variant of papillary renal cell carcinoma. These tumors characteristically contain tubular elements, like those of type I papillary renal cell carcinoma, with similar immunohistochemical staining characteristics, including labeling for AMACR and cytokeratin 7. However, these elements are admixed with other areas of spindle-shaped cell morphology and areas of stromal mucin

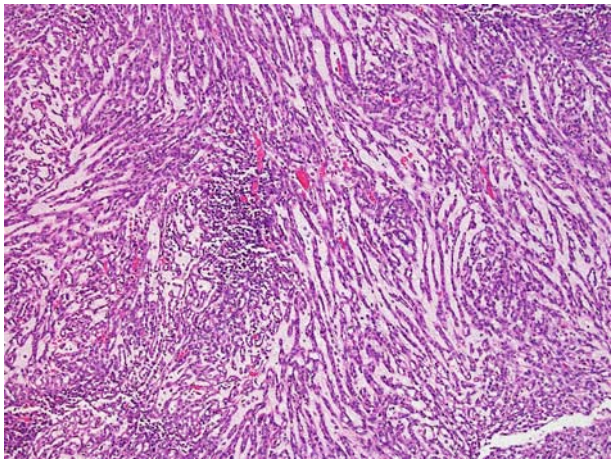

Fig. 11 Mucinous tubular and spindle cell carcinoma is a rare variant of renal cell carcinoma that shares some overlapping features with papillary renal cell carcinoma, but exhibits compressed, elongated tubular structures with a spindle-shaped appearance and intercellular mucinous material.

deposition, together making up the three components of the tumor's name (Fig. 11). Although the spindle-shaped cell component may be mistaken for sarcomatoid dedifferentiation, this is typically hypothesized to represent compressed, elongated tubular structures with epithelial differentiation, rather than a true mesenchymal component. The mucinous component can be highlighted with histochemical stains for mucin. Despite the resemblance of the tubular component to that of type I papillary renal cell carcinoma, studies have found multiple chromosomal losses involving chromosomes 1, 4, 6, 8, 9, 13, 14, 15, and 22, in contrast to the trisomy (or gain) of chromosomes 7 or 17 that is characteristic of papillary renal cell carcinoma. However, some tumors with overlapping morphologic features of papillary renal cell carcinoma have been reported to show a combination of chromosomal alterations of both entities. Behavior of mucinous tubular and spindle cell carcinoma is largely nonaggressive; however, metastases have been described.

Tubulocystic Renal Cell Carcinoma

Tubulocystic renal cell carcinoma is another subtype of renal cancer that is considered a distinct tumor entity, although again it may have some overlapping characteristics with papillary renal cell carcinoma, and there may be some cases of papillary renal cell carcinoma that overlap in morphology and mimic this entity. This tumor type was initially reported as low-grade collecting duct carcinoma, based on the presence of some areas of similar morphology in collecting duct carcinoma; however, current thinking is that there is no true relationship between these entities. The characteristic gross appearance of this tumor has been described as resembling bubble wrap, in that the cut surface contains multiple cystic spaces. Histologically, tumors are composed of tubular and cystic structures, both of which are lined with eosinophilic cells with prominent nucleoli (Fig. 12). Immunohistochemically, staining is characteristically positive for AMACR, like papillary renal cell carcinoma. Cytokeratin 7 staining may be limited, although as noted previously, this is not necessarily a discriminator from papillary renal cell carcinoma, as those with eosinophilic cells are

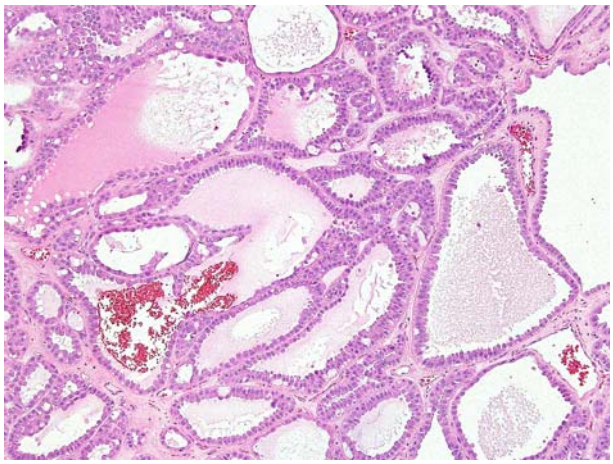

Fig. 12 Tubulocystic renal cell carcinoma is composed of cystic and tubular structures lined by uniform cells with eosinophilic cytoplasm and prominent nucleoli.

characteristically less positive for cytokeratin 7 than type I tumors. Like mucinous tubular and spindle cell carcinoma, there is some debate as to whether this entity is a variant of papillary renal cell carcinoma. Tumors with pure morphology (entirely tubular and cystic) have been found to lack trisomy 7 and 17; however, tumors with mixed papillary and tubulocystic morphology have been found to have gains of chromosomes 7 and 17.

Acquired Cystic Disease Associated Renal Cell Carcinoma

Acquired cystic kidney disease-associated renal cell carcinoma is another tumor subtype that is now acknowledged as a distinct entity in the current 2016 WHO Classification of Tumours; however, like mucinous tubular and spindle cell carcinoma and tubulocystic renal cell carcinoma, it again has overlapping features with papillary renal cell carcinoma and raises debate as to whether it represents a morphologic variant. In contrast to other subtypes of renal cell carcinoma, this histology is thus far considered to be restricted to the setting of end-stage kidney disease with acquired cystic disease (cystic disease usually occurring after long-term dialysis), unless papillary renal cell carcinoma with eosinophilic cells or tubulocystic renal cell carcinoma is considered the non-end-stage renal disease counterpart. The unique morphologic findings that define this entity include a cribriform architecture (tubular growth with multiple secondary glandular lumens) and calcium oxalate crystals within the tumor (Fig. 13). Like tubulocystic renal cell carcinoma, these tumors characteristically stain immunohistochemically for AMACR but may be variable or negative for cytokeratin 7, again not necessarily representing a robust distinction from papillary renal cell carcinoma due to the tendency for papillary tumors with eosinophilic cells to have less labeling for this marker. Behavior of this entity is usually nonaggressive, but metastases have been reported. Like tubulocystic renal cell carcinoma there may be overlapping chromosomal alterations with those of papillary renal cell carcinoma in some cases; however, other chromosomal changes that have been reported include chromosomes 1, 2, 3, 6, 7, 10, and 16, particularly gains and losses of 3 and 16.

Renal Cell Carcinoma, Unclassified

Renal cell carcinoma, unclassified, is not a specific diagnostic entity but rather represents a category for tumors that are interpreted as of renal cell origin but cannot be confidently given a definitive diagnosis. This is often due to either mixed patterns of multiple entities (Fig. 14), unusual morphology or immunohistochemical staining characteristics that do not correspond well to a single entity, or high-grade or sarcomatoid features that obscure any diagnostic low-grade histology. As such, this is a heterogeneous category that cannot be necessarily assigned a specific prognosis or treatment. Some studies have reported that prognosis for these tumors is worse than for conventional renal cell carcinoma types, although this may be influenced by thresholds at a given institution for assigning a diagnosis of renal cell carcinoma unclassified vs categorization with a defined entity. However, as classification of renal cancer is continually being refined, it is likely that more and more tumors from this group will be interpreted as novel diagnostic entities.

Hereditary Renal Cancer Syndromes

Several hereditary renal cancer syndromes are now recognized, and it may be relevant to consider the possibility of a syndrome when encountering a renal tumor in patients of a young age (under age 46 has been proposed as one cutoff) or with multiple tumors. VHL disease, hereditary papillary renal cell carcinoma, and Birt–Hogg–Dubé syndromes were discussed previously with clear cell, papillary, and chromophobe renal cell carcinomas, respectively.

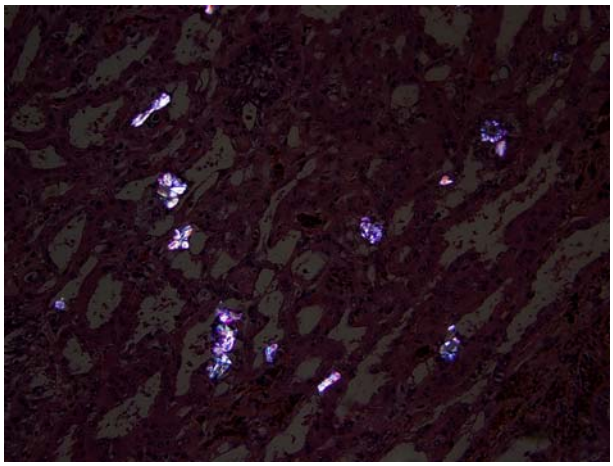

Fig. 13 The prototypical feature of acquired cystic kidney disease-associated renal cell carcinoma is the presence of calcium oxalate within the tumor, highlighted here by polarization microscopy.

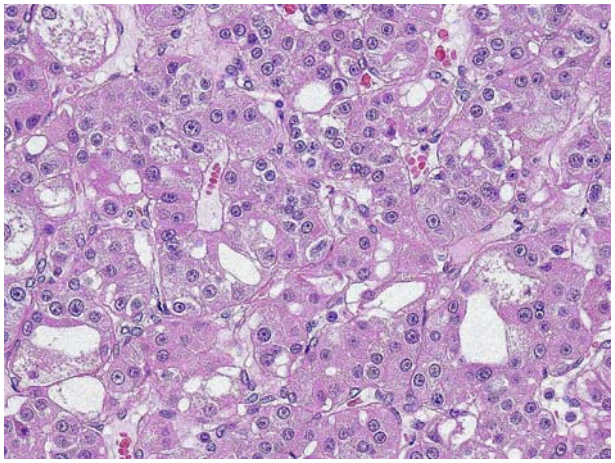

Fig. 14 Unclassified renal cell carcinoma is a category for tumors regarded to be of renal cell origin that do not fit cleanly into a specific category. This example contains cells with eosinophilic cytoplasm and prominent nucleoli.

Although the HLRCC syndrome (germline mutation of *FH*) has been previously considered to be associated with type II papillary renal cell carcinoma, it is now considered that the renal cancers in these patients are a distinct tumor type, that may exhibit not only papillary, but also tubular, solid, cystic, and diffusely infiltrative morphology. The characteristic morphologic feature that may draw attention to this diagnostic possibility is the presence of very large, prominent nucleoli with perinucleolar clearing, although this may be focal. Until recently, diagnostic tumor markers for this syndrome were not specific, other than undertaking germline testing; however, two recent immunohistochemical markers that have been reported as helpful for recognizing tumors associated with this syndrome include abnormal negative staining for fumarate hydratase (FH) protein, corresponding to the same gene that harbors germline mutation in these patients, and increased staining for 2-succinocysteine (2SC), a succinated protein that accumulates in the setting of increased fumarate. The behavior of these tumors is highly aggressive, and in contrast to VHL disease, in which tumors are typically conservatively managed and enucleated (surgically removed with almost no rim of normal tissue) only upon reaching a certain size, current recommendations are to manage HLRCC tumors aggressively, often with radical nephrectomy even for relatively small and clinically localized-appearing tumors.

Another hereditary renal cancer syndrome that is now increasingly recognized is associated with germline mutations of the succinate dehydrogenase (SDH) subunit genes. The syndromes resulting from these mutations are usually referred to as the hereditary pheochromocytoma–paraganglioma syndromes, although patients with these mutations also develop gastrointestinal stromal tumors (GIST) with deficiencies of SDH subunits and renal cancers, usually with deficiency of SDH subunit B (SDHB). Only recently it has been recognized that SDHB-deficient renal cancers have distinctive morphology, typically characterized by solid growth of monomorphic eosinophilic cells, often without much glandular configuration (**Fig. 15**). The tumor cells contain cytoplasmic vacuoles or inclusions that likely correspond to enlarged abnormal mitochondria. Immunohistochemical features of these tumors often

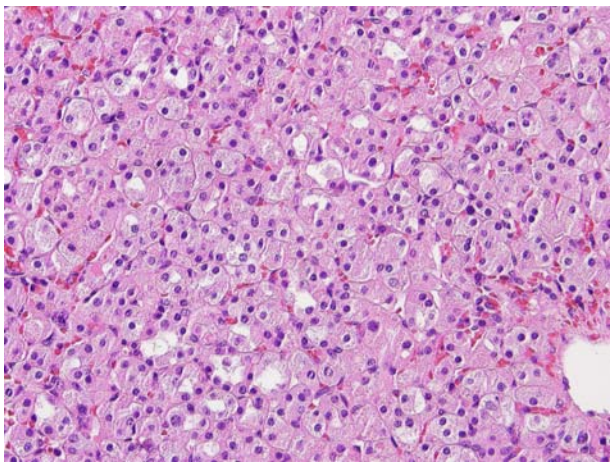

Fig. 15 Succinate dehydrogenase-deficient renal cell carcinoma has been recently recognized to often have distinctive morphology, including uniform, often bland, eosinophilic cells, with cytoplasmic vacuoles or inclusions that may represent defective mitochondria. Recognition of these tumors is important, as most are thought to be associated with inherited mutations in the succinate dehydrogenase subunit genes.

include negative staining for KIT (contrasting to oncocytoma and chromophobe renal cell carcinoma) and cytokeratin 7, and positive staining for kidney-specific cadherin and PAX8. Other epithelial markers may be underwhelming, such as general cytokeratin or epithelial membrane antigen; however, the abnormal absence of SDH protein can be detected by negative staining for SDHB (usually even if the mutation involves another subunit, especially SDHA).

Renal Cancer Grading

For the last few decades, the Fuhrman grading system has been used for renal cell carcinoma, based on several different parameters of the tumor cell nuclei; however, recent refinements by the International Society of Urological Pathology (ISUP) have emphasized the tumor cell nucleoli as the most important parameter, leading to a recent revised ISUP grading system based on this finding. In this system, the presence of nucleoli visible at 100× magnification (10× microscope objective combined with 10× ocular magnification) justifies a modified ISUP grade 3. Nucleoli that are visible only at higher magnification would then correspond to grade 2, and nuclei that are small and condensed without nucleoli at higher magnification correspond to grade 1. Grade 4 conversely represents markedly atypical nuclei that are irregular or multilobated. Rhabdoid cells, as discussed with sarcomatoid features, are also currently classified as grade 4. Other grading systems that incorporate tumor necrosis into a multi-tier system have also been proposed. The ISUP modified grading system appears to work well for clear cell renal cell carcinoma and perhaps papillary renal cell carcinoma. However, there is currently no compelling evidence that it is helpful for chromophobe renal cell carcinoma, since these tumors by their characteristic cytology would often by grade 3 or higher, yet their behavior, as discussed previously, tends to be nonaggressive. Therefore, current recommendations are against grading of chromophobe carcinoma (grading not applicable). Alternative grading schemes for chromophobe tumors have been proposed, based on features such as nuclear crowding and overlap; however, this is not universally agreed upon at present and therefore not widely used. Given the more recent increased utilization of renal mass biopsy, algorithms incorporating tumor histology and grade on tumor biopsy into patient management are now being used in some centers.

Renal Cancer Pathologic Staging

As noted previously, pathologic stage criteria for renal cancer may be subtler than other cancers, as renal tumors typically begin as round or spherical masses. With increasing size, these masses can bulge well beyond the shape of the normal kidney, which does not necessarily indicate invasion alone; however, as size increases, the likelihood of invasion of various structures dramatically increases, especially for clear cell renal cell carcinoma. The pathologic pT stage category (in the TNM system of staging) depends first on tumor size, with pT1a including tumors of 4.0 cm or less, pT1b including tumors >4.0–7.0 cm, pT2a including >7.0–10.0 cm, and pT2b including tumors >10.0 cm without invasion of structures. However, for clear cell renal cell carcinoma, recent data indicate that tumors larger than 7.0 cm without any such invasion are very rare. The pT3a stage category is used when tumors invade either (1) the main renal vein or its branches, (2) the renal sinus (the loose tissue and adipose tissue located in the hilum of the kidney), or (3) the perinephric fat. Interestingly, renal cell carcinoma can extend with elongated, tongue-like extensions (like a finger in a glove) into large blood vessels (Fig. 16), occasionally extending in this manner from the renal vein to the inferior vena, rarely even to the level of heart. Despite this, surgery can sometimes still be attempted by pulling back the finger-like thrombus to a level at which it can be removed. Higher pT stage categories are based on these levels of extension, including pT3b (extending to

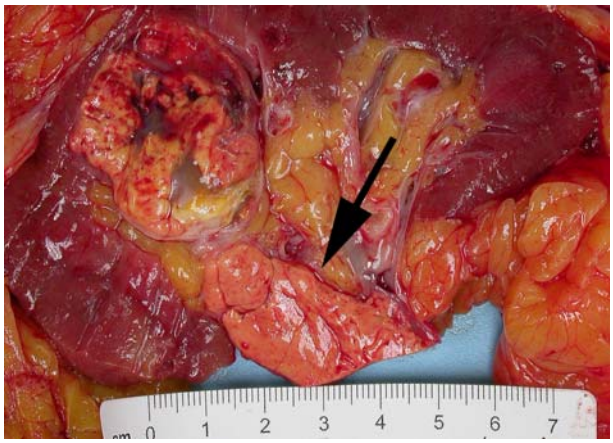

Fig. 16 Higher stage in renal cell carcinoma is somewhat different from other cancers, in that the primary tumor is often spherical or round (upper left), but with invasion manifesting as tongue-like extension into veins (*arrow*) or soft tissue.

vena cava below the diaphragm) and pT3c (extending to vena cava above the diaphragm or invading the wall of the vena cava). The pT4 category includes extension beyond the Gerota fascia or invasion of the adrenal gland (directly). The American Joint Commission on Cancer (AJCC) staging system, which has been revised for implementation in 2018, has made a few refinements to these categories, most critical of which include removing the requirement that vein branches contain muscle to be considered pT3a and that this invasion be identified grossly. Therefore, any vein branch invasion, even if identified microscopically and without a muscular wall, can be regarded as evidence of pT3a pathologic stage category.

Prospective Vision

The future of pathology and genetics of renal cell carcinoma likely lies in continued integration of tumor histology with immunohistochemistry and genetics, to define tumor entities with distinct treatment options and behavior. Although knowledge of tumor genetics has facilitated recognition of multiple new tumor types in the latest classification schemes, it is in most cases possible to extrapolate this knowledge of genetics and recognize these tumor types based on subtle distinctions in their histology. Some have substantial importance for prognosis, and others have implications for inherited tumor predisposition syndromes.

See also: Kidney Cancer: Diagnosis and Treatment.

Further Reading

Brugarolas, J., 2014. Molecular genetics of clear-cell renal cell carcinoma. Journal of Clinical Oncology 32, 1968–1976.

Cancer Genome Atlas Research Network, 2013. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 499, 43–49.

Davis, C.F., Ricketts, C.J., Wang, M., Yang, L., Cherniack, A.D., Shen, H., et al., 2014. The somatic genomic landscape of chromophobe renal cell carcinoma. Cancer Cell 26, 319–330.

Moch, H., Amin, M., Argani, P., Cheville, J., Delahunt, B., Martignoni, G., et al., 2016. Renal cell tumors. In: Moch, H., Humphrey, P., Ulbright, T., Reuter, V. (Eds.), WHO classification of tumours of the urinary system and male genital organs. International Agency for Research on Cancer, Lyon, pp. 14–17.

Srigley, J.R., Delahunt, B., Eble, J.N., Egevad, L., Epstein, J.I., Grignon, D., et al., 2013. The International Society of Urological Pathology (ISUP) Vancouver classification of renal neoplasia. American Journal of Surgical Pathology 37, 1469–1489.

The Cancer Genome Atlas Research Network, 2016. Comprehensive molecular characterization of papillary renal-cell carcinoma. New England Journal of Medicine 374, 135–145.

Role of DNA Repair in Carcinogenesis and Cancer Therapeutics

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Introduction and Scope

Every day, each cell in the human body is subjected to tens of thousands of DNA damage lesions, either through environmental exposure or through internal chemical reactions. Unrepaired, these lesions can impair transcription, stall replication, and induce senescence, a permanent state of cell cycle arrest, or apoptosis, a process that results in cell death. If persistent, DNA damage can introduce mutations or genomic instability that can trigger cancer development. This article provides an overview of the major DNA repair pathways that identify and remove substrate lesions, including their associated cancer syndromes and disease predispositions, and summarizes the DNA repair targets that are currently under clinical therapeutic evaluation in the treatment of malignancies.

Sources of DNA Damage

The majority of DNA damage acquired by a cell (see overview in **Fig. 1**) is the result of normal physiological processes. Spontaneous hydrolysis of chemical bonds within DNA produces ~10,000 depurinated (abasic) sites per cell per day, while spontaneous deamination of cytosine nucleotides is an important source of inappropriate uracil or thymine base-pairing (Lindahl, 1993). Endogenously produced reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals, can result in the modification of all cellular macromolecules, including the formation of various oxidized DNA base lesions, abasic sites, and strand breaks (Cadet and Wagner, 2013). The major source of cellular ROS is mitochondria, generated during oxidative phosphorylation, with minor mechanisms of ROS formation including endoplasmic reticulum stress and peroxisome metabolism (Murphy, 2009; Turrens and Boveris, 1980). Other intracellular metabolites capable of DNA modification include *N*-nitroso compounds (which also arise from exogenous sources such as cigarette smoke) (Murata and Kawanishi, 2011), or methyl groups generated by the activity of the methyl group donor *S*-adenosylmethionine (Rydberg and Lindahl, 1982), both of which induce a range of alkylation adducts. Failure of enzymatic activity that specifically targets DNA, such as during repair or replication (discussed more later), is also a common source of endogenous genomic damage. For example, single base mismatches may be introduced by DNA polymerases during replication or repair, or larger insertions or deletions may result from polymerase slippage and subsequent realignment of the duplex (Lange et al., 2011). Resolution of damage lesions by processes such as base excision (BER) or nucleotide excision repair (NER) features obligate strand breaks that may persist if repair is interrupted, or be converted to DNA double-strand breaks (DSBs) if encountered by a replication fork (Edenberg et al., 2014). Likewise, reversal of the transient strand nicking activity of topoisomerase 1 (TOP1), which relaxes supercoiled DNA ahead of transcription or replication, can fail if the TOP1-DNA cleavage complex encounters a replication fork or DNA damage lesion, resulting in the formation of a complex strand break intermediate (Pommier et al., 2003).

Besides the many natural mechanisms that can generate DNA modifications, there are numerous environmental physical and chemical agents that have the potential to induce DNA damage (**Fig. 1**). A major external source of DNA damage is radiation. Ultraviolet (UV) radiation commonly induces intrastrand crosslinks, such as cyclobutane pyrimidine dimers and 6,4-photoproducts, while ionizing radiation can introduce a wide range of lesions, including clustered base damage and strand breaks (Cadet and Wagner, 2013). Many ingested or inhaled substances can also have effects on DNA. For example, tobacco smoke contains ~60 recognized carcinogens, including polycyclic aromatic hydrocarbons (PAHs), such as benzo[α]pyrene, which intercalates into DNA to induce bulky base lesions, and a variety of alkylating chemicals that cause a range of base adducts (Phillips and Venitt, 2012). Heterocyclic amines, produced by over-cooking meat, generate bulky adducts that are several-fold more mutagenic than tobacco PAHs (Murata and Kawanishi, 2011). Infectious agents also cause DNA damage; for example, the aflatoxin mold induces formation of ring-opened formamidopyrimidine or depurinated base lesions (Bedard and Massey, 2006). In addition to the mostly inadvertent exposure to genotoxins, targeted use of DNA-damaging agents is employed therapeutically, such as via radiotherapy, radioisotopes, and chemotherapy in cancer treatment (see later).

Consequences of Unrepaired DNA Damage

The presence of a DNA lesion triggers a complex DNA damage response (DDR; **Fig. 1**) that is characterized by damage recognition, activation of downstream signaling pathways, and coordination of cellular responses that may include activation of cell cycle checkpoints, induction of DNA repair pathways, persistent cessation of replication (e.g., in senescence), or triggering of programmed mechanisms of cell death, such as apoptosis, autophagy, or necroptosis (Jackson and Bartek, 2009; Sirbu and Cortez, 2013). Alternatively, unrepaired DNA damage may be "mishandled" during replication, leading to mutations and subsequent malignant

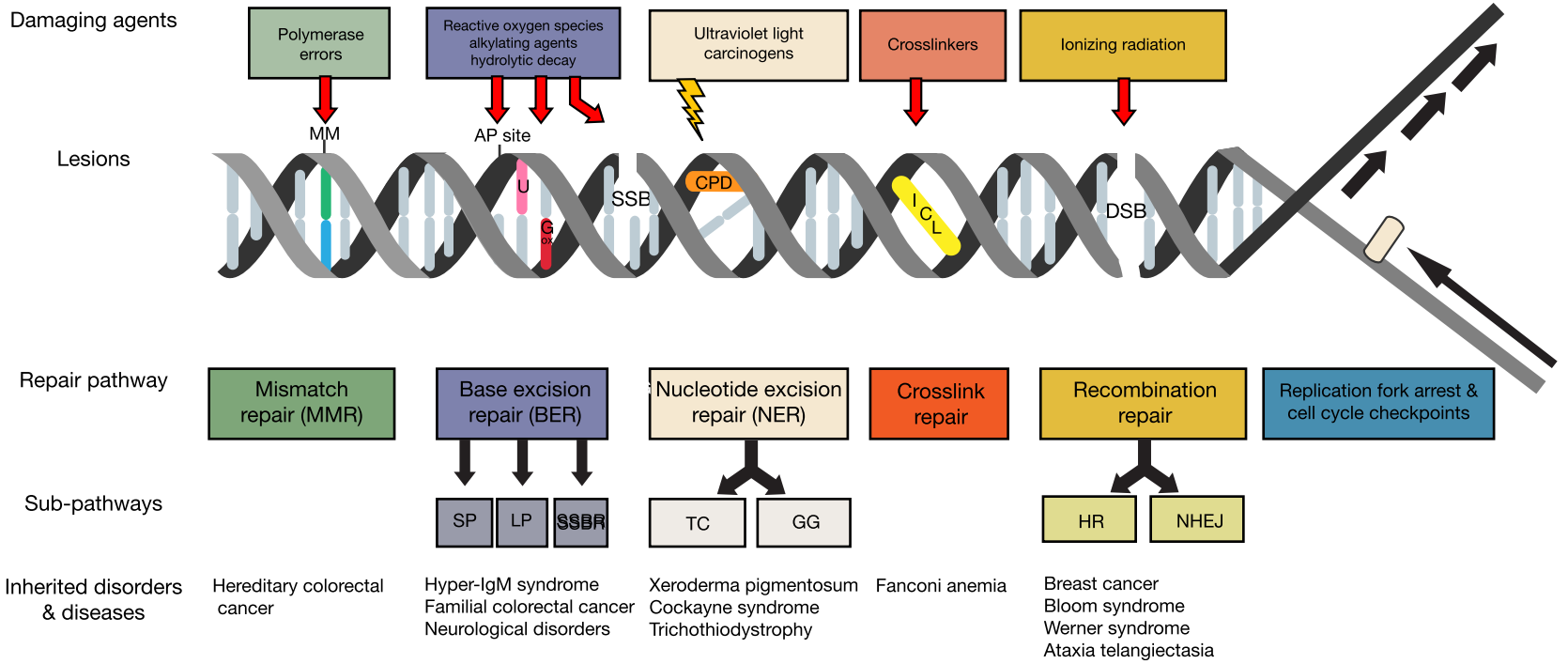

Fig. 1 Overview of DNA damage and DNA repair responses. DNA damage can be introduced by a wide range of internal and external damaging agents. Generally speaking, each type of DNA lesion is recognized and processed by a specific DNA repair pathway, or one of its associated subpathways, in some instances in connection with transcription, replication, or cell cycle checkpoint machinery. Due to the harmful consequences of unresolved DNA damage (see text), mutations in DNA repair genes, within each of the major DNA repair pathways, have been linked to a spectrum of inherited disorders or diseases (listed), which typically involve neurological, immunological, malignant and/or premature aging phenotypes. *MM*, mismatch; *AP*, apurinic/apyrimidinic; *U*, uracil; *Gox*, 8-oxoguanine; *SSB*, single-strand break; *CPD*, cyclobutane pyrimidine dimer; *ICL*, interstrand crosslink; *DSB*, double strand break; *SP*, short patch; *LP*, long patch; *SSBR*, SSB repair; *TC*, transcription-coupled; *GG*, global genome; *HR*, homologous recombination; *NHEJ*, nonhomologous end joining. *Note*: direct reversal is not shown.

transformation. Small, nonhelix-distorting lesions, such as uracil or 8-oxoguanine (8-oxoG), are particularly susceptible to this process. Deamination converts cytosine to a uracil or thymine, ultimately leading to C → T transitions following chromosome duplication, a signature mutation recorded during the sequencing of many cancer genomes (Olinski et al., 2010). Similar mispairings can occur opposite oxidized bases, such as thymine glycol or 8-oxoG, giving rise to T → C or G → T mutations, respectively. These single base mispairings often occur within noncoding regions and are well tolerated; however, in coding regions they can cause a variety of deleterious effects including missense, nonsense, or truncating mutations. Larger DNA base adducts can also be highly mutagenic. For example, benzo[*a*]pyrene, a carcinogen in cigarette smoke, induces G → T transversions that may specifically target genomic regions, such as p53, resulting in inactivation of its tumor suppressor function (Yoon et al., 2001).

Noncoding abasic sites, which primarily arise from purine base hydrolysis, are typically potent blocks to replicative polymerases, but can be bypassed efficiently by one of several error-prone translesion polymerases. In the former situation, the replication fork collapses resulting in a complex DNA intermediate that can promote cell death (Edenberg et al., 2014), whereas in the latter scenario, the polymerase inserts adenine (typically) opposite the abasic site, allowing replication to continue at the expense of potential mutagenesis. Error-prone DNA polymerases are in fact often employed during DNA damage tolerance, which directs bypass of a damage lesion by the replication fork, permitting cell survival at the cost of genomic stability. Polymerases may also suffer slippage during replication or repair synthesis, particularly in highly repetitive DNA or if in close proximity to a damage lesion or stalled fork, which can lead to insertion/deletion events (Lange et al., 2011).

Besides base substitutions and small genomic changes, which often require nucleotide sequencing to identify, larger chromosomal aberrations can also arise from persistent DNA damage. For example, strand breaks, both those induced by exogenous sources such as ionizing radiation and those that are generated as DNA processing intermediates, can promote chromosomal fusions, rearrangements, and large-scale deletions, which are typically visible by simple Giemsa staining of metaphase spreads (Fenech, 2002; Forment et al., 2012). Certain regions of the genome are particularly prone to carcinogenic rearrangements, such as the fusion of immunoglobulin heavy chain genes to the proto-oncogene *c-Myc* during B cell class switch recombination (see later) (Haberl et al., 2016).

Recent studies by Tomasetti et al. have challenged the existing paradigm that the mutations driving carcinogenesis arise from two primary sources, inherited and acquired errors, where the latter are attributed mainly to environmental factors. Instead, the authors describe a model that integrates more robustly unavoidable mutations induced by internal processes (namely, base mispairing, polymerase slippage or errors, base deamination, and endogenous ROS lesions), arguing that mechanisms associated mainly with copying mistakes are responsible for up to two-thirds of carcinogenic driver mutations (Tomasetti et al., 2017). Ultimately, of course, the carcinogenic potential of a mutation is dependent on where in the genome it occurs. One of the major theories behind carcinogenesis is the "mutator phenotype," wherein a cell acquires mutations in genes that are involved in the DDR, thereby driving DNA damage accumulation, genome-wide mutagenesis, and ultimately the activation of oncogenes or inactivation of tumor suppressor genes, accelerating the process of malignant transformation (Loeb, 2016). This phenomenon seemingly explains the susceptibility observed in homozygous cancer syndromes associated with mutations in DNA repair genes (discussed later). It also forms the basis of the "second hits" of the Knudson hypothesis, wherein mutations, loss of heterozygosity (LOH), or promoter inactivation at a second (initially wild type/normal) allele can trigger carcinogenesis in cells with a heterozygous germline or somatic mutation at a DNA repair gene locus (Knudson, 1971).

DNA Repair Mechanisms

Collectively, the many forms of exogenous and endogenous DNA damage pose a tremendous threat to the functional stability of the cell and the entire organism. DNA damage-induced mutations arising in key regulatory genes, for example, can initiate a multistep cascade that ultimately ends in a cancer phenotype. Thus, to maintain genomic stability and cellular integrity in the face of DNA damage, a number of DNA repair mechanisms have evolved (see overview in **Fig. 1**).

DNA Damage Response

The protein kinases ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) are central to DDR signaling, being recruited and activated by DSBs and single-stranded DNA (ssDNA), respectively (Awasthi et al., 2015). These proteins activate the serine/threonine-specific protein checkpoint kinases Chk1 (by ATR) and Chk2 (by ATM), which phosphorylate and inactivate the Cdc phosphotyrosine phosphatases (Cdc25A, -B, and -C), thereby preventing downstream signaling to activate the cyclin-dependent kinases that promote cell cycle transition (Jackson and Bartek, 2009). Functionally, this phenomenon arrests the cell cycle and allows DNA repair to operate prior to the potential deleterious effects associated with unchecked cell cycle progression, such as strand break formation associated with stalling and collapse of replication forks on encountering damage lesions. Three main checkpoints exist: G1/S, intra-S phase, and G2/M. The G1/S checkpoint is activated by damage that prevents initiation of replication, via the ATM-Chk2-Cdc25A or ATR-Chk1-Cdc25A cascades, and is maintained by Chk1 or Chk2-mediated phosphorylation of p53, which leads to p21-mediated inactivation of Cdk2 (Bartek and Lukas, 2001). The intra-S phase checkpoint is activated by replication fork stalling, and is probably initiated both by the specialized checkpoint sensors (via inactivation of Cdk2) and by various repair proteins such as the MRE11/Rad50/NBN (MRN) complex (see section "**Homologous Recombination**") and BRCA1 (Iyer and Rhind, 2017). Activation by the latter group of factors is thought to activate a second pathway via phosphorylation

of SMC1 and SMC3, which promotes recombination repair (see later) to recover stalled or collapsed replication forks. The G2/M checkpoint, which prevents initiation of mitosis in the presence of DNA damage, operates via ATM and ATR activation to regulate G2/M transition via inactivation of Cdc25C. Maintenance of the G2/M arrest requires transcriptional repression of Cdc25 expression, mediated via activation of p53 and p21 (Lobrich and Jeggo, 2007).

Ataxia telangiectasia (AT) is a rare autosomal recessive disorder caused by biallelic mutation of the *ATM* gene (Nissenkorn and Ben-Zeev, 2015). Over 300 causative *ATM* mutations have been recognized, with the majority (>80%) resulting in truncation mutants with little to no residual protein expression, although missense mutants with variable expression and kinase activity have been described. Consequently, AT presents with a phenotypically heterogenous group of symptoms dependent on the degree of residual ATM kinase activity (Teive et al., 2015). The most common and most severe form, resulting from biallelic ATM-null or kinase inactivating mutations, typically presents with early onset, progressive ataxia (~2 months to 2 years of age), associated with dysarthria, abnormal ocular movements, and oculocutaneous telangiectasia. Other features include lymphopenia and agammaglobulinemia (particularly IgA and IgG2), and respiratory dysfunction secondary to neuromuscular impairment and immunodeficiency. Malignancies are the main cause of mortality, specifically lymphoid and hematological in children, and solid tumors (including breast, hepatocellular, endocrine, and brain) in adults (Hecht and Hecht, 1990).

The importance of ATM in the maintenance of genomic stability and suppression of carcinogenesis, mediated through its role in DDR and checkpoint signaling, is illustrated by the increased frequency of chromosomal translocations observed in AT patient cells. ATM plays a particularly important role during class switch recombination, the process in which activated B cell immunoglobulin production is modified to alter function without affecting antigenic specificity. During this recombination event, DSB formation upstream of genes encoding the immunoglobulin constant region leads to the removal of unwanted heavy chain exons, with the free ends subsequently rejoined via nonhomologous end joining (NHEJ, see later) (Hecht and Hecht, 1987). In keeping with the important role that ATM plays in initiating the response to DSBs, mutation is associated with a high rate of chromosomal rearrangements around the Ig heavy chain loci, particularly oncogenic *IgH* to *c-Myc* translocations commonly found in AT-associated B cell lymphomas (Hathcock et al., 2015). Indeed, even a heterozygous *ATM* mutation is sufficient to induce tumorigenic genomic instability, as observed by the increased breast cancer susceptibility of the female relatives of AT patients (van Os et al., 2016).

Compared to ATM, which is activated by the comparatively rare event of DSB formation, a wide range of substrates can activate ATR, including not only damage lesions (such as ssDNA associated with stalled replication forks or resected DSBs) but also replication origins arising during S phase in a mechanism that prevents premature onset of mitosis. Given that ATR performs a similar role to ATM in DDR signaling, it also represents a plausible cancer susceptibility gene. This concept is supported by evidence in cell and mouse models that ATR heterozygosity increases genomic instability and tumor incidence. Sequencing of sporadic endometrial, colon, and stomach tumors has demonstrated the acquisition of somatic mutations or LOH in *ATR* exon 10, a region thought to be required for ATR activation of p53 (Lewis et al., 2005). These mutations were observed on a background of microsatellite instability (MSI), suggesting they arose as a consequence of mismatch repair (MMR) deficiency (see later)—a phenotype that has been replicated in double knockout MMR/ATR mouse models (Fang et al., 2004). To date, however, germline *ATR* mutations have not been convincingly shown to increase cancer risk. Specifically, human mutation analysis studies in a variety of familial cancers, including breast and ovarian cancers, have identified a small number of novel sequence variants in ATR, but established no definitive segregation with cancer risk (Heikkinen et al., 2005). The rarity of *ATR* mutation is likely a consequence of the critical importance of protein function, as observed by the early embryonic mortality of *ATR*-null mouse models (Brown and Baltimore, 2000).

Significantly, biallelic *ATR* mutations have been described in a small number of families with the recessively inherited Seckel syndrome, which presents with dwarfism, microcephaly, and mental retardation (O'Driscoll et al., 2003). In these patients, the defect was determined to be a synonymous hypomorphic mutation that generates a splicing variant that produced markedly reduced expression of normal ATR transcript and protein. Despite patient fibroblasts exhibiting an impaired response to DNA damage and increased genomic instability, no association between Seckel syndrome and cancer predisposition has been established (Alderton et al., 2004). Interestingly, Seckel syndrome shares clinical features with Nijmegen breakage syndrome (NBS), which does have increased susceptibility to hematological malignancies. NBS is caused by mutation of the DSB repair protein nibrin (NBN), which makes up part of the MRN complex with MRE11 and Rad50 (see section "**Homologous Recombination**") (Antoccia et al., 2006). MRN acts as a molecular sensor for DSBs, playing a pivotal role in initiating repair, and recruiting and activating ATM and ATR. The precise role of each component of MRN still requires further elucidation to understand why NBS phenotypically segregates with Seckel syndrome, while mutations in *MRE11* cause an ataxia telangiectasia-like disorder (Stracker and Petrini, 2011).

Direct Repair

Several proteins possess the ability to directly repair DNA damage lesions via a single-step mechanism, including reversal of O^6-methylguanine by O^6-alkylguanine DNA alkyltransferase (MGMT), and alkylation adducts (such as 1-methyladenine and 1-ethyladenine) by AlkB homologs 2 (ABH2) and 3 (ABH3). MGMT, in particular, has been investigated in the context of cancer development, as unrepaired substrate lesions can result in G → A transitions or crosslinks. However, *MGMT* knockout mouse models exhibit no cancer phenotype due to the presence of backup repair mechanisms such as NER, BER, and MMR (Glassner et al., 1999). Accordingly, no cancer-related human *MGMT* mutation has been reported, and common polymorphic variants (such as Ile143Val and Lys178Arg) have been found to neither impact protein function nor conclusively segregate with cancer risk (Du et al., 2013). However, MGMT likely plays an important role in the suppression of tumorigenesis following alkylation

damage, with knockout animals developing thymic lymphomas following methylnitrosourea exposure (Kawate et al., 1998), and transgenic *MGMT* overexpression suppressing cancer development in various mouse models of alkylation-induced malignancy (Dumenco et al., 1993). This protective effect is also relevant in the therapeutic setting, where efficient repair of alkylation damage by MGMT is associated with resistance to alkylating agents and poor patient outcomes (Kaina and Christmann, 2002). Promoter methylation resulting in loss of *MGMT* expression has been recognized as a common event in several tumor types, with MGMT expression levels now routinely used as a biomarker for chemotherapy response in glioblastoma and melanoma (Wick et al., 2014).

Mismatch Repair

MMR resolves errors introduced during replication that are not recognized by the proofreading capacity of DNA polymerases (Fishel, 2015; Goellner et al., 2015) (**Fig. 2**). Base mismatches and 1–2 nucleotide insertions/deletions are detected by MutSα (MSH2-MSH6 heterodimer), while larger mismatches (up to 16 nucleotides) are recognized by MutSβ (MSH2-MSH3). Upon binding to DNA, an ATP-dependent conformational change permits MutS to recruit multiple MLH1-PMS2 heterodimers (MutLα), forming sliding clamp complexes that dissociate from the DNA and translocate in either direction. MLH1-PMS1 activation is dependent upon proliferating cell nuclear antigen (PCNA), which may either be recruited by MutS, or may be spatially available if mispair recognition is associated with replication. MLH1-PMS1-mediated nicking of the DNA 5′ to the mispair initiates excision, either by recruitment of the 5′ to 3′ exonuclease EXO1 or by an EXO1-independent mechanism (**Fig. 2**). In EXO1-dependent excision, EXO1 is loaded at the 5′ nick and excises the DNA in a 3′ direction. Processivity is maintained through interaction with the multiple MutLα heterodimers loaded around the mispair, until EXO1 dissociation occurs due to the absence of further 3′ MutLα or presence of a strand terminus such as an Okazaki fragment. Subsequent repair synthesis and ligation completes repair across the newly generated gap. In the EXO1-independent pathway, excision may proceed via multiple nicking events catalyzed by MutLα (generating an identical gapped product as the EXO1-dependent mechanism), or by polymerase loading at a nicked site with subsequent strand displacement synthesis.

Germline mutations in genes involved in MMR (particularly *MSH2* and *MLH1*) are associated with hereditary nonpolyposis colorectal cancer (HNPCC; also known as Lynch syndrome), which imparts a 70%–85% lifetime risk of colorectal carcinoma, along with an elevated risk of endometrial and other cancers (Papadopoulos and Lindblom, 1997). HNPCC is inherited in an autosomal dominant pattern, although a somatic mutation event or epigenetic silencing at the second allele is required to trigger tumorigenesis; in the rare situation where two mutant alleles are inherited, cancers develop during childhood. A small number of familial cases with normal MMR gene sequences are recognized to result from germline "epimutations," wherein inherited promoter methylation of *MLH1* or *MSH2* silences gene expression (Hitchins et al., 2005); approximately 15% of sporadic colorectal cancers (CRC) also exhibit promoter methylation at *MLH1* (Li et al., 2013).

HNPCC is associated with increased variability in the length of short (1–6 nucleotides) tandem repeated DNA tracts—a state known as MSI that arises from failure to repair MMR substrates, such as base mismatches or insertion–deletion loops resulting from DNA polymerase slippage. MSI is a hallmark of a mutator phenotype state, wherein MMR deficiency results in a 100–1000-fold increased mutation rate, associated particularly with base substitutions and frameshift mutations that occur throughout the genome, contributing to malignant transformation when localized at tumor suppressor genes, such as APC, KRAS, and p53 (Loeb, 1994). Although MSI-positive tumors are histologically aggressive, they are associated with favorable clinical outcomes including increased tumor-infiltrating lymphocytes, reduced metastatic potential, and increased overall survival (Gelsomino et al., 2016). This survival benefit persists despite an association between MMR-deficient tumors and alkylating agent resistance. In repair-proficient cells, misincorporation of thymine opposite alkylative guanine lesions (such as O^6-methylguanine) is repeatedly recognized, corrected, and erroneously reestablished, preserving the original lesion and directing futile MMR cycling that eventually causes lethal strand breaks via ATM/ATR-induced apoptosis. Loss of MMR, in contrast, is associated with damage tolerance that does not trigger a cellular response, conferring drug resistance (Colella et al., 1999; Fink et al., 1998).

Base Excision Repair

BER is the primary mechanism by which nonbulky base lesions and abasic sites are resolved (Krokan and Bjoras, 2013; Wallace, 2014) (**Fig. 3**). BER is initiated by one of a group of DNA glycosylases, each of which recognizes a specific collection of base modifications. The substrate base is flipped out of the double helix into the glycosylase active site pocket, permitting cleavage of the *N*-glycosidic bond to yield an abasic site (Dizdaroglu et al., 2017). Apurinic/apyrimidinic endonuclease 1 (APE1) breaks the DNA phosphodiester backbone at the abasic intermediate, producing a single-strand interruption flanked by a 3′ hydroxyl (3′-OH) and a 5′ deoxyribosephosphate (5′-dRP) (Li and Wilson, 2013). Additionally, some members of the glycosylase family ("bifunctional" glycosylases) possess AP lyase activity and can perform strand incision by β- or β,δ-elimination of the abasic site, resulting in a 3′ α,β-unsaturated aldehyde or 3′ phosphate, respectively, adjacent to a 5′ phosphate group. These terminal groups block polymerase extension or nick ligation, and must be resolved to a 3′-OH/5′-P configuration by end-processing enzymes, including polynucleotide kinase-phosphatase (PNKP) (Weinfeld et al., 2011), APE1 (Demple and Harrison, 1994), DNA polymerase β (POLβ) (Matsumoto and Kim, 1995), tyrosyl-DNA phosphodiesterase 1 (TDP1) (Cortes Ledesma et al., 2009), and aprataxin (APTX) (Ahel et al., 2006). Subsequent repair proceeds by one of two mechanisms of polymerase-mediated gap-filling, with pathway choice dependent on factors such as initial damage substrate, local DNA structure, cell cycle, and cellular replicative status. During short patch repair (SP-BER), a single nucleotide is inserted by POLβ, and the remaining nick is ligated by the X-ray cross-complementing 1

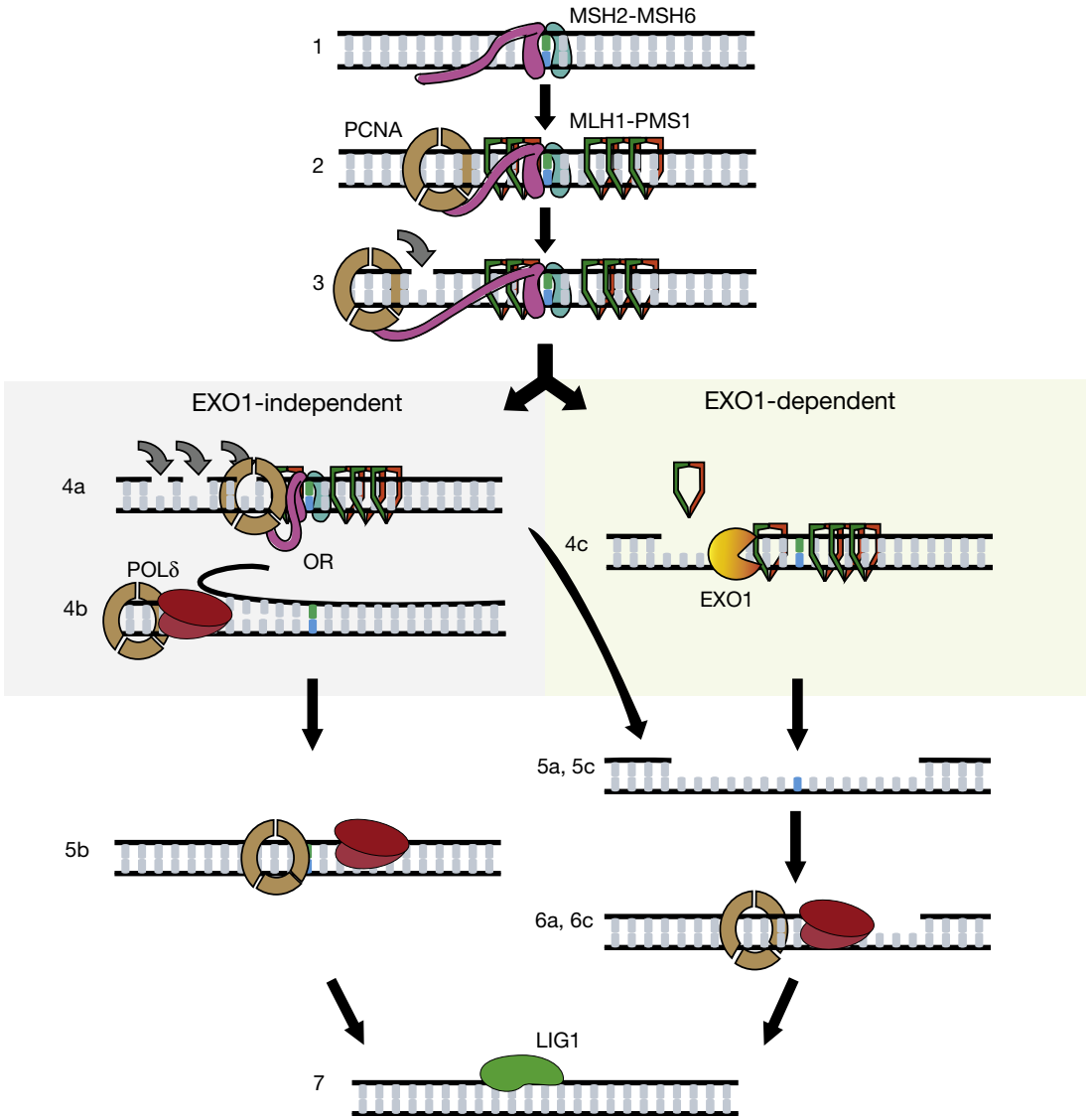

Fig. 2 Mismatch repair (MMR) recognizes and resolves polymerase errors introduced during replication, with MSH2-MSH6 targeting base mismatches and 1–2 nucleotide insertions/deletions (1) and MSH2-MSH3 commencing repair of larger mismatched sections (not shown). MSH2-MSH6 triggers the ATP-dependent recruitment of the MLH1-PMS1 sliding clamp and recruitment/retention of PCNA (2). Following endonucleolytic cleavage by PCNA-activated MLH1-PMS1 (3), MMR proceeds via an EXO1-independent (left) or EXO1-dependent (right) subpathway. One model of EXO1-independent MMR hypothesizes that PCNA stimulates MLH1-PMS1 to perform several endonucleolytic cleavages, leading to degradation of the DNA past the mispair (4a), thereby generating a product similar to that produced by the exonuclease activity of EXO1 (5a). The resulting gap is filled by POLδ (6a), and ligation occurs via LIG1 (7). In the second EXO1-independent model (4b), a 5′ nick is made by MLH1-PMS1, permitting strand displacement synthesis by POLδ past the nucleotide mismatch (5b). The 5′ flap is then cleaved before the nick is sealed by LIG1 (7). In EXO1-dependent MMR, a 5′ nick enables EXO1 recruitment (4c), followed by excision of the newly synthesized strand to a position past the mispair (5c). Gap-filling subsequently proceeds by POLδ (6c), followed by ligation by LIG1 (7). Adapted from Goellner, E. M., Putnam, C. D. and Kolodner, R. D. (2015). Exonuclease 1-dependent and independent mismatch repair. *DNA Repair (Amst)* **32**, 24–32. https://doi.org/10.1016/j.dnarep.2015.04.010, with permission.

(XRCC1)-ligase 3α (LIG3α) heterodimer (Dianov and Lindahl, 1994). Alternatively, long patch repair (LP-BER) involves generation of a 2–10 nucleotide 5′ flap by strand displacement synthesis primarily by POLδ/ϵ (supported by the PCNA sliding clamp), and subsequent excision by flap endonuclease 1 (FEN1), prior to ligase 1 (LIG1) nick sealing (Fortini et al., 1998). A third related pathway (Fig. 3), single strand break repair (SSBR), resolves single-strand DNA breaks induced by ROS or failed enzymatic reactions (such as BER interruptions or incomplete resolution of DNA TOP1 cleavage complexes during replication or transcription). Strand breaks are typically detected and bound by poly(ADP-ribose) polymerase 1 (PARP1), activating poly(ADP-ribosyl)ation to recruit a complex of factors that includes XRCC1, the above end-processing enzymes, DNA polymerases, and DNA ligases that complete repair via a manner analogous to classic BER (Abbotts and Wilson, 2017).

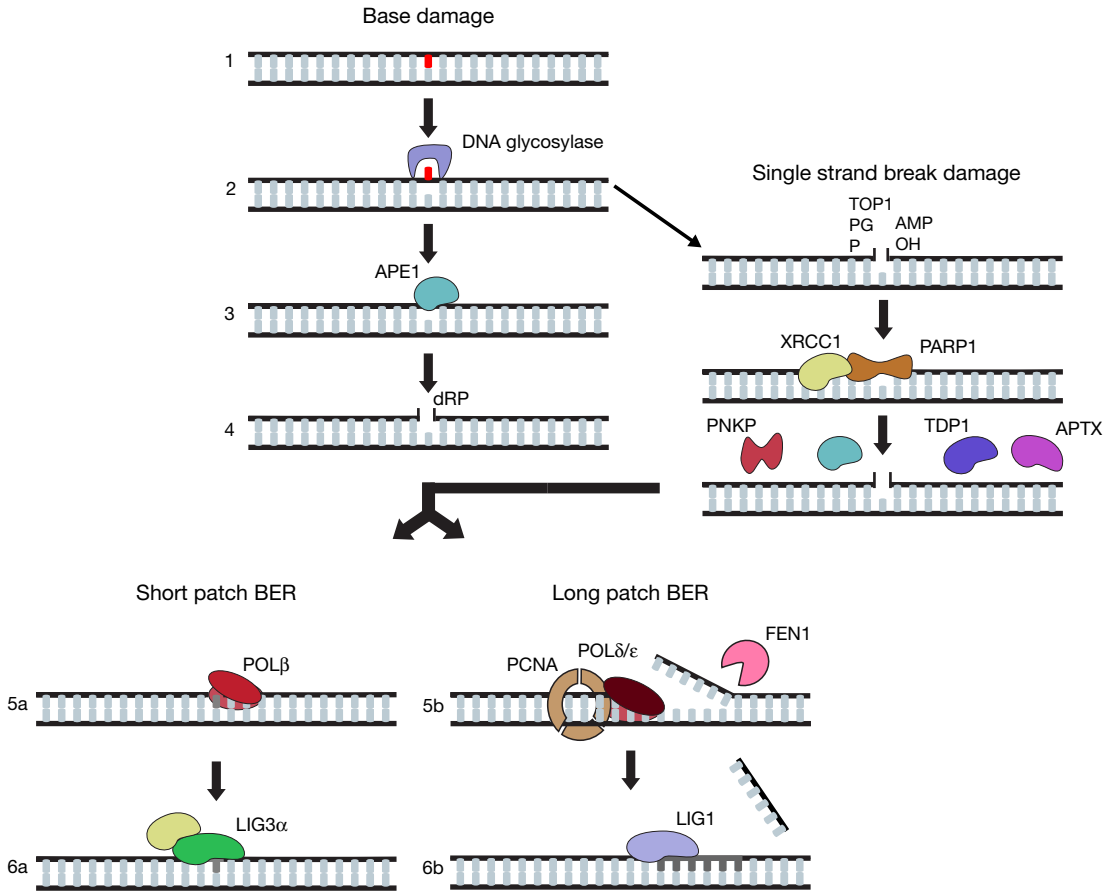

Fig. 3 Base excision repair (BER) typically begins at sites of small, nonhelix-distorting base damages, such as uracil or 8-oxoguanine (1). Damage-specific DNA glycosylases recognize and "flip-out" the base lesion to allow cleavage of the *N*-glycosidic bond and production of an abasic site (2). Most commonly, APE1 incises the DNA backbone at the abasic site (3), creating a single-strand interruption (4). During short patch BER (bottom, left), DNA POLβ removes the 5'-dRP residue and inserts a single nucleotide into the gap (5a), followed by ligation by LIG3α in complex with XRCC1 (6a). Repair synthesis in long patch BER (bottom, right) is mainly performed by DNA POLδ/ϵ (5b), which generates a 2–10 nucleotide flap by strand displacement. FEN1 excises the flap structure (5b) prior to LIG1 nick sealing (6b). Single-strand break damage (some examples shown) is resolved by a third pathway, SSBR, which shares overlap with components of BER, but serves a specialized DNA "clean-up" function. In classic SSBR (top, right), break recognition by PARP1 activates poly(ADP-ribosyl)ation, which recruits XRCC1 and associated end-processing enzymes that function to generate required 3′-OH and 5′-P groups for subsequent polymerase gap-filling and nick ligation. *dRP*, deoxyribose phosphate; *TOP1*, topoisomerase cleavage complex; *PG*, phosphoglycolate; *P*, phosphate; *AMP*, adenosine monophosphate; *OH*, hydroxyl.

The fundamental importance of BER is highlighted by the number of base and sugar damages that arise under normal cellular conditions (>10,000 lesions per cell per day). Consequently, germline mutations in core BER factors are commonly associated with embryonic or early perinatal lethality in mouse models. Where BER-related syndromes are recognized, for example with mutation of end-processing factors (such as *APTX*, *PNKP*, and *TDP1*), or the recently reported case of biallelic mutations in *XRCC1* (Hoch et al., 2017), the associated phenotype is one of neurological deficit rather than cancer predisposition, reflecting the critical role that BER (or more accurately SSBR) plays in the resolution of genomic damage in terminally differentiated cells, such as neurons, where other repair pathways (particularly those associated with replication) are downregulated (Leandro et al., 2015; Sykora et al., 2013).

Numerous polymorphic variants of BER factors have been described, with considerable effort devoted to determining the impact on BER proficiency and cancer risk. Variants of OGG1, a repair enzyme involved in recognition and removal of 8-oxoG, are the best characterized among the DNA glycosylases. Mouse models for Ogg1 are associated with 8-oxoG accumulation and an increase in spontaneous mutation frequency (Larsen et al., 2006), although no increased cancer predisposition is described without exogenous stress (e.g., skin tumors following UVB exposure), or deletion of both *Ogg1* and *MutY* (see later) simultaneously, which is associated with lymphomas, lung and ovarian tumors (Xie et al., 2004). In humans, the OGG1 polymorphic variant Ser326Cys is observed at a population frequency of ~32%. Functional studies suggest a minor catalytic defect (~63% of wild type) that does not impact the rate of 8-oxoG removal (Simonelli et al., 2013), and the ability of the variant to complement the spontaneous mutation frequency of *E. coli mutY*$^{-/-}$ has been under debate. Accordingly, an associated cancer risk has not been clearly established, with epidemiological studies producing inconsistent correlations (Zou et al., 2016), although an association with lung cancer risk seems to be better supported (Hung et al., 2005). More recently, polymorphism of a second BER glycosylase, thymine DNA glycosylase (TDG), has

also been reported as a possible tumor-associated variant (Sjolund et al., 2014). Functionally, this Gly199Ser substitution increases TDG binding of both the damage substrate and the abasic site product, preventing downstream repair. Collapse of replication forks at these lesions creates DSBs that risk genomic instability; consequently, cells expressing the Gly199Ser variant exhibit cellular transformation, although a cancer association has not yet been demonstrated in population studies.

The MUTYH (a.k.a. MutY) DNA glycosylase functions to remove adenine that is incorrectly inserted opposite 8-oxoG by a replicative polymerase (a mismatch that can also be resolved by MMR). MUTYH-associated polyposis (MAP) is a subtype of familial adenomatous polyposis (FAP), a CRC syndrome that presents with colonic polyps, an increased lifetime risk of CRC, and an association with other malignancies, including of the duodenum, ovary, bladder, and skin (Yamaguchi et al., 2014). MAP is almost always associated with somatic mutations in *APC*, the gene responsible for FAP, resulting from GC → TA transversions introduced by failure to remove misincorporated adenine opposite 8-oxoG (Al-Tassan et al., 2002). Inheritance is autosomal recessive, and usually the result of biallelic mutations, with over 30 truncating mutations and over 50 missense variants of *MUTYH* being described (Cheadle and Sampson, 2007). Functionally, the most common missense variants, Tyr165Cys and Gly382Asp, are associated with greatly reduced substrate binding, glycosylase activity, and ability to complement *E. coli mutY* mutation (Al-Tassan et al., 2002). A similar cancer susceptibility syndrome is attributed to mutations in *NTHL1*, which encodes a bifunctional DNA glycosylase that recognizes oxidized pyrimidine bases. As with MAP, NTHL1-associated polyposis is associated with an extended spectrum of cancers, including endometrium, duodenum, skin, breast, and pancreas (Talseth-Palmer, 2017).

The multifunctional protein APE1 accounts for >95% of total cellular endonuclease activity, and has important (and separable) roles in redox regulation of gene expression and nonredox transcriptional repression (Li and Wilson, 2013). While the essentiality of the redox function is unclear, knockout of the endonuclease function rapidly induces apoptosis in a number of cell models, emphasizing the critical importance of APE1 in cell viability (Fung and Demple, 2005). Consistently, nullizygous mouse models are associated with early embryonic lethality, while heterozygous animals, though generally phenotypically normal, exhibit reduced BER activity, increased frequency of spontaneous mutation, sensitivity to oxidative stress, elevated cancer risk, and reduced survival (Ludwig et al., 1998). Cancer susceptibility with several common polymorphisms (particularly Asp148Glu, found in ~46% of the population) has been extensively studied, without definitive evidence of risk association (Karahalil et al., 2012). Functionally, several rare variants (such as Leu104Arg and Arg237Ala) have been shown to exhibit impaired endonuclease activity, although no association with cancer has been reported for these proteins (Hadi et al., 2000). Moreover, while the endometrial cancer-associated variant Arg237Cys displays in vitro impairment of function (including reduced AP-DNA complex stability, 3′-5′ exonuclease activity and 3′-damage processing, and AP site cleavage within nucleosome complexes (Illuzzi et al., 2013)), it was not found to induce cancer cell phenotypes when expressed exogenously in certain cell models (Illuzzi et al., 2017; Lirussi et al., 2016). This suggests that the APE1 variants characterized to date represent possible susceptibility factors, but are not likely to be drivers of carcinogenesis.

The SP-BER polymerase POLβ is associated with embryonic or perinatal lethality in knockout mouse models, with heterozygotes accumulating DNA damage and exhibiting sensitivity to oxidative stress (Ray et al., 2013). A germline polymorphism of *POLβ*, Arg137Gln, has been associated with significant impairment of polymerase activity, DNA damage accumulation, and damaging agent sensitivity, although an association with cancer susceptibility has not been conclusively demonstrated (Guo et al., 2009). Somatic mutations in POLβ have been observed in ~40% of CRC, and have been reported in many other tumor types (Sobol, 2012). Most frequently observed is an 87 bp deletion mutant that produces a truncated protein at the catalytic domain. Tumor cells expressing this mutant exhibit significantly decreased BER capacity as a result of a dominant negative effect of the truncated protein that suppresses the catalytic activity of wild-type POLβ (encoded by the intact allele), possibly through competition for XRCC1 binding (Bhattacharyya and Banerjee, 1997). It is thought that acquisition of this mutation early in tumorigenesis is an important step in the development of a mutator phenotype that promotes further oncogenic mutations. Additional frameshift and missense mutations that similarly alter polymerase activity (e.g., increased infidelity) and/or reduce BER capacity have been implicated in breast and prostate cancer (Sobol, 2012).

As with other central BER factors, XRCC1 nullizygosity is associated with early embryonic lethality (Tebbs et al., 1999). Heterozygote (haploinsufficient) animals, while largely normal phenotypically for many parameters related to disease and aging, develop liver toxicity and precancerous colonic lesions following alkylating agent exposure (McNeill et al., 2011). In addition to the rare pathogenic mutants associated with neurological disease noted above, polymorphic germline variants in XRCC1 that have been extensively studied include Arg194Trp, Arg280His, and Arg399Gln (Karahalil et al., 2012). Reports are mixed regarding possible association of Arg194Trp and Arg399Gln with functional alterations (such as protein interactions or subcellular localization), and although both have been implicated by meta-analyses as possible susceptibility factors for cancer (including breast, prostate, and bladder), no consensus has been reached. Conversely, Arg280His appears to more consistently exhibit impaired function in terms of repair capacity, such as DNA binding or retention at sites of DNA damage (Berquist et al., 2010), and has been associated with a generalized overall increased cancer risk (albeit not observed when stratified by cancer type) (Zhang et al., 2012). For each variant, however, it is interesting to note that subgroup analysis often indicates that XRCC1 genotype–lifestyle interactions (particularly smoking) are strongly associated with cancer risk, including for gastrointestinal, colorectal, and head and neck malignancies.

Nucleotide Excision Repair

NER resolves bulky base lesions associated with distortion of the DNA helical structure (Marteijn et al., 2014) (**Fig. 4**). Two subpathways have been identified: global-genome NER (GG-NER), which removes lesions throughout the actively transcribed and nontranscribed genome; and transcription-coupled NER (TC-NER), which provides a more rapid mechanism for removing lesions from the

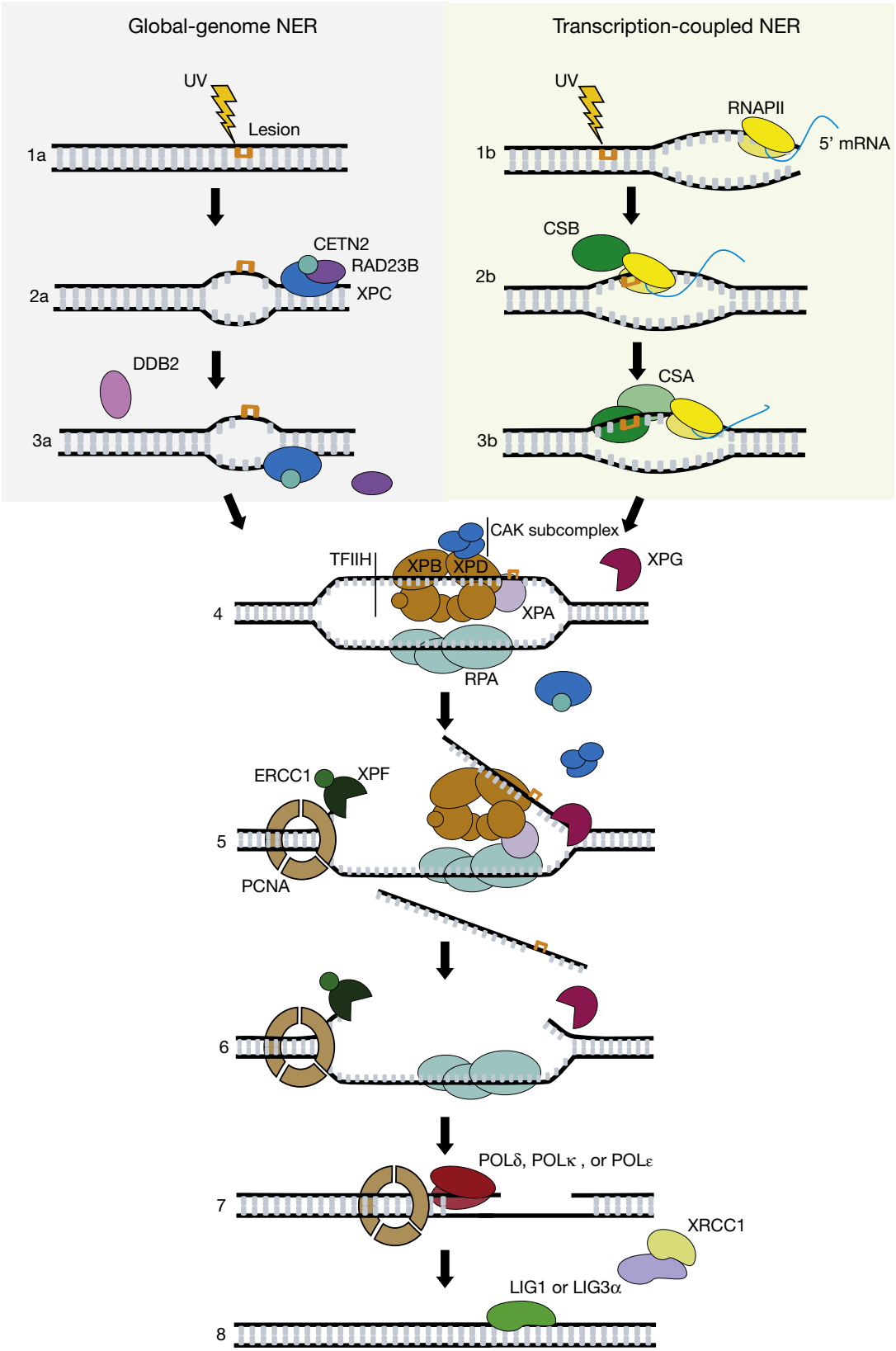

Fig. 4 Nucleotide excision repair (NER). Bulky, helix-distorting lesions (e.g., UV photoproducts) are recognized and repaired by one of two subpathways of NER: global-genome (left) or transcription-coupled (right). GG-NER (1a) functions throughout the genome, and is initiated by a detection complex that includes XPC, RAD23B, CETN2, and possibly an E3 ubiquitin ligase complex (DDB1-CUL4-RBX1) that contains DDB2 (2a); RAD23B dissociates from this assembly upon binding of XPC to the site of damage (3a). Rapid removal of lesions from actively transcribed strands in the genome is performed by TC-NER (1b), which is activated by stalling of RNAPII and subsequent recruitment of CSB and additional factors (not shown) (2b). CSB likely communicates with an E3 ubiquitin ligase complex (DDB1-CUL4-RBX1) that contains CSA (instead of DDB2), resulting in

transcribed strand of active genes. GG-NER is initiated by the xeroderma pigmentosum (XP) complementation factor XPC, in complex with RAD23B and centrin 2 (CETN2), which constantly scans the genome for helix-distorting lesions (Sugasawa et al., 1998). Substrate lesions are also recognized and bound by DDB2, the substrate recognition factor in an E3 ligase complex (also comprised of DDB1-CUL4-RBX1), which ubiquitylates both histones and NER factors (including DDB2 and XPC) to coordinate localized chromatin relaxation and repair factor interactions at the site of damage (Kapetanaki et al., 2006; Scrima et al., 2008). Damage recognition in TC-NER is initiated by stalling of RNA polymerase II (RNAPII) at the site of a lesion (Fousteri et al., 2006). Arrest of the transcription complex increases the affinity of RNAPII for the transcription elongation factor CSB (Cockayne syndrome complementation protein B). CSB is responsible for displacing (or repositioning) RNAPII and coordinating recruitment of downstream NER factors including CSA, which in TC-NER replaces DDB2 as the substrate recognition factor within the DDB1-CUL4-RBX1 E3 ligase complex (Fousteri et al., 2006).

Following damage recognition by either pathway (**Fig. 4**), the transcription initiation factor IIH (TFIIH) complex and the stability factor XPG are recruited to the lesion (Volker et al., 2001). Two TFIIH components, the ATPases/helicases XPB and XPD, induce localized DNA unwinding to create a 20–30-nucleotide bubble (Winkler et al., 2000). XPA is recruited and binds near the 5′ side of the bubble, where it verifies the damage and coordinates the structure-specific endonuclease XPF-ERCC1 to create a 5′ incision (Sugasawa et al., 2009). This in turn activates the endonuclease activity of XPG to incise the 3′ end of the bubble, excising the lesion in a 22–30 nucleotide DNA fragment (Fagbemi et al., 2011). PCNA is loaded at the 5′ end to facilitate gap-filling synthesis by DNA polymerases δ, κ and/or ϵ, and repair is completed by nick ligation via DNA LIG1 or XRCC1-LIG3α (Moser et al., 2007).

Defects in several NER proteins give rise to the autosomal recessive disorder XP (Dupuy and Sarasin, 2015). XP is subdivided into seven complementation groups, which reflect the pathogenic genetic defect: XPC and XPE/DDB2 (involved in GG-NER damage recognition); XPA, XPG, XPB, and XPD (involved in DNA unwinding); and XPF (involved, along with XPG, in excision). A further, milder, variant caused by defects in the translesion synthesis (TLS) polymerase POLH/XPV is also recognized. XP is associated with accumulation of unrepaired NER substrate lesions throughout the genome, where tolerance of this damage by TLS enables cell survival, but markedly increases the mutagenesis rate. Given that UV radiation is a major source of damage repaired by NER, the XP phenotype characteristically manifests with cutaneous symptoms, including extreme UV hypersensitivity, altered skin pigmentation, and a vastly elevated ($>10{,}000\times$) risk of sun-induced skin cancers. The risk of other solid tumors is also elevated, presumably due to the role of NER in the repair of ROS-induced DNA damage, such as cyclopurine lesions.

The precise clinical phenotype of XP depends upon the complementation group affected and the degree to which the associated protein is involved in both GG-NER and TC-NER (Berquist and Wilson, 2012). Accordingly, mutations in XPC or XPE/DDB2, which function as damage sensors in GG-NER, produce a "pure" XP phenotype of UV sensitivity and skin cancer. This contrasts with mutations in TC-NER genes, that is *CSA* and *CSB*, which produce Cockayne syndrome (CS), a progeroid phenotype with significant neurological features (including microcephaly, mental retardation, and sensorineural deafness), cachexia, and a markedly reduced life expectancy (Laugel, 2013). The predominance of neurological features in CS has been proposed to reflect the reliance of post-mitotic cells (such as neurons) on active TC-NER to maintain gene expression by removing transcription-blocking lesions, while compensatory repair mechanisms such as GG-NER are downregulated (Iyama and Wilson, 2013). In this context, damage accumulation due to the loss of TC-NER creates increased stalled RNAP complexes that can activate apoptotic mechanisms that accelerate the segmental premature aging phenotype and simultaneously provide protection from tumorigenic mutagenesis. Predictably, then, mutations in core downstream NER factors that play roles in both GG-NER and TC-NER, such as the TFIIH subunits XPD and XPB, manifest with complex phenotypes that may encompass both XP and CS features (Natale and Raquer, 2017).

Importantly, some factors have roles outside of NER that impact clinical presentation. For example, mutations affecting the endonuclease function of XPG mainly affect NER, resulting in an XP phenotype. However, XPG truncation mutants, which affect the structural role that XPG plays in other repair processes (such as the ability to trigger BER at oxidative damage sites, and interactions with homologous recombination (HR) factors during replication-associated repair), produce a mixed XP/CS disorder. Similarly, TFIIH plays an important role in transcription initiation, with perturbation of this function in certain XPB or XPD mutants underlying trichothiodystrophy, a CS-like disorder that also features brittle hair and scaling of the skin (Yew et al., 2016). Lastly, the CSA and CSB proteins have been shown to play various roles in general transcription regulation, possibly via chromatin remodeling mechanisms.

Interstrand Crosslink Repair

Interstrand crosslinks (ICLs) are a highly toxic form of damage that covalently links both strands of DNA, creating a potent block to transcription and replication. Efficiency of recognition and repair of ICLs varies depending on cell cycle phase and the presence of

reverse translocation or expulsion of RNAPII, creating accessibility for the DNA repair machinery (3b). Following damage verification by XPA in both GG-NER and TC-NER, the TFIIH complex is recruited to the site of the lesion (4), possibly in cooperation with the E3 ligase complex. TFIIH member helicases (XPB and XPD) produce localized DNA unwinding, resulting in RPA binding to single-stranded DNA (4). Incision occurs on both sides of the unwound DNA, first by XPF-ERCC1 at the 5′ end (5), and second by XPG at the 3′ end (6), releasing a damage-containing oligonucleotide of 22–30 nucleotides in length. Gap-filling synthesis is performed by DNA polymerases δ/κ/ϵ in cooperation with PCNA (7), prior to ligation by LIG1 or XRCC1-LIG3α (8). *CAK*, cdc-activating kinase.

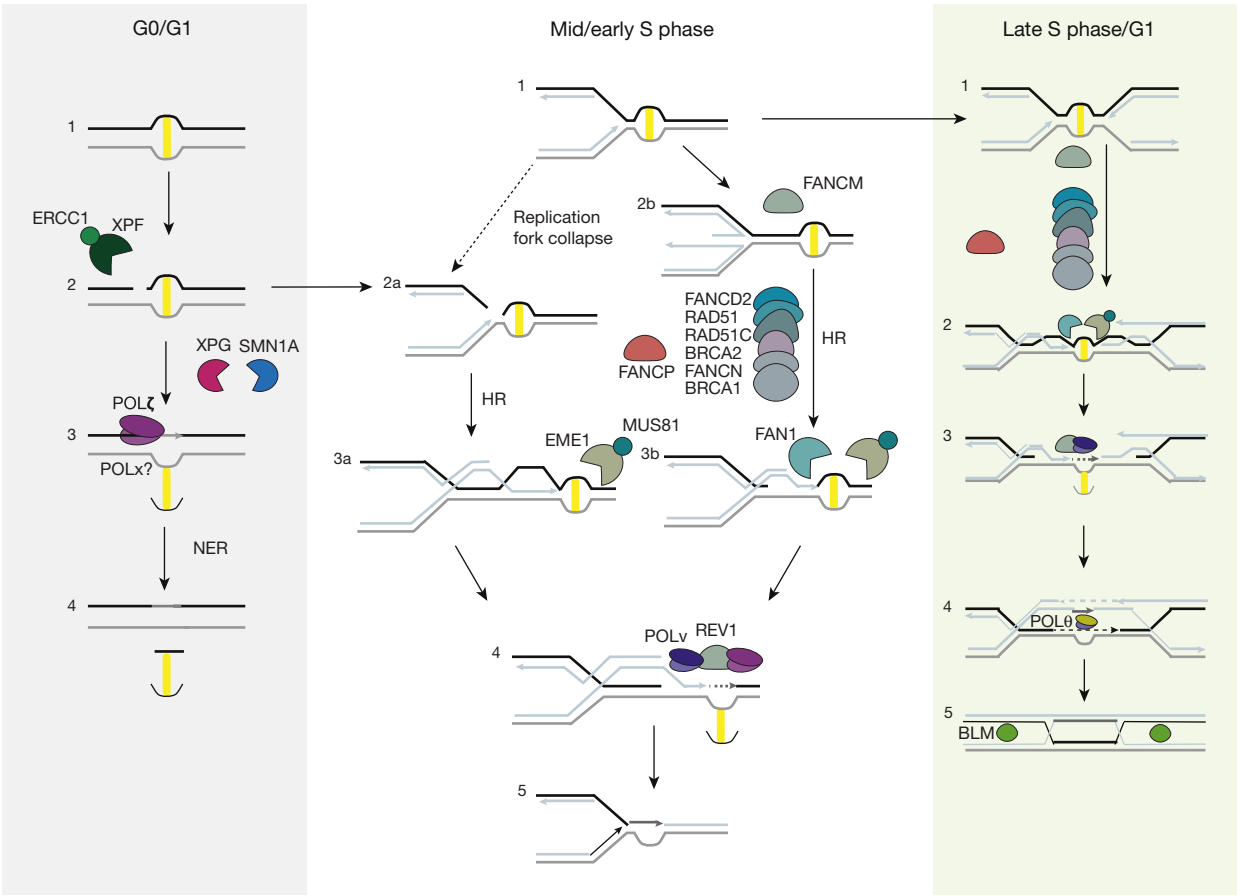

Fig. 5 Interstrand crosslink (ICL) repair. Covalent linkage of bases on opposing DNA strands, that is ICLs (depicted by *yellow line*) are repaired by ICL repair mechanisms. In G0/G1 phase (left panel), or in noncycling cells, ICLs are recognized by one of the two NER subpathways (1; see **Fig. 4**), ultimately resulting in XPF-ERCC1 incision (2), followed by unhooking by one of several nucleases, such as XPG or SNM1A, and gap-filling across the crosslink remnant by a TLS polymerase (3). A second round of classic NER excises the remaining damage from the opposing strand to complete repair (4). In mid/early S phase (center), repair is initiated when the replisome encounters a ICL (1). Collapsed replication forks, which result in the formation of a one-ended DSB (2a), can be avoided by FANCM-mediated fork regression (formation of the so-called chicken foot) and stabilization of the intermediate by the FA protein complex (depicted, 2b). In either case, homologous recombination (HR) and processing by various structure-specific nucleases (e.g., EME1-MUS81 or FAN1) results in unhooking (3a, 3b) and facilitates generation of a stable substrate for TLS polymerases (4). HR then completes the process, reestablishing replication, prior to excision of the crosslink remnant (5). In late S or G1 (right), the ICL is often encountered by a dual replication fork (1), resulting in FA protein-mediated HR and subsequent dual incision by structure-specific nucleases to carry out unhooking (2). TLS polymerases then perform gap-filling synthesis across the remaining crosslink damage (3), before remnant removal and replication completion (4). The Holliday junction intermediate is resolved by the BLM-containing complex (5) or a combination of nucleases/resolvases (see **Fig. 7**). Adapted from Deans, A. J. and West, S. C. (2011). DNA interstrand crosslink repair and cancer. *Nature Review. Cancer* **11**(7), 467–480. https://doi.org/10.1038/nrc3088, with permission.

active replication or transcription (Fig. 5). In G phase, NER proteins play a particularly critical role in damage recognition and excision, although the associated responses can of course take place in any cell cycle phase (Deans and West, 2011). The GG-NER factor XPC has been implicated in ICL recognition throughout the genome, probably in a structure-specific manner that detects extensively kinked or unwound lesions (Muniandy et al., 2009). This function may be supported, particularly for less distorted lesions, by MMR proteins, such as MSH2 (Vasquez, 2010). When a transcribing RNA polymerase collides with an ICL, recruitment of TC-NER proteins, such as CSA and CSB, can initiate repair (Iyama et al., 2015). In either scenario, dual incisions by XPF-ERCC1 and possibly XPG on a single strand 5′ and 3′ to (or SNM1A exonuclease processing across) the ICL "unhook" the covalent linkage, and synthesis by a TLS polymerase (such as DNA polymerase κ, ζ, or REV1) bypasses the remaining lesion and fills the gap prior to ligation. A second round of NER on the opposite strand is required to excise the crosslink remnant, allowing for the repair process to be complete (Kottemann and Smogorzewska, 2013). Notably, studies have suggested that the progressive degeneration in postmitotic (noncycling) tissue, such as the brain, or the broad premature aging phenotypes associated with defects in the XPF-ERCC1 nuclease complex (due to mutation of *XPF* (Niedernhofer et al., 2006) or *ERCC1* (Jaspers et al., 2007)) or the CS proteins may stem specifically from impaired ICL resolution (Iyama and Wilson, 2013).

In S phase of cycling cells (Fig. 5), stalling of helicase progression ahead of a replication fork by an ICL is a substrate for recognition by FANCM, which recruits the Fanconi anemia (FA) core complex, comprised of the FA complementation group proteins

FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCL (Palovcak et al., 2017). This complex ubiquitinates (via the E3 ubiquitin ligase activity of FANCL) two further FA complementation group factors, FANCD2 and FANCI. This event leads to the recruitment of a number of nucleases, including XPF-ERCC1, MUS81-EME1, SLX4-SLX1, FAN1, and SNM1A, which carry out processing to unhook the lesion and allow bypass via TLS. The critical recruitment of DSB repair (DSBR) factors BRCA1, BRCA2, and RAD51 suggests that the HR pathway (described later) is required for the completion of repair. The mechanism for the generation of a suitable DSB substrate has not been precisely delineated, but may stem from nuclease unhooking, resulting in replication fork collapse or replication fork regression to generate a "chicken foot" structure possessing a double-stranded DNA end (Haynes et al., 2015).

Mutation of one of the genes comprising the 17 FA complementation groups forms the genetic basis for FA, a rare autosomal recessive disorder that commonly causes short stature, subfertility, progressive bone marrow failure, and an increased risk of hematological (particularly acute myeloid leukemia) and solid (head and neck, esophageal, and gynecological) malignancies (Walden and Deans, 2014). Despite the genetic heterogeneity, the clinical presentation of FA is broadly similar. Specific exceptions include biallelic mutations of *PALB2* or *BRCA2*, which present with childhood malignancies and markedly reduced lifespan (5 years of age, compared to approximately 40 years in FANCA, which represents 65% of FA cases). Notably, FA is diagnosed by cellular sensitivity to crosslinking agents, and as such, chemotherapeutic agents of this mode of action are contraindicated in FA patients (Deans and West, 2011). Mechanistically, failure by the FA pathway to repair the DSB produced when an ICL is encountered by a progressing replication fork is associated with a high risk of genetic recombination, including oncogene translocations or tumor suppressor gene deletions that may drive carcinogenesis (Thompson and Hinz, 2009). Unrepaired ICLs are also potent inducers of apoptosis, leading in FA patients to depletion of the hemopoietic stem cell pool that creates a selection pressure for tumorigenic clones that are resistant to apoptotic signaling.

Nonhomologous End Joining

Two major pathways are responsible for the resolution of DNA DSBs. Outside of S/G2 phase, when the sister chromatid is not closely aligned to permit homology-driven repair (see later), canonical NHEJ (c-NHEJ) is the most common mechanism of repair, characteristically joining DNA ends possessing little (<10 bp) or no regions of microhomology (Bunting and Nussenzweig, 2013; Radhakrishnan et al., 2014) (**Fig. 6**). Mechanistically, c-NHEJ is highly flexible, able to recognize and initiate repair on a wide range of terminal substrates, including those resulting from ionizing radiation. DSBs are often recognized by the Ku heterodimer (comprised of KU70 and KU80 subunits), which forms a ring structure that encircles the DSB end and acts as a scaffold to recruit other NHEJ factors (Difilippantonio et al., 2000). The DNA-dependent protein kinase (DNA-PK_{CS}) is recruited early into a complex with Ku (collectively known as DNA-PK) that stabilizes and protects the substrate DSB termini by inhibiting ligation until the DNA ends are appropriately processed and juxtaposed for resolution (Uematsu et al., 2007). In order to remove complex damage lesions that may be present, a number of substrate-specific end-processing enzymes are known to be recruited by Ku to the strand break. These include PNKP (which processes nonligatable 5′-OH or 3′-P termini), Aprataxin (which removes adenylate groups from 5′-termini), polymerases μ and λ (which perform gap-filling activities), and the end resection nuclease Artemis, PNKP-related protein (APLF), and WRN (Davis and Chen, 2013). Also recruited to the site of DNA end joining is XRCC4, a nonenzymatic scaffold protein that plays an additional role in factor recruitment, XRCC4-like factor (XLF), which interacts with XRCC4 to form a filament that bridges the DNA ends, and DNA ligase 4 (LIG4), which ultimately seals the DSB (Kusumoto et al., 2008).

Knockout mouse models of various c-NHEJ proteins have been described, generally characterized by genomic instability, radiosensitivity, and severe combined immunodeficiency (SCID). The SCID phenotype arises from the role of c-NHEJ in the repair of programmed DSBs during V(D)J recombination, the process which generates diversity within the immune repertoire (Roth, 2014). In germ line cells, immunoglobulin and T cell receptor genes contain variable (V), diversity (D), and joining (J) segments, with each segment possessing a coding region flanked by recombination signal sequences (RSS). During immune development, cleavage at the RSS generates blunt-ended DSBs that are processed, rearranged, and rejoined by c-NHEJ in an ordered fashion to introduce recombination diversity, which is amplified by additional variability introduced at the segment junctions during the end-processing step of c-NHEJ. Impairment of V(D)J recombination limits this immune diversity, resulting in immunodeficiency, as well as increasing the risk of inappropriate recombination events that promote lymphoid malignancies. In humans, radiosensitive SCID phenotypes associated with c-NHEJ impairment are rare, but have been described in mutations of DNA-PKcs and Artemis (Le Deist et al., 2004). Mutation of proteins involved in the c-NHEJ ligation complex (XRCC4, XLF, and LIG4) also produces immunodeficiency, but within the context of a syndrome similar to NBS or Seckel syndrome, exhibiting microcephaly, facial dysmorphism, and growth retardation (Woodbine et al., 2014). This phenotypic difference stems from the precise role of each factor in c-NHEJ: for example, mutation of end-processing enzymes affects repair of a subset of DSBs only, with V(D)J-associated breaks being disproportionately affected, whereas mutation in a ligation-associated protein limits the rate of repair of all DSBs processed through the c-NHEJ pathway and hence produces a profound developmental defect and increased malignancy risk.

Due to the absence of homologous DNA to guide repair, NHEJ has a reputation for being an error-prone mechanism. However, the degree of repair accuracy is determined by the complexity of the strand break chemistry and the processing steps required to produce ligatable termini. Accordingly, repair of complementary strand breaks without blocking termini can dispense with the requirement for Ku recruitment and can be directly ligated by LIG4 with minimal introduced error (Frit et al., 2014). Conversely, repair of noncomplementary complex DSBs, such as those introduced by ionizing radiation, requires processing (including resection and/or gap-filling) by a repertoire of NHEJ proteins that introduce genomic alterations (Aparicio et al., 2014; Pannunzio et al., 2014). Furthermore, when c-NHEJ is unavailable (due to mutation or downregulation), a DNA-PK-independent alternative NHEJ pathway (alt-NHEJ) is engaged that is constitutively associated with profound genomic rearrangements (Iliakis et al., 2015) (**Fig. 6**).

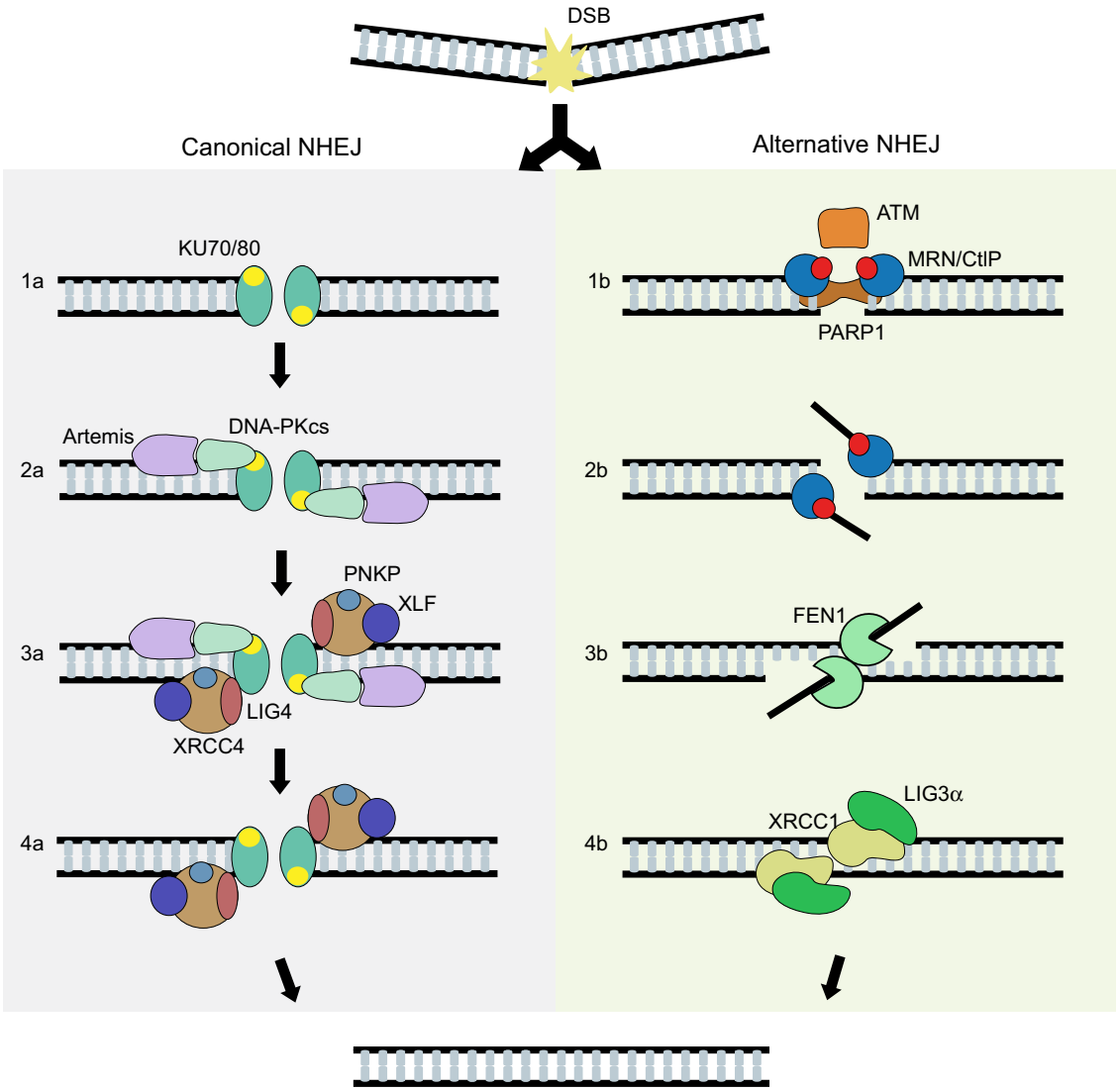

Fig. 6 Nonhomologous end joining (NHEJ) resolves a double-strand break (DSB) that arises typically outside of S/G2 phase. The KU70/KU80 heterodimer recognizes and initiates repair of canonical NHEJ (1a, left), recruiting DNA-PKcs to protect the DNA ends (2a). A range of end-processing enzymes are also recruited, such as Artemis and PNKP (3a), to resect the ends, remove blocking termini, and fill nucleotide gaps to produce ligatable ends, with often loss of genetic material. A ligation complex that includes XLF, XRCC4, and LIG4 seals the processed ends (4a). In the absence of canonical NHEJ, a second alternative pathway is carried out (right). PARP1 is involved in recognition and factor recruitment (1b), including MRE11 and CtIP, which resect the DNA ends to uncover 5–25 bp stretches of microhomology (2b) that are used to align the strand ends, before a nuclease (e.g., FEN1) excises the overhangs (3b) and XRCC1-LIG3α ligates the break (4b). As this microhomology-guided repair results in larger nucleotide deletions than c-NHEJ, the rate of mutagenesis is increased. Furthermore, due to the absence of strand tethering by the DNA-PK complex, alt-NHEJ risks errant recombination with other localized strand breaks and thus is associated with profound genomic rearrangements.

This association is mostly explained by the absence of Ku-mediated strand tethering, leading to increased mobility of DNA ends. DNA synapsis may instead be performed by PARP1, which likely also performs a factor recruitment role (Wang et al., 2006). Furthermore, alt-NHEJ is commonly guided by relatively long (5–25 bp) stretches of microhomology, which are uncovered by end resection by the HR exonuclease MRE11. The strand ends are aligned around the microhomologous regions and the overhangs excised by the flap-endonuclease FEN1, translating to local deletions around the original break. Ligation in alt-NHEJ is believed to require the XRCC1-LIG3α heterodimer (Della-Maria et al., 2011).

To limit the potentially deleterious outcomes (namely genomic instability and cancer manifestation), the presence of c-NHEJ factors such as Ku and DNA-PK suppresses the alt-NHEJ pathway (Bennardo et al., 2008). The importance of this process is exemplified by Werner syndrome (WS), a cancer-associated syndrome that is associated with dysregulation of NHEJ pathway choice. WS is a rare autosomal recessive progeria that manifests with a hierarchical decline in connective tissue integrity, as well as endocrine, immune, and nervous system function (Oshima et al., 2017). Premature mortality (~50 years of age) commonly results from cardiovascular disease or cancer, particularly thyroid, melanoma, meningioma, sarcoma, bone tumors, or leukemias (Lauper et al., 2013). Patient-derived WS cells are hypersensitive to agents that cause DSBs (such as ionizing radiation) and exhibit evidence of genomic instability in the form of chromosomal translocations, large deletions, and telomere fusions (Salk et al., 1981).

Genetically, WS results from defects in the *WRN* gene, of which >70 mutations have been described (Yu et al., 1996). WRN is a RecQ family helicase that also possesses strand annealing and exonuclease activities. It acts preferentially on complex DNA structures, such as G4 quadruplexes, Holliday junctions, and DNA bubble structures, suggesting a role in resolution of complex intermediates formed during DNA metabolism (Opresko et al., 2003). Functionally, WRN was first ascribed a role in end processing in NHEJ, observed via its interactions with c-NHEJ proteins such as Ku, DNA-PK, XRCC4, and LIG4 (Karmakar et al., 2002a,b; Kusumoto et al., 2008). That role has been refined to indicate that it is a regulator of pathway choice between c-NHEJ and alt-NHEJ; specifically, it suppresses recruitment of proteins (such as MRE11 and the CtIP endonuclease) that are involved in end resection and would channel the damage substrate toward alt-NHEJ (Shamanna et al., 2016). By protecting DSBs from end resection and promoting c-NHEJ, WRN therefore limits DNA end mobility associated with potentially oncogenic chromosomal translocations, deletions, and telomere fusions.

Homologous Recombination

The second major pathway for resolving DSBs is HR, which functions in concert with replication when sister chromatids are available to provide homologous guide sequences (Jasin and Rothstein, 2013; Mehta and Haber, 2014; **Fig. 7**). Initiation of repair requires chromatin remodeling, which involves at least two mechanisms: SIRT6 phosphorylation and mobilization to the damage site, which in turn activates PARP1 to subsequently recruit the chromatin remodeler ALC1; and phosphorylation of the histone

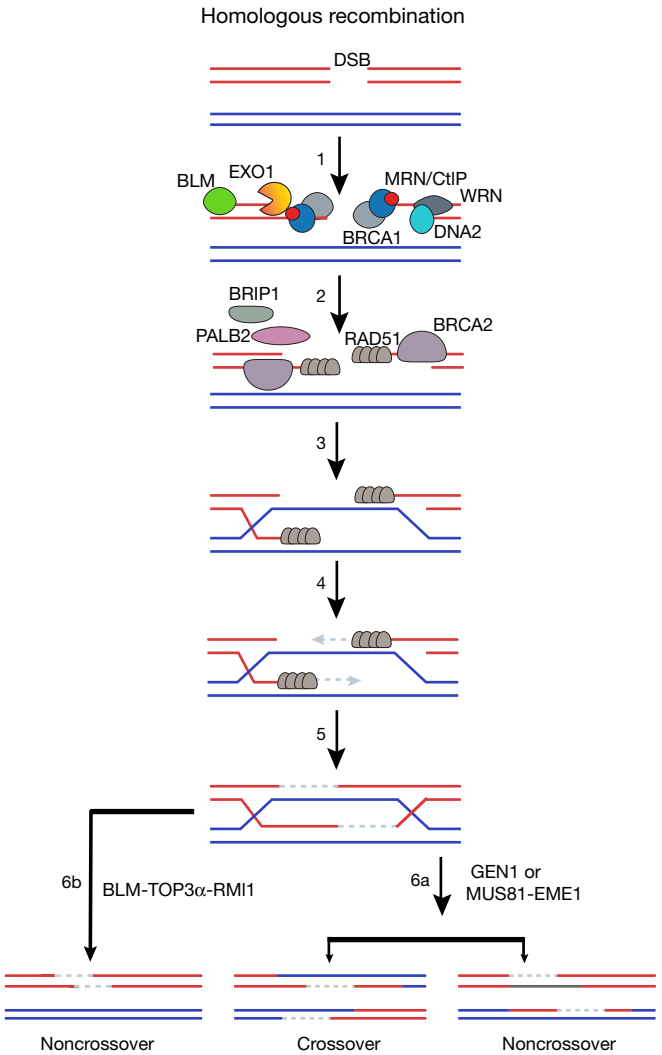

Fig. 7 Homologous recombination (HR). During S phase in replicating cells, a DNA double-strand break (DSB) is repaired by HR. Recruitment of the MRN (MRE11-RAD50-NBS1) complex and CtIP initiates 5′ end resection to generate 3′ overhangs, a process that is stimulated by BRCA1 and assisted by the EXO1 and DNA2 nucleases, in cooperation with the BLM and WRN helicases, respectively (1). RAD51 is then loaded onto the single-stranded DNA overhangs by BRCA2 and PALB2, in cooperation with BRIP1 (2). The RAD51 filament guides strand invasion into the intact duplex DNA of the adjacent sister chromatid or homologous chromosome (3). Polymerase synthesis (likely by POL δ/ϵ/ζ) using the homologous DNA sequences as a template extends the 5′ end to fill the gap (4). End ligation (5) generates double Holliday junctions that are resolved by GEN1 or MUS81-EME1 endonuclease incisions (6a) or topoisomerase-mediated branch migration (6b).

protein H2AX, which stimulates RNF8-mediated chromatin decondensation (Price and D'Andrea, 2013). A 3′ ssDNA overhang is generated by end resection of the 5′ ends flanking the DSB, initiated by the MRN complex in combination with the CtIP endonuclease, and continued by the activities of the BLM helicase and the EXO1 and DNA2 nucleases (Nimonkar et al., 2011; Sartori et al., 2007). End resection also requires the presence of BRCA1, which appears to interact with (and possible recruit) activated CtIP to the damage site, as well as to antagonize 53BP1, a potent inhibitor of resection and promoter of NHEJ (Panier and Boulton, 2014; Yu et al., 1998). CtIP is also important in the recruitment of RPA, which binds and protects the ssDNA 3′ overhangs prior to strand exchange (Sartori et al., 2007).

The process of end resection is an important determinant in DSBR pathway choice: binding of the NHEJ recognition factor Ku inhibits resection to favor c-NHEJ, but can be overcome by CtIP endonuclease clipping of bound Ku from the DNA strand termini (Symington and Gautier, 2011). Furthermore, the production of 3′ overhangs can initiate several repair pathways, including alt-NHEJ and the similar (and equally mutagenic) single-strand annealing (SSA) pathway, in which end resection around a DSB between two repeat sequences is sufficient to allow RAD52-guided annealing (Bhargava et al., 2016). When conditions favor HR, such as in S/G2, RAD51 is recruited and bound to the ssDNA, displacing RPA to form a nucleoprotein filament. Loading of RAD51 requires the presence of mediator proteins PALB2 and BRCA2, which are recruited via an interaction with BRCA1, which persists at the damage site following the end resection activities (Sy et al., 2009). Both RAD51 and BRCA2 are important in directing repair toward the HR pathway, as formation of the nucleoprotein filament directly suppresses SSA and alt-NHEJ (Ahrabi et al., 2016).

Specific to the HR pathway, the RAD51-guided ssDNA filament then invades duplex DNA—most commonly the closely localized sister chromatid ("inter-sister" HR), but also potentially the homologous chromosome ("inter-homolog" HR). The invading 3′ end functions as a primer to initiate repair synthesis using the homologous region as a template. The strand invasion intermediate forms a displacement loop (D loop) that can be resolved by a number of mechanisms. In mitotic cells, synthesis-dependent strand annealing (SDSA) is thought to predominate. The invading strand dissociates from the D loop and anneals to the other 3′ overhang on the damaged chromosome, with the newly synthesized region acting as template for repair synthesis on the opposing strand prior to gap-filling and nick ligation (Morrical, 2015). In contrast, the canonical mechanism of DSBR involves capture of the second 3′ end to form double Holliday junctions (dHJ) that can be resolved by nicking endonucleases (such as EXO1 in meiotic cells, or MUS81-EME1 or SLX1-SLX4 in mitotic cells), or dissolved by the branch migration activity of a BLM-TOP3α-RMI1 complex (Jasin and Rothstein, 2013).

The genetic outcome of HR is dependent on a number of factors. Inter-sister HR, by virtue of identical homology, is genetically silent, while inter-homolog HR can result in both noncrossover and crossover events (**Fig. 7**). In the context of inter-homolog HR, the mechanism of D loop resolution produces a particular outcome: SDSA, for example, always produces a noncrossover product, while Holliday junction resolution can result in both noncrossover and crossover events depending upon the dHJ resolvase involved and the orientation of Holliday junction incision (Moynahan and Jasin, 2010). Noncrossover events (such as in SDSA and most dHJ resolution in mitosis) produce only short stretches of gene conversion that span the homologous template regions. In contrast, crossover events affect the entire chromosome distal to the DSB, thereby forming the basis of genetic recombination during meiosis (Gray and Cohen, 2016), but risk large-scale LOH during subsequent mitotic cell divisions. LOH at various tumor suppressor and oncogene loci, including p53, RB1, MET, and BRCA1 and BRCA2 (see later), has been widely implicated in carcinogenesis, particularly in cancer predisposition syndromes (Thiagalingam et al., 2002).

The most well-known familial cancer predispositions associated with DSBR defects result from mutations in *BRCA1* and *BRCA2* (Paul and Paul, 2014). Heterozygous inheritance of a germline mutant *BRCA1* allele is a major cause of hereditary breast cancer, conferring a disease risk of over 60% by age 70 (and a lifetime risk of ~40% for ovarian cancer, along with rarer pancreatic, prostate and other cancer types), while somatic mutation is a common finding in sporadic breast and ovarian carcinomas (Janatova et al., 2005). *BRCA2* mutation also represents an important inherited cause of breast and ovarian cancer susceptibility, while mutation of its partner *PALB2* contributes to ~1% of hereditary disease (Economopoulou et al., 2015). Homozygous *BRCA* mutation in humans is extremely rare: the few reported cases of homozygous *BRCA1* or *BRCA2* mutations present with congenital abnormalities and hypersensitivity to crosslinking agents, and as such, are considered (along with biallelic mutation of *PALB2* and *RAD51C*) to be distinct subtypes of FA (see earlier) (Howlett et al., 2002; Sawyer et al., 2015). In tumor cells, however, mutation in *BRCA* genes (whether inherited or acquired) is commonly associated with inactivation of the second allele through promoter hypermethylation or LOH (Esteller et al., 2000).

Additionally, certain non-HR genes have been reported to impact HR efficiency. Mutation of *CDK12* has been well established in sporadic ovarian cancer, with loss of function impairing phosphorylation of RNAPII that is required to promote expression of HR pathway genes, such as *BRCA1* (Chila et al., 2016). The tumor suppressor *PTEN*, which encodes a major regulator of the prosurvival phosphoinositide 3-kinase/Akt pathway, acquires somatic mutations in numerous tumor types, including breast and ovarian cancer (Song et al., 2012). PTEN has more recently been ascribed a role in genomic stability that appears to be mediated through interactions with the HR pathway, although this role is controversial and the precise mechanism remains unclear (Ming and He, 2012). Also controversial is the role of EMSY, which is amplified in a subset of sporadic breast and ovarian cancers. EMSY colocalizes with BRCA2 at sites of DNA damage, where it may interact with the BRCA2 N-terminal domain to inactivate BRCA2-mediated RAD51 loading onto ssDNA; however, it is unclear whether this interaction significantly impacts HR efficiency. Given that EMSY maps to chromosome 11q13, a region that harbors a number of potential oncogenes and is often amplified in cancer, it remains to be ascertained whether it genuinely harbors an oncogenic function (Haber, 2003).

As described earlier, WS is caused by mutation in the RecQ family helicase, WRN. A second RecQ helicase, BLM, is also associated with a rare autosomal recessive disorder, Bloom syndrome (BS) (Arora et al., 2014). While BLM and WRN share sequence homology and structural similarity, the clinical manifestations are largely distinct, with BS presenting with growth retardation, immunodeficiency, and photosensitivity. Like WS, however, BS is associated with cancer predisposition (albeit across a considerably wider spectrum of malignancies), arising from marked genomic instability. Characteristically, BLM is associated with a 10-fold elevated rate of sister chromatid exchange (not observed in WS), a likely indication of a defect in HR regulation (Manthei and Keck, 2013). Specifically, the helicase function of BLM plays a critical role in resolution of dHJs to produce exclusively noncrossover products, via branch migration activity that brings together two Holliday junctions into an intermediate structure that is a substrate for dissolution by topoisomerase TOP3α (Wu and Hickson, 2003). In terms of DSBR pathway involvement, the disparate functions of BLM (in HR) and WRN (in NHEJ) likely account for the distinct clinical presentations (Kitano, 2014). However, the two proteins do broadly exhibit functional redundancy in 5′-3′ end resection, wherein their helicase function produces a substrate for nuclease activity during generation of 3′ overhangs for RAD51 strand invasion. It is likely, though, that this activity is nuclease and/or structure specific: BLM is able to specifically stimulate the end resection activities of both the DNA2 endonuclease and the EXO1 exonuclease, whereas WRN interacts with DNA2 alone, but is preferentially recruited over BLM during resection around crosslinked DNA or stalled replication forks (Sturzenegger et al., 2014).

Targeting DNA Repair in Cancer Therapy

The efficacy of many conventional anticancer therapies relies on their ability to induce toxic levels of DNA damage, specifically in the context of a rapidly dividing malignant cell (Cheung-Ong et al., 2013). Ionizing radiation, for example, induces a complex, lethal spectrum of lesions, including base modifications, strand breaks, bulky adducts, and crosslinks, and thus requires careful dosing and targeting to reduce toxicity to healthy cells. Acute but self-limiting toxicity primarily occurs in rapidly proliferating cells, where a high burden of damage lesions induces apoptosis, causing symptoms such as dermatitis, mucositis, proctitis, cystitis, and hair loss. Late effects, which include tissue fibrosis or atrophy, vascular damage, infertility, and secondary malignancies, have a more insidious onset, reflecting their basis in more slowly proliferating tissues, but are often permanent (White and Joiner, 2006). Many conventional chemotherapy agents also rely on DNA damage induction for therapeutic efficacy (**Table 1**). Like radiotherapy, dose management is required to prevent significant off-target toxicity. Acute effects represent the primary concern, because the systemic nature of chemotherapy results in damage accumulation in most body tissues. With respect to the toxicity, as with radiotherapy, rapidly proliferating cells (including tumor cells) are disproportionately affected by DNA damage induction, accounting for the classic side effects, such as hair loss, mucositis, and cytopenia. Secondary malignancies also represent a risk following chemotherapy, particularly those involving proliferative cells, such as found in the bone marrow or lymphatic system. This risk is elevated in certain therapeutic classes (such as alkylating agents or topoisomerase inhibitors), and by patient background (such as the use of crosslinking agents in FA) (Diatloff-Zito et al., 1986).

Given that many anticancer agents elicit their toxicity through the generation of lethal DNA damage, there has been a growing effort to develop targeted small molecule inhibitors against molecular targets in each DNA repair pathway, with the idea that these inhibitory compounds would enhance the efficacy of relevant genotoxins (Gavande et al., 2016). The best known of these compounds are the PARP inhibitors, which mimic the PARP1 substrate NAD^+ to compete for the catalytic pocket, and, with variable

Table 1 Common DNA-damaging chemotherapeutic agents

Class	*Subclass*	*Examples*	*Cytotoxic mechanism*	*Pathway involved*
Alkylating agents	Nitrogen mustards	Chlorambucil, cyclophosphamide, ifosfamide, melphalan	DNA alkylation	BER
	Triazenes	Dacarbazine, temozolomide	DNA alkylation	BER
Dialkylating agents	Nitrosoureas	Carmustine, lomustine, streptozotocin	Bulky DNA adducts, crosslink formation	BER, NER, ICL repair
Alkylating-like	Platinums	Cisplatin, carboplatin, oxaliplatin	Bulky DNA adducts, crosslink formation	NER, ICL repair
Topoisomerase inhibitors	Type I topoisomerase inhibitor	Camptothecin, irinotecan, topotecan	Topo-DNA adducts, DSBs	HR, NHEJ
	Type II topoisomerase inhibitor	Etoposide	Topo-DNA adducts, DSBs	HR, NHEJ
	Anthracyclines	Doxorubicin, daunorubicin	Topo-DNA adducts, intercalation	
Radiomimetics		Bleomycin, enediyne antibiotics	DSBs	HR, NHEJ
Antimetabolites	Pyrimidine analogs	5-Fluorouracil, capecitabine, gemcitabine	Intercalation, replication stalling	DNA replication
	Purine analogs	6-Mercaptopurine, fludarabine	Intercalation, replication stalling	DNA replication
	Antifolates	Methotrexate, pemetrexed	Inhibition of nucleotide synthesis	DNA replication

See text for abbreviations.

efficiency, trap PARP1 on DNA to create toxic PARP–DNA complexes that interfere with replication. Initial strategic development of PARP inhibitors focused on their ability to potentiate the cytotoxicity of DNA-damaging agents classically repaired by PARP-dependent mechanisms (Annunziata and O'Shaughnessy, 2010). As such, several trials evaluating a combinatorial approach have reached the clinic. In glioblastoma, combining the alkylating agent temozolomide, which shows some efficacy as a single agent due to its ability to cross the blood–brain barrier, with an early generation PARP inhibitor (veliparib) produced inconclusive results in terms of overall survival in a phase II study, likely because veliparib is a relatively poor PARP trapper (Robins et al., 2016). Similarly, inconclusive results have been observed in a combination involving the PARP inhibitor olaparib and docetaxel, a taxane. Taxanes, which disrupt microtubule formation during mitosis, produce a response rate benefit in advanced gastric cancers. Over 60% of this group of tumors exhibit mutation in *ATM*, which has been shown in vitro to render cells sensitive to PARP inhibitors due to impairment of DSBR (Bang et al., 2015). Dual therapy, therefore, is hypothesized to improve outcomes by additive cytotoxicities of: (a) gold standard taxane chemotherapy and (b) "synthetic lethality" interaction of *ATM* mutation with PARP inhibitor (see more below). In a phase III study evaluating an olaparib/docetaxel combination in late-stage gastric cancer, however, no survival benefit was established (Drean et al., 2016). Radiotherapy also offers potential benefit in a combination approach, given that generation of DSBs (among other DNA damage lesions) can be specifically targeted to a tumor site. In combination with radiotherapy, phase I secondary outcomes of radiological response and median survival were improved with the use of veliparib in primary breast and nonsmall cell lung cancers (Mehta et al., 2015).

A number of other phase I and II studies assessing various PARP inhibitor/conventional agent combinations remain in recruitment stage, but the value of this approach currently remains limited by the nonspecificity of conventional therapy with regard to normal cell toxicity. In the last decade, focus has instead shifted to examine the use of PARP inhibitors as a targeted monotherapy. This approach was born out of the exciting preclinical observation that PARP inhibitors can be effectively employed in a synthetic lethality context to specifically target cancer cells (Bryant et al., 2005). Synthetic (or synergistic) lethality exploits intergene relationships wherein loss of function of either gene is nonlethal, but loss of both is cytotoxic. The connection between BRCA1/2 and PARP1 represents the first described synthetic lethality relationship. In the context here, inhibition of PARP1-mediated repair prevents SSB resolution, which, along with stable PARP-DNA complex formation, causes replication fork collapse and DSB generation. In a HR-deficient background, such as in the case of a *BRCA* mutation, unrepaired DSBs rapidly activate apoptotic pathways and induce cell death (Lord and Ashworth, 2017). Early phase I and II trials supported a role for PARP inhibition in a *BRCA* mutant background, but results in phase III trials have been mixed—presumably due to PARP inhibitor choice (with one candidate agent iniparib later proved to not inhibit PARP function) and the composition of the patient cohort, which, regardless of tumor type, has generally been expanded to include a range of later stage cancers due to the relative scarcity of BRCA-associated malignancies. More recently, a dual cohort phase III study that specifically recruited a large *BRCA* mutant population ($n = 203$) has reported a statistically significant improvement in the progression-free survival of ovarian cancer patients treated with the PARP inhibitor niraparib (Mirza et al., 2016). Similar results have also been described in phase III trials of metastatic *BRCA*-mutated breast cancers treated with olaparib, again highlighting the importance of appropriate cohort selection (Robson et al., 2017). Thus, with promising results based on the concept of synthetic lethality in place, future efforts will explore in greater depth the ability to clinically target the genetics and functionality of cancer cells with relevant DNA repair inhibitors.

One strategy to evaluate PARP inhibitor efficacy in broader clinical trials has been to target tumors with histopathologically and/or clinically similar phenotypes (BRCAness) (Lord and Ashworth, 2016). For example, *BRCA1*-mutant breast cancers are commonly basal-like and do not express estrogen or progesterone receptors or undergo human epidermal growth factor 2 (HER2) amplification. This "triple negative" pathological presentation is observed in patients without germline *BRCA* mutation, but is often enriched for other mechanisms that give rise to HR deficiency, including *BRCA1* or *RAD51* promoter methylation or somatic mutation. To date, PARP inhibitors have not been successful in phase III studies of triple negative breast cancers; results utilizing later generation, highly specific PARP inhibitors (such as talazoparib) are expected soon (Lord and Ashworth, 2017). Sensitivity to platinum chemotherapy agents may also be a marker of BRCAness, given that *BRCA*-mutant tumors are highly sensitive to platinum compounds, which induce crosslinks that require effective HR for resolution. Accordingly, a placebo-controlled phase II study in platinum-sensitive ovarian cancer reported a doubling in progression-free survival with maintenance olaparib, and phase III trials are now ongoing (Oza et al., 2015).

An additional anticancer agent that targets the BER pathway is methoxyamine, which reacts with abasic sites to create an adduct that indirectly inhibits APE1 activity, producing a lesion capable of inducing cytotoxicity by stalling replication forks. Preclinically, it is able to potentiate the effects of alkylating agents such as temozolomide (Taverna et al., 2001), and a phase II trial testing the combination of methoxyamine/temozolomide in glioblastoma is currently in the recruitment stage. Additionally, methoxyamine enhances the effects of pemetrexed, an antifolate agent that interrupts purine and thymidine synthesis, resulting in uracil misincorporation in DNA. Recognition and excision by the BER protein uracil DNA glycosylase creates an abasic site that is bound by methoxyamine, with the combination increasing the damage load (and hence cytotoxicity) compared to either single agent alone (Bulgar et al., 2012). This therapeutic strategy, along with modified regimens including cisplatin and/or radiotherapy, is currently in phase I/II. In contrast, identification of specific APE1 active site inhibitors has not progressed beyond an early preclinical development stage (due to potency and selectivity issues), although DNA glycosylase inhibition may offer more promise (Jacobs et al., 2013).

Other DNA repair inhibitors are much less advanced in the development pipeline. Small molecules have been designed that target DNA-PK function by stabilizing the protein complex at DSB ends, preventing initiation of downstream NHEJ, and blocking other repair processes. In preclinical studies, one such compound, NU7441, sensitizes cells and xenografts to agents that induce

DSBs (such as ionizing radiation and topoisomerase inhibitors), without significant cytotoxicity as a monotherapy (Zhao et al., 2006). Structure activity relationship analysis of this compound has identified related inhibitors that are currently in phase I trials, both as monotherapy and in combination with radiotherapy or doxorubicin. ATM inhibitors, currently in later stages of development, also target the initiating stages of DSBR. However, the mechanism of action of these compounds is less clear, given that ATM's role in the DDR extends beyond DNA repair initiation. Regardless, preclinical evidence indicates that ATM inhibitors are able to sensitize cells to DSB-inducing agents, such as ionizing radiation and topoisomerase inhibitors (including camptothecin and etoposide) (Hickson et al., 2004). Furthermore, targeting ATM inhibits DSBR, creating a synthetic lethality partner for PARP inhibition, and phase I trials of ATM inhibitor in combination with olaparib are ongoing. Design of ATR inhibitors is following a different strategy, focusing on ATR's activation by ssDNA around a stalled replication fork, which allows the exciting potential to combine ATR inhibitors with agents that target cell cycle checkpoints in a synthetic lethality paradigm. As described earlier, the checkpoint regulator Chk1 is triggered by a signaling cascade downstream of activated ATR, but also has a role in S-phase checkpoint activation via an ATR-independent backup pathway (Sanjiv et al., 2016). As a result, impaired initiation of repair secondary to ATR inhibition rapidly induces cytotoxicity in the context of ATR- and Chk1-deficient checkpoint signaling. Although this combination has not yet been tested in clinical trials, phase I/II studies to evaluate both ATR and Chk1 inhibitors in combination with agents inducing replication-associated DNA damage (such as platinum agents or gemcitabine) are currently underway. Taken together, the many preclinical studies indicate that DNA repair inhibition is a promising therapeutic strategy, although drug development for most targets is currently at an early stage. The challenges faced by PARP inhibitors, whose strong in vitro performance has been tempered by variable success in the clinic, highlight the importance of compound validation and patient cohort selection (Brown et al., 2017).

Summary

The volume of DNA damage, both endogenous and secondary to external insults, that affects every cell each day, has necessitated the evolution of a sophisticated DDR to prevent deleterious outcomes, such as mutagenesis and apoptosis, that underlie carcinogenesis, disease, or aging. Central to this global response is a number of highly conserved DNA repair pathways that can resolve a wide range of damage substrates at various stages of the cell cycle. Defects in members of these pathways, or associated signaling processes, give rise to a variety of inherited syndromes that are associated with an elevated risk of cancer, neurological disease, immunodysfunction, or premature aging phenotypes. Moreover, haploinsufficiency in DNA repair, possibly through heterozygous mutations or single nucleotide polymorphisms, can affect disease risk, likely in a life style- or exposure-dependent manner. The molecular mechanisms of the DNA repair systems, including the interrelationships between pathways, are a current area of therapeutic focus, particularly given the frequent use of genotoxins as anticancer agents. It is therefore anticipated that in the near future tailored treatment regimens will greatly improve cancer outcomes.

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See also: Hereditary Risk of Breast and Ovarian Cancer: BRCA1 and BRCA2.

References

Abbotts, R., Wilson 3rd., D.M., 2017. Coordination of DNA single strand break repair. Free Radical Biology & Medicine 107, 228–244. https://doi.org/10.1016/j.freeradbiomed.2016.11.039.

Ahel, I., Rass, U., El-Khamisy, S.F., Katyal, S., Clements, P.M., McKinnon, P.J., et al., 2006. The neurodegenerative disease protein aprataxin resolves abortive DNA ligation intermediates. Nature 443 (7112), 713–716. https://doi.org/10.1038/nature05164.

Ahrabi, S., Sarkar, S., Pfister, S.X., Pirovano, G., Higgins, G.S., Porter, A.C., Humphrey, T.C., 2016. A role for human homologous recombination factors in suppressing microhomology-mediated end joining. Nucleic Acids Research 44 (12), 5743–5757. https://doi.org/10.1093/nar/gkw326.

Alderton, G.K., Joenje, H., Varon, R., Borglum, A.D., Jeggo, P.A., O'Driscoll, M., 2004. Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway. Human Molecular Genetics 13 (24), 3127–3138. https://doi.org/10.1093/hmg/ddh335.

Al-Tassan, N., Chmiel, N.H., Maynard, J., Fleming, N., Livingston, A.L., Williams, G.T., et al., 2002. Inherited variants of MYH associated with somatic G:C → T:A mutations in colorectal tumors. Nature Genetics 30 (2), 227–232. https://doi.org/10.1038/ng828.

Annunziata, C.M., O'Shaughnessy, J., 2010. Poly (ADP-ribose) polymerase as a novel therapeutic target in cancer. Clinical Cancer Research 16 (18), 4517–4526. https://doi.org/10.1158/1078-0432.CCR-10-0526.

Antoccia, A., Kobayashi, J., Tauchi, H., Matsuura, S., Komatsu, K., 2006. Nijmegen breakage syndrome and functions of the responsible protein, NBS1. Genome Dynamics 1, 191–205. https://doi.org/10.1159/000092508.

Aparicio, T., Baer, R., Gautier, J., 2014. DNA double-strand break repair pathway choice and cancer. DNA Repair (Amst) 19, 169–175. https://doi.org/10.1016/j.dnarep.2014.03.014.

Arora, H., Chacon, A.H., Choudhary, S., McLeod, M.P., Meshkov, L., Nouri, K., Izakovic, J., 2014. Bloom syndrome. International Journal of Dermatology 53 (7), 798–802. https://doi.org/10.1111/ijd.12408.

Awasthi, P., Foiani, M., Kumar, A., 2015. ATM and ATR signaling at a glance. Journal of Cell Science 128 (23), 4255–4262. https://doi.org/10.1242/jcs.169730.
Bang, Y.J., Im, S.A., Lee, K.W., Cho, J.Y., Song, E.K., Lee, K.H., et al., 2015. Randomized, double-blind phase II trial with prospective classification by ATM protein level to evaluate the efficacy and tolerability of olaparib plus paclitaxel in patients with recurrent or metastatic gastric cancer. Journal of Clinical Oncology 33 (33), 3858–3865. https://doi.org/10.1200/JCO.2014.60.0320.
Bartek, J., Lukas, J., 2001. Mammalian G1- and S-phase checkpoints in response to DNA damage. Current Opinion in Cell Biology 13 (6), 738–747.
Bedard, L.L., Massey, T.E., 2006. Aflatoxin B1-induced DNA damage and its repair. Cancer Letters 241 (2), 174–183. https://doi.org/10.1016/j.canlet.2005.11.018.
Bennardo, N., Cheng, A., Huang, N., Stark, J.M., 2008. Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair. PLoS Genetics 4 (6), e1000110. https://doi.org/10.1371/journal.pgen.1000110.
Berquist, B.R., Wilson 3rd., D.M., 2012. Pathways for repairing and tolerating the spectrum of oxidative DNA lesions. Cancer Letters 327 (1–2), 61–72. https://doi.org/10.1016/j.canlet.2012.02.001.
Berquist, B.R., Singh, D.K., Fan, J., Kim, D., Gillenwater, E., Kulkarni, A., et al., 2010. Functional capacity of XRCC1 protein variants identified in DNA repair-deficient Chinese hamster ovary cell lines and the human population. Nucleic Acids Research 38 (15), 5023–5035. https://doi.org/10.1093/nar/gkq193.
Bhargava, R., Onyango, D.O., Stark, J.M., 2016. Regulation of single-strand annealing and its role in genome maintenance. Trends in Genetics 32 (9), 566–575. https://doi.org/10.1016/j.tig.2016.06.007.
Bhattacharyya, N., Banerjee, S., 1997. A variant of DNA polymerase beta acts as a dominant negative mutant. Proceedings of the National Academy of Sciences of the United States of America 94 (19), 10324–10329.
Brown, E.J., Baltimore, D., 2000. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes & Development 14 (4), 397–402.
Brown, J.S., O'Carrigan, B., Jackson, S.P., Yap, T.A., 2017. Targeting DNA repair in cancer: beyond PARP inhibitors. Cancer Discovery 7 (1), 20–37. https://doi.org/10.1158/2159-8290.CD-16-0860.
Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., et al., 2005. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 434 (7035), 913–917. https://doi.org/10.1038/nature03443.
Bulgar, A.D., Weeks, L.D., Miao, Y., Yang, S., Xu, Y., Guo, C., et al., 2012. Removal of uracil by uracil DNA glycosylase limits pemetrexed cytotoxicity: overriding the limit with methoxyamine to inhibit base excision repair. Cell Death & Disease 3, e252. https://doi.org/10.1038/cddis.2011.135.
Bunting, S.F., Nussenzweig, A., 2013. End-joining, translocations and cancer. Nature Reviews. Cancer 13 (7), 443–454. https://doi.org/10.1038/nrc3537.
Cadet, J., Wagner, J.R., 2013. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harbor Perspectives in Biology 5 (2). https://doi.org/10.1101/cshperspect.a012559.
Cheadle, J.P., Sampson, J.R., 2007. MUTYH-associated polyposis—from defect in base excision repair to clinical genetic testing. DNA Repair (Amst) 6 (3), 274–279. https://doi.org/10.1016/j.dnarep.2006.11.001.
Cheung-Ong, K., Giaever, G., Nislow, C., 2013. DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology. Chemistry & Biology 20 (5), 648–659. https://doi.org/10.1016/j.chembiol.2013.04.007.
Chila, R., Guffanti, F., Damia, G., 2016. Role and therapeutic potential of CDK12 in human cancers. Cancer Treatment Reviews 50, 83–88. https://doi.org/10.1016/j.ctrv.2016.09.003.
Colella, G., Marchini, S., D'Incalci, M., Brown, R., Broggini, M., 1999. Mismatch repair deficiency is associated with resistance to DNA minor groove alkylating agents. British Journal of Cancer 80 (3–4), 338–343. https://doi.org/10.1038/sj.bjc.6690360.
Cortes Ledesma, F., El Khamisy, S.F., Zuma, M.C., Osborn, K., Caldecott, K.W., 2009. A human 5′-tyrosyl DNA phosphodiesterase that repairs topoisomerase-mediated DNA damage. Nature 461 (7264), 674–678. https://doi.org/10.1038/nature08444.
Davis, A.J., Chen, D.J., 2013. DNA double strand break repair via non-homologous end-joining. Translational Cancer Research 2 (3), 130–143. https://doi.org/10.3978/j.issn.2218-676X.2013.04.02.
Deans, A.J., West, S.C., 2011. DNA interstrand crosslink repair and cancer. Nature Reviews. Cancer 11 (7), 467–480. https://doi.org/10.1038/nrc3088.
Della-Maria, J., Zhou, Y., Tsai, M.S., Kuhnlein, J., Carney, J.P., Paull, T.T., Tomkinson, A.E., 2011. Human Mre11/human Rad50/Nbs1 and DNA ligase IIIalpha/XRCC1 protein complexes act together in an alternative nonhomologous end joining pathway. Journal of Biological Chemistry 286 (39), 33845–33853. https://doi.org/10.1074/jbc.M111.274159.
Demple, B., Harrison, L., 1994. Repair of oxidative damage to DNA: enzymology and biology. Annual Review of Biochemistry 63, 915–948. https://doi.org/10.1146/annurev.bi.63.070194.004411.
Dianov, G., Lindahl, T., 1994. Reconstitution of the DNA base excision-repair pathway. Current Biology 4 (12), 1069–1076.
Diatloff-Zito, C., Papadopoulo, D., Averbeck, D., Moustacchi, E., 1986. Abnormal response to DNA crosslinking agents of Fanconi anemia fibroblasts can be corrected by transfection with normal human DNA. Proceedings of the National Academy of Sciences of the United States of America 83 (18), 7034–7038.
Difilippantonio, M.J., Zhu, J., Chen, H.T., Meffre, E., Nussenzweig, M.C., Max, E.E., et al., 2000. DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. Nature 404 (6777), 510–514. https://doi.org/10.1038/35006670.
Dizdaroglu, M., Coskun, E., Jaruga, P., 2017. Repair of oxidatively induced DNA damage by DNA glycosylases: mechanisms of action, substrate specificities and excision kinetics. Mutation Research 771, 99–127. https://doi.org/10.1016/j.mrrev.2017.02.001.
Drean, A., Lord, C.J., Ashworth, A., 2016. PARP inhibitor combination therapy. Critical Reviews in Oncology/Hematology 108, 73–85. https://doi.org/10.1016/j.critrevonc.2016.10.010.
Du, L., Wang, H., Xiong, T., Ma, Y., Yang, J., Huang, J., et al., 2013. The polymorphisms in the MGMT gene and the risk of cancer: a meta-analysis. Tumour Biology 34 (5), 3227–3237. https://doi.org/10.1007/s13277-013-0893-x.
Dumenco, L.L., Allay, E., Norton, K., Gerson, S.L., 1993. The prevention of thymic lymphomas in transgenic mice by human O6-alkylguanine-DNA alkyltransferase. Science 259 (5092), 219–222.
Dupuy, A., Sarasin, A., 2015. DNA damage and gene therapy of xeroderma pigmentosum, a human DNA repair-deficient disease. Mutation Research 776, 2–8. https://doi.org/10.1016/j.mrfmmm.2014.08.007.
Economopoulou, P., Dimitriadis, G., Psyrri, A., 2015. Beyond BRCA: new hereditary breast cancer susceptibility genes. Cancer Treatment Reviews 41 (1), 1–8. https://doi.org/10.1016/j.ctrv.2014.10.008.
Edenberg, E.R., Downey, M., Toczyski, D., 2014. Polymerase stalling during replication, transcription and translation. Current Biology 24 (10), R445–452. https://doi.org/10.1016/j.cub.2014.03.060.
Esteller, M., Silva, J.M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., et al., 2000. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. Journal of the National Cancer Institute 92 (7), 564–569.
Fagbemi, A.F., Orelli, B., Scharer, O.D., 2011. Regulation of endonuclease activity in human nucleotide excision repair. DNA Repair (Amst) 10 (7), 722–729. https://doi.org/10.1016/j.dnarep.2011.04.022.
Fang, Y., Tsao, C.C., Goodman, B.K., Furumai, R., Tirado, C.A., Abraham, R.T., Wang, X.F., 2004. ATR functions as a gene dosage-dependent tumor suppressor on a mismatch repair-deficient background. EMBO Journal 23 (15), 3164–3174. https://doi.org/10.1038/sj.emboj.7600315.
Fenech, M., 2002. Biomarkers of genetic damage for cancer epidemiology. Toxicology 181–182, 411–416.
Fink, D., Aebi, S., Howell, S.B., 1998. The role of DNA mismatch repair in drug resistance. Clinical Cancer Research 4 (1), 1–6.
Fishel, R., 2015. Mismatch repair. Journal of Biological Chemistry 290 (44), 26395–26403. https://doi.org/10.1074/jbc.R115.660142.

Forment, J.V., Kaidi, A., Jackson, S.P., 2012. Chromothripsis and cancer: causes and consequences of chromosome shattering. Nature Reviews. Cancer 12 (10), 663–670. https://doi.org/10.1038/nrc3352.

Fortini, P., Pascucci, B., Parlanti, E., Sobol, R.W., Wilson, S.H., Dogliotti, E., 1998. Different DNA polymerases are involved in the short- and long-patch base excision repair in mammalian cells. Biochemistry 37 (11), 3575–3580. https://doi.org/10.1021/bi972999h.

Fousteri, M., Vermeulen, W., van Zeeland, A.A., Mullenders, L.H., 2006. Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. Molecular Cell 23 (4), 471–482. https://doi.org/10.1016/j.molcel.2006.06.029.

Frit, P., Barboule, N., Yuan, Y., Gomez, D., Calsou, P., 2014. Alternative end-joining pathway(s): bricolage at DNA breaks. DNA Repair (Amst) 17, 81–97. https://doi.org/10.1016/j.dnarep.2014.02.007.

Fung, H., Demple, B., 2005. A vital role for Ape1/Ref1 protein in repairing spontaneous DNA damage in human cells. Molecular Cell 17 (3), 463–470. https://doi.org/10.1016/j.molcel.2004.12.029.

Gavande, N.S., VanderVere-Carozza, P.S., Hinshaw, H.D., Jalal, S.I., Sears, C.R., Pawelczak, K.S., Turchi, J.J., 2016. DNA repair targeted therapy: the past or future of cancer treatment? Pharmacology & Therapeutics 160, 65–83. https://doi.org/10.1016/j.pharmthera.2016.02.003.

Gelsomino, F., Barbolini, M., Spallanzani, A., Pugliese, G., Cascinu, S., 2016. The evolving role of microsatellite instability in colorectal cancer: a review. Cancer Treatment Reviews 51, 19–26. https://doi.org/10.1016/j.ctrv.2016.10.005.

Glassner, B.J., Weeda, G., Allan, J.M., Broekhof, J.L., Carls, N.H., Donker, I., et al., 1999. DNA repair methyltransferase (Mgmt) knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents. Mutagenesis 14 (3), 339–347.

Goellner, E.M., Putnam, C.D., Kolodner, R.D., 2015. Exonuclease 1-dependent and independent mismatch repair. DNA Repair (Amst) 32, 24–32. https://doi.org/10.1016/j.dnarep.2015.04.010.

Gray, S., Cohen, P.E., 2016. Control of meiotic crossovers: from double-strand break formation to designation. Annual Review of Genetics 50, 175–210. https://doi.org/10.1146/annurev-genet-120215-035111.

Guo, Z., Zheng, L., Dai, H., Zhou, M., Xu, H., Shen, B., 2009. Human DNA polymerase beta polymorphism, Arg137Gln, impairs its polymerase activity and interaction with PCNA and the cellular base excision repair capacity. Nucleic Acids Research 37 (10), 3431–3441. https://doi.org/10.1093/nar/gkp201.

Haber, D.A., 2003. The BRCA2-EMSY connection: implications for breast and ovarian tumorigenesis. Cell 115 (5), 507–508.

Haberl, S., Haferlach, T., Stengel, A., Jeromin, S., Kern, W., Haferlach, C., 2016. MYC rearranged B-cell neoplasms: impact of genetics on classification. Cancer Genetics 209 (10), 431–439. https://doi.org/10.1016/j.cancergen.2016.08.007.

Hadi, M.Z., Coleman, M.A., Fidelis, K., Mohrenweiser, H.W., Wilson 3rd., D.M., 2000. Functional characterization of Ape1 variants identified in the human population. Nucleic Acids Research 28 (20), 3871–3879.

Hathcock, K.S., Padilla-Nash, H.M., Camps, J., Shin, D.M., Triner, D., Shaffer 3rd, A.L., et al., 2015. ATM deficiency promotes development of murine B-cell lymphomas that resemble diffuse large B-cell lymphoma in humans. Blood 126 (20), 2291–2301. https://doi.org/10.1182/blood-2015-06-654749.

Haynes, B., Saadat, N., Myung, B., Shekhar, M.P., 2015. Crosstalk between translesion synthesis, Fanconi anemia network, and homologous recombination repair pathways in interstrand DNA crosslink repair and development of chemoresistance. Mutation Research, Reviews in Mutation Research 763, 258–266. https://doi.org/10.1016/j.mrrev.2014.11.005.

Hecht, F., Hecht, B.K., 1987. Chromosome changes connect immunodeficiency and cancer in ataxia-telangiectasia. American Journal of Pediatric Hematology/Oncology 9 (2), 185–188.

Hecht, F., Hecht, B.K., 1990. Cancer in ataxia-telangiectasia patients. Cancer Genetics and Cytogenetics 46 (1), 9–19.

Heikkinen, K., Mansikka, V., Karppinen, S.M., Rapakko, K., Winqvist, R., 2005. Mutation analysis of the ATR gene in breast and ovarian cancer families. Breast Cancer Research 7 (4), R495–501. https://doi.org/10.1186/bcr1037.

Hickson, I., Zhao, Y., Richardson, C.J., Green, S.J., Martin, N.M., Orr, A.I., et al., 2004. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer Research 64 (24), 9152–9159. https://doi.org/10.1158/0008-5472.CAN-04-2727.

Hitchins, M., Williams, R., Cheong, K., Halani, N., Lin, V.A., Packham, D., et al., 2005. MLH1 germline epimutations as a factor in hereditary nonpolyposis colorectal cancer. Gastroenterology 129 (5), 1392–1399. https://doi.org/10.1053/j.gastro.2005.09.003.

Hoch, N.C., Hanzlikova, H., Rulten, S.L., Tetreault, M., Komulainen, E., Ju, L., et al., 2017. XRCC1 mutation is associated with PARP1 hyperactivation and cerebellar ataxia. Nature 541 (7635), 87–91. https://doi.org/10.1038/nature20790.

Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., et al., 2002. Biallelic inactivation of BRCA2 in Fanconi anemia. Science 297 (5581), 606–609. https://doi.org/10.1126/science.1073834.

Hung, R.J., Hall, J., Brennan, P., Boffetta, P., 2005. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. American Journal of Epidemiology 162 (10), 925–942. https://doi.org/10.1093/aje/kwi318.

Iliakis, G., Murmann, T., Soni, A., 2015. Alternative end-joining repair pathways are the ultimate backup for abrogated classical non-homologous end-joining and homologous recombination repair: implications for the formation of chromosome translocations. Mutation Research, Genetic Toxicology and Environmental Mutagenesis 793, 166–175. https://doi.org/10.1016/j.mrgentox.2015.07.001.

Illuzzi, J.L., Harris, N.A., Manvilla, B.A., Kim, D., Li, M., Drohat, A.C., Wilson 3rd., D.M., 2013. Functional assessment of population and tumor-associated APE1 protein variants. PLoS One 8 (6), e65922. https://doi.org/10.1371/journal.pone.0065922.

Illuzzi, J.L., McNeill, D.R., Bastian, P., Brenerman, B., Wersto, R., Russell, H.R., et al., 2017. Tumor-associated APE1 variant exhibits reduced complementation efficiency but does not promote cancer cell phenotypes. Environmental and Molecular Mutagenesis 58 (2), 84–98. https://doi.org/10.1002/em.22074.

Iyama, T., Wilson 3rd., D.M., 2013. DNA repair mechanisms in dividing and non-dividing cells. DNA Repair (Amst) 12 (8), 620–636. https://doi.org/10.1016/j.dnarep.2013.04.015.

Iyama, T., Lee, S.Y., Berquist, B.R., Gileadi, O., Bohr, V.A., Seidman, M.M., et al., 2015. CSB interacts with SNM1A and promotes DNA interstrand crosslink processing. Nucleic Acids Research 43 (1), 247–258. https://doi.org/10.1093/nar/gku1279.

Iyer, D.R., Rhind, N., 2017. The intra-S checkpoint responses to DNA damage. Genes (Basel) 8 (2). https://doi.org/10.3390/genes8020074.

Jackson, S.P., Bartek, J., 2009. The DNA-damage response in human biology and disease. Nature 461 (7267), 1071–1078. https://doi.org/10.1038/nature08467.

Jacobs, A.C., Calkins, M.J., Jadhav, A., Dorjsuren, D., Maloney, D., Simeonov, A., et al., 2013. Inhibition of DNA glycosylases via small molecule purine analogs. PLoS One 8 (12), e81667. https://doi.org/10.1371/journal.pone.0081667.

Janatova, M., Zikan, M., Dundr, P., Matous, B., Pohlreich, P., 2005. Novel somatic mutations in the BRCA1 gene in sporadic breast tumors. Human Mutation 25 (3), 319. https://doi.org/10.1002/humu.9308.

Jasin, M., Rothstein, R., 2013. Repair of strand breaks by homologous recombination. Cold Spring Harbor Perspectives in Biology 5 (11), a012740. https://doi.org/10.1101/cshperspect.a012740.

Jaspers, N.G., Raams, A., Silengo, M.C., Wijgers, N., Niedernhofer, L.J., Robinson, A.R., et al., 2007. First reported patient with human ERCC1 deficiency has cerebro-oculo-facio-skeletal syndrome with a mild defect in nucleotide excision repair and severe developmental failure. American Journal of Human Genetics 80 (3), 457–466. https://doi.org/10.1086/512486.

Kaina, B., Christmann, M., 2002. DNA repair in resistance to alkylating anticancer drugs. International Journal of Clinical Pharmacology and Therapeutics 40 (8), 354–367.

Kapetanaki, M.G., Guerrero-Santoro, J., Bisi, D.C., Hsieh, C.L., Rapic-Otrin, V., Levine, A.S., 2006. The DDB1-CUL4ADDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites. Proceedings of the National Academy of Sciences of the United States of America 103 (8), 2588–2593. https://doi.org/10.1073/pnas.0511160103.

Karahalil, B., Bohr, V.A., Wilson 3rd., D.M., 2012. Impact of DNA polymorphisms in key DNA base excision repair proteins on cancer risk. Human & Experimental Toxicology 31 (10), 981–1005. https://doi.org/10.1177/0960327112444476.
Karmakar, P., Piotrowski, J., Brosh Jr., R.M., Sommers, J.A., Miller, S.P., Cheng, W.H., et al., 2002a. Werner protein is a target of DNA-dependent protein kinase in vivo and in vitro, and its catalytic activities are regulated by phosphorylation. Journal of Biological Chemistry 277 (21), 18291–18302. https://doi.org/10.1074/jbc.M111523200.
Karmakar, P., Snowden, C.M., Ramsden, D.A., Bohr, V.A., 2002b. Ku heterodimer binds to both ends of the Werner protein and functional interaction occurs at the Werner N-terminus. Nucleic Acids Research 30 (16), 3583–3591.
Kawate, H., Sakumi, K., Tsuzuki, T., Nakatsuru, Y., Ishikawa, T., Takahashi, S., et al., 1998. Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes. Proceedings of the National Academy of Sciences of the United States of America 95 (9), 5116–5120.
Kitano, K., 2014. Structural mechanisms of human RecQ helicases WRN and BLM. Frontiers in Genetics 5, 366. https://doi.org/10.3389/fgene.2014.00366.
Knudson Jr., A.G., 1971. Mutation and cancer: statistical study of retinoblastoma. Proceedings of the National Academy of Sciences of the United States of America 68 (4), 820–823.
Kottemann, M.C., Smogorzewska, A., 2013. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature 493 (7432), 356–363. https://doi.org/10.1038/nature11863.
Krokan, H.E., Bjoras, M., 2013. Base excision repair. Cold Spring Harbor Perspectives in Biology 5 (4), a012583. https://doi.org/10.1101/cshperspect.a012583.
Kusumoto, R., Dawut, L., Marchetti, C., Wan Lee, J., Vindigni, A., Ramsden, D., Bohr, V.A., 2008. Werner protein cooperates with the XRCC4-DNA ligase IV complex in end-processing. Biochemistry 47 (28), 7548–7556. https://doi.org/10.1021/bi702325t.
Lange, S.S., Takata, K., Wood, R.D., 2011. DNA polymerases and cancer. Nature Reviews. Cancer 11 (2), 96–110. https://doi.org/10.1038/nrc2998.
Larsen, E., Reite, K., Nesse, G., Gran, C., Seeberg, E., Klungland, A., 2006. Repair and mutagenesis at oxidized DNA lesions in the developing brain of wild-type and Ogg1 −/− mice. Oncogene 25 (17), 2425–2432. https://doi.org/10.1038/sj.onc.1209284.
Laugel, V., 2013. Cockayne syndrome: the expanding clinical and mutational spectrum. Mechanisms of Ageing and Development 134 (5–6), 161–170. https://doi.org/10.1016/j.mad.2013.02.006.
Lauper, J.M., Krause, A., Vaughan, T.L., Monnat Jr., R.J., 2013. Spectrum and risk of neoplasia in Werner syndrome: a systematic review. PLoS One 8 (4), e59709. https://doi.org/10.1371/journal.pone.0059709.
Le Deist, F., Poinsignon, C., Moshous, D., Fischer, A., de Villartay, J.P., 2004. Artemis sheds new light on V(D)J recombination. Immunological Reviews 200, 142–155. https://doi.org/10.1111/j.0105-2896.2004.00169.x.
Leandro, G.S., Sykora, P., Bohr, V.A., 2015. The impact of base excision DNA repair in age-related neurodegenerative diseases. Mutation Research 776, 31–39. https://doi.org/10.1016/j.mrfmmm.2014.12.011.
Lewis, K.A., Mullany, S., Thomas, B., Chien, J., Loewen, R., Shridhar, V., Cliby, W.A., 2005. Heterozygous ATR mutations in mismatch repair-deficient cancer cells have functional significance. Cancer Research 65 (16), 7091–7095. https://doi.org/10.1158/0008-5472.CAN-05-1019.
Li, M., Wilson III, D.M., 2013. Major human apurinic/apyrimidinic endonuclease, APE1. Antioxidants & Redox Signaling 20, 678–707. https://doi.org/10.1089/ars.2013.5492.
Li, X., Yao, X., Wang, Y., Hu, F., Wang, F., Jiang, L., et al., 2013. MLH1 promoter methylation frequency in colorectal cancer patients and related clinicopathological and molecular features. PLoS One 8 (3), e59064. https://doi.org/10.1371/journal.pone.0059064.
Lindahl, T., 1993. Instability and decay of the primary structure of DNA. Nature 362 (6422), 709–715.
Lirussi, L., Antoniali, G., D'Ambrosio, C., Scaloni, A., Nilsen, H., Tell, G., 2016. APE1 polymorphic variants cause persistent genomic stress and affect cancer cell proliferation. Oncotarget 7 (18), 26293–26306. https://doi.org/10.18632/oncotarget.8477.
Lobrich, M., Jeggo, P.A., 2007. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nature Reviews. Cancer 7 (11), 861–869. https://doi.org/10.1038/nrc2248.
Loeb, L.A., 1994. Microsatellite instability: marker of a mutator phenotype in cancer. Cancer Research 54 (19), 5059–5063.
Loeb, L.A., 2016. Human cancers express a mutator phenotype: hypothesis, origin, and consequences. Cancer Research 76 (8), 2057–2059. https://doi.org/10.1158/0008-5472.CAN-16-0794.
Lord, C.J., Ashworth, A., 2016. BRCAness revisited. Nature Reviews. Cancer 16 (2), 110–120. https://doi.org/10.1038/nrc.2015.21.
Lord, C.J., Ashworth, A., 2017. PARP inhibitors: synthetic lethality in the clinic. Science 355 (6330), 1152–1158. https://doi.org/10.1126/science.aam7344.
Ludwig, D.L., MacInnes, M.A., Takiguchi, Y., Purtymun, P.E., Henrie, M., Flannery, M., et al., 1998. A murine AP-endonuclease gene-targeted deficiency with post-implantation embryonic progression and ionizing radiation sensitivity. Mutation Research 409 (1), 17–29.
Manthei, K.A., Keck, J.L., 2013. The BLM dissolvasome in DNA replication and repair. Cellular and Molecular Life Sciences 70 (21), 4067–4084. https://doi.org/10.1007/s00018-013-1325-1.
Marteijn, J.A., Lans, H., Vermeulen, W., Hoeijmakers, J.H., 2014. Understanding nucleotide excision repair and its roles in cancer and ageing. Nature Reviews. Molecular Cell Biology 15 (7), 465–481. https://doi.org/10.1038/nrm3822.
Matsumoto, Y., Kim, K., 1995. Excision of deoxyribose phosphate residues by DNA polymerase beta during DNA repair. Science 269 (5224), 699–702.
McNeill, D.R., Lin, P.C., Miller, M.G., Pistell, P.J., de Souza-Pinto, N.C., Fishbein, K.W., et al., 2011. XRCC1 haploinsufficiency in mice has little effect on aging, but adversely modifies exposure-dependent susceptibility. Nucleic Acids Research 39 (18), 7992–8004. https://doi.org/10.1093/nar/gkr280.
Mehta, A., Haber, J.E., 2014. Sources of DNA double-strand breaks and models of recombinational DNA repair. Cold Spring Harbor Perspectives in Biology 6 (9), a016428. https://doi.org/10.1101/cshperspect.a016428.
Mehta, M.P., Wang, D., Wang, F., Kleinberg, L., Brade, A., Robins, H.I., et al., 2015. Veliparib in combination with whole brain radiation therapy in patients with brain metastases: results of a phase 1 study. Journal of Neuro-Oncology 122 (2), 409–417. https://doi.org/10.1007/s11060-015-1733-1.
Ming, M., He, Y.Y., 2012. PTEN in DNA damage repair. Cancer Letters 319 (2), 125–129. https://doi.org/10.1016/j.canlet.2012.01.003.
Mirza, M.R., Monk, B.J., Herrstedt, J., Oza, A.M., Mahner, S., Redondo, A., et al., 2016. Niraparib maintenance therapy in platinum-sensitive, recurrent ovarian cancer. New England Journal of Medicine 375 (22), 2154–2164. https://doi.org/10.1056/NEJMoa1611310.
Morrical, S.W., 2015. DNA-pairing and annealing processes in homologous recombination and homology-directed repair. Cold Spring Harbor Perspectives in Biology 7 (2), a016444. https://doi.org/10.1101/cshperspect.a016444.
Moser, J., Kool, H., Giakzidis, I., Caldecott, K., Mullenders, L.H., Fousteri, M.I., 2007. Sealing of chromosomal DNA nicks during nucleotide excision repair requires XRCC1 and DNA ligase III alpha in a cell-cycle-specific manner. Molecular Cell 27 (2), 311–323. https://doi.org/10.1016/j.molcel.2007.06.014.
Moynahan, M.E., Jasin, M., 2010. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nature Reviews. Molecular Cell Biology 11 (3), 196–207. https://doi.org/10.1038/nrm2851.
Muniandy, P.A., Thapa, D., Thazhathveetil, A.K., Liu, S.T., Seidman, M.M., 2009. Repair of laser-localized DNA interstrand cross-links in G1 phase mammalian cells. Journal of Biological Chemistry 284 (41), 27908–27917. https://doi.org/10.1074/jbc.M109.029025.
Murata, M., Kawanishi, S., 2011. Mechanisms of oxidative DNA damage induced by carcinogenic arylamines. Frontiers in Bioscience (Landmark Ed) 16, 1132–1143.
Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochemical Journal 417 (1), 1–13. https://doi.org/10.1042/BJ20081386.
Natale, V., Raquer, H., 2017. Xeroderma pigmentosum-Cockayne syndrome complex. Orphanet Journal of Rare Diseases 12 (1), 65. https://doi.org/10.1186/s13023-017-0616-2.
Niedernhofer, L.J., Garinis, G.A., Raams, A., Lalai, A.S., Robinson, A.R., Appeldoorn, E., et al., 2006. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature 444 (7122), 1038–1043. https://doi.org/10.1038/nature05456.
Nimonkar, A.V., Genschel, J., Kinoshita, E., Polaczek, P., Campbell, J.L., Wyman, C., et al., 2011. BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. Genes & Development 25 (4), 350–362. https://doi.org/10.1101/gad.2003811.

Nissenkorn, A., Ben-Zeev, B., 2015. Ataxia telangiectasia. Handbook of Clinical Neurology 132, 199–214. https://doi.org/10.1016/B978-0-444-62702-5.00014-7.
O'Driscoll, M., Ruiz-Perez, V.L., Woods, C.G., Jeggo, P.A., Goodship, J.A., 2003. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nature Genetics 33 (4), 497–501. https://doi.org/10.1038/ng1129.
Olinski, R., Jurgowiak, M., Zaremba, T., 2010. Uracil in DNA—its biological significance. Mutation Research 705 (3), 239–245. https://doi.org/10.1016/j.mrrev.2010.08.001.
Opresko, P.L., Cheng, W.H., von Kobbe, C., Harrigan, J.A., Bohr, V.A., 2003. Werner syndrome and the function of the Werner protein; what they can teach us about the molecular aging process. Carcinogenesis 24 (5), 791–802.
Oshima, J., Sidorova, J.M., Monnat Jr., R.J., 2017. Werner syndrome: clinical features, pathogenesis and potential therapeutic interventions. Ageing Research Reviews 33, 105–114. https://doi.org/10.1016/j.arr.2016.03.002.
Oza, A.M., Cibula, D., Benzaquen, A.O., Poole, C., Mathijssen, R.H., Sonke, G.S., et al., 2015. Olaparib combined with chemotherapy for recurrent platinum-sensitive ovarian cancer: a randomised phase 2 trial. The Lancet Oncology 16 (1), 87–97. https://doi.org/10.1016/S1470-2045(14)71135-0.
Palovcak, A., Liu, W., Yuan, F., Zhang, Y., 2017. Maintenance of genome stability by Fanconi anemia proteins. Cell & Bioscience 7, 8. https://doi.org/10.1186/s13578-016-0134-2.
Panier, S., Boulton, S.J., 2014. Double-strand break repair: 53BP1 comes into focus. Nature Reviews. Molecular Cell Biology 15 (1), 7–18. https://doi.org/10.1038/nrm3719.
Pannunzio, N.R., Li, S., Watanabe, G., Lieber, M.R., 2014. Non-homologous end joining often uses microhomology: implications for alternative end joining. DNA Repair (Amst) 17, 74–80. https://doi.org/10.1016/j.dnarep.2014.02.006.
Papadopoulos, N., Lindblom, A., 1997. Molecular basis of HNPCC: mutations of MMR genes. Human Mutation 10 (2), 89–99. https://doi.org/10.1002/(SICI)1098-1004(1997)10:2<89::AID-HUMU1>3.0.CO;2-H.
Paul, A., Paul, S., 2014. The breast cancer susceptibility genes (BRCA) in breast and ovarian cancers. Frontiers in Bioscience (Landmark Ed) 19, 605–618.
Phillips, D.H., Venitt, S., 2012. DNA and protein adducts in human tissues resulting from exposure to tobacco smoke. International Journal of Cancer 131 (12), 2733–2753. https://doi.org/10.1002/ijc.27827.
Pommier, Y., Redon, C., Rao, V.A., Seiler, J.A., Sordet, O., Takemura, H., et al., 2003. Repair of and checkpoint response to topoisomerase I-mediated DNA damage. Mutation Research 532 (1–2), 173–203 doi:S002751070300201X [pii].
Price, B.D., D'Andrea, A.D., 2013. Chromatin remodeling at DNA double-strand breaks. Cell 152 (6), 1344–1354. https://doi.org/10.1016/j.cell.2013.02.011.
Radhakrishnan, S.K., Jette, N., Lees-Miller, S.P., 2014. Non-homologous end joining: emerging themes and unanswered questions. DNA Repair (Amst) 17, 2–8. https://doi.org/10.1016/j.dnarep.2014.01.009.
Ray, S., Menezes, M.R., Senejani, A., Sweasy, J.B., 2013. Cellular roles of DNA polymerase beta. Yale Journal of Biology and Medicine 86 (4), 463–469.
Robins, H.I., Zhang, P., Gilbert, M.R., Chakravarti, A., de Groot, J.F., Grimm, S.A., et al., 2016. A randomized phase I/II study of ABT-888 in combination with temozolomide in recurrent temozolomide resistant glioblastoma: an NRG oncology RTOG group study. Journal of Neuro-Oncology 126 (2), 309–316. https://doi.org/10.1007/s11060-015-1966-z.
Robson, M., Im, S.A., Senkus, E., Xu, B., Domchek, S.M., Masuda, N., et al., 2017. Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. New England Journal of Medicine 377 (6), 523–533. https://doi.org/10.1056/NEJMoa1706450.
Roth, D.B., 2014. V(D)J recombination: mechanism, errors, and fidelity. Microbiology spectrum 2 (6). https://doi.org/10.1128/microbiolspec.MDNA3-0041-2014.
Rydberg, B., Lindahl, T., 1982. Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. EMBO Journal 1 (2), 211–216.
Salk, D., Au, K., Hoehn, H., Martin, G.M., 1981. Cytogenetics of Werner's syndrome cultured skin fibroblasts: variegated translocation mosaicism. Cytogenetics and Cell Genetics 30 (2), 92–107.
Sanjiv, K., Hagenkort, A., Calderon-Montano, J.M., Koolmeister, T., Reaper, P.M., Mortusewicz, O., et al., 2016. Cancer-specific synthetic lethality between ATR and CHK1 kinase activities. Cell Reports 14 (2), 298–309. https://doi.org/10.1016/j.celrep.2015.12.032.
Sartori, A.A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., et al., 2007. Human CtIP promotes DNA end resection. Nature 450 (7169), 509–514. https://doi.org/10.1038/nature06337.
Sawyer, S.L., Tian, L., Kahkonen, M., Schwartzentruber, J., Kircher, M., University of Washington Centre for Mendelian Genomics, et al., 2015. Biallelic mutations in BRCA1 cause a new Fanconi anemia subtype. Cancer Discovery 5 (2), 135–142. https://doi.org/10.1158/2159-8290.CD-14-1156.
Scrima, A., Konickova, R., Czyzewski, B.K., Kawasaki, Y., Jeffrey, P.D., Groisman, R., et al., 2008. Structural basis of UV DNA-damage recognition by the DDB1-DDB2 complex. Cell 135 (7), 1213–1223. https://doi.org/10.1016/j.cell.2008.10.045.
Shamanna, R.A., Lu, H., de Freitas, J.K., Tian, J., Croteau, D.L., Bohr, V.A., 2016. WRN regulates pathway choice between classical and alternative non-homologous end joining. Nature Communications 7, 13785. https://doi.org/10.1038/ncomms13785.
Simonelli, V., Camerini, S., Mazzei, F., Van Loon, B., Allione, A., D'Errico, M., et al., 2013. Genotype-phenotype analysis of S326C OGG1 polymorphism: a risk factor for oxidative pathologies. Free Radical Biology & Medicine 63, 401–409. https://doi.org/10.1016/j.freeradbiomed.2013.05.031.
Sirbu, B.M., Cortez, D., 2013. DNA damage response: three levels of DNA repair regulation. Cold Spring Harbor Perspectives in Biology 5 (8), a012724. https://doi.org/10.1101/cshperspect.a012724.
Sjolund, A., Nemec, A.A., Paquet, N., Prakash, A., Sung, P., Doublie, S., Sweasy, J.B., 2014. A germline polymorphism of thymine DNA glycosylase induces genomic instability and cellular transformation. PLoS Genetics 10 (11), e1004753. https://doi.org/10.1371/journal.pgen.1004753.
Sobol, R.W., 2012. Genome instability caused by a germline mutation in the human DNA repair gene POLB. PLoS Genetics 8 (11), e1003086. https://doi.org/10.1371/journal.pgen.1003086.
Song, M.S., Salmena, L., Pandolfi, P.P., 2012. The functions and regulation of the PTEN tumour suppressor. Nature Reviews. Molecular Cell Biology 13 (5), 283–296. https://doi.org/10.1038/nrm3330.
Stracker, T.H., Petrini, J.H., 2011. The MRE11 complex: starting from the ends. Nature Reviews. Molecular Cell Biology 12 (2), 90–103. https://doi.org/10.1038/nrm3047.
Sturzenegger, A., Burdova, K., Kanagaraj, R., Levikova, M., Pinto, C., Cejka, P., Janscak, P., 2014. DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA end resection in human cells. Journal of Biological Chemistry 289 (39), 27314–27326. https://doi.org/10.1074/jbc.M114.578823.
Sugasawa, K., Ng, J.M., Masutani, C., Iwai, S., van der Spek, P.J., Eker, A.P., et al., 1998. Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. Molecular Cell 2 (2), 223–232.
Sugasawa, K., Akagi, J., Nishi, R., Iwai, S., Hanaoka, F., 2009. Two-step recognition of DNA damage for mammalian nucleotide excision repair: directional binding of the XPC complex and DNA strand scanning. Molecular Cell 36 (4), 642–653. https://doi.org/10.1016/j.molcel.2009.09.035.
Sy, S.M., Huen, M.S., Chen, J., 2009. PALB2 is an integral component of the BRCA complex required for homologous recombination repair. Proceedings of the National Academy of Sciences of the United States of America 106 (17), 7155–7160. https://doi.org/10.1073/pnas.0811159106.
Sykora, P., Wilson 3rd, D.M., Bohr, V.A., 2013. Base excision repair in the mammalian brain: implication for age related neurodegeneration. Mechanisms of Ageing and Development 134 (10), 440–448. https://doi.org/10.1016/j.mad.2013.04.005.
Symington, L.S., Gautier, J., 2011. Double-strand break end resection and repair pathway choice. Annual Review of Genetics 45, 247–271. https://doi.org/10.1146/annurev-genet-110410-132435.
Talseth-Palmer, B.A., 2017. The genetic basis of colonic adenomatous polyposis syndromes. Hereditary Cancer in Clinical Practice 15, 5. https://doi.org/10.1186/s13053-017-0065-x.
Taverna, P., Liu, L., Hwang, H.S., Hanson, A.J., Kinsella, T.J., Gerson, S.L., 2001. Methoxyamine potentiates DNA single strand breaks and double strand breaks induced by temozolomide in colon cancer cells. Mutation Research 485 (4), 269–281.

Tebbs, R.S., Flannery, M.L., Meneses, J.J., Hartmann, A., Tucker, J.D., Thompson, L.H., et al., 1999. Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. Developmental Biology 208 (2), 513–529. https://doi.org/10.1006/dbio.1999.9232.
Teive, H.A., Moro, A., Moscovich, M., Arruda, W.O., Munhoz, R.P., Raskin, S., Ashizawa, T., 2015. Ataxia-telangiectasia—a historical review and a proposal for a new designation: ATM syndrome. Journal of the Neurological Sciences 355 (1–2), 3–6. https://doi.org/10.1016/j.jns.2015.05.022.
Thiagalingam, S., Foy, R.L., Cheng, K.H., Lee, H.J., Thiagalingam, A., Ponte, J.F., 2002. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. Current Opinion in Oncology 14 (1), 65–72.
Thompson, L.H., Hinz, J.M., 2009. Cellular and molecular consequences of defective Fanconi anemia proteins in replication-coupled DNA repair: mechanistic insights. Mutation Research 668 (1–2), 54–72. https://doi.org/10.1016/j.mrfmmm.2009.02.003.
Tomasetti, C., Li, L., Vogelstein, B., 2017. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. Science 355 (6331), 1330–1334. https://doi.org/10.1126/science.aaf9011.
Turrens, J.F., Boveris, A., 1980. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochemical Journal 191 (2), 421–427.
Uematsu, N., Weterings, E., Yano, K., Morotomi-Yano, K., Jakob, B., Taucher-Scholz, G., et al., 2007. Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks. Journal of Cell Biology 177 (2), 219–229. https://doi.org/10.1083/jcb.200608077.
van Os, N.J., Roeleveld, N., Weemaes, C.M., Jongmans, M.C., Janssens, G.O., Taylor, A.M., et al., 2016. Health risks for ataxia-telangiectasia mutated heterozygotes: a systematic review, meta-analysis and evidence-based guideline. Clinical Genetics 90 (2), 105–117. https://doi.org/10.1111/cge.12710.
Vasquez, K.M., 2010. Targeting and processing of site-specific DNA interstrand crosslinks. Environmental and Molecular Mutagenesis 51 (6), 527–539. https://doi.org/10.1002/em.20557.
Volker, M., Mone, M.J., Karmakar, P., van Hoffen, A., Schul, W., Vermeulen, W., et al., 2001. Sequential assembly of the nucleotide excision repair factors in vivo. Molecular Cell 8 (1), 213–224.
Walden, H., Deans, A.J., 2014. The Fanconi anemia DNA repair pathway: structural and functional insights into a complex disorder. Annual Review of Biophysics 43, 257–278. https://doi.org/10.1146/annurev-biophys-051013-022737.
Wallace, S.S., 2014. Base excision repair: a critical player in many games. DNA Repair (Amst) 19, 14–26. https://doi.org/10.1016/j.dnarep.2014.03.030.
Wang, M., Wu, W., Wu, W., Rosidi, B., Zhang, L., Wang, H., Iliakis, G., 2006. PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Research 34 (21), 6170–6182. https://doi.org/10.1093/nar/gkl840.
Weinfeld, M., Mani, R.S., Abdou, I., Aceytuno, R.D., Glover, J.N., 2011. Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. Trends in Biochemical Sciences 36 (5), 262–271. https://doi.org/10.1016/j.tibs.2011.01.006.
White, J., Joiner, M.C., 2006. Toxicity from radiation in breast cancer. Cancer Treatment and Research 128, 65–109.
Wick, W., Weller, M., van den Bent, M., Sanson, M., Weiler, M., von Deimling, A., et al., 2014. MGMT testing—the challenges for biomarker-based glioma treatment. Nature Reviews. Neurology 10 (7), 372–385. https://doi.org/10.1038/nrneurol.2014.100.
Winkler, G.S., Araujo, S.J., Fiedler, U., Vermeulen, W., Coin, F., Egly, J.M., et al., 2000. TFIIH with inactive XPD helicase functions in transcription initiation but is defective in DNA repair. Journal of Biological Chemistry 275 (6), 4258–4266.
Woodbine, L., Gennery, A.R., Jeggo, P.A., 2014. The clinical impact of deficiency in DNA non-homologous end-joining. DNA Repair (Amst) 16, 84–96. https://doi.org/10.1016/j.dnarep.2014.02.011.
Wu, L., Hickson, I.D., 2003. The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature 426 (6968), 870–874. https://doi.org/10.1038/nature02253.
Xie, Y., Yang, H., Cunanan, C., Okamoto, K., Shibata, D., Pan, J., et al., 2004. Deficiencies in mouse Myh and Ogg1 result in tumor predisposition and G to T mutations in codon 12 of the K-ras oncogene in lung tumors. Cancer Research 64 (9), 3096–3102.
Yamaguchi, S., Ogata, H., Katsumata, D., Nakajima, M., Fujii, T., Tsutsumi, S., et al., 2014. MUTYH-associated colorectal cancer and adenomatous polyposis. Surgery Today 44 (4), 593–600. https://doi.org/10.1007/s00595-013-0592-7.
Yew, Y.W., Giordano, C.N., Spivak, G., Lim, H.W., 2016. Understanding photodermatoses associated with defective DNA repair: photosensitive syndromes without associated cancer predisposition. Journal of the American Academy of Dermatology 75 (5), 873–882. https://doi.org/10.1016/j.jaad.2016.03.044.
Yoon, J.H., Smith, L.E., Feng, Z., Tang, M., Lee, C.S., Pfeifer, G.P., 2001. Methylated CpG dinucleotides are the preferential targets for G-to-T transversion mutations induced by benzo[a]pyrene diol epoxide in mammalian cells: similarities with the p53 mutation spectrum in smoking-associated lung cancers. Cancer Research 61 (19), 7110–7117.
Yu, C.E., Oshima, J., Fu, Y.H., Wijsman, E.M., Hisama, F., Alisch, R., et al., 1996. Positional cloning of the Werner's syndrome gene. Science 272 (5259), 258–262.
Yu, X., Wu, L.C., Bowcock, A.M., Aronheim, A., Baer, R., 1998. The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. Journal of Biological Chemistry 273 (39), 25388–25392.
Zhang, K., Zhou, B., Wang, Y., Rao, L., Zhang, L., 2012. The XRCC1 Arg280His polymorphism contributes to cancer susceptibility: an update by meta-analysis of 53 individual studies. Gene 510 (2), 93–101. https://doi.org/10.1016/j.gene.2012.08.039.
Zhao, Y., Thomas, H.D., Batey, M.A., Cowell, I.G., Richardson, C.J., Griffin, R.J., et al., 2006. Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. Cancer Research 66 (10), 5354–5362. https://doi.org/10.1158/0008-5472.CAN-05-4275.
Zou, H., Li, Q., Xia, W., Liu, Y., Wei, X., Wang, D., 2016. Association between the OGG1 Ser326Cys polymorphism and cancer risk: evidence from 152 case-control studies. Journal of Cancer 7 (10), 1273–1280. https://doi.org/10.7150/jca.15035.

Senescence and Cellular Immortality

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Introduction

Somatic cells undergo cell proliferation in response to growth stimuli. However, cells have a finite replicative lifespan, rendering these cells permanently unable to divide even when exposed to mitogenic signals. This state of essentially irreversible cell cycle arrest was first observed by Hayflick and Moorhead in 1961, and termed cellular senescence. Senescent cells remain metabolically active and survive in culture indefinitely when supplied with sufficient nutrients.

Through the induction of irreversible growth arrest, cellular senescence acts as an important block against tumorigenesis. Random DNA mutations due to excessive DNA damage may activate proto-oncogenes or inactivate tumor suppressor genes, leading to the formation of tumor cells—cells that proliferate uncontrollably. Cellular senescence can prevent formation of tumor cells by inhibiting their capacity to proliferate. Impairing the senescence pathways causes damaged cells to escape this inhibition, resulting in the development of cancer. While senescent cells play an important role in inhibiting cancer growth, the continuous exposure of cells to DNA damaging agents results in a steady supply of cells undergoing cellular senescence. Since senescent cells are able to survive for extended periods of time, they accumulate in tissues with age. A striking phenotype associated with senescent cells is the expression and secretion of several pro-inflammatory cytokines and growth factors. Through the secretion of such factors, senescent cells actively participate in tissue remodeling and repair after damage. However, when senescent cells accumulate and persist in tissues, they might contribute to chronic inflammation and generate an environment favorable to cell growth. In accordance, several studies have shown that senescent cells contribute to the onset and progression of many age-related diseases, including cancer.

In this article, we will describe the different inducers and effectors of cellular senescence, the phenotypes and features of senescent cells, and the pleiotropic contribution of senescent cells to tumorigenesis. We will further explore potential therapeutic approaches to dampen the effects of cellular senescence as a mean to treat cancer.

Cellular Senescence

Senescence Stimuli

Senescence can be induced by different stimuli promoting DNA or mitochondrial damages, chromatin changes or perturbations of mitosis (Fig. 1). DNA damage is triggered by a number of signals, including telomere shortening, excessive oxidative and genotoxic stress, and oncogene activation. Telomere shortening, a main cause of the replicative lifespan of human cells (replicative senescence), was the first described inducer of senescence. Telomeres are regions at the ends of the chromosome which protects

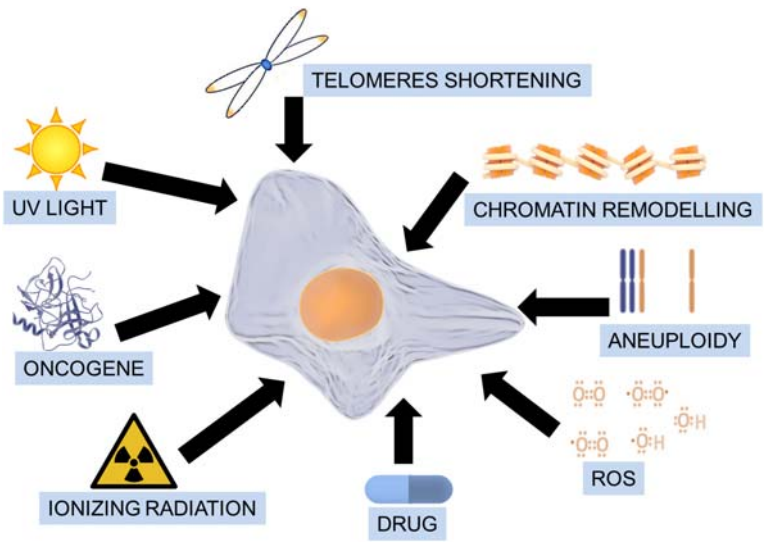

Fig. 1 Inducers of cellular senescence. Different stresses can induce senescence in normal and cancerous cells. Telomeres shortening is the first inducer of senescence being described. Some of the stresses are environmental and include UV light, genotoxic drugs and ionizing radiations.

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chromosomes from deterioration or from fusion with other chromosomes. However, due to the intrinsic mechanism of DNA replication, cellular divisions promote a progressive decrease in telomeres length, which eventually become critically short and dysfunctional. This telomere shortening activates a DNA damage response without effective repair, leading to replicative senescence. In addition to replicative senescence, cells may also be induced to senesce prematurely by acute DNA damages.

Reactive oxygen species can cause DNA damage by targeting DNA bases and other associated macromolecules. Reactive oxygen species (ROS), such as superoxides, hydrogen peroxides, and hydroxyl radicals, are produced in the cell through different metabolic reactions. While low levels of ROS may act as signaling molecules, excessive ROS production may destroy the integrity of different proteins, carbohydrates, lipids, and nucleic acids which maintain proper cellular functions. Damage to these macromolecules may inhibit cell cycle progression and cause a cellular senescence response. Moreover, in the presence of oxidative stress, damaged telomeres are repaired inefficiently relative to other parts of the chromosome. Strong genotoxic stress, such as ionizing radiation or drugs that interfere with DNA synthesis, directly induces DNA double strand breaks (DSBs) and activates a persistent DNA damage response (DDR), which then promotes the senescence program and arrests cell proliferation.

Overexpression of oncogenic mitogenic signals also promotes premature senescence in cells through generation of DNA damage. For example, activation of Ras oncogene induces an acute hyperproliferation, which is subsequently followed by a permanent cell cycle arrest as early as 3 days after Ras induction. Ras induces cellular senescence by increasing expression of Cdc6, suppressing nucleotide metabolism, inducing DSBs, and activating the DDR pathway. BRAF(V600E) triggers cellular senescence by upregulating mitochondrial pyruvate dehydrogenase, independent of the DDR pathway. Prolonged exposure to the mitotic signal interferon-β also activates the cellular senescence response.

The contribution of metabolic changes to establishing cellular senescence is further exemplified in cells with mitochondrial dysfunction. Mitochondrial dysfunction can induce cellular senescence, called mitochondrial dysfunction-associated senescence (MiDAS). MiDAS is characterized by reduced NAD^+/NADH levels and increased AMPK and p53 activation, which are important for the establishment of cellular senescence.

Opening chromatin structures in the genome activates the senescence pathway. Inhibition of histone deacetylases can induce formation of euchromatin structures in the genome, resulting in expression of the cell cycle inhibitors $p21^{Cip1}$ and $p16^{INK4a}$. Bmi1 polycomb complex protein and histone H2A K119 are responsible for hypermethylation and inducing heterochromatin structure of the $p16^{INK4a}$ promoter, preventing transcription of the $p16^{INK4a}$ gene. Hence, inhibition of the Bmi1 polycomb complex protein and ubiquitination of the histone H2A K119 results in de-repression of the $p16^{INK4a}$ gene.

Inhibition of mitosis and spindle formation can also induce cellular senescence. Decreased expression of TACC3, BubR1, Bub3, and Rae1 can activate p53/$p21^{Cip1}$ pathway and $p16^{INK4a}$ in the absence of a DDR. BubR1-insufficient mice have elevated levels of p53, $p21^{Cip1}$, $p16^{INK4a}$, and $p19^{Arf}$. These progeroid mouse model elicit early onset of aging-associated phenotypes, including decrease in lifespan, loss of subcutaneous fat, delay in wound healing, and development of cachectic dwarfism, lordokyphosis, and cataracts. BubR1 progeroid mice also develop progressive aneuploidy, which is thought to play a role in the development of senescence in these mice.

Effector Pathways

The main effector mechanisms for the establishment of cellular senescence are p53/$p21^{Cip1}$ and $p16^{INK4a}$/pRB pathways (Fig. 2). DNA damage response activates p53 by the ATM/ATR DNA damage signaling pathway. In senescent cells, sustained p53 activity results in persistent elevated expression of the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1}$. Inhibition of the cyclin-dependent complexes cyclinD/CDK4/6 and cyclinE/CDK2 prevents phosphorylation of retinoblastoma (Rb) protein. This

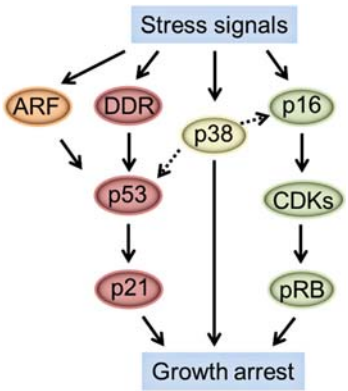

Fig. 2 Pathways associated to cellular senescence. Stress signals can activate distinct pathways that either induce or maintain the senescence phenotype. Many signals promote DNA damage which, in turn, activates a chronic DNA damage response (DDR) dependent on p53 and p21. Other effectors can be activated independent of the DDR. ARF (p19) regulates and activates the p53/p21 pathway. P38 and p16 directly interfere with cellular proliferation.

stabilizes binding of Rb to E2F, preventing E2F from transcribing genes associated with cell cycle progression, resulting in permanent cell cycle arrest.

The CDK inhibitor $p16^{INK4a}$ is an important player for the maintenance of cell cycle arrest during cellular senescence. $p16^{INK4A}$ inhibits CDK4/6 kinase activity and prevents Rb phosphorylation and degradation. Rb remains associated with E2F1 and prevents transcription of E2F1 target genes that are crucial for cell cycle progression. $p16^{INK4A}$ can also crosstalk with the $p21^{CIP1}$ pathway. However, in contrast to p53 activity, $p16^{INK4A}$ activation is less dependent on DDR. The $p19^{Arf}$ protein, an alternate reading frame product of the same locus of $p16^{INK4A}$ (CDKN2A), can activate p53 via DDR-independent mechanisms. The senescence pathways of murine cells in contrast to human cells are more dependent on $p19^{Arf}$, suggesting a species-specific difference in senescence establishment.

Signaling pathways that trigger cellular senescence activates Rb and its family members, p107 and p130. Increasing expression of Rb proteins is sufficient to induce a permanent cell cycle arrest. Moreover, activation of Rb proteins renders cells permanently growth arrested, even after subsequent Rb inactivation. In contrast, ablation of all Rb proteins prevents cells from undergoing cellular senescence. Other signaling pathways may also affect the ability of $p16^{INK4A}$/Rb to promote cellular senescence. For example, PI3K/AKT activation establishes cellular senescence and not quiescence when $p16^{INK4A}$ is induced. This is partly due to increased DNA damage foci and ROS production caused by decreased expression of the mitochondrial antioxidant superoxide dismutase 2 (SOD2). Elevated ROS levels activate protein kinase C delta (PKC delta), which in turn, creates a positive feedback loop between ROS and PKC delta that leads to cellular senescence. In contrast, contact inhibition and serum starvation have decreased DNA damage foci and low ROS production, partly due to increased SOD2 expression and increased FoxO3a expression, which results in quiescence instead of cellular senescence.

Because of the role of $p53/p21^{Cip1}$ and $p16^{INK4A}$/Rb pathways, persistent activation of these signaling molecules is a widely used marker of senescent cells. In addition to DNA damage, activation of RAS oncogene (Ha-RASv12) also increases expression of p53 and $p16^{INK4a}$ to induce permanent cell cycle arrest. Ras activates the Raf1/MEK/ERK pathway, which targets the transcription factors AP-1 and Ets and increases p38δ expression. p38MAPK activity is required for oncogene-induced senescence and is sufficient to induce growth arrest p38α and p38γ are required for RAS-induced senescence in a p53 and $p16^{INK4A}$ dependent manner, while p38δ promotes cellular senescence in RAS-induced senescence independent of p53 and a $p16^{INK4A}$.

Loss of certain tumor suppressors may also trigger a senescent growth arrest phenotype. For example, loss of PTEN, NF1, and VHL induces cellular senescence through $p19^{Arf}$ and $p16^{INK4a}$, independent of DDR.

Phenotypes of Senescent Cells

A number of phenotypical changes accompany the establishment of cellular senescence (Fig. 3). Senescent cells are classically defined by their inability to undergo cell proliferation even in the presence of proliferative signals. Senescent cells are permanently arrested at G1 and G2 phases of the cell cycle and they are more resistant to apoptotic signals than normal cells.

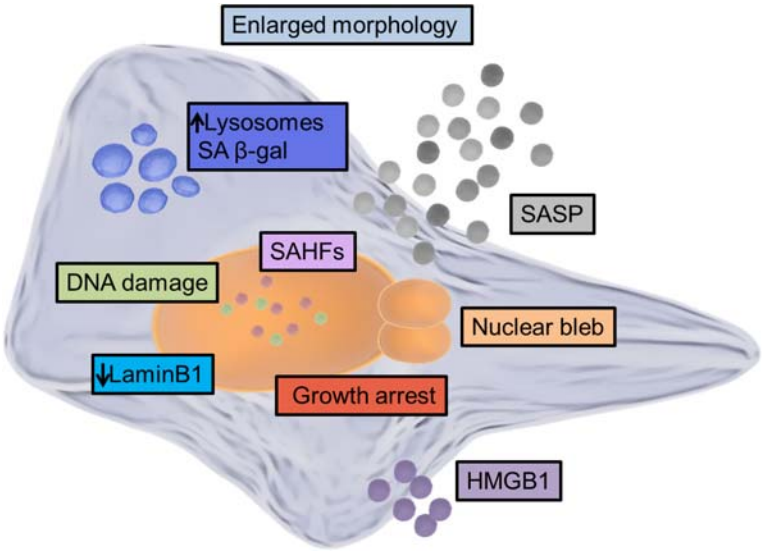

Fig. 3 Markers of cellular senescence. Induction of the senescence phenotype is accompanied by a number of molecular anf morphological changes, and by a permanent growth arrest. Senescent cells display enlarged size and increased number of lysosomes with activation of lysosomal enzymes such as senescence-associated β-galactosidase (SA β-gal). They are also characterized by changes in nuclear composition, measured by loss of LaminB1, and nuclear blebbing. Senescent cells show persistent DNA damage and characteristic senescence-associated heterochromatin foci (SAHFs). Modifications in nuclear composition and/or DNA induce extracellular release of the nuclear alarmin protein HGMB1 and activation of peculiar gene expression patterns, including transcriptional activation of secreted factors (senescence-associated secretory phenotype or SASP).

Another feature of senescent cells is their distinct morphology. Senescent cells have enlarged cell size and flattened appearance. They have increased lysosomal mass, accompanied by increased activity of a lysosomal β-galactosidase, called galactosidase beta 1 (GLB1). This increase in β-galactosidase activity distinguishes senescent cells from proliferating, quiescent, and terminally differentiated cells. Hence, it is termed as senescence-associated β-galactosidase (SA β-gal). SA β-gal activity assay has become a widely used assay to detect senescent cells in culture and in vivo. While SA β-gal activity increases in senescent cells, this activity does not seem to be important for the establishment of cellular senescence. It is also important to note that while SA β-gal is a popular biomarker for cellular senescence the specificity and selectivity of the assay to detect senescent cells have also been questioned. SA β-gal activity may also be detected in non-senescent cells when cultured at low serum conditions for long periods of time or incubated at a very high cell density. Nonetheless, SA β-gal activity is still a well accepted marker for cellular senescence.

Senescent cells are associated with polyploidy. They have increased nuclear size and nuclear bleb formation. Downregulation of lamin B1 is thought to contribute to nuclear blebbing in senescent cells. Lamin B1 are downregulated in senescent cells but not in non-senescent cells. Activation of p53 and pRb pathways induces loss of Lamin B1 independent of p38MAPK, NF-κB, ATM or ROS. Unlike SA β-gal activity, decrease in lamin B1 is an early feature of cellular senescence, suggesting that lamin B1 may be an important step in the development of senescence.

Senescent cells have distinct chromatin regions associated with gene repression called senescence-associated heterochromatin foci (SAHF). Loss of Lamin B1 promotes formation of SAHF in senescent cells. SAHF formation is induced by several proteins known to promote gene silencing. These proteins localize in promyelocytic leukemia (PML) nuclear bodies to initiate chromatin condensation by recruiting proteins such as heterochromatin protein 1 (HP1), methylated lysine 9 of histone H3 (H3K9Me), and macroH2A. SAHF formation is partly activated by Rb and p53 pathways.

Senescent cells have persistent DNA damage foci called DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS). DNA-SCARS are comprised of DNA damage repair proteins, such as ataxia telangiectasia mutated (ATM), Rad3, γH2AX, 53BP1, MDC1, and NBS1, that are unable to fully repair DNA strand breaks. DNA-SCARS are also associated with PML nuclear bodies. DNA damage activates ATM kinase, which phosphorylates histone H2AX (γH2AX). In senescent cells, the activated DNA repair proteins remain bound to the DNA damaged sites forming the persistent DNA damage foci. Unresolved DNA damage foci prevent the cell from completing DNA replication and progressing through the S phase of the cell cycle. DNA-SCARS are distinct from transient DNA damage foci which gets resolved after DNA repair.

Another key feature that distinguishes senescent cells from quiescent and terminally differentiated cells is their ability to secrete factors that may elicit a pro-inflammatory response in the surrounding tissue microenvironment. HMGB1, a member of the Alarmin family, is secreted by senescent cells. HMGB1 is a nuclear protein which translocates outside of the cell shortly after DNA damage in a p53-dependent and ATM- or $p16^{INK4a}$ -independent manner. HMGB1 secretion stimulates a senescence-associated inflammation response by signaling through the toll-like receptor-4 (TLR-4). In addition to HMGB1 release, senescent cells also secrete several cytokines, chemokines, and growth factors, collectively termed as senescence-associated secretory phenotype (SASP). However, in contrast to HMGB1, expression of the SASP gradually develops over time after sustained DNA damage. Components of the SASP include interleukin 6 (IL6), IL1A, IL1B, chemokine C-X-C motif ligand 1 (CXCL1, also known as GRO-α), CXCL8, vascular endothelial growth factor (VEGF), and matrix metalloproteinase-1 (MMP1).

DNA damage, oncogene activation, and telomere shortening can all elicit the SASP. But, it is important to indicate that not all senescent cells express the same SASP expression profile. For example, senescent cells induced by $p16^{INK4A}$ over-expression without DNA damage have reduced expression of SASP, while senescent cells induced by mitochondrial dysfunction show a distinct set of SASP factors.

Cellular Senescence as a Tumor Suppressive Mechanism

Since the first description as an irreversible inhibitor of cell cycle progression, cellular senescence has been considered a potentially powerful tumor suppressor. Currently, senescent cells are thought to prevent tumorigenesis via cell autonomous (growth arrest) and non-cell autonomous (SASP) mechanisms (Fig. 4).

Growth Arrest

Cellular senescence acts as a strong tumor suppressor mechanism. It acts as a brake to prevent tumor cells from further replicating. Unlike apoptosis, cellular senescence does not cause cancer cells to die. They remain in their microenvironment unless cleared by the immune system. Tumor cells must first escape the cellular senescence mechanisms in order to gain their proliferative characteristics. Indeed, senescent cells are more common in premalignant tumors than advanced malignant tumors.

Senescence growth arrest depends on cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p16^{INK4a}$. Persistent upregulation of either the $p19^{Arf}$/p53/$p21^{Cip1}$ and $p16^{INK4a}$/Rb tumor suppressor mechanisms result in a permanent growth arrest state. The tumor suppressor pathways inhibit the expression and/or function of genes that promote cell cycle progression. Hence, mutation or epigenetic silencing of at least one crucial regulator of these tumor suppressor pathways will render cells to bypass the senescence checkpoint and progress towards tumorigenesis. Decreased expression or gene silencing of at least one of the tumor suppressor pathways allows cancer cells to escape cellular senescence and develop tumors. Escape from the senescence pathways occurs even in cells with activation of Ras oncogene, a known promoter of cellular senescence. Indeed, Ras mutations are found in 20%–30% of all tumors.

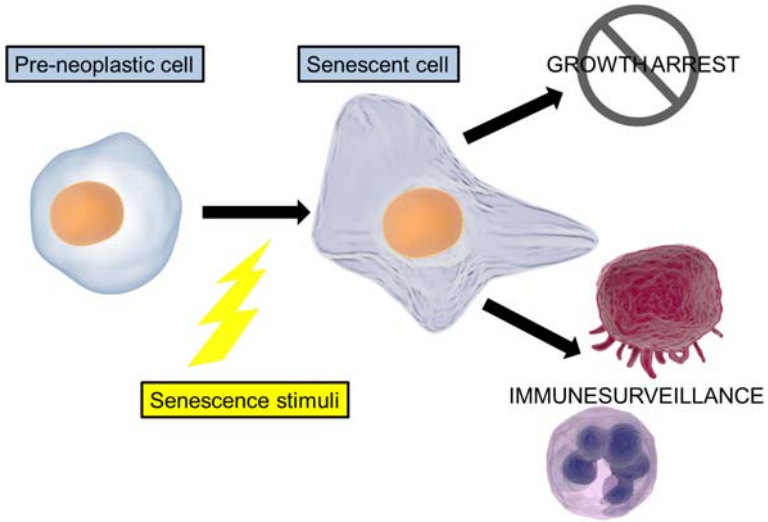

Fig. 4 Tumor suppression. Induction of cellular senescence acts as a potent tumor suppressor via cell and non-cell autonomous mechanisms. The growth arrest (cell autonomous) prevents propagation of potentially pre-neoplastic cells. The secretion of several pro-inflammatory factors (non-cell autonomous) allows for the recruitment of different immune cells that control tumor propagation (immunesurveillance).

Hras mutation is often observed in papillary thyroid cancer, Nras mutation in hepatocellular carcinoma, and Kras mutations in pancreatic, colorectal, and non-small-cell lung carcinomas. In some instances, loss of $p21^{Cip1}$ may also cause tumor cells to go through additional S phases, resulting in polyploidy.

Consistent with the ability of tumor suppressors to induce cellular senescence and prevent tumorigenesis, many human tumors have inactivated tumor suppressor genes. $p16^{INK4a}$ and Rb are inactivated in a large number of human tumors, but their levels may vary depending on the tumor cell. Indeed, tumors that have low $p16^{INK4a}$ expression usually elicit high Rb expression, whereas tumors that have reduced Rb expression show high $p16^{INK4a}$ levels. Tumors that retain both $p16^{INK4a}$ and Rb are accompanied by overexpression of CDK4 or cyclin D1. CDK4 is amplified in sarcomas and gliomas. Mutations that cause resistance to $p16^{INK4}$-mediated senescence are found in melanomas. Overexpression of cyclin D1 are observed in breast tumors and mantle-cell lymphomas.

Tumors also have high expression of telomerase. Telomerase maintains telomere length in tumor cells, preventing these cells from reaching replicative senescence. This causes tumor cells to become immortal, allowing them to proliferate indefinitely. Tumor cells share this high telomerase activity with germ cells, embryonic stem cells, and adult stem cells, which also have very long replicative lifespan. It is important to note though, that high telomerase activity is not the only factor for cellular immortality. For example, DNA damage can still cause cellular senescence in cells with high telomerase activity. Indeed, mouse cells can still senesce after treatment of DNA damaging agents despite high telomerase activity.

SASP factors, such as chemokine (C-X-C motif) receptor 2 (CXCR2) (also called interleukin 8 receptor 2 (IL8R2)), plasminogen activator inhibitor-1 (PAI-1), and the pleiotropic protein insulin-like growth factor binding protein-7 (IGFBP-7), can reinforce the senescence growth arrest in an autocrine manner.

Immunesurveillance

The SASP attracts and activates innate and adaptive immune cells, such as natural killer (NK) cells and T cells. Because senescent cells are also found in premalignant tumors, the SASP produced by senescent cells can also induce immune clearance of premalignant senescent cells and surrounding tumor cells. In premalignant liver cells, senescent cells can activate an immune-dependent clearance, termed "senescence surveillance" through a $CD4^+$ T cell-mediated response. This adaptive immune response is accompanied by recruitment of monocytes and macrophages to eliminate premalignant senescent cells. NK cell-mediated clearance of senescent stellate cells restricts the progression of fibrosis in liver tissue. When senescent cells are surrounded by established liver cancer cells, senescence clearance is facilitated by an innate immune response, composed of macrophages, neutrophils, and natural killer cells. Hence, senescent cells play an important role in immunesurveillance. While senescent cells may elicit different immune responses depending on the tumor microenvironment, the mechanisms involved in senescence surveillance remain unclear.

Senescent Cells and Tumor Promotion

Senescent cells can survive for extended periods of time when supplied with sufficient nutrients. Persistence of cells in a senescence state increases the odds to acquire additional mutations, thus potentially escaping from growth arrest, but also generate an environment rich in pro-inflammatory and pro-growth factors with the potential of generating a pro-tumorigenic niche (Fig. 5).

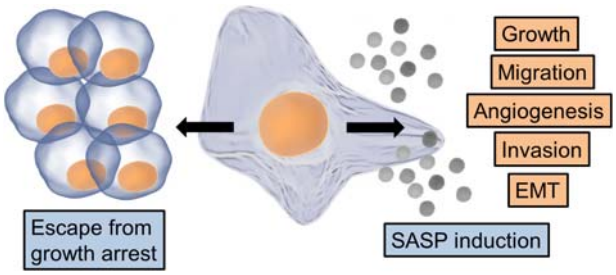

Fig. 5 Tumor promotion. Persistent senescent cells that are not efficiently eliminated can acquire additional mutations and escape from the state of growth arrest. In parallel, secretion of SASP factors can potentiate the development of aggressive phenotype of cancer cells.

SASP

The SASP is thought to promote inflammation. SASP factors may affect cell growth, motility, inflammation, differentiation, tissue repair, vascularization, and angiogenesis. SASP factors can reinforce cellular senescence through paracrine and autocrine signaling and cause surrounding cells to senesce through a bystander effect. However, they may also promote proliferation of surrounding cancer cells which have escaped cellular senescence. Sustainable supply of growth factors in the tumor microenvironment can permit malignant tumorigenesis. Some components of the SASP enhance proliferation of surrounding cancer epithelial cells. Indeed, senescent stromal fibroblasts can alter tissue microenvironment and cause abnormal epithelial cell proliferation in the mammary gland. Senescent fibroblasts also induce hyperproliferation of surrounding prostate epithelial cells. The SASP factors GROα and IL-8 stimulates proliferation of malignant melanocytes, which express high levels of C-X-C chemokine receptor-2 (CXCR2). The SASP factor MMP3 activates mitogenic signals and stimulates tumorigenicity and proliferation of epithelial cells. MMPs may induce proteolysis of the extracellular matrix and the release of mitogens.

The SASP induces neoplastic transformation and tumor vascularization in epithelial cells. Epithelial cancer cells surrounding senescent cells become invasive and less differentiated. They undergo epithelial-mesenchymal transition (EMT), which is an important step for cancer progression. Indeed, the SASP increases growth and invasiveness of surrounding cancer cells in mouse xenograft models. MMPs expressed by senescent cells promote EMT, induce invasiveness, and cause metastasis of epithelial cancer cells. Vascular endothelial growth factor (VEGF), which is also expressed by senescent cells, stimulates endothelial cell proliferation and increases vessel formation in tumors to drive cancer progression. Tumors grown with senescent cells have more blood vessels density than those grown with non-senescent cells in a breast cancer xenograft mouse model. Tumors with oncogenic RAS activation also contain highly vascularized structures. While senescent cells may promote vascularization of surrounding tumor microenvironment, the contribution of the SASP on tumor angiogenesis may depend on cell type.

DNA-damage response increases expression of the SASP factors by activating ATM. Increased expression of IL-1α in senescent cells activates the transcription factors NF-κB and C/EBPβ, which are important for transcription of several SASP factors, including IL-6 and IL-8. IL-1α also promotes its own expression by an NF-κB-mediated positive-feedback loop. High levels of IL-6 enhances invasiveness of breast cancer cells.

Induction of Cellular Senescence During Anti-Cancer Treatments

Chemotherapy and radiation therapy are classical anti-cancer therapies inducing cellular senescence in cancer cells and surrounding normal cells. Chemotherapy drugs promoting senescence include doxorubicin, docetaxel, cisplatin, fluorouracil, etoposide, camptothecin, and bleomycin. Radiation therapy also causes cellular senescence as a consequence of unrepairable DNA damage. Ionizing radiation produces charged particles which ionizes DNA directly or indirectly. Therapy-induced senescence (TIS) can be observed in colon carcinoma, breast cancer, osteosarcoma, and prostate cancer cells.

Induction of TIS is dependent on activation of p53, $p21^{CIP1}$, and $p16^{INK4A}$ pathways. TIS prevents cancer cells from further replicating and stimulates immunosurveillance to remove surrounding tumor cells. While TIS is a tumor suppressive mechanism, TIS may cause proliferation, chronic inflammation, and drug resistance in surrounding tumor cells. TIS may also cause neighboring cells to undergo cellular senescence through a bystander effect and further alter the tumor microenvironment.

Many cancer therapies have side effects. Cancer survivors exposed to chemotherapy develop several age-associated diseases. Side effects of cancer therapy limit the treatment dose that can be used in patients and may even result in the discontinuation of the treatment altogether. Cancer therapies may have off target effects that have not yet been completely explored. TIS may partly contribute to the side effects caused by cancer therapy. Indeed, removing senescent cells minimizes the side effects of chemotherapy. These short-term and long-term side effects, include impaired cardiac function, low bone marrow cell count, increased cancer recurrence, reduced physical activity, and diminished grip strength.

Accumulation of Senescent Cells During Aging

While it is rare to find senescent cells in tissues of young organisms, senescent cells accumulate with age in somatic tissues of several animal species, including humans, primates, and rodents. Aged human skin have increased levels of the senescent

marker SA β-gal. Skin from aging baboons also show markers of senescence, such as shortened telomere, increased heterochromatin foci formation, activated ATM, and elevated $p16^{INK4A}$ expressions. Moreover, several tissues in aging rats and mice also show increased $p16^{INK4A}$ expression. It is still unclear why senescent cells accumulate with age. It is possible that several inducers of senescence also increase with age. Aged tissues might be more prone to cellular damage than would younger tissues. On the other hand, it is also possible that senescent cells are not readily eliminated in older individuals. During aging, the declining function of the immune system may fail to recognize senescent cells, allowing the steady increase in the number of senescent cells.

Senescent cells are associated with age-related diseases, such as osteoarthritis, cardiovascular diseases, diabetes, and Alzheimer's disease. Patients with osteoarthritis have high $p16^{INK4a}$ and $p21^{Cip1}$ expression, decreased telomere length, and increased SA β-gal activity. Mutations in $p16^{INK4a}$ and $p14/p19^{ARF}$ are linked to coronary heart disease and type 2 diabetes. Atherosclerotic plaques have increased SA β-galactosidase activity and $p16^{INK4a}$ and $p21^{Cip1}$ expression. Adipose tissues in obese mice have increased p53 expression and develop insulin resistance. Pyramidal neurons of the hippocampus in patients with amyloid plaques have high levels of $p16^{INK4A}$ expression. Astrocytes of older individuals and those with Alzheimer's disease also have elevated levels of $p16^{INK4A}$. Moreover, induction of senescence by telomere shortening can accelerate aging phenotypes in mice. Genetic elimination of persistent age-related senescent cells is sufficient to improve life span and delay the onset of a subset of age-related diseases in mouse models, including cardiac and renal dysfunctions, atherosclerosis, and osteoarthritis. Aging is associated with a number of loss-of-function phenotypes and diseases, but also marked by an increase in hyperplasias. According to this idea, the rate of cancer exponentially increases with age. Hence, the role of senescent cells in tumor initiation and/or progression could be an interesting target for anti-cancer therapy.

Targeting Senescent Cell

Considering the implication of cellular senescence during aging and cancer, there is mounting interest in developing strategies that can interfere with the deleterious effects of senescent cells (Fig. 6).

Pro-apoptotic Strategy

Identifying the signaling pathways that stabilizes senescent cells may help design therapeutic strategies to mitigate senescence-associated cancer. Drugs that selectively eliminate senescent cells are being developed as means of therapy. Dasatinib and quercetin are effective in synergistically removing senescent cells. Dasatinib, an ephrin B (EFNB) inhibitor, selectively eliminates senescent adipocytes, whereas quercetin, a phosphoinositide 3-kinase inhibitor, effectively removes human endothelial cells. ABT-263 (also Navitoclax) and ABT-737, inhibitors of the Bcl-2 family of anti-apoptotic proteins, are also effective drugs that remove senescent cells. Interestingly, ABT-263 reduces cancer relapse and improves physical activity in mice exposed to doxorubicin. Knockdown of EFNB1 and EFNB3 mRNA transcripts and phosphatidylinositol-4,5-bisphosphate 3-kinase delta catalytic subunit (PIK3CD) will trigger cell death in senescent cells with little effect on proliferating, quiescent, and differentiated nonsenescent cells.

Senescent cells have increased expression of the transcription factor FOXO4 compared to normal cells. Senescent cells depend on FOXO4 for survival. Downregulation of FOXO4 but not other FOXO family members induces apoptosis in senescent but not in normal cells. FOXO4-p53 interaction is important for survival of senescent cells. Pharmacologic inhibition of FOXO4-p53 interaction by the peptide FOXO4-D-retro-inverso (FOXO4-DRI) causes apoptosis in senescent but not normal cells. FOXO4-DRI also suppresses age-associated pathology and doxorubicin-induced tissue dysfunction.

Modulation of the SASP

Another approach to reduce the negative effects of senescent cells is to decrease the expression of the SASP. The tumor suppressor p53, microRNAs miR-146a/b, and membrane protein Klotho are capable of reducing the SASP. C/EBPγ by heterodimerizing with C/EBPβ can also suppress the SASP. Moreover, inhibition of the NF-κB pathway can reduce SASP expression. Indeed, compounds such as resveratrol, apigenin, kaempferol, and wogonin can suppress the SASP by increasing expression of IκBζ and activation of NF-κB. Resveratrol inhibits NF-κB activity and SASP expression. Apigenin, kaempferol, and wogonin also diminish NF-κB activity and exert anti-inflammatory effects.

Summary

The study of cellular senescence in mouse models and human tissues has revealed the complexity of this phenotype and of its biological function. For a long time, senescent cells have been considered potent gatekeepers against initiation of tumors. However, their accumulation during aging and after cancer treatments may cause deleterious effects, including cancer promotion and progression. This has attracted several researchers to develop anti-senescence therapeutic strategies to minimize the negative effects of senescent cells.

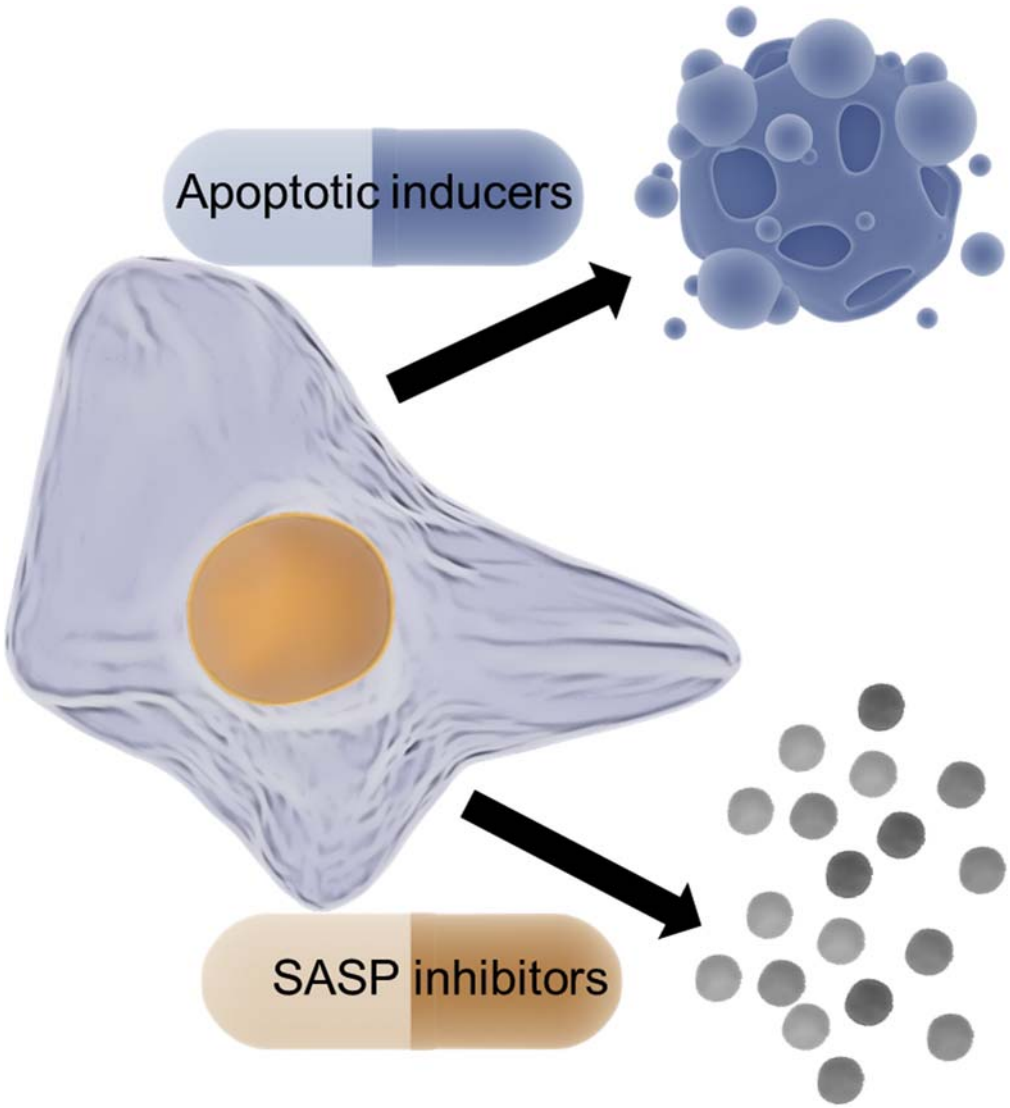

Fig. 6 Therapy against senescence. Interventions against senescent cells can promote apopoptosis or interfere with the secretion of SASP factors.

Prospective Vision

Cellular senescence is a potent block for tumorigenesis. However, recent evidences strongly support the idea that accumulation of senescent cells could be a promoter of pathologies. More interest is growing towards targeting persistent senescent cells. A number of ongoing studies aims at dissecting the importance of senescence for various diseases one-by-one. Current therapeutics against senescence carries intrinsic toxicities which essentially disqualify long-term and systemic treatments. However, efforts will be made for the generation of more safe and less toxic compounds which could extend life span and improve healthspan by delaying a basic mechanism of aging such as cellular senescence.

Further Reading

Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., van Deursen, J.M., 2011. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature 479, 232–236. https://doi.org/10.1038/nature10600.

Baker, D.J., Childs, B.G., Durik, M., Wijers, M.E., Sieben, C.J., Zhong, J., Saltness, R.A., Jeganathan, K.B., Casaclang Verzosa, G., Pezeshki, A., et al., 2016. Naturally occurring p16 Ink4a-positive cells shorten healthy lifespan. Nature 530. https://doi.org/10.1038/nature16932.

Campisi, J., 2005. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 120, 513–522. https://doi.org/10.1016/j.cell.2005.02.003.

Campisi, J., 2011. Cellular senescence: putting the paradoxes in perspective. Current Opinion in Genetics and Development 21, 107–112. https://doi.org/10.1016/j.gde.2010.10.005.

Campisi, J., d'Adda di Fagagna, F., 2007. Cellular senescence: when bad things happen to good cells. Nature Reviews Molecular Cell Biology 8, 729–740. https://doi.org/10.1038/nrm2233.

Chang, J., Wang, Y., Shao, L., Laberge, R.-M., Demaria, M., Campisi, J., Janakiraman, K., Sharpless, N.E., Ding, S., Feng, W., et al., 2015. Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. Nature Medicine 22, 78–83. https://doi.org/10.1038/nm.4010.

Coppé, J.-P., Patil, C.K., Rodier, F., Sun, Y., Muñoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.-Y., Campisi, J., 2008. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biology 6, e301. https://doi.org/10.1371/journal.pbio.0060301.

Demaria, M., O'Leary, M., Chang, J., Shao, L., Liu, S., Alimirah, F., Koenig, K., Le, C., Mitin, N., Deal, A.M., et al., 2017. Cellular senescence promotes adverse effects to chemotherapy and cancer relapse. Cancer Discovery 7, 165–176. https://doi.org/10.1158/2159-8290.CD-16-0241.

d'Adda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., Jackson, S.P., 2003journal. A DNA damage checkpoint response in telomere-initiated senescence. Nature 426, 194–198. https://doi.org/10.1038/nature02118.

Hayflick, L., 1974journal. The longevity of cultured human cells. Journal of the American Geriatrics Society 22, 1–12. https://doi.org/10.1111/j.1532-5415.1974.tb02152.x.

Herbig, U., Jobling, W.A., Chen, B.P.C., Chen, D.J., Sedivy, J.M., 2004. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21CIP1, but not p16INK4a. Molecular Cell 14, 501–513. https://doi.org/10.1016/S1097-2765(04)00256-4.

Nelson, G., Wordsworth, J., Wang, C., Jurk, D., Lawless, C., Martin-Ruiz, C., von Zglinicki, T., 2012. A senescent cell bystander effect: senescence-induced senescence. Aging Cell 11, 345–349. https://doi.org/10.1111/j.1474-9726.2012.00795.x.

Rodier, F., Coppé, J.-P., Patil, C.K., Hoeijmakers, W.A., Muñoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., Campisi, J., 2009. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nature Cell Biology 11, 973–979. https://doi.org/10.1038/ncb1909.

Velarde, M.C., Demaria, M., Campisi, J., 2013. Senescent cells and their secretory phenotype as targets for cancer therapy. Interdisciplinary Topics in Gerontology 38, 17–27.

Waldman, T., Lengauer, C., Kinzler, K.W., Vogelstein, B., 1996. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. Nature 381, 713–716. https://doi.org/10.1038/381713a0.

Zhu, Y., Tchkonia, T., Pirtskhalava, T., Gower, A.C., Ding, H., Giorgadze, N., Palmer, A.K., Ikeno, Y., Hubbard, G.B., Lenburg, M., et al., 2015. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell 14, 644–658. https://doi.org/10.1111/acel.12344.

Sleep Disturbances and Misalignment in Cancer

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Glossary

Actigraphy Commonly used, easily applicable, and noninvasive research instrument to measure sleep duration and activity levels. Units are typically worn on the wrist and look like wrist watches.
Chronotype A trait that distinguishes among people who are definite morning types (colloquially referred to as "larks"), are definite evening types ("owls"), and who prefer sleep times that are more intermediate compared to these two more extreme forms of sleep timing. Can be assessed via questionnaire (e.g., the Horne–Ostberg Morningness-Eveningness Questionnaire (MEQ)).
Circadian system Comprised of neurons located in the anterior hypothalamus called the suprachiasmatic nuclei (SCN), which acts as the "master clock" and coordinates various "peripheral clocks" throughout tissues of the body. Together, this clock system regulates 24 h ("circadian") rhythms in humans.
Melanopsin receptor Receptor located in the retina that transmits information about environmental light to the SCN.
Melatonin A hormone produced by the pineal gland, which is secreted primarily at night during darkness and is acutely sensitive to light exposure.
Polysomnography An evaluation of sleep-related biophysical changes (e.g., brain waves (EEG), skeletal muscle activation, and heart rhythm (ECG)), which requires individuals to be connected to multiple electrodes overnight.
Sleep apnea A disturbance that affects breathing at night and is tightly linked to obesity.

Abbreviations

AHI Apnea-hypopnea index
CDC Centers for Disease Control and Prevention
DSM-V Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (released in 2013)
EEG Electroencephalography
ECG Electrocardiography
MEQ Morningness-Eveningness Questionnaire (Horne–Ostberg)
MT1, MT2 Melatonin receptors 1 and 2
PSG Polysomnography
REM Rapid eye movement
SCN Suprachiasmatic nuclei
SDB Sleep-disordered breathing
US United States

Sleep Disturbance and Circadian Misalignment

Characteristics of Sleep

Sleep is a state of unconscious changes in brain wave activity and other physiologic parameters (e.g., body temperature, heart rate, and breathing), which occurs primarily at night in darkness. When an individual sleeps, there is a progression through multiple sleep cycles with various distinct stages: stages 1–4 are referred to as "nonrapid eye movement", or non-REM, sleep; stage 5 is referred to as REM sleep, and is characterized by higher sleep quantity and quality (see Fig. 1).

Average sleep durations at night vary across individuals, depending on their age. Currently, the National Sleep Foundation recommends sleep durations of 7–9 h per day for adults ages 24–64. Subjective recall of sleep duration is not always accurate, and objective measures via actigraphy or polysomnography (PSG) provide a less biased method of ascertaining this information. Sleep timing is defined as the times at which a person goes to bed and gets up, and this parameter varies across individuals as well. These differences in preferred sleep timing are referred to as chronotype when they are not driven by social and/or occupational circumstances. In Western society, roughly 15%–20% of the population classify themselves as "definite morning types," 10% as "definite evening types," and the remainder as having an intermediate sleep timing preference. In adolescence, chronotype tends

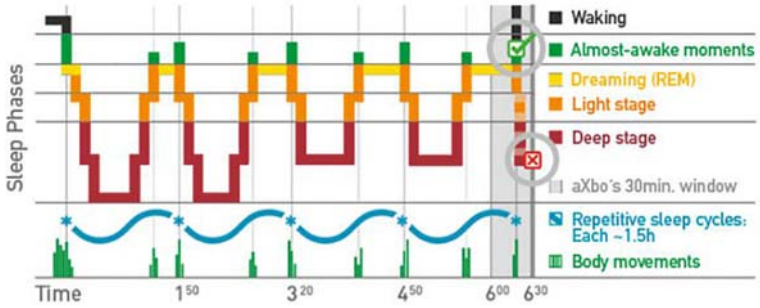

Fig. 1 Typical sleep behavior.

to be more skewed toward evening types across the population, after which time the population gravitates toward morning-type preferences as people age; however, any particular individual will tend to track with their same sleep timing preference throughout their life relative to other individuals of comparable age.

Sleep quality is another important sleep parameter, which has several measurable indicators: (1) time slept in bed (ideally, more than 85% of total sleep time); (2) time in bed before falling asleep (ideally, 30 min or less); (3) number of times waking up at night (ideally, not more than once); and (4) duration of time awake after initially falling asleep (ideally, less than 20 min). Currently, actigraphy and polysomnography are most frequently used to obtain objective data on these sleep quality indicators, although questionnaire-based assessments can be used (most notably, the Pittsburgh Sleep Quality assessment).

Types of Sleep Disturbances

Shortened sleep duration and other sleep disorders have significantly increased over the past century, reaching epic proportions: it has been estimated that sleep disturbances may affect up to 44% of all adults in the United States (see Fig. 2). Sleep disturbances can occur acutely or chronically, and can affect single or multiple sleep characteristics. The most frequently described sleep disorders include: insomnia, sleep-disordered breathing (SDB), restless leg syndrome, and circadian rhythm sleep disorders. Less well-known and rarer sleep disorders include parasomnias (e.g., bed-wetting, tooth grinding, sleep terror) and hypersomnias (e.g., narcolepsy). Medical and psychiatric conditions can also induce sleep disorders.

Insomnia is a condition in which people experience chronic sleeplessness. Their symptoms are characterized by difficulties falling or staying asleep, and early morning awakening with an inability to fall back asleep. DSM-V criteria require these symptoms to recur for at least 3 months before a diagnosis can be made.

Sleep-disordered breathing (SDB) includes conditions such as sleep apnea, snoring, and upper airway resistance syndrome. People with SDB suffer from sleep fragmentation, and as a result spend less time in REM sleep; they can also experience reduced oxygen saturation levels, leading to daytime sleepiness and fatigue.

Restless leg syndrome manifests as a strong urge to move one's legs due to unpleasant sensations in them; these sensations typically occur at rest. Intentional or involuntary leg movements (i.e., periodic limb movement disorder, which involves repetitive cramping of the legs during sleep) relieve the urge, but can make it difficult to initiate or maintain sleep.

Circadian rhythm sleep disorders comprise delayed and advanced sleep phase disorder, as well as non 24 h sleep–wake disorder.

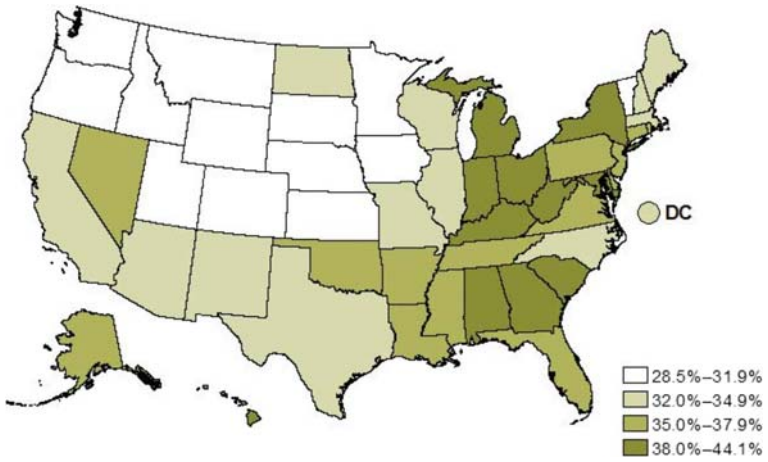

Fig. 2 Prevalence of short sleep duration among adults in the US (CDC, 2014).

The Circadian System

In the brain, the intricate and highly complex interplay between retinal melanopsin receptors, the retinohypothalamic tract, suprachiasmatic nuclei (SCN), and the pineal gland (i.e., the main site of melatonin secretion), as well as receptors and clock genes expressed in virtually every cell of the human body makes up the circadian system (see Fig. 3). This system is acutely sensitive to a small number of "zeitgebers," which includes environmental light (most importantly) as well as meal timing and activity. It directs many bodily functions to follow distinct 24 h rhythms, and keeps them in sync with the environmental light cycles. These functions include: the sleep/wake cycle, body temperature, blood pressure, and production of certain hormones (e.g., cortisol, melatonin, excretion).

Melatonin

Melatonin is a hormone marker of circadian rhythmicity, which also demonstrates cancer-protective properties.

Physiology and pharmacology

Melatonin production is stimulated by darkness and suppressed by light. Production increases in the evening and peaks between 2 and 4 a.m., then gradually declines. It does not vary with the menstrual cycle in premenopausal women. Melatonin diffuses from the pineal gland into the cerebrospinal fluid and capillary blood, freely crossing the blood-brain barrier. The effects of melatonin in the periphery can be roughly divided into direct effects (such as scavenging of free radicals) and receptor mediated effects (via its two receptors, MT_1 and MT_2). Melatonin is metabolized by the liver (90% at first pass) and mainly excreted in the urine. Bioavailability

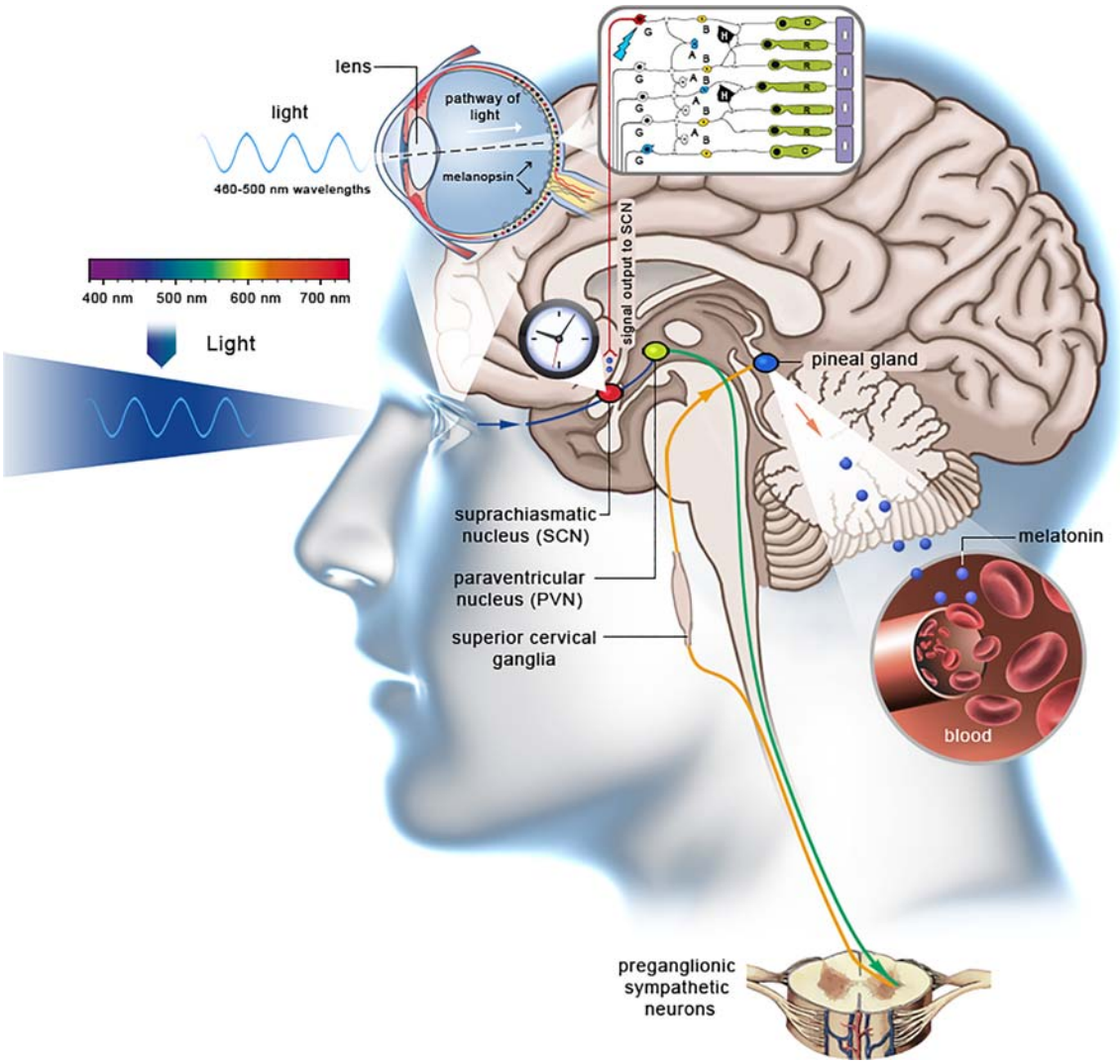

Fig. 3 The circadian system.

of oral melatonin varies, but doses of 1–5 mg generally result in supraphysiologic concentrations (10–100 times the usual peak) within 1 h after ingestion, followed by a decline to baseline within 4–8 h. Melatonin's half-life after oral administration varies between 30 and 60 min. Selective beta-adrenergic blockers (e.g., atenolol, propranolol) have been shown to significantly reduce melatonin release, which is stimulated by norepinephrine effects on beta receptors.

Anticarcinogenic effects

Relevant functions of melatonin related to cancer risk appear to be manifold and complementary. The most prominent mechanisms currently being studied involve melatonin's effects: (1) on estradiol; (2) as an indirect antioxidant and free radical scavenger; (3) on cytokine-mediated immune system function (thereby inhibiting growth of tumor cells); (4) suppression of fatty acid uptake and metabolism; (5) on increasing degradation of calmodulin (a key player in cell proliferation); and (6) on induction of apoptosis (possibly acting as a natural antiangiogenic molecule).

What Is Circadian Misalignment?

To varying degrees, exposure to light at night (as experienced by night workers) or circadian disruption induced by other means (e.g., traveling across time zones, moving to daylight savings time) can disturb synchronization of the central pacemaker in the SCN with peripheral clocks throughout the brain and body. This disruption is commonly referred to as circadian misalignment, and can be felt physically as prolonged fatigue or fatigue/hunger at odd times of the day. For example, once a person has settled into a stable new time zone, it takes the internal clock (i.e., SCN) 5–7 days to adjust to the new time, after which bodily functions return to normal. However, chronic circadian misalignment (as experienced, e.g., by long-term rotating night-shift workers) has been associated with a number of negative health effects on mood, metabolism, the immune system, and cancer risk (see Fig. 4).

Shift work is common, and is becoming increasingly prevalent in the so-called "24/7" society. According to the International Agency for Research on Cancer, about 15%–30% of the working population in Europe and the United States is engaged in shift work. The International Labor Organization defines shift work as "a method of organization of working time in which workers succeed one another at the workplace so that the establishment can operate longer than the hours of work of individual workers"). In the scientific literature and general public, the term "shift work" has been widely used and usually includes any arrangement of daily working hours outside of regular daytime hours (7/8 am–5/6 pm). Therefore, shift workers include all individuals working evening shifts, night shifts, rotating shifts, split shifts, or irregular or on-call schedules both during the week and on weekends. Obviously, people who work shift schedules may be subject to desynchronized circadian rhythm, particularly those working during the night on a rotating schedule. Emerging scientific evidence suggests that daily sleep/wake cycles, hormone regulation, and many physiological processes are often misaligned with behavioral patterns during shift work, especially rotating night shift work, leading to an increased risk of developing cancer and cardiometabolic disorders, including obesity, metabolic syndrome, type 2 diabetes, and cardiovascular disease.

Sleep and Cancer Etiology

Sleep disorders, including short or long sleep duration, sleep disruptions, and sleep-disordered breathing, have emerged as highly prevalent conditions in cancer patients, and have gained increased attention in the past decades. The high prevalence of sleep problems and their potential link with tumorigenesis and cancer prognosis has stimulated interest in both clinical and experimental

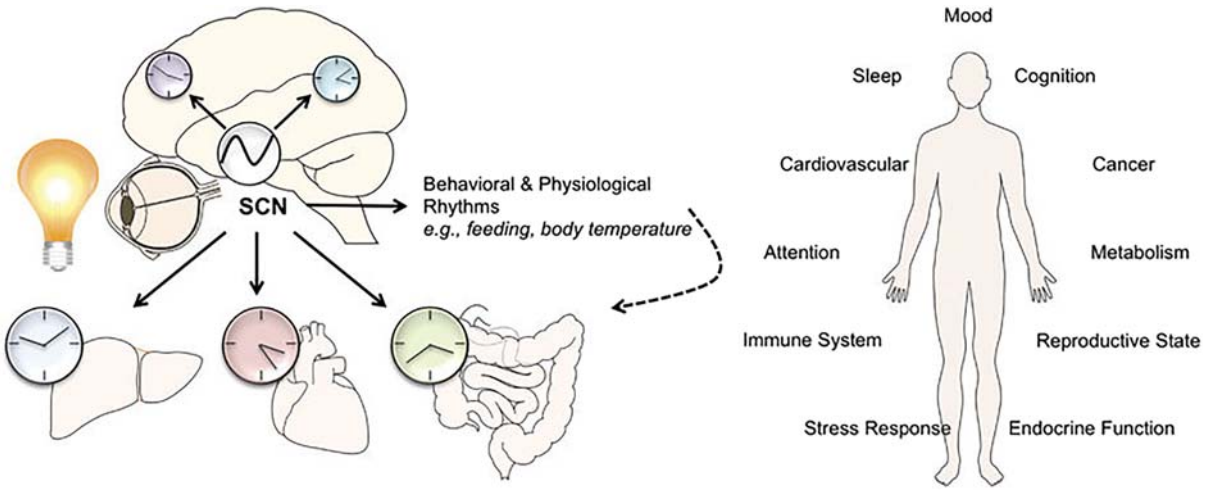

Fig. 4 Health effects of aberrant light exposure.

research. A deeper understanding of epidemiologic links between sleep disturbances, higher cancer incidence, and poorer cancer survival is warranted to improve evidence-based treatment for sleep disruption in the oncology setting. A number of large-scale prospective studies have been conducted worldwide, aiming to investigate this issue based on specific types of sleep problems in populations with and without cancer. While additional studies are still needed to fully elucidate these associations, confirmation of these associations between sleep disorders and cancer would have major clinical implications.

Sleep as Regulator of Carcinogenic Processes

The tight coupling of sleep and the circadian system suggests that circadian misalignment and related pathways essential for cancer development will be similarly central to the link between sleep and cancer. In addition, various general mechanisms of a potential link between particularly long sleep and all-cause as well as cancer mortality have been proposed, including sleep fragmentation, lack of physiological challenge (e.g., exercise), depression, and underlying disease processes (e.g., sleep apnea, heart disease).

Epidemiological Evidence Linking Sleep and Cancer

Due to the growing interest in the role of sleep in carcinogenesis, to date, multiple prospective studies have evaluated sleep duration in relation to cancer incidence and mortality. In these studies, individuals with self-reported short sleep duration (less than 5 or 6 h) or long sleep duration (9 h or more) have been compared to individuals with "average" sleep duration (7–8 h), although studies have classified sleep duration differently (either by number of hours per night or number of hours per 24 h period). This difference in classification might imply that studies examining 24 h sleep durations include napping times in their sleep duration estimates, whereas studies of nighttime sleep durations do not. Results have been inconsistent across individual studies, indicating a range of associations: positive, negative, and null. In meta-analyses, summary estimates across studies have suggested no overall association of short or long sleep duration with total cancer risk, and no association between sleep duration and specific cancer endpoints (breast, prostate, endometrial, thyroid, and ovarian), except that long sleep duration may be associated with an increased risk of colorectal cancer. However, the reason for this particular association is unclear, and could be due to either underlying biology or confounding bias. In addition, associations have been observed to be similar across studies according to gender, sample size, geographic location, participants' occupation, and definition of sleep duration. Furthermore, studies on sleep duration and cancer mortality generally have reported that long, not short, sleep durations may be related to an increased risk of total cancer mortality, although confounding bias may play a role in this association; moreover, studies have produced mixed results when mortality due to specific cancers (e.g., breast, colorectal, prostate) has been considered. Additional large-scale, prospective studies of sleep duration and cancer endpoints, particularly cancer-specific mortality, are needed to better understand these associations.

Epidemiology of Sleep-Disordered Breathing and Cancer

Sleep-disordered breathing and cancer is a related topic of interest that has gained attention. Of particular interest, obstructive sleep apnea is a common type of sleep-disordered breathing, characterized by recurrent bouts of upper airway obstruction while sleeping, resulting in sleep fragmentation and intermittent hypoxia that could have implications for cancer risk. Several studies have explored the associations of sleep-disordered breathing and/or obstructive sleep apnea in relation to cancer incidence and mortality, with exposure assessment relying on various metrics: the apnea-hypopnea index (AHI), the respiratory disturbance index (RDI), or a hypoxia index based on oxygen saturation level. To date, most individual studies have indicated that sleep-disordered breathing/sleep apnea may be associated with a somewhat increased cancer risk, and recent meta-analyses suggested an overall modest association when studies were considered together; however, meta-analysis found no apparent association when these data were stratified according to severity of sleep apnea (mild, moderate, and severe). Still, interpretation of this literature is limited due to study design issues, including lack of prospectively collected data, differences in exposure assessment, and insufficient information on important confounding factors. More well-conducted, prospective studies are needed to evaluate associations of sleep-disordered breathing and both cancer incidence and mortality.

Special Case: (Night) Shift Workers

In 2007, the International Agency for Research on Cancer classified shift work as a possible carcinogen, based on "convincing experimental evidence and suggestive epidemiologic data" to this effect. Indeed, experimental data on the association between shift work and cancer has consistently demonstrated that simulated shift work can lead to a higher incidence of cancer in animal models; specifically, light entrains the biological clock, as the SCN receives environmental dark/light information directly from the retina. This mechanism (also regulated by genes) appears to have evolved to detect changes in daylength/season, perhaps useful for promoting survival through migration and hibernation. Consistent with biologic evidence, the epidemiologic literature suggests that shift work is probably associated with a modestly increased risk of breast cancer. There may also be such an association with prostate cancer, although research on this and other cancers is still limited. A key distinguishing feature of prior studies is the method of exposure assessment: retrospective studies have relied heavily on either company records of shift work, or the

coupling of brief employment histories and job matrices (which make broad assumptions about the amount of shift work involved in specific occupations or occupational settings); in contrast, prospective studies have tended to ascertain more detailed shift work information from participants. Because exposure misclassification is more likely when shift work history is derived from employment records and job matrices, studies that have used this approach tend to be more limited in their ability to detect associations with shift work. This limitation may explain why some studies have reported either no association or a weak association between shift work and cancer. Thus, future research should focus on prospective collection of detailed information on shift work exposure, and investigation of shift work in relation to risk of cancers other than breast cancer.

Sleep and Cancer Survival

One of the most commonly reported complaints in oncology is poor sleep quality; for example, up to 70% of nonmetastatic breast cancer patients report poor sleep quality within the first months following diagnosis. Chronically poor sleep may influence cancer outcomes through impaired immune function, metabolic pathways leading to obesity, and altered melatonin release.

Epidemiology of Sleep and Cancer Survival

Because many cancer patients experience disrupted sleeping patterns, several recent studies have explored the association of postdiagnosis sleep characteristics and survival among cancer patients. Overall, results have indicated that sleep disturbances (defined as extreme sleep durations, subjective report of poor quality sleep, or objectively-measured sleep disruptions) may reduce survival time across multiple cancer populations: individuals with various stages of breast, colorectal, lung, liver/pancreatic, and head/neck cancer. For example, a recent prospective study of breast cancer patients suggested that long sleep durations (nine or more hours per night) and subjective, regular sleeping difficulties at postdiagnosis assessment, as well as increased sleep durations over time (comparing pre- and postdiagnosis assessments), might be related to greater risks of death due to breast cancer. However, as in all observational studies, it is possible that observed associations may reflect some degree of reverse causation (i.e., declining health may have contributed to sleep disturbances, as well as vice versa). More prospective research is necessary to refine our understanding of which sleep parameters are associated with survival across different cancer populations.

Oral Melatonin in Cancer Treatment/Patients

In vitro studies, although not entirely consistent, indicate that both pharmacologic and physiologic doses of melatonin reduce the growth of malignant cells of the breast and other tumors. In cancer patients, melatonin has been used extensively both alone and in combination with chemotherapy, with no significant adverse effects reported thus far. Overall, these reports suggested a statistically significant improvement in clinical response rate and overall survival among people with metastatic solid tumors randomly assigned to chemotherapy and melatonin compared to chemotherapy alone; they also indicated a decrease in chemotherapy-induced toxicity, such as neurotoxicity, thrombocytopenia, cachexia, and asthenia in the melatonin arm. However, larger scale double blind randomized trials are currently still lacking.

Several small uncontrolled pilot studies lend direct and indirect support for an inverse association between melatonin and estradiol levels, although these studies are not entirely consistent. Notably, in a small placebo-controlled, randomized study of the effects of 3 mg melatonin daily on several plasma markers of breast cancer in postmenopausal women with a history of breast cancer, short-term melatonin treatment did not influence circulating estradiol and IGF-1/IGBP-3 levels. However, subjective sleep quality was improved in these breast cancer survivors, which, in combination with the virtual lack of side effects (especially also regarding hormonal side effects), may suggest it is a suitable sleep remedy in these patients.

Melatonin in Cancer Prevention

Does Circulating Melatonin Predict Cancer Risk?

Several retrospective studies have examined melatonin levels in cancer patients, with inconsistent results. The main limitation of these retrospective studies is that blood samples for melatonin are collected after a diagnosis of cancer, thus allowing no assessment of the hormone's predictive value for breast cancer risk. However, more recently, evidence from prospective case–control studies nested in larger cohorts has emerged, largely focused on breast cancer. In these studies, urine was collected in healthy women (to measure the overnight excretion of 6-sulfatoxymelatonin—melatonin's major metabolite), and women were followed for several years until they developed breast cancer. Currently existing prospective studies of melatonin levels and breast cancer risk in postmenopausal women tend to indicate an inverse association. However, results are somewhat mixed, and whether these differences are driven by biology (e.g., effects in subgroups defined by hormone receptor status of the tumor) or methodologic issues (e.g., differences in laboratory methods, or varying times between urine collection and cancer diagnosis) requires larger, pooled studies, which currently do not exist. Evidence for an association between urinary melatonin and breast cancer risk among premenopausal women is also mixed, though also largely pointing toward a lower risk of breast cancer among women with higher

melatonin levels. More recently, some preliminary evidence for inverse associations between higher melatonin secretion and prostate cancer risk has been published, although larger studies are warranted to more fully appreciate the role of melatonin in cancer prevention.

Role of Melatonin in Cancer Prevention?

To date, no studies have examined the potential of oral melatonin, or one of the melatonin receptor agonists, in cancer prevention. These agonists include ramelteon, agomelatine, and tasimelteon, all of which are approved and primarily applied for sleep disorder treatment. Some indirect evidence suggests that any relevant effects of melatonin for breast cancer risk may perhaps not operate through circulating melatonin given that a randomized trial of 3 mg oral melatonin did not demonstrate an effect of melatonin on circulating sex steroids; however, it might operate at the tissue level. Still, a small study of physiologically circulating melatonin did not reveal important differences in the effect of melatonin on breast cancer risk stratified by melatonin receptor status in the tumor tissue, although clearly more research is needed. To date, few short-term side effects of melatonin have been reported in randomized trials of up to 6 months duration; however, longer-term studies have yet to be conducted in humans, to fully eliminate any remaining concerns regarding the long-term potential for melatonin to negatively affect reproduction or exert other adverse health effects.

Summary

In summary, sleep, the circadian system, and their related biomarkers represent a highly active, although fairly young, research area; this is especially true for these factors in regards to cancer endpoints. Despite sparse literature, some studies have suggested a role for sleep and the circadian system in cancer prevention, treatment, and survival.

Limitations of Existing Studies

General limitations of existing studies should be noted and improved upon in future work. The biologic complexity of sleep, the circadian system, and cancer makes it difficult to collect and analyze data that can be used to obtain straightforward answers about causality in these relationships. For example, studies of sleep duration used different definitions/categories to define short and long sleep, which can lead to heterogeneity of results. In addition, most studies have assessed vital sleep variables such as sleep duration and sleep efficiency based on self-reported information from participants (instead of objective measurements), which may lead to potential information bias. Confounding is a major bias that precludes inferences about causality of associations identified in existing studies. Individual studies often take into account information on traditional cancer risk factors (e.g., age, gender, smoking habits, alcohol consumption, and body mass index); however, additional information is needed to adjust for the influence of other potential confounding factors (e.g., sleep apnea, altered immune functioning, shortened light exposure, fatigue). Moreover, it is impossible to account for unknown or unmeasured confounders, which are possible in observational studies and can lead to undetected or unmeasurable bias. It is also true that sleep disorders may affect different malignancies differently, such that studies of overall cancer outcomes may mask associations with specific types of cancer. Finally, the number of high-quality studies that can be utilized in systematic reviews and meta-analyses is relatively small, making it difficult to avoid publication bias when summarizing previous literature.

Future Research Areas

Growing evidence has yielded mixed results but indicates some potential associations of sleep disorders with cancer incidence and mortality, although existing data are limited and more research is necessary. As research progresses, it will be critical to update systematic reviews and meta-analyses to further clarify this body of literature. Large, prospective studies based on population-based cohorts with long-term follow up should be conducted to further examine associations of sleep disorders (using subjective and objective assessments) with specific cancer subtypes across diverse populations. In addition, randomized controlled trials may be warranted to evaluate sleep-related treatment options to determine their effectiveness in reducing cancer incidence and/or mortality.

Prospective Vision (Where the Field Is Moving)

The circadian system and its main correlates, including sleep, are rapidly gaining recognition in medicine as important factors and contributors to virtually every major disease outcome. Research on the circadian clock and sleep is a relatively young discipline, where central findings such as the discovery of a melanopsin-containing receptor in the retina, which transmits information about environmental light (the main zeitgeber for the circadian system) to the SCN, have only been made at the beginning of the 21st century. The 2017 Nobel Prize in medicine for the discovery of genes that drive the circadian rhythm is further testimony to an increasing relevance of this young discipline. It appears likely that, with the emergence of new findings in this highly active research field, and a growing understanding of the mechanisms involved, sleep problems and circadian disruption will be recognized and

added to the small list of key lifestyle factors that account for the majority of adjustable disease risk in humans, and as such will also alter cancer practice.

Further Reading

Devore, E., Schernhammer, E., 2013. Shift work, obesity, and cancer. Impact of sleep and sleep disturbances on obesity and cancer. In: Redline, S., Nate, B. (Eds.), Energy balance and cancer, pp. 137–154. Chapter 7.

IARC, 2010. Monograph working group. IARC monographs on the evaluation of carcinogenic risks to humans. Volume 98. Painting, fire-fighting, and shift work. World Health Organization, International Agency for Research on Cancer, Lyon, France.

Kryger, M.H., Roth, T., Dement, W.C. (Eds.), 2017. Principles and practices of sleep medicine. Elsevier, Philadelphia, PA.

Pandi-Perumal, S.R. (Ed.), 2007. Melatonin: From molecules to therapy. Nova Science Publishers, NY, USA.

Relevant Website

https://sleepfoundation.org—National Sleep Foundation.

Small-Cell Cancer of the Lung: Pathology and Genetics

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Definition

Initially called oat-cell carcinoma or small-cell anaplastic carcinoma, small-cell lung cancer (SCLC) has been classified in the 1981 WHO classification as typical SCLC or intermediate variant of SCLC. Since the 2004 WHO classification, only two types were considered: pure SCLC and combined SCLC, when associated with nonsmall-cell carcinoma (adenocarcinomas, squamous-cell carcinoma, large-cell carcinoma, large-cell neuroendocrine carcinoma (LCNEC), or sarcomatoid carcinoma). By definition, SCLCs are malignant epithelial tumors composed of small cells with a scant cytoplasm and a typical granular chromatin. This tumor belongs to the group of neuroendocrine (NE) tumors of the lung along with carcinoids and LCNEC, with whom it shares morphological, immunohistochemical and molecular features; however, SCLC exhibits the highest grade of malignancy and the poorest prognosis.

Burden

Geographic differences in smoking habits combined with gender and ethnic differences still prevent accurate worldwide estimates. While the incidence of SCLC is decreasing in countries with effective smoking cessation policies, it is increasing in low-income and middle-income countries. According to Surveillance, Epidemiology and End Results database, the incidence of SCLC is 10 cases per 100,000 men, and 8 cases per 100,000 women. SCLC remains the seventh most common cause of cancer-related death in the United States. The frequency of SCLC as a proportion of all lung cancer cases peaked at 17%–20% in the late 1980s, but is now ranging from 13% to 15%. Only modest improvements have been seen in SCLC detection, therapy or survival over the past 30 years. From 1973 to 2002, the 5-year overall survival rate only increased from 4.3% to 6.3%. Overall, SCLC is estimated to kill 250,000 people worldwide yearly.

Risk Factors

The primary cause of SCLC is tobacco use, with $>95\%$ of patients being current or former smokers. In the United States, the decreasing prevalence of cigarette smoking together with the changes in the doses and types of carcinogens inhaled, related to the characteristics of cigarettes, has resulted in a decrease in the incidence of SCLC over the past 30 years.

Pathology

Histogenesis

Both carcinoids and SCLC were believed initially to arise from normal bronchial NE cells called Kulchitsky-type cells, acting as airways chemoreceptors involved in lung growth and differentiation. However, given recent gene expression profiling studies, the hypothesis of common multipotent precursor cells for SCLC/LCNEC and non-NE pulmonary tumors is favored, based on the existence of combined tumors. Sutherland et al. have shown in transgenic mice that all lung carcinomas could derive from a common epithelial precursor cell, ASH1-positive cells giving rise either to bronchiolo-alveolar stem cells (BASC) and adenocarcinomas, or to basal-cell progenitor and squamous-cell carcinoma, or to neuroendocrine cells and LCNEC/SCLC. In addition, SCLC could also arise directly from alveolar type II cells through NE markers re-expression, as demonstrated in resistant *Egfr*-mutated adenocarcinomas after oncogenic Kras expression. However, to date, no preneoplastic lesion has been identified for LCNEC or SCLC.

Macroscopy

SCLCs are typically proximal tumors, forming a soft tan-yellow endobronchial mass with extensive necrosis, invading directly peribronchial lymph nodes. However, in 5% of the cases, SCLC can be peripheral, presenting as soft ill-defined and necrotic nodules. It would then be misdiagnosed clinically as early-stage nonsmall-cell lung cancer (NSCLC), leading to surgical resection.

Histology

At low magnification, SCLC presents as a dense proliferation of small size tumor cells, arranged in diffuse sheets or ribbons, and with extensive areas of necrosis. Less frequently, neuroendocrine (organoid) characteristics such as rosettes, palisades and nests

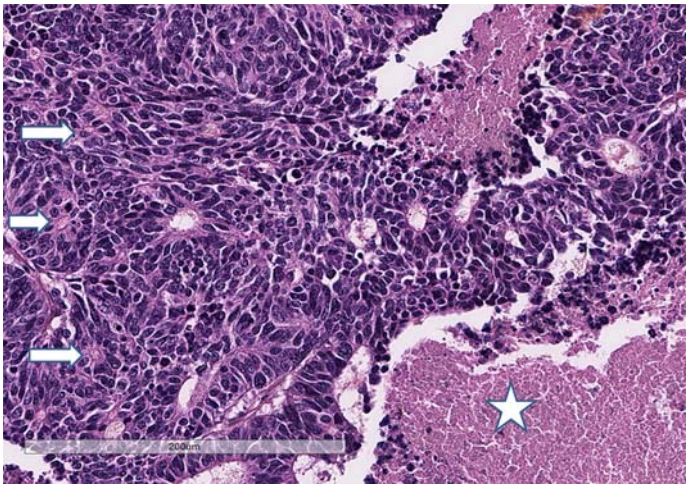

Fig. 1 Surgical sample of SCLC: large sheet of small tumor cells with scant cytoplasm and a typical salt and pepper nucleus. Note the numerous rosettes (arrows), necrosis (star) and mitoses (Hematoxylin Eosin Saffron stain; original magnification $\times 200$).

can be found. At high power fields, tumor cells are round to spindle and measure usually < 3 resting lymphocytes. They present with a scant cytoplasm and a typical salt and pepper nucleus. Chromatin is finely granular with inconspicuous nucleoli. Nuclear molding is frequent as well as nuclear debris known as Azzopardi effect and apoptotic bodies. Mitoses are numerous with an average of 80 mitoses per 2 mm^2 areas (Fig. 1). Those features are more obvious on surgical samples, where tumors cells seem larger with distinct cytoplasm and focal vesicular chromatin (Fig. 2), but on small biopsies, crushed artifacts can be predominant, and necrosis or rosettes and palisades absent. Several studies have shown a considerable overlap between SCLC and LCNEC when nuclear size or chromatin features were considered.

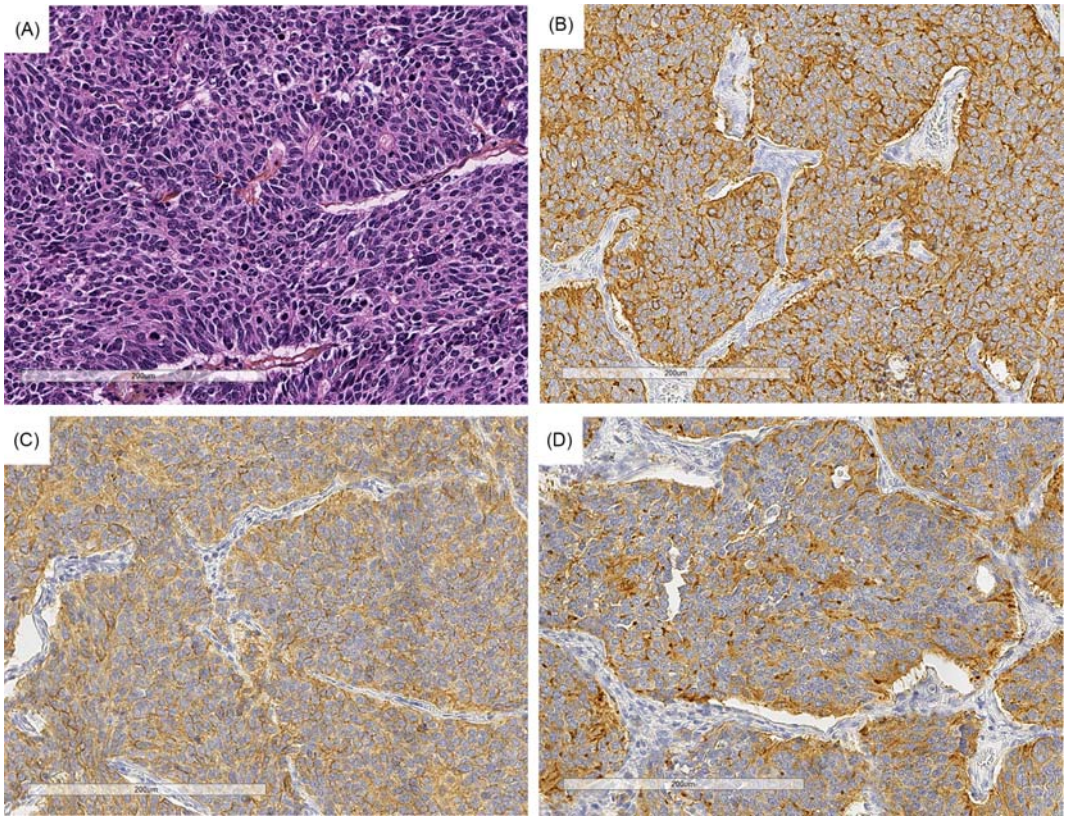

Fig. 2 (A) Surgical sample of SCLC (HES stain; original magnification $\times 200$). (B) Synaptophysin expression by tumor cells (immunoperoxydase; original magnification $\times 200$). (C) CD56 expression by tumor cells with a typical membranar staining (immunoperoxydase; original magnification $\times 200$). (D) Chromogranin A expression by tumor cells (immunoperoxydase; original magnification $\times 200$).

Combined SCLCs are defined by a mixture of pure SCLC areas and of adenocarcinomas, squamous-cell carcinoma, large-cell carcinoma or sarcomatoid (spindle or giant cells) areas, whatever the amount of NSCLC component. In contrast, for combined LCNEC and SCLC, a minimum of 10% of LCNEC is required. Combined SCLCs are more frequently diagnosed on surgical specimens widely sampled in comparison to biopsies. Of note, as for SCLC, there are some variations of interpretation and the overall kappa value for the identification of a SCLC component in SCLC and combined SCLC was 0.41 (moderate agreement). Combined SCLC share with pure SCLC the same epidemiology and clinical presentation, even if combined SCLCs tend to be more peripheral. Regarding prognosis, combined SCLC could harbor a worse prognosis than pure SCLC, possibly due to relative chemoresistance of non-SCLC components. From a clinical standpoint, the hypothesis of combined SCLC has to be raised when facing a SCLC tumor with heterogeneous response to first-line chemotherapy.

Cytology

On sputum and bronchial washings, SCLC cells are loosely arranged in rows or small groups, but maybe more cohesive on brushings and fine needle aspiration. They are often larger than three lymphocytes but cytoplasms are restricted to small rims. Nuclei are finely "salt and pepper," with inconspicuous nucleoli; nuclear molding is often prominent particularly on air-dried preparations. Mitoses are easily found, as well as a necrotic background. In daily practice, most SCLCs are diagnosed on the combination of cytology and biopsy, which increases dramatically the diagnostic yield.

Immunophenotype

SCLC is easily recognized on cytological samples, but on small biopsies a confirmation by immunohistochemistry can be of great help, particularly when tumor cells are crushed. The vast majority (95%) of SCLC diffusely express hASH1 and CD56 (membrane staining), whereas synaptic vesicle protein synaptophysin and dense-core associated protein chromogranin A cytoplasmic stainings are observed in 54% and in 37% of the cases, respectively, and often focally. Interestingly, CD56 expression is often retained in crushed specimens; in contrast, TTF1 is expressed classically in 90% of SCLC, but it may be absent on crushed biopsies (Fig. 3).

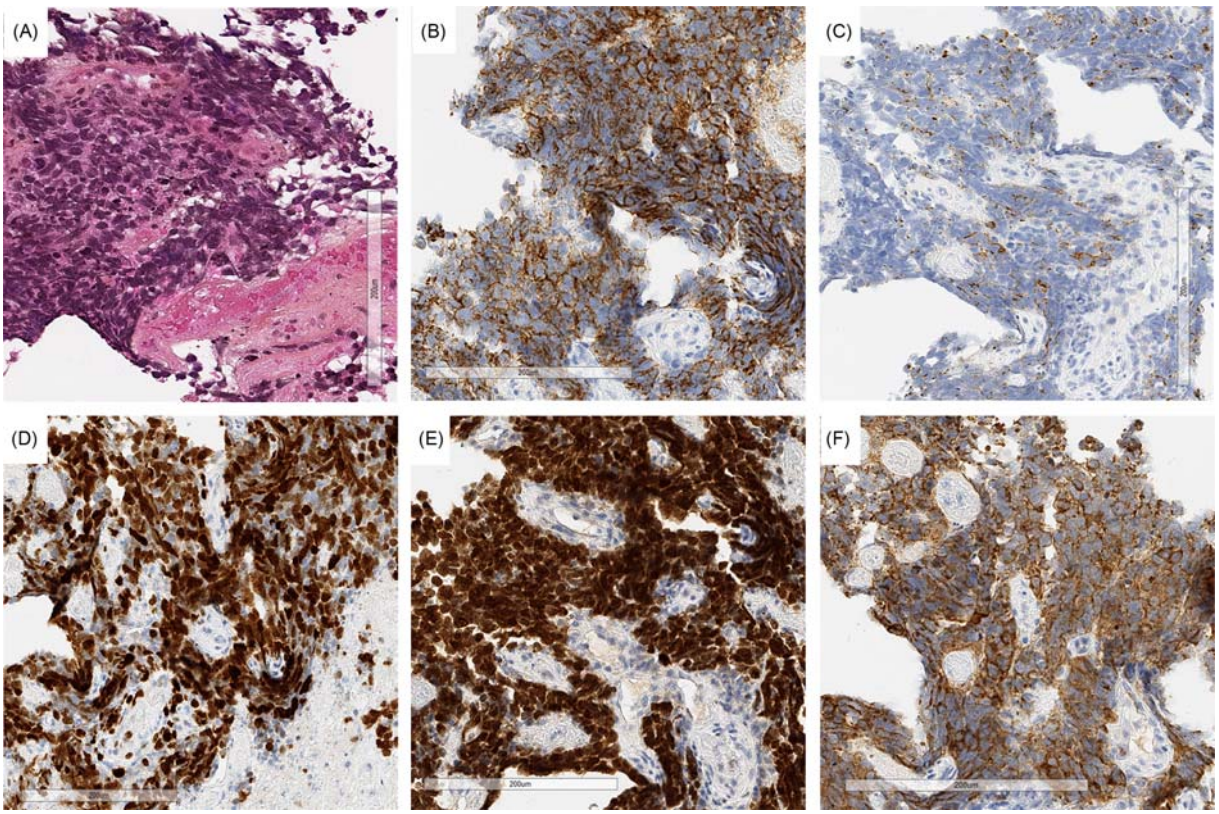

Fig. 3 (A) SCLC on a small bronchial biopsy. Note the crush artifacts (HES stain; original magnification ×200). (B) The same SCLC case: tumor cells express CD56 with a typical membranar staining (immunoperoxydase; original magnification ×200). (C) Chromogranin A expression by tumor cells (immunoperoxydase; original magnification ×200). (D) High Ki67 expression (nuclear staining with MiB1 Ab) (immunoperoxydase; original magnification ×200). (E) TTF1 expression by all tumor cells (nuclear staining with 8G7G3/1 Ab) (immunoperoxydase; original magnification ×200). (F) Synaptophysin expression by tumor cells (immunoperoxydase; original magnification ×200).

The expression of TTF1 in SCLC remains unexplained but it might be a cell lineage-specific phenomenon involving the developing neural cell-specific homeoprotein BRN2. In SCLC, nearly 50% of the nuclei are positively stained for KI67 (MIB1). Accordingly, Pelosi et al. have proposed a new grading system including three categories Lu-NET G1, Lu-NET G2, and Lu-NET G3 based on mitotic count, necrosis and KI67 index.

A recent international study by a group of pathologists of the IASLC has evaluated the impact of immunohistochemistry on the diagnostic reproducibility for SCLC. On selected cases, the agreement rate between pathologists increased from 0.55 to 0.60 when a panel of markers, including NE markers (CD56, chromogranin A and synaptophysin), TTF1 and KI67, was used. The use of such panel also increased the confidence of the pathologists. SCLC always express CK17 and CK19 cytokeratins, and are frequently positive with cocktails of cytokeratins, with a peculiar "punctate and paranuclear dot" pattern, but a diffuse cytoplasmic pattern does not exclude the diagnosis of SCLC. P63 and CK7 stains are of poor help as a minority of SCLC can be positive, but P40 is always negative. Interestingly, P16 nuclear staining is observed in 95%–100% of the SCLC, contrasting with RB1, which is never expressed. The usefulness in clinical practice of other putative diagnostic markers such as BAI3, CDX2, STK11, VIL1 needs to be further evaluated.

Differential Diagnoses

Differential diagnoses include other NE lung tumors, such as typical carcinoids (TC), atypical carcinoids (AC), and LCNEC, other NSCLC with small basal-like cells (basaloid carcinoma, NUT1 carcinoma) and more rarely, small round cell sarcoma, metastatic breast carcinoma and non-Hodgkin lymphoma. LCNEC can present morphological overlaps with SCLC but obvious NE architecture is mandatory for its diagnosis and cells are classically larger, with a vesicular nucleus and prominent nucleoli (Fig. 4A). Immunohistochemistry is of little help as SCLC and LCNEC share the same phenotype regarding NE markers, cytokeratins, P40, P16, and KI67 expression. Conversely, TTF1 is expressed in nearly half of the cases and LCNEC can be positive or negative for RB1.

Carcinoids in crushed or small specimen can also mimic SCLC, particularly when they are composed of spindle cells; however (Fig. 4B), clinical presentation differs dramatically and NE markers as well as cytokeratins are more diffusely expressed. In addition, carcinoids only focally and weakly express TTF1, and are RB1 positive. More interestingly, KI67/MIB1 antibody stains $<25\%$ of the tumor nuclei.

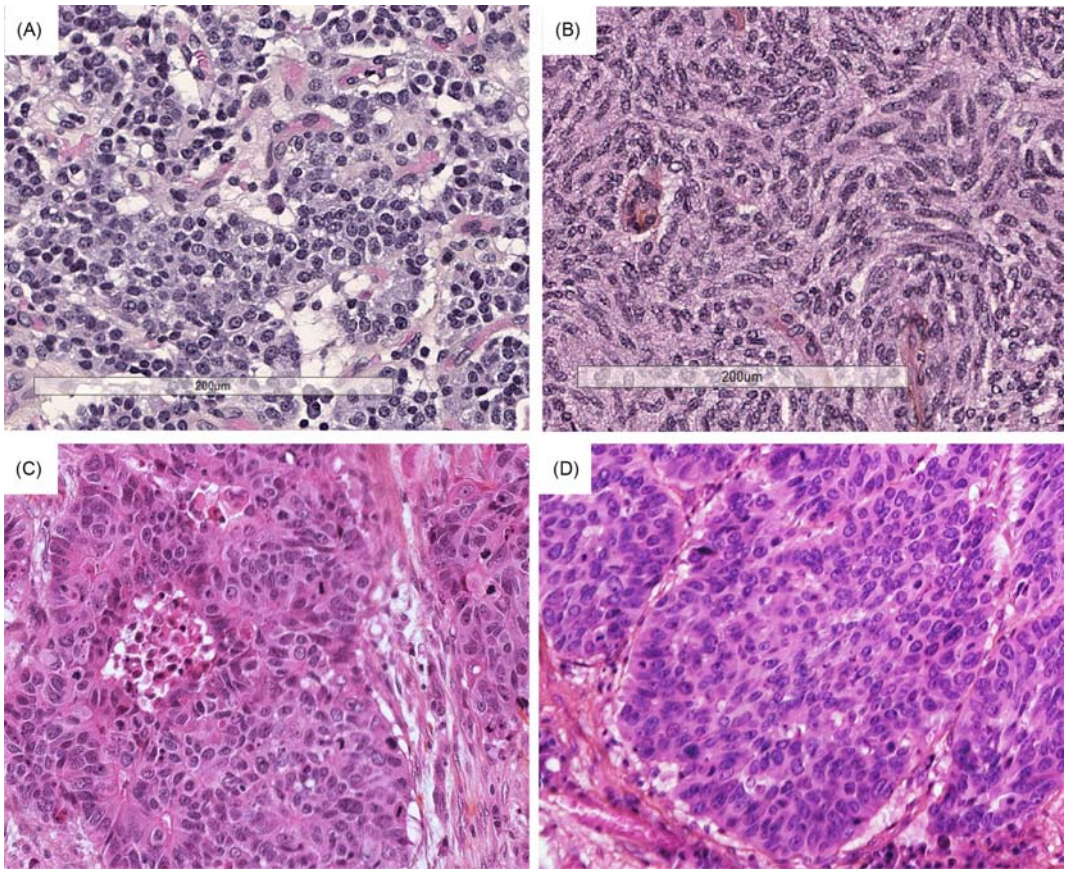

Fig. 4 Differential diagnoses for SCLC: (A) Typical carcinoid tumor (HES stain; original magnification $\times 200$). (B) Spindle cell carcinoid tumor (HES stain; original magnification $\times 100$). (C) Large-cell neuroendocrine carcinoma (HES stain; original magnification $\times 200$). (D) Basaloid variant of squamous-cell carcinoma (HES stain; original magnification $\times 100$).

Distinction of SCLC form basaloid carcinoma can be tricky on small specimens, particularly when they are poorly preserved or crushed (Fig. 4C). Immunohistochemistry is very helpful, with no NE markers and TTF1 expression, contrasting with strong and diffuse P63/P40 and high-molecular weight keratins (CK5–6 or CK1,5,10,14) stainings. NUT-rearranged carcinoma can be either P40 or TTF1 positive, but they are positive with NUT1 antibody and show typical *NUT1* gene rearrangement by FISH (Fig. 4D).

SCLC never expresses estrogen and progesterone receptors, which can be useful for the differential diagnosis with metastatic breast carcinoma. At least, SCLC needs sometimes to be distinguished from reactive or malignant lymphoid infiltrates on small crushed bronchial biopsies, justifying in doubtful cases the use of a panel of antibodies including lymphoid markers.

Molecular Pathology and Genetics

As mentioned above, SCLC is strongly associated with smoking exposure, with only 2% of cases occurring in never-smokers. Accordingly, SCLCs have a high load of somatic mutations induced by tobacco carcinogens (C:G > A:T transversions). The mean mutation rate of SCLC estimated to 8.62 nonsynonymous mutations per-million-base-pairs is similar to that of other tobacco-associated (lung) cancers.

Despite the difficulties in obtaining material suitable for genomic studies, large sequencing efforts have been made in recent years to molecularly characterize this deadly and untreatably disease. The genomes/exomes of around 200 resected SCLCs have been sequenced to date. These studies have revealed that SCLC is a disease of *TP53* and *RB1* since bi-allelic inactivation of these two genes is near ubiquitous in these tumors. In most cases, the inactivation of these genes happens due to bi-allelic inactivation through mutation, loss of heterozygosity (LOH), and inactivating rearrangements. In few cases the inactivation of *RB1* can occur through chromothripsis events leading to overexpression of cyclin D1 (encoded by the *CCND1* gene). In addition, homozygous loses in the *CDKN2A* locus (indirect activator of TP53 and RB1) and inactivating mutations and translocations in *RBL1* and *RBL2* (closely related to *RB1*) were also identified (Fig. 5A). These data suggest that the loss of the tumor suppressors *TP53* and *RB1* is required for the development of SCLC. In fact, this has already been inferred from mice studies, in which the inactivation of these two genes in pulmonary cells led to the development of SCLC tumors (Fig. 5B). Interestingly, both neuroendocrine (NE), and alveolar type 2 (SPC-expressing) cells led to SCLC upon inactivation of *Tp53* and *Rb1*, albeit SPC-expressing cells at a lesser efficiency. These data suggest that, although NSCLC and SCLC are commonly thought to be different diseases originating from different pulmonary cells (Alveolar type 1 and 2 and Clara cells for NSCLC, and neuroendocrine cells for SCLC), these malignant disorders might share common cells of origin. This idea has been supported by the unexpected findings that one of the resistant mechanisms of *EGFR*-mutated NSCLCs treated with EGFR tyrosine kinase inhibitors (TKI) is through transdifferentiation to SCLC (Fig. 5B). In a study on nine patients, which NSCLC tumors underwent transdifferentiation to SCLC at the time of acquired

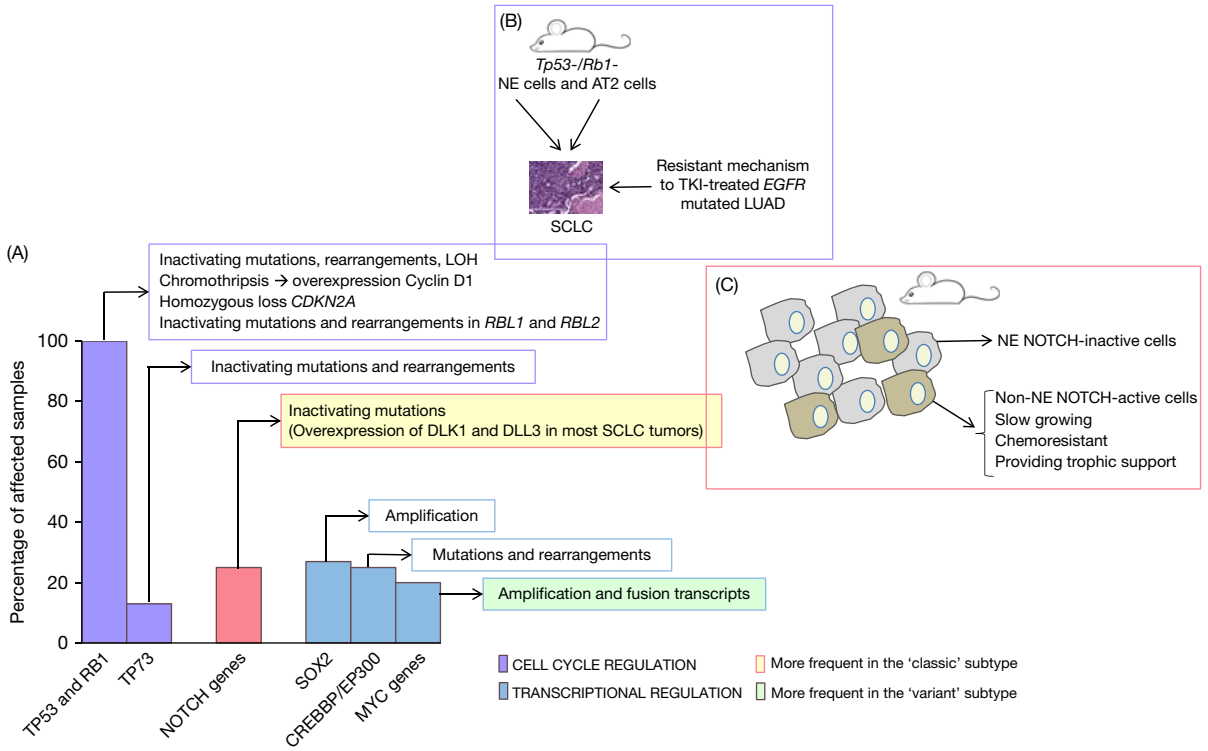

Fig. 5 Overview of the main biological and molecular characteristics of SCLC tumors.

resistance to EGFR TKI therapy, *RB1* has been found lost in 100% of these SCLC transdifferentiated cases. This rarely happened in those that remained NSCLC. Moreover, in another study with genome data on 21 patients with advanced *EGFR*-mutant NSCLCs that were transdifferentiated into EGFR TKI-resistant SCLCs, the authors found that EGFR TKI-resistant SCLCs branched out early from the NSCLC clones that harbored completely inactivated *RB1* and *TP53*.

The TP53 family of proteins also includes TP63 and TP73 based on their gene structures and functions; however, the functions of these three transcription factors diversified in the higher vertebrates. It has being found that TP73 is somatically inactivated by mutations and genomic rearrangements in 13% of the SCLC cases (**Fig. 5**A). Transcriptome analyses proved that the identified somatic genomic rearrangements generated the N-terminally truncated transcript variants p73Dex2 and p73Dex2/3, as well as p73Dex10. TP73 with N-terminal truncations are oncogenic through the dominant-negative functions on wild-type TP73 and TP53. C-terminal truncations, such as p73Dex10, can similarly exert dominant-negative effects on wild-type TP73.

Notch is inactivated in the majority of SCLC tumors (especially in those expressing the full set of NE cell markers), either by expression of the Notch inhibitors DLK1 and DLL3 or through mutations in Notch genes, which are detected in 25% of human SCLC (**Fig. 5**A). Although these receptors have been validated as tumor suppressors in mouse models of SCLC, it is known that the Notch signaling pathway mediates cell fate decisions and is tumor-suppressive or oncogenic depending on the context. In this context, a recent study has shown that Notch signaling might also be pro-tumorigenic in SCLC (**Fig. 5**C). The authors showed in mouse models that endogenous activation of the Notch pathway resulted in a neuroendocrine to nonneuroendocrine fate switch is 10%–50% in tumor cells. These nonneuroendocrine Notch-active SCLC cells are slow growing, consistent with the tumor-suppressive role, but these cells are also relatively chemoresistant and provide trophic support to neuroendocrine tumor cells, consistent with a pro-tumorigenic role. These data suggest that SCLC tumors generate their own microenvironment via activation of Notch signaling in a subset of tumor cells. This switch was partially mediated by the RE1-silencing transcription factor (Rest). Interestingly, in the first genome-scale analyses of methylation changes in SCLC tumors, it has been found that methylated gene promoters are enriched in binding sites for the neurogenic transcription factors NEUROD1, heart and neural crest derivatives-expressed protein 1 (HAND1), zinc finger protein 423 (ZNF423), and REST, which the authors interpreted as being indicative of a defect in neuroendocrine differentiation.

Another group of genes frequently inactivated in SCLC are those implicated in transcriptional regulation, including the transcription factors SOX2 and the MYC family of proteins. *SOX2* is amplified in ~27% of SCLC cases and MYC genes are also mostly altered through copy number amplification (9% *MYCL1*, 4% *MYCN*, and 6% *MYC*) and fusion transcripts (RLF-MYCL1) (**Fig. 5**A). Also included in transcriptional regulation are the histone modifying enzymes CREBBP and EP300 altered by mutation and genomic rearrangements in 15% and 13% of SCLC cases, respectively (**Fig. 5**A). Alterations in these genes suggest a role for epigenetic regulators in SCLC. In line with this, a recent study reported specific overexpression of the histone methyl-transferase EZH2 in primary SCLCs. EZH2 plays a major role in SCLC via regulation of stem cells maintenance, apoptosis, cell proliferation, expression of ASCL1, and induction of chemoresistance. It might also be that both groups of transcriptional regulators interact in this disease since it has been recently reported that an aberrant ATP-dependent chromatin remodeling SWI/SNF complex–MYC network is essential for SCLC development. In normal conditions, MYC forms a heterodimer with its primary partner MAX to bind gene promoters and activate transcription. Romero and colleagues detected mutations in *MAX* in 6% of the SCLC tumors and cell lines they analyzed, and these mutations were mutually exclusive with amplifications in genes of the MYC family and with *BRG1* mutations. BRG1, the ATPase of the SWI/SNF complex and regulator of MAX, seems also to upregulate MYC targets, both events required for the activation of the neuroendocrine transcriptional programs.

Finally, recurrent somatic alterations in receptor kinases and members of the PI3K signaling (KIT, FGFR1, IRS2, PIK3CA, and PTEN) and regulators of the actin cytoskeleton (SLIT2, EPHA7, and ROBO1) have also been detected in SCLC.

Due to its tendency for early dissemination, two-thirds of SCLC are diagnosed with extensive-stage disease (ES-SCLC, with distant metastasis); however, the genomic comprehensive studies above described are enriched for SCLC with limited-stage disease since these are probably the cases suitable for surgical resection and therefore providing the necessary material for such studies. A recent study analyzing 50 ES-SCLC found lower frequency of *TP53* (86%) and *RB1* (58%) mutations in comparison with the above-mentioned studies. In addition, it has recently been found that *NFIB* is almost universally overexpressed in human metastatic high-grade neuroendocrine lung tumors. Similarly to *Mycl1*, *Ezh* is frequently amplified in SCLC mouse models based on the inactivation of *Rb1* and *Tp53*. In this models, Nfib promotes metastatic spread and its high levels are associated with expansive growth of a poorly differentiated and almost exclusively E-cadherin (CDH1)-negative invasive tumor cell population.

Expression Profiles

The "classic" SCLC subtype (75% of the cases) expresses the transcription factor ASCL1, which is required for the survival and growth of these cells. ASCL1 is a master regulator that induces neuronal and NE differentiation. It regulates the expression of *DLL3*, which encodes an inhibitor of the Notch pathway, and the expression of *REST*, which encodes an inhibitor of neuronal and NE differentiation. ASCL1 also drives the expression of many proto-oncogenes implicated in SCLC progression and cell survival, including *MYCL1*, *RET*, *SOX2*, *NFIB*, and *BCL2*. The so-called "variant" subtype is formed by SCLC tumors with high expression of NEUROD1, often in association with ASCL1. ASCL1 and NEUROD1 target different gene sets for NE function, being one of the NEUROD1 targets, the gene *MYC*. In a genetically engineered mouse model (GEMM), conditional Cre-mediated excision of the *Tp53* and *Rb1* genes in lung epithelial cells gave rise to lung tumors resembling the classic, but not to the variant, subtype. Ascl1, but not NeuroD1, would be required for tumor initiation in this model. The switch from ASCL1 to NEUROD1 expression

might be associated with a change from the classic to the variant form. Overexpression of Myc in a GEMM of SCLC promoted the development of SCLC tumors with high NeuroD1 and low Ascl1 expression. Finally, there is a third and minor SCLC subtype that contains those tumors on which neither ASCL1 nor NEUROD1 are expressed. The clinical implications of these three distinct SCLC groups are still unknown.

Microenvironment Including Immune Response

The immune cell microenvironment has not been extensively studied in SCLC. Presence of CD45+ immune cells within the SCLC stroma, so-called tumor infiltrating lymphocytes TILs, correlates with a better overall survival, independently of stage and performance status. A high number of intratumoral T cells and CD8 cells is associated with a low tumor size (<3 cm) and low tumor stage (stages I–II) and effector T cells are more abundant in LD-SCLC. In addition, $CD8^{(high)}CD57^{(+)}$T-cells in the peripheral blood stroma could be an independent predictor of response to chemoradiation therapy in ES-SCLC. As regulatory T cells (Tregs) downregulate immune response to tumor cells in SCLC, in the same way than in other tumor types, low effector-to-regulatory T-cell ratio is associated with a shorter survival. SCLC could also evade the immune response by reducing the transcription of HLA-A, B, and C and beta 2m genes more effectively than other lung cancer types.

High macrophage content correlates with a better prognosis. Those macrophages harbor a CD68, CD163, CD14 and CD47 receptor SIRPalpha expression in relation with an immunosuppressive M2 phenotype and SCLC tumor cells strongly express CD47, justifying the use of CD47 blockade agents to enhance SCLC phagocytosis.

PD-1 is another key immune checkpoint receptor expressed by activated T cells after binding to its ligand PD-L1 that mediates immunosuppression. PD-L1 expression has been demonstrated in nearly 5% of SCLC (and in 10% of LCNEC and in none of carcinoids) using E1L3N clone. In another study involving 74 SCLC, PD-L1 expression was observed in 19% of cases using the same clone and the same threshold. Interestingly, in both studies, PD-L1 expression was associated with significantly longer overall survival. Using SP142 and 28-8 clones, the percentage of positive cases was around 16% with a cut-off of 1%. Conversely, Schultheis et al. found no PD-L1 protein expression in tumor cells using E1L3N clone in 94 SCLC (pulmonary and extrapulmonary), but 48% of the cases exhibited PD-1 positive lymphocytes. Overall all those discrepancies seem mainly related to the antibody used and need to be further reevaluated with PD-L1 pharmDx assays.

Clinical Presentation, Staging and Grading

Symptoms reflect central location and loco-regional spread, as well as rapid tumor growth. While hemoptysis are rare, superior vena cava and vocal cord paralysis are common. However, clinical symptoms more often reflect disseminated disease. Brain metastases are frequent. The average age at diagnosis is 70. At imaging, SCLC appears as hilar or perihilar masses often with mediastinal lymphadenopathy and lobar atelectasis. Paraneoplastic syndromes are also a hallmark of SCLC. Most fall into endocrine or neurological classes, with the endocrine symptoms due to ectopic hormonal production by tumor cells and the neuronal symptoms largely due to the production of antineuronal antibodies. The most common endocrine syndromes include inappropriate antidiuretic hormone production and adrenocorticotropic hormone (ACTH)-associated Cushing syndrome.

The International Association for the Study of Lung Cancer (IASLC) recommends the recently published 8th edition of the TNM Classification for Lung cancer for SCLC staging. However, the 1957 classification system developed by the Veterans Affairs Lung Study Group is still the most relevant staging system clinically; this classification divides SCLC into limited-stage (LS) disease and extensive-stage (ES) disease, depending on whether or not the affected area can be included in the radiotherapy (RT) field. Unfortunately, only 20%–30% of patients are diagnosed with LS-SCLC. Even with treatment, prognosis remains poor, with a median survival of 16–22 months in LS-SCLC and only 10 months in ES-SCLC. The proportion of patients diagnosed with ES-SCLC has increased substantially in recent years due to the inclusion of positron-emission tomography computed tomography (PET-CT) and magnetic resonance imaging (MRI) in the routine practice setting. A brain magnetic resonance imaging scan will detect brain metastases in 10%–15% of newly diagnosed patients without neurologic symptoms. A systematic review of studies comparing FDG-PET imaging with conventional imaging procedures reported that 16% of patients with LS-SCLC by conventional imaging were up-staged to ES by FDG-PET imaging, whereas 11% of patients with ES-SCLC by conventional imaging were down-staged to LS.

Prognostic and Predictive Biomarkers

A number of multivariate analyses of adverse prognostic factors have been performed in SCLC. Poor outcome has been associated with poor performance status, extensive disease, elevated lactate dehydrogenase, high alkaline phosphatase, low sodium, low serum albumin, high aspartate aminotransferase and low bicarbonate; those factors have been included in the Manchester prognostic score. A potential novel prognostic factor might be the "CpG-island methylator phenotype" (CIMP). In a recent study on which SCLC samples were stratified according to CIMP status, patients with CIMP-positive tumors had a poorer prognosis than those with CIMP-negative disease, consistent with observations among other lung carcinoma epitypes.

SCLC is rarely diagnosed at early stages. However, these patients have considerably improved survival rates relative to late-stage patients, principally as the localized nature of the disease affords the widest range of treatment options. The routine use of FDG-PET and brain MRI has also led to a better selection of patients with LS-SCLC, associated with better outcome.

Unfortunately, predictive markers that might increase the number of SCLC patients diagnosed with limited-stage disease are widely unknown. In a study aiming at the characterization of the SCLC methylome, the authors found that among the hundreds of tumor-specifically methylated genes discovered, 73 gene targets were methylated in >77% of primary SCLC tumors, most of which have never been linked to aberrant methylation in tumors. These methylated targets might have potential for biomarker development for early detection. Another study has focused on the study of the potential use of cell-free DNA (cfDNA) for the early detection of SCLC and, although they were able to detect *TP53* mutations in the cfDNA of ~36% early-stage SCLC patients, they also detected mutations on this gene in 11% of the noncancer controls they analyzed, posing a serious specificity problem for the development of screening tests.

Treatment

For LS-SCLC, standard of care is concurrent chemoradiation, while for ES-SCLC chemotherapy alone is used; the most commonly recommended chemotherapy regimen is platinum-etoposide (PE), which has been proven to increase survival with less toxicity than other regimens that combine anthracyclines, vinca-alkaloids, methotrexate, and/or cyclophosphamide. SCLC shows chemosensitivity in the first-line setting, as response rate range from 70% to 80% with up to 50% complete responses. Many other systemic approaches have been assessed, but none have proven superior to PE, including combination of platin plus irinotecan or topotecan. Still the majority of patients die from recurrences, which are refractory to chemotherapy. Recurrent SCLC is divided into two categories: refractory/resistant (progression <3 months from completion of initial therapy) or relapsed/sensitive (progression >3 months). Rates of response to second-line therapy are substantially lower in patients with refractory/resistant disease. The reinitiation of the front-line chemotherapy regimen is proposed if the initial response duration is 6 months or more based on reported response rates of 50%–60%. The benefit of second-line chemotherapy in recurrent SCLC was evaluated in a randomized trial comparing oral topotecan with best supportive care. Although topotecan induced response in only 7% of patients, it did significantly improve overall survival.

As for thoracic radiotherapy, the results available so far have not yet answered the questions concerning optimum timing, schedule and doses. The randomized controlled study published by Turrisi et al. in 1999 is considered a landmark. A total of 471 patients were randomized to either 45 Gy in 5 weeks (25 daily sessions of 1.8 Gy) or 45 Gy in 3 weeks (1.5 Gy, twice daily, 30 fractions) beginning with the first of four platin and etoposide cycles. The 5-year OS in the hyper-fractionated group was 26% versus 16% in the conventional-treatment group ($P = 0.04$). The more recent CONVERT phase III trial compared the standard 45 Gy in 30 fractions (twice daily for 3 weeks) to 66 Gy in 33 fractions (once daily over 6.5 weeks) starting on day 22 of chemotherapy; no significant between-group differences were found in 1-, 2- or 3-year overall survival. After response to chemotherapy, the opportunity to deliver prophylactic cranial irradiation (PCI) is highly debated. The use of PCI in the treatment of LS-SCLC is mostly supported by the fact that approximately 50% of patients with SCLC develop brain metastases.

SCLC Developing From Adenocarcinoma

EGFR-mutant lung adenocarcinoma can trans-differentiate to SCLC, especially when resistance to tyrosine kinase inhibitors (TKIs) develops. This phenomenon has repeatedly been described in several case reports and small patient series, and may occur in other oncogene-driven adenocarcinoma subsets. This may also explain reports of *EGFR*-mutant SCLCs. In a pooled analysis of 39 of those cases, the median time from initial diagnosis of lung adenocarcinoma to the transformation to SCLC was 19 months (range 1–61 months). Median survival after SCLC diagnosis was 6 months. Male gender and smoking were associated with poor outcome. The management of SCLC developing from *EGFR*-mutant adenocarcinoma may rely on chemotherapy; meanwhile, SCLC retain the same *EGFR* mutation, and transformation may be associated with other mechanisms of resistance, such as T790M or C797S. SCLC-trans-differentiated tumors also harbored activating mutations in *PIK3CA*, inactivating mutations of *TP53*, and *RB1* loss that are common features associated with the SCLC phenotype.

Immunotherapy

Immune checkpoint inhibitors targeting PD-1 and CTLA-4 have been showing promise in SCLC. In a phase I/II trial, 216 patients with recurrent SCLC were treated with nivolumab alone ($n = 98$), or one of two combination regimens: nivolumab 3 mg/kg with ipilimumab 1 mg/kg every 3 weeks, or conversely, nivolumab 1 mg/kg with ipilimumab 3 mg/kg on the same schedule. In general, the toxicity spectra observed were similar to those reported in other studies of these agents in patients with cancer, with still some patients experiencing exacerbation of paraneoplastic neurological syndromes. Nivolumab alone was associated with a 10% response rate and a 32% disease-control rate, while the combination treatment cohorts had response rates of 19%–23% and disease-control rates of 36%–42%. The 1-year survival was 33% with nivolumab alone, and 35%–43% in the combination arms. While PD-L1 positivity is rare in SCLCs, and does not seem to correlate with benefit from PD-1 directed therapy, ancillary

analyses indicated that tumor mutation burden may represent a predictive biomarker in this setting: an elevated tumor mutational burden was associated with a 22 months median overall survival with nivolumab and ipilimumab.

Other available results with immunotherapy are reports with the use of pembrolizumab, in the second-line or even the maintenance setting. Of note, although most SCLC show low expression of PD-L1, a subset (1.9%) of human SCLC patient cases exhibits massive expression of PD-L1 caused by focal amplification of CD274. Such tumors may be particularly susceptible to immune checkpoint blockade.

DLL3 Targeting

Targeting of the Notch pathway in SCLC relies on inhibiting the Notch inhibitor DLL3, which is expressed as a neoantigen on the cell surface of SCLC cells. Rovalpituzumab tesirine (ROVA T) is a DLL3 targeted antibody–drug conjugate that, in a phase I study, was shown to have highly encouraging single-agent antitumor activity in DLL3 expressing SCLC. This trial enrolled a total of 74 patients with recurrent metastatic SCLC; among the patients with SCLC treated at active dose levels, an overall objective response rate of 18% was observed. DLL3 expression may represent a predictive biomarker as among all patients in this DLL3 high cohort (around two-thirds of patients with SCLC) the confirmed objective response rate was 38% (10 of 26 patients), the disease-control rate 88% (23 of 26 patients), and 1-year survival rate was 32%. Phase III trials are ongoing.

Additional Therapeutic Opportunities

In vitro targeted drug screening has revealed that SCLC with high MYC expression is vulnerable to Aurora kinase inhibition, which, combined with chemotherapy, strongly suppressed tumor progression and increased survival.

Further Reading

Brierley, J.D., Gospodarowicz, M.K., Wittekind, C., 2017. TNM classification of malignant tumours. In: Union for International Cancer Control (UICC), 8th edn. John Wiley & Sons, Ltd.

Früh, M., De Ruysscher, D., Popat, S., Crinò, L., Peters, S., et al., 2013. Small-cell lung cancer (SCLC): ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Annals of Oncology 24 (Suppl. 6), vi99–vi105.

Gazdar, A.F., Bunn, P.A., Minna, J.D., 2017. Small-cell lung cancer: What we know, what we need to know and the path forward. Nature Reviews. Cancer (12), 725–737.

George, J., Lim, J.S., Jang, S.J., Cun, Y., Ozretic, L., et al., 2015. Comprehensive genomic profiles of small cell lung cancer. Nature 524, 47–53.

Govindan, R., Page, N., Morgensztern, D., Read, W., Tierney, R., et al., 2006. Changing epidemiology of small-cell lung cancer in the United States over the last 30 years: Analysis of the surveillance, epidemiologic, and end results database. Journal of Clinical Oncology 24, 4539–4544.

Kalemkerian, G.P., Schneider, B.J., 2017. Advances in small cell lung cancer. Hematology/Oncology Clinics of North America 31, 143–156.

Navada, S., Lai, P., Schwartz, A.G., Kalemkerian, G.P., 2006. Temporal trends in small cell lung cancer: Analysis of the national surveillance, epidemiology, and end-results (SEER) database. Journal of Clinical Oncology 24, 7082.

Sabari, J.K., Lok, B.H., Laird, J.H., Poirier, J.T., Rudin, C.M., 2017. Unravelling the biology of SCLC: Implications for therapy. Nature Reviews. Clinical Oncology (9), 549–561.

Travis, W.D., Brambilla, E., Burke, A.P., Marx, A., Nicholson, A.G. (Eds.), 2015. WHO classification of tumours of the lung pleura, thymus and heart, 4th edn. International Agency for Research on Cancer, Lyon.

Squamous Cell and Basal Cell Carcinoma of the Skin: Diagnosis and Treatment

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Abbreviations

BCC Basal cell carcinoma
CT Computed tomography
EGFR Epidermal growth factor receptor
FNA Fine-needle aspiration
HPV Human papillomavirus
ICD-O International Classification of Diseases for Oncology
IMRT Intensity-modulated radiotherapy
LOH Loss of heterozygosity
MMS Mohs micrographic surgery
MRI Magnetic resonance imaging
NMSC Non-melanoma skin cancer
PDT Photodynamic therapy
RT Radiotherapy
SCC Squamous cell carcinoma
UV Ultraviolet radiation

Definition and Classification

Basal cell and squamous cell carcinoma are the two most common types of skin malignancies. They belong to keratinocytic tumors (also called keratinocyte tumors), one of the families of non-melanoma skin cancers (NMSCs).

Basal cell carcinomas (BCC) of the skin, or cutaneous basal cell carcinomas (ICD-O code: 8090/3; also called basal cell epithelioma and trichoblastic carcinoma), are a group of malignant tumors which arise from basal cells of the epidermis and pilosebaceous units and are histologically characterized by the presence of lobules, columns, bands or cords of basaloid ("germinative") cells surrounded by an outer palisade of cells. The associated stroma is loose and fibromucinous. There are several BCC subtypes. The most common of them is nodular BCC (ICD-O code: 8097/3) which occurs in approximately 60% of cases, followed by multifocal superficial BCC (8091/3; also called multicentral BCC; 30% of BCC cases) and infiltrating sclerosing BCC (8092/3; also called morpheic BCC or morpheaform BCC; 5%–10% of cases). The less common subtypes are cystic, linear, and micronodular BCC. A BCC variant which is frequently confused with SCC is basosquamous carcinoma (ICD-O code: 8094/3), also called mixed basal squamous carcinoma. It arises from basal cells but displays squamous differentiation with a more abundant cytoplasm and more marked keratinization than those of typical basal cell carcinomas, and has a more aggressive behavior and a higher metastatic capacity than other BCC types, thus resembling SCC. That is why it is often incorrectly referred to as a particular variant of squamous cell carcinoma.

Squamous cell carcinomas (SCC) of the skin, or cutaneous squamous cell carcinomas (ICD-O code: 8070/3) are malignant neoplasms of epidermal and mucous membrane keratinocytes characterized by variable degrees of squamous differentiation, with atypical, often pleomorphic squamous cells. SCCs are graded as well, moderately, or poorly differentiated. Well differentiated carcinomas are usually associated with keratin production and the presence of intercellular bridges between adjacent cells.

A particular form of SCC in situ is Bowen disease (ICD-O code: 8081/2; also called intraepidermal SCC, Bowen type). It is a distinct clinicopathological entity which arises from the skin or the mucocutaneous junction and is characterized by the presence of hyperkeratosis, parakeratosis, dyskeratosis, and acanthosis.

Presentation and Diagnosis

Keratinocytic tumors vary in their clinical presentation based on the type of lesion and the stage of development.

BCCs develop on hair-bearing skin, most commonly on radiation-exposed areas, with a majority occurring on the head and neck, followed by the trunk and extremities. Less frequent sites of involvement are penis and vulva. Nodular BCC arises predominantly on the head and neck as a well-circumscribed nodule with pearly or rolled borders and telangiectasias. Some lesions are pigmented and clinically indistinguishable from melanoma. Larger tumors may develop central necrosis and ulceration, forming the so-called rodent ulcer. Superficial BCC usually arises on the trunk, often as multiple lesions. It appears as red, scaly patches

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with areas of brown or black pigmentation, which spread over the skin surface and may have areas of nodularity. Sclerosing BCCs usually affect the face. The tumors resemble scars and may have an ivory-colored, poorly delineated, indurated border. Histologically, the cancer cells are surrounded by a dense bed of fibrosis ("morphealike"). Despite excellent prognosis, BCC can produce substantial local destruction with disfigurement and may involve extensive areas of soft tissue, cartilage, and bone.

SCCs may arise on all cutaneous surfaces and mucous membranes but they most commonly develop in areas of direct exposure to the sun, such as the forehead or ears. The lesions present as shallow ulcers, often with a keratinous crust and elevated, indurated surrounds, or as plaques or nodules. Actinic damage to the surrounding skin is typical. Histologically, cutaneous squamous cell carcinomas are characterized by nests, sheets and strands of squamous epithelial cells which arise from the epidermis and extend into the dermis. Sixty percent of SCCs arise from actinic keratoses. In case of Bowen disease, the tumor most frequently develops on sun-exposed glabrous skin areas, particularly on the head, face and neck, but it may also arise in mucous membranes. It presents as an erythematous, scaly, keratotic patch or plaque, with frequent multiple contiguous lesions. Metastatic SCC most frequently involves the draining lymph nodes but in late stages distant organs may also be involved.

Patients presenting with suspicious skin lesions are offered a physical examination, with an emphasis on a complete skin examination. The latter is particularly important as individuals with skin cancer often have additional, concurrent lesions, either precancerous or cancerous, at other, usually sun-exposed sites. For the same reason, an interview with an emphasis on history of cancerous lesions should be conducted. All suspicious lesions are biopsied, including deep reticular dermis if the lesion is suspected to be more than a superficial process. This procedure allows to detect infiltrative histology which is present only at the deeper advancing margin of the tumor and may thus be missed by a superficial biopsy. In high-risk populations, clinical evaluation of suspected lesions is more difficult and so a low threshold of performing biopsies is necessary. If the lesion suggests SCC, examination of regional lymph nodes should additionally be performed. Imaging analyses should be conducted when extensive disease is suspected, such as bone involvement, perineural invasion or deep soft tissue involvement. Magnetic resonance imaging (MRI) with contrast is preferred over computed tomography (CT) in case of suspected perineural disease or deep soft tissue involvement.

Epidemiology ad Risk Factors

Burden

Non-melanoma skin cancers (NMSCs) are the most common type of malignancy but they account for less than 0.1% of cancer-related deaths. Among those, keratinocytic tumors are the most common skin neoplasms, accounting for approximately 90% of all skin malignancies. Basal cell and squamous cell carcinomas (BCCs and SCCs, respectively) are by far the most prevalent, with BCC being four to five times more frequent than SCC. In lower latitudes, the proportion of SCC is higher and some studies show that the overall SCC incidence is rising quicker than that of BCC. The exact incidence is not known as the data on these two tumor types are typically not reported to cancer registries. It is estimated that in the US alone 2 million new BCC cases and 2.5 million new SCC cases occurred in the year 2012. The BCC incidence rates are estimated to have been rising by 2% a year in the United States and 5% a year in Europe in recent decades. This increase may be partly due to increasing awareness among the general population and among physicians as well as to the fact that more surgical treatments are followed by a histopathological confirmation of the diagnosis. The highest BCC rates are reported in Australia where one in two inhabitants will be diagnosed with BCC by the age of 70.

Etiology and Risk Factors

The major risk factor for developing NMSC is exposure to ultraviolet radiation (UV), in particular in childhood and adolescence, with 90% of BCC cases being attributable to UV exposure. However, the skin's ability to tan modulates the UV-induced risk. Also indoor tanning has been shown to be associated with an increased risk of BCC. This can also explain a higher prevalence of BCC in young women compared to men. In the same line, UV-based therapies have been associated with a moderately increased risk of BCC. Fair skin, red or blond hair, and bright eyes are independent risk factors for both BCC and SCC as they increase susceptibility to UV-induced damage. Not surprisingly, BCC incidence is inversely correlated with the latitude combined with the pigment status of the population. Hereditary conditions resulting in an increased sensitivity to sunlight: xeroderma pigmentosum (impaired DNA damage repair) and oculocutaneous albinism (generalized decrease in pigmentation), predispose to UV-induced skin cancers from early childhood.

Individuals who were exposed to ionizing radiation (uranium miners, radiation-treated patients, cancer survivors), in particular those exposed at young age, have a higher risk of NMSC. The risk of radiation-induced BCC seems to also increase with lighter pigmentation.

Ingestion of inorganic arsenic and its arsenic compounds predisposes to the development of Bowen disease, multiple BCC, and SCC. From among other putative environmental risk factors, some studies have pointed out to smoking (and/or benzo(a)pyrene exposure) and alcohol consumption but the evidence is not conclusive.

Treatment with psoralens (used for psoriasis) combined with phototherapy is also associated with an increased risk of BCC. The same association has been suggested for other photosensitizing drugs but the evidence is still limited.

Table 1 Risk factors for basal cell and squamous cell carcinoma of the skin[a]

Environmental factors and medical conditions
Exposure to ultraviolet radiation (UVA and UVB), including indoor tanning and UV therapies (BCC)
Exposure to ionizing radiation
Inorganic arsenic exposure
Chronic immunosuppression and chronic use of glucocorticoids (SCC)
Psoralens combined with phototherapy (BCC)
Scars and chronic wounds (SCC)
Infection with some HPV types (SCC; conflicting reports for BCC)
Lifestyle and phenotypic factors
Living at low latitude
Light pigmentation
Age
Male gender
Hereditary conditions
Xeroderma pigmentosum
Oculocutaneous albinism
Gorlin syndrome (BCC)

BCC, basal cell carcinoma of the skin; *HPV*, human papillomavirus; *SCC*, squamous cell carcinoma of the skin.
[a]Association shown for both cancer types unless indicated otherwise.

Chronic immunosuppression, in particular in the context of organ transplantation, lymphoma, chronic lymphocytic leukemia and HIV, and chronic use of glucocorticoids, is associated with the risk of developing SCC. Organ transplant recipients have a 60- to 250-fold higher risk of developing the disease than the general population.

SCC is also known to develop in association with scars and chronic wounds (e.g., Marjolin's ulcer) as well as with thermal burns and chronic draining osteomyelitis. It is the most common malignancy arising in chronic ulcers.

Moreover, infections with human papillomaviruses (HPV), in particular type 5, 8, 16, and 18, have been associated with SCC. HPV type 5 and 8 cause epidermodysplasia verruciformis, which may lead to the development of SCC in situ and invasive SCC, synergistically with other carcinogens, such as sunlight. Sexually transmitted HPV infections increase the risk SCC of the genitals and the anal regions.

Finally, Gorlin syndrome (basal cell nevus syndrome), characterized by germline mutations in the *PTCH* gene, is associated with multiple BCC lesions over the face, arms, and trunk, which appear during the late teenage years. **Table 1** lists all confirmed risk factors for developing BCC and SCC.

Pathology and Genetics

As NMSC carcinogenesis is associated with exposure to radiation and resulting DNA damage, genetic alterations in pathways involved in DNA damage repair and in the control of cell cycle and apoptosis are the most prevalent events. Somatic mutations are frequent, with a mutational signature characteristic for UV-induced cancers (tandem CC to TT transitions) affecting multiple oncogenes, tumor-suppressor genes and important housekeeping genes, which results in deregulation of keratinocyte cell cycle and substantially contributes to carcinogenesis.

Alterations in the sonic hedgehog signaling pathway seem to play a pivotal role in the pathogenesis of BCC and are found in up to 90% of cases (**Table 2**). Mutations in the *PTCH1* (patched 1) encoding the sonic hedgehog transmembrane receptor are the underlying cause of the nevoid BCC syndrome and are also found in 30%–90% of sporadic BCC. Loss of heterozygosity on chromosome 9q22, encompassing the *PTCH1* gene, is the most frequent genetic alteration in sporadic BCC. About half of *PTCH1* mutations are C to T and tandem CC to TT transitions. However, the UV radiation origin of *PTCH1* mutations is controversial since other factors, such as oxidative stress, have been implicated in the mutagenesis of this gene. The second frequently altered gene of the hedgehog pathway is *SMO* which is activated by gain-of-function mutations. Also *PTCH2* and *SUFU* have been reported to be mutated in BCCs, however in a low proportion.

Specific UV-induced mutation in *TP53* are also common events in BCC pathogenesis, with a high prevalence of characteristic UV-induced CC to TT tandem mutations. Mutations in the *TP53* gene have been reported in about half of sporadic BCC cases, whereas LOH has been described with a much lower frequency in BCC than in other tumors. Hot spots occurring specifically in BCC have been found at codons 177, 196, and 245. Codon 177 seems to be specific for BCC since it is not frequently mutated in other malignancies. In a mouse model investigating BCC pathogenesis, loss of *TP53* has been shown to upregulate the activity of the hedgehog pathway by increasing *SMO* expression and rendering the mouse interfollicular keratinocytes susceptible to develop X-ray induced BCCs.

Recent genomic analyses have identified new driver genes for BCC, suggesting the existence of a more complex genetic network of cancer-associated genes than it had been hypothesized. It is noteworthy that there are discrepancies regarding the list of driver

Table 2 Gene loci that are most frequently altered in basal cell (BCC) and squamous cell skin carcinoma (SCC)

Pathway	*Gene (locus)*	*Alteration type*	*Prevalence*
p53 signaling (cell-cycle control)	*TP53* (17p13.1)	Mostly point mutations (high prevalence of CC to TT transitions in BCC)	About half of BCC cases and up to 85% of SCC cases
	CDKN2A (9p21.3)	Point mutations and deletions	About 60% of SCC cases
Telomere maintenance	*TERT* (5p15.33)	Promoter alterations and point mutations	Found in all skin cancers, including BCC (up to 40%) and SCC (20%–40%)
Translation fidelity	*DPH3-OXNAD1* (3p25.1)	Promoter alterations	Common in all skin cancers, including BCC
Sonic hedgehog signaling	*PTCH1* (9q22.32)	Inactivating point mutations, loss of heterozygosity (LOH), and copy-neutral LOH (due to uniparental disomy)	Point mutations in 10%–75% of BCCs, LOH in 40%–70% of BCCs; germline mutations in nevoid BCC syndrome
	SMO (7q32.1)	Gain-of-function mutations	BCC
Hippo–YAP pathway (organ size control)	*LATS1* (6q25.1)	Inactivating mutations	BCC
	PTPN14 (1q32-q41)	Inactivating mutations	BCC
Histone modifications	*MLL2* (*KMT2D*; 12q13.12)	Point mutations	27%–50% of SCC cases
NOTCH signaling	*NOTCH1* (9q34.3)	Point mutations	43%–57% of SCCs
Low density lipoprotein (LDL) receptors	*LRP1B* (2q21.2)	Point mutations	50% of BCC and 20%–65% of SCC cases

genes identified across different studies, probably reflecting the clinico-pathological heterogeneity of analyzed tumors. Inactivating mutations in two key components of the Hippo-YAP pathway: *LATS1* and *PTPN14*, have been identified in BCC. In addition, three candidate genes known to be associated with melanoma development: MYCN, PPP6C, and STK19, have emerged as putative new BCC driver genes. Finally, some studies report that somatic, non-coding mutations within promoter regions of *TERT* and *DPH3-OXNAD1* genes are common in all types of skin cancer, including BCC (Table 2).

Only few studies have analyzed comprehensive genetic alteration profiles in cutaneous SCC. In a recent study by the Cancer Genome Atlas Research Network, which analyzed 122 tumors, the most frequently altered gene was *TP53*, followed by *CDKN2A*, *NOTCH1*, *MLL2*, *LRP1B*, and *TERT* (Table 2). *TP53*, *NOTCH1*, and *TERT* alterations have been systematically found in various studies, albeit with variable prevalence. Overall, the major difference between SCC and BCC appears to be a less frequent involvement of genes of the hedgehog and Hippo pathways in SCC carcinogenesis.

Management and Therapy

Most patients with a primary cutaneous NMSC have an excellent prognosis. BCCs are usually indolent tumors. However, if untreated, they may invade subcutaneous fat, skeletal muscle and bone. Still, distant metastases are extremely rare, with the exception of basosquamous tumors which are usually more aggressive and may produce regional or widespread metastases. SCCs may be more aggressive but most of them just locally.

The primary goal of both BCC and SCC treatment is the complete removal of the tumor while preserving the maximum of skin function and cosmesis. The therapeutic approach is customized to the particular factors, and the individual needs and preferences of the patient.

Surgery is often the most effective mean to achieve cure. Mohs micrographic surgery (MMS) with intraoperative frozen section assessment has the highest primary tumor cure rate (99% for BCC and 96% for SCC) and excellent cosmetic effects. As it allows to assess 100% of the surgical margins, it is also the recommended option for high-risk tumors. Standard surgical excision with postoperative margin assessment is also an option. However, intraoperative assessment of all tissue margins is key to complete curative removal of high-risk tumors. Curettage (scraping the tumor tissue with a curette down to a layer of normal dermis) and electrodessication may be effective for low-risk tumors with free caveats appearing in hairless areas. However, some studies suggest that it may be associated with higher recurrence rates than surgery. In order to reduce that risk, a biopsy taken during curettage should be evaluated. Moreover, surgical excision should follow if the subcutaneous layer is reached.

Cosmesis and patient's preferences may lead to choosing radiotherapy (RT) as primary and definitive treatment of localized BCC or SCC without lymph node involvement. RT is also recommended as an adjuvant therapy following surgery in patients with high-risk tumors with the large nerve and extensive perineural involvement, and in all patients in whom clear surgical margins could not be achieved. Intensity-modulated RT (IMRT) has been gaining popularity is this respect. Given that exposure to radiation is one of the risk factors for developing NMSCs, potential benefits and risks of using RT need to be carefully weighed up. Generally, RT is not recommended in patients below 40 years of age or with tumors recurring after previous RT, and in individuals with inherited syndromes that increase skin's sensitivity to radiation, like xeroderma pigmentosum.

SCC occasionally spreads to regional lymph nodes. In case of enlarged lymph nodes, fine-needle aspiration (FNA) or biopsy should be performed. If lymph node involvement is confirmed, elective lymph node dissection follows. Sentinel lymph node biopsy has been used to try to identify SCC patients likely to benefit from completion lymph node dissection or adjuvant RT. However, the results are conflicting and also the criteria of selecting patients for this procedure are not clear.

In patients with low-risk superficial tumors in whom surgery and radiotherapy are contraindicated or simply not preferred, topical therapies with 5-fluorouracil or imiquimod, photodynamic therapy, or vigorous cryotherapy using liquid nitrogen may be considered. However, the cure rates may be lower than those achieved with surgery.

Systemic treatment is indicated as an adjuvant therapy—in combination with RT or alone—for BCC patients after surgery without clear surgical margins. In particular, two FDA-approved inhibitors of the sonic hedgehog pathway: vismodegib and sonidegib, give promising results in the treatment of locally advanced BCC. Using these molecular therapeutics is also associated with reduced recurrence rates in metastatic BCC. Of note, vismodegib has also been tested as a BCC treatment and prophylaxis in patients with nevoid BCC syndrome, with promising results. However, a key limitation of using hedgehog inhibitors is that advanced BCC may become resistant to these treatments, mainly due to de novo mutations in the gene encoding the drug target, *SMO*. Other hedgehog pathway inhibitors are currently under clinical trials testing whether they would give higher and more durable response rates. A phase II clinical trial evaluating the efficacy of combining a hedgehog pathway inhibitor (vismodegib) with immunotherapy (pembrolizumab, anti-PDL1) for treatment of metastatic or unresectable BCC is currently under way.

Regional SCC has been shown to respond to systemic cytotoxic therapies and to inhibitors of the epidermal growth factor receptor (EGFR), most commonly cetuximab, in several studies. However, it remains unclear whether these treatments provide any additional clinical benefit when used postoperatively with RT. Due to low metastasis rates, experience with systemic treatment of metastatic disease is very limited. Metastatic SCC patients have been shown to respond to a combination of cytotoxic (cisplatin-based) and EGFR inhibitor therapy, and this remains the most common approach.

About 40% of NMSC patients will develop another NMSC within 5 years. These individuals are also at a higher risk of developing melanoma. Therefore, the success of secondary prevention depends on a regular follow-up and patient education in order to allow for early detection and treatment of pre-cancerous lesions and early-stage cancers. Actinic keratoses (precancerous lesions for SCC) may be treated with cryosurgery, surgery, photodynamic therapy (PDT), or topical treatments, such as 5-fluorouracil or imiquimod. Using nicotinamide may be effective in reducing the risk of developing BCC, whereas oral retinol and retinoids reduce the incidence of SCC in high-risk individuals, such as transplant recipients, psoriasis and xeroderma pigmentosum patients, and patients with a history of multiple lesions and/or extensive diffuse actinic keratosis.

See also: Malignant Skin Adnexal Tumors: Pathology and Genetics.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia, PA.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Basal Cell Skin Cancer. Version 1.2018 – September 18, 2017: https://www.nccn.org/professionals/physician_gls/default.aspx#site

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Sqaumous Cell Skin Cancer. Version 2.2018 – October 5, 2017: https://www.nccn.org/professionals/physician_gls/default.aspx#site

Pellegrini, C., et al., 2017. Understanding the molecular genetics of basal cell carcinoma. International Journal of Molecular Sciences 18 (11), E2485.

Verkouteren, J.A.C., et al., 2017. Epidemiology of basal cell carcinoma: Scholarly review. The British Journal of Dermatology 177 (2), 359–372.

Symptom Control

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Glossary

Activities of daily living Activities in self-care done routinely without needing assistance, including eating, bathing, toileting, dressing, walking or transferring.

Patient-reported outcomes A report directly from the patient of their own health condition, including symptoms, physical and social function, mental health and wellbeing.

Patient-reported outcome measurements (PROMs) Instruments used in clinical and research settings for the measurement of patient-reported outcomes.

Prophylactic Treatment given to prevent symptom occurrence.

Quality of life A multidimensional concept that encompasses an individual's sense of physical, emotional, and social wellbeing and ability to carry out various activities.

Rescue Fast-acting treatment given as needed for immediate symptom relief.

Symptom cluster A group of two or more concurrent, interrelated symptoms.

Introduction

On the eve of its 50th anniversary in 2014, the American Society of Clinical Oncology conducted a poll wherein healthcare providers and patients across the country voted on what they perceived to be the top medical advancements in modern oncology. Alongside pivotal discoveries in prevention and chemotherapy, antiemetics were voted among the top five for their role in improving quality of life in patients undergoing treatment. With a range of more effective and convenient therapies available, patients may continue to lead active lives outside of, and following their treatment. In recognition of this, there has been increasing attention on identification and management of symptoms, and on patient-reported outcomes in quality of life.

A symptom is defined as a subjective experience reflecting changes in the biopsychosocial functioning, sensations, or cognition of an individual. Symptoms are multidimensional and are described using a patient's perception of prevalence, intensity and distress. Patients with advanced cancer have been reported to experience an average of 11 concurrent symptoms, either due to the disease itself or as a consequence of anticancer or supportive therapies.

Symptoms are often multifactorial and interrelated, posing challenges in measurement and elucidation of underlying pathophysiology. Patient-reported outcomes or "self-report" are considered primary sources of information, as opposed to caregiver- or physician-reported symptoms. Often generated through extensive literature reviews and interviews with patients and health care professionals, several questionnaires have been validated for measurement of patient-reported outcomes such as symptoms, function, and quality of life. In addition to assessment of traditional biomedical outcomes, patient-reported outcomes are widely measured and reported on in oncological clinical trials, and are also clinically significant for their value in prognostication.

Despite advances in symptom research and control, a large proportion of cancer patients continue to report inadequate treatment. Contributing to this issue is underestimation of symptom intensity by healthcare providers. In addition to being a major detriment in patients' function, wellness and quality of life, symptom distress may also lead to dose reductions or termination of treatment, or negatively affect post-treatment rehabilitation. Basic principles in symptom management in oncology include active symptom evaluation and treatment at each encounter, starting at diagnosis through to survivorship or end-of-life care.

This article will outline some of the most prevalent symptoms in this patient population, current approaches in symptom management, and patient-reported outcome measures available for clinicians and investigators.

Common Symptoms in Oncology

Pain

The International Association for the Study of Pain defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. A systematic review conducted by van den Beuken-van Everdingen et al. of 40 years of literature reported prevalence of pain at 33% in patients following curative treatment and at 64% in patients with advanced disease. Persistent pain can significantly compromise patient function, wellbeing and overall quality of life. Almost 70% of patients who experience pain at least weekly reported pain-related difficulties in activities of daily living on the European Pain in Cancer Survey.

There are several sources of pain throughout the course of the disease and associated treatments. *Malignancy-related pain* can arise from the primary tumor, metastases, or complications such as spinal cord compression, pathologic fracture or malignant bowel obstruction. *Iatrogenic pain* follows diagnostic or surgical procedures (e.g., mastectomies and axillary resection, thoracotomy), or as a side effect of chemotherapy, hormone therapy, radiation therapy or pharmaceuticals (e.g., osteonecrosis of the jaw from bisphosphonates). Patients undergoing chemotherapy may report specific forms of pain, such as infusion-related pain syndrome, taxane-induced arthralgia and myalgia, peripheral neuropathy, and palmar plantar erythrodysesthesia. Similarly, patients undergoing radiation to the bone may report "pain flare" or transient worsening of bone pain at the site of irradiation.

Nociceptive pain is caused by tissue damage in the body, and can be somatic (e.g., skin, bone, muscle), or visceral (internal organs). *Neuropathic pain* is caused by dysfunctional or injured nervous tissue. *Psychogenic pain* is related to psychological factors such as anxiety and depression. Pain can be further classified by duration, as acute (several weeks to months) or chronic (>6 months). *Breakthrough pain* is a transient increase of pain with rapid onset arising at moderate or severe intensity from background pain.

Pain can be managed with a variety of non-pharmaceutical and pharmaceutical methods depending on its pathophysiology. The World Health Organization first presented the analgesic ladder in 1986 as a framework for management of cancer-related pain. The analgesic ladder is three-step, with the first offering non-opioids such as acetaminophen or nonsteroidal antiinflammatory drugs (NSAIDs) with or without adjuvants. With persistent, moderate or severe pain, the second step suggests use of a weak opioid (e.g., codeine) with or without non-opioids or adjuvants, and the last step involves substitution with a strong opioid (e.g., morphine, oxycodone). Since its publication, several groups such as the National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology have composed guidelines that are available to clinicians for assessment and management of the symptom.

Adjuvant medications which can be used with non-opioids and opioids for added relief include steroids, antidepressants, anticonvulsants, and topical anesthetics. At present, there is insufficient evidence for the support of cannabinoids as a first or second line therapy. Its action on endocannabinoid receptors may make it effective in treating cancer-related pain; however, this requires more investigation.

In the management of neuropathic pain, which is considered insensitive to opioids, recommended treatments include antidepressants (e.g., tricyclic antidepressants, dual serotonin-norepinephrine reuptake inhibitors), and anticonvulsants (e.g., gabapentin, pregabalin). The American Society of Clinical Oncology provides a moderate recommendation for the use of duloxetine in chemotherapy-induced peripheral neuropathy after positive results from a phase III trial.

It is estimated that 10% of patients will experience insufficient pain relief despite treatment in adherence to the World Health Organization analgesic ladder principles. Some alternatives include anesthetic interventions such as nerve blocks, epidural and intrathecal infusions, and neurosurgical procedures (e.g., cordotomy). Patients with life expectancies exceeding 3 months may benefit from the mobility and convenience of implanted intrathecal drug delivery (ITDD) pumps. Surgical procedures may relieve localized pain through resection of tumors. Patients with bone pain from metastases may benefit from external beam radiation, surgical stabilization or procedures such as vertebroplasty which will be revisited later in this article. Although the evidence for complementary treatment of cancer-related pain remains weak, patients may also undergo therapies such as massage, acupuncture, transcutaneous electrical nerve stimulation or hypnotherapy. The NCCN recommends use of acupuncture as part of integrative interventions, delivered as needed with pharmacologic interventions.

Nausea and Vomiting

Nausea is a sensation in the back of the throat, or a feeling of sickness or discomfort in the stomach, which may or may not lead to vomiting, the expulsion of gastric contents. There are several emetogenic pathways that may be impacted, depending on the cause(s). Structures for coordinating nausea and vomiting include the vomiting center and the chemoreceptor trigger zone in the medulla oblongata.

Patients with advanced disease may experience multifactorial nausea, and six common causes identified by Stephenson and Davies include:

1. *Chemical:* Triggered by drugs, chemotherapy, metabolic issues (e.g., renal failure, liver failure, hypercalcemia, hyponatremia, ketoacidosis), and toxins (e.g., ischemic bowel, tumor products, infection).
2. *Impaired gastric emptying:* Triggered by drugs, tumor ascites, hepatomegaly, autonomic dysfunction, tumor infiltration.
3. *Visceral/serosal:* Triggered by bowel obstruction, severe constipation or fecal impaction, liver capsule stretch, ureteric distention, mesenteric metastases, difficult expectoration or pharyngeal stimulation.
4. *Cranial:* Triggered by raised intracranial pressure, meningeal infiltration, radiotherapy.
5. *Vestibular:* Triggered by drugs, motion sickness, tumor in the base of the skull.
6. *Cortical:* Triggered by anxiety or pain.

Prior to the introduction of antiemetics to clinical practice, patients undergoing chemotherapy were often debilitated by chemotherapy-induced nausea and vomiting (CINV). Without prophylaxis, it is estimated that up to 80% of patients are at risk of experiencing the symptom. Chemotherapy-induced nausea can be classified as: *acute*, occurring within the first 24 h of initiation of therapy; *delayed*, occurring within 2–5 days following therapy; *breakthrough*, occurring despite prophylactic treatment; *anticipatory*, occurring before initiation of therapy as a conditioned response from previous experience; and *refractory*, occurring in subsequent

cycles of therapy. Patients who are female, <50 years old, and have a history of chronic heavy alcohol abuse or motion sickness are at a higher risk of developing CINV. There are several different signaling mechanisms involved in CINV, with the main three neurotransmitters being serotonin or 5-hydroxytryptoamine (5-HT_3), substance P, and dopamine with bind to 5-HT_3 receptors, neurokinin-1 (NK_1) receptors and dopamine receptors respectively. Damage of the enterochromaffin cells in the gastrointestinal mucosa cause the release of 5-HT_3 by these cells. Substance P and dopamine act in auxiliary capacities to evoke the CINV response.

Oral and intravenous chemotherapeutic agents are differentiated as highly ($>90\%$ risk), moderately ($>30\%$–90% risk), low (10%–30% risk), and minimally ($<10\%$) emetic. Several organizations have provided antiemetic guidelines for the management of CINV, namely using one or a combination of 5-HT_3 receptor antagonists (RAs) (e.g., palonosetron, ondansetron, granisetron), NK_1 RAs (e.g., aprepitant, rolapitant, netupitant), dopamine RAs (e.g., metoclopromide) and dexamethasone. As a function of their pharmacological profile and mechanism of action, it is understood that different antiemetic classes offer protection in acute versus delayed phases of chemotherapy. Dual action medications such as NEPA are thought to provide coverage in both acute (through 5-HT_3 RA palonosetron) and delayed (through NK_1 RA netupitant) phases.

Based on site of irradiation, radiotherapy is differentiated into the following levels of risk: high ($>90\%$), moderate ($>30\%$–90%), low (10%–30%), and minimal ($<10\%$). Several organizations have compiled guidelines in the treatment of RINV.

The Multinational Association for Supportive Care in Cancer, European Society for Medical Oncology, and the National Comprehensive Cancer Network recommend the following regimens for RINV:

- High (total body irradiation): prophylaxis with a 5-HT_3 RA and dexamethasone
- Moderate (upper abdomen, craniospinal): prophylaxis with a 5-HT_3 RA with optional dexamethasone
- Low (cranium): prophylaxis or rescue with dexamethasone
- Low (head, neck, thorax, pelvis): prophylaxis or rescue with dexamethasone, a dopamine RA or 5-HT_3 RA
- Minimal (extremities, breast): rescue with dexamethasone, a dopamine RA, or 5-HT_3 RA

In addition to chemotherapy and radiotherapy, opioid analgesics are another chemical trigger of nausea and vomiting. Opioid rotation and route switching are recommended in patients who experience nausea and/or vomiting as a consequence of the analgesic. No recommendations have been made about specific antiemetics in the management of this side effect.

There are several non-chemical causes of nausea and vomiting that can be corrected. Nausea and vomiting due to hypercalcemia may subside with bisphosphonates. Cancer-related hyponatremia may be precipitated by cancer treatments, or the syndrome of inappropriate antidiuretic hormone (SIADH) which is frequently observed in patients with small-cell lung cancer. Tumor-related SIADH may resolve with treatment of the malignancy. Otherwise, fluid restriction or pharmacologic treatment with vasopressin antagonists can be delivered. Patients who experience nausea or vomiting as a consequence of delayed gastric emptying may benefit from domperidone or metoclopramide. Nausea or vomiting due to vestibular causes may be relieved with agents such as cyclizine or levomepromazine.

Non-pharmacologic aspects of treatment include patient and caregiver education about the symptom, consultations on appropriate dietary or nutritional advice, environmental modification (e.g., elimination of strong smells), and counselling on oral hygiene.

Fatigue

Cancer-related fatigue, with feelings of tiredness, weakness and lack of energy, is characteristic in its lack of relief with rest and sleep, and disproportionality with the patient's level of exertion. This type of fatigue is distressing in its impairment of usual functioning. Approximately 40% of patients report the symptom at diagnosis, and its prevalence increases to 80%–90% during chemotherapy and radiotherapy. It persists chronically in more than a third of patients following completion of treatment.

Despite the universality of this symptom in patients with cancer, there is no effective treatment for fatigue, due largely in part to the unclear pathophysiology of the symptom. Several mechanisms for cancer-related fatigue have been proposed, including activation of the immune system by the disease or treatment leading to a release of pro-inflammatory cytokines and subsequent peripheral inflammation. Downstream effects of immune cell activation and release of pro-inflammatory cytokines include impairment of endocrine function, function of the hypothalamic–pituitary axis and mitochondrial activity in the peripheral and central nervous system.

In the National Comprehensive Cancer Network (NCCN) guidelines for management of cancer-related fatigue, Berger et al. advocate for a focused history and physical examination in patients reporting moderate-to-severe levels (≥ 4 out of 10). Such an assessment would include an evaluation of physical, emotional and cognitive symptoms, as well as social support. Other contributing factors that should be assessed include pain, emotional distress, sleep disturbance, poor sleep hygiene, anemia, nutrition, activity level, medication side effects, alcohol/substance abuse and co-morbidities.

In patients undergoing treatment, Berger et al. recommend education and counseling on strategies. Purposeful management of personal energy resources to prevent depletion, or "energy conservation," helps to maintain realistic expectations. Habits and practices conducive to sleep, or "sleep hygiene" can be improved by sleeping in a dark room, reducing stress (e.g., yoga, reading, listening to music), limiting electronic use and limiting naps in the daytime.

Specific and manageable causes of fatigue can include infection, fluid and electrolyte imbalances, and cardiac dysfunction. If cause(s) of fatigue remain unidentified, the NCCN recommends appropriate pharmacological and nonpharmacological treatment.

Although the evidence of effectiveness is limited, several organizations suggest use of non-pharmacologic interventions such as physical activity during and after treatment, physical-based therapies and complementary therapies (e.g., acupuncture, massage therapy, yoga, and muscle relaxation), psychosocial interventions (e.g., cognitive behavioral therapy), sleep therapy and nutrition consultation. In patients who are able to engage in physical activity, at least 30 min of moderate intensity exercise on at least 5 days of the week, or at least 20 min of vigorous exercise on at least 3 days of the week is recommended. There is sufficient evidence for psychosocial or educational interventions wherein patients are equipped with information on the symptom, self-care, coping techniques and activity management. Additionally, group psycho-education related to self-management of fatigue in patients and survivors may allow for positive peer-enforcement, and the opportunity to share similar experiences. There is also sufficient evidence for use of cognitive behavioral therapy facilitated by fatigue clinics. The effectiveness of pharmacologic treatment of fatigue (e.g., methylphenidate) in this patient population is limited.

Although its effectiveness in treating cancer-related fatigue requires more investigation, use of modafinil benefited patients with severe baseline fatigue undergoing chemotherapy in a randomized controlled trial. Short-term (up to 14 days) use of glucocorticoids may improve fatigue in patients with terminal disease near the end of their lives.

Dyspnea

Dyspnea is defined by shortness of breath or difficulty breathing. Patients with dyspnea may report physical tiredness from expending effort on breathing, and tightness in the chest. The inability to breath and the feeling of suffocating or choking can be frightening for patients. Over 60% of patients with cancer report dyspnea in palliative care.

There are several possible specific underlying causes of dyspnea, including issues in the respiratory system (e.g., airway obstruction, pleural effusion), cardiovascular system (e.g., atrial fibrillation, congestive heart failure, superior vena cava obstruction), anemia or severe ascites. The sensation of hypercapnia by the medullary chemoreceptors, and hypoxemia by carotid and aortic body chemoreceptors can work on the respiratory centre to cause dyspnea. However, the majority of patients with dyspnea are not hypoxemic. Another hypothesized mechanism is the dissociation between the brain's desired respiration and the sensory information it receives.

The symptom can exist continuously in the background for prolonged periods of time, and/or can arise acutely with high intensity in durations typically <20 min, as episodic dyspnea or breathlessness. Episodic dyspnea can be especially distressing for patients and caregivers, impairing quality of life through anxiety.

Dyspnea is often undertreated, compared to other symptoms of advanced cancer such as pain or nausea. The European Society for Medical Oncology advocates that non-pharmacological interventions be offered first if possible, and these include proper positioning, breathing exercises, and teaching of techniques (e.g., cooling the face). Education about the symptom, management, and supportive measures can help to reduce accompanying anxiety. Oxygen supplementation may provide relief in patients with hypoxemia, or those who have comorbid chronic obstructive pulmonary disease. The Supportive Care Guidelines Group of the Cancer Care Ontario Program in Evidence-Based Care conducted a systematic review of pharmacological therapies: opioids, benzodiazepines, phenothiazines, and systemic corticosteroids. Controlled trials using opioids produced contradictory results, however overall, favored a benefit in dyspnea and exercise tolerance. The action of opioids on opioid receptors in the cardio-respiratory system and central nervous system are hypothesized to reduce dyspnea. There is less controlled trial evidence to support the use of non-opioid pharmacological therapies, however the group recommended use of phenothiazine or promethazine as an alternative in the event systemic opioids could not be used. Although several benzodiazepines (e.g., lorazepam, midazolam) are used in this cancer population in an anxiolytic capacity, they have been considered to reduce the intensity of the symptom through muscle relaxation. The review found no evidence to support the effectiveness of benzodiazepines on dyspnea. Bronchodilators, mast cell stabilizers, leukotriene inhibitors and/or corticosteroids can be used in patients with bronchospasms.

Since the publication of this review, a few studies have been conducted using similar treatments for dyspnea in patients with cancer. A randomized controlled trial of 20 patients with cancer observed significant improvements in breakthrough dyspnea with prophylactic fentanyl. Rubiales et al. reported patients pre-medicated with subcutaneous fentanyl had significantly improved scores in dyspnea at the end of a 6 minute walk test. In another randomized controlled trial of 41 patients, dexamethasone was administered at 8 mg twice daily for 4 days then 4 mg twice daily for 3 days. Although there were was no statistical significant between-arm difference, those in the treatment arm demonstrated a significant reduction in dyspnea by day 4. This pilot study suggests improvement in dyspnea, however the findings require further investigation.

Constipation and Diarrhea

Treatment or disease-induced gastrointestinal complications include diarrhea or constipation, which can be described in terms of stool bulk/water content, as well as frequency or difficulty of stool passage. Diarrhea is often described as passing more than three watery stools in a 24 h period. Under the Rome III Diagnostic Criteria for functional constipation, there must be at least of two of the following criteria in at least 25% of defecations: straining; lumpy or hard stools; sensation of incomplete evacuation; sensation of anorectal obstruction; requirement of manual maneuvers; or fewer than three defecations per week. In addition, there must be insufficient criteria for irritable bowel syndrome, and a lack of loose stools without laxatives. It is recommended that clinicians consider the patient's perceptions of the symptoms as well gather a history of bowel patterns, dietary changes and medications.

Constipation can be caused or influenced by medications (e.g., chemotherapy, opioids, antiemetics, excessive use of laxatives/enemas), reduced food or fluid intake, neuromuscular disorders of bowel innervation, reduced mobility, metabolic disorders (e.g., hypercalcemia), depression, inability to increase intra-abdominal pressure, muscular atony, environmental factors (e.g., excess heat, bathroom inaccessibility due to disability) and bowel obstruction. It can be accompanied by nausea and/or abdominal pain.

Patients can be encouraged to integrate natural laxatives into their diet, and increase dietary fiber and fluid intake where possible. Bulk producers (e.g., methylcellulose, psyllium) are natural or semisynthetic polysaccharide and cellulose. Osmotic laxatives (e.g., magnesium sulfate, polyethylene glycol) attract water to the intestinal lumen; stimulant laxatives (e.g., Senekot) directly increase motor activity of the bowels; and lubricant laxatives (e.g., mineral oil) soften stool by lubricating the intestinal mucosa. Physical activity, appropriate to the patient's performance status and health status, is also recommended.

The MD Anderson Cancer Center practice algorithm for cancer pain recommends starting patients on a laxative bowel regimen (e.g., stimulant laxative plus stool softener) and providing education on bowel management upon initiation of opioids. The guidelines outlines four steps: (1) assessing the potential cause of constipation with consideration of changes in medications; (2) increasing senekot-S and supplementing with milk of magnesia oral concentrate and/or polyethylene glycol; (3) performing a digital rectal exam to rule out low impaction if there continues to be no response; and (4) prescribing methylnaltrexone for patients with suspected opioid-induced constipation.

Diarrhea can also be caused by treatments (e.g., chemotherapy, intestinal surgery, pelvic radiation) and by certain cancers (e.g., colon cancer). It can be induced by stress and anxiety.

It is encouraged that patients increase their daily liquid intake, and avoid irritants such as caffeine or alcohol. Pharmacologic treatments include loperamide and octreotide. Parenteral hydration may be required for fluid replenishment in patients with severe diarrhea.

Radiation enteritis, inflammation of the small intestine, is a risk associated with radiation to the abdomen or pelvis. Patients with radiation enteritis may experience nausea, vomiting, abdominal cramping, rectal pain and diarrhea. Acute enteritis (<3 weeks) may be treated with antidiarrheal agents (e.g., kaopectate, paregoric, immodium), donnatal (anticholinergic agent), cholestyramine (bile salt sequestering agent) or lomotil. Chronic enteritis may present up to 6–18 months following radiation therapy, and if severe, can be surgically managed with an intestinal bypass or resection.

Chemotherapy may induce diarrhea through acute damage to the intestinal mucosa, causing an imbalance between absorption and secretion. Loperamide is commonly prescribed to decrease motility and increases anal sphincter tone. Somatostatin analog octreotide decreases hormone secretion and reduces motility to promote absorption.

Loss of Appetite and Weight Loss

Appetite loss, weight loss and malnutrition are often observed preceding diagnosis, and can arise or worsen with treatment and/or disease progression. Although these symptoms can go hand in hand, malnutrition, weight loss, and appetite loss are not interchangeable. Despite its association with survival, the clinical utility of weight loss without the context of time course or type of tissue lost (e.g., fat, muscle) is limited. Anorexia is the abnormal loss of appetite for food, which is present in at least 15% of patients at diagnosis and has been observed in a quarter of patients during chemotherapy. Anorexia is an important predictor of symptom burden and survival in patients with cancer, compared to those with weight loss alone. Malnutrition is marked by inadequacy of key vitamins and minerals, caused by altered or reduced food intake and/or impaired absorption. Observational studies have placed prevalence of malnutrition in patients with cancer at around 30%, and identified pre-existing obesity and primary cancers of the head and neck or upper digestive tract as risk factors. Common nutrition impact symptoms such as dry mouth, nausea, and constipation negatively impact nutritional status, increasing risk of malnutrition and are associated with lower quality of life in patients treated with chemotherapy.

It is estimated that over half of patients with advanced cancer develop cachexia, or continued loss of adipose and skeletal muscle mass despite nutritional support leading to progressive functional impairment. Diagnostic criteria of muscle sarcopenia or weight loss $>5\%$ (or $>2\%$ in individuals with a BMI <20 kg/m^2) in the past 6 months were established in an international consensus published by Fearon et al. A two-group model using the later criterion of weight loss was validated, and demonstrated an ability to distinguish between cachectic versus non-cachectic patients in all domains of food intake, catabolism and function, as well as in survival. Cancer cachexia can be graded using BMI and weight loss. Martin et al. observed that the prognostic discrimination of the grading system was clinically significant, with median survival ranging from 4.3 months (grade 4) to 29 months (grade 0), irrespective of factors such as type of cancer or stage, age or performance status. Secondary cachexia may be due to correctable causes. Patients who do not meet the diagnostic criteria for cachexia, may be defined as pre-cachectic if they exhibit unintentional weight loss in the past 6 months, anorexia or anorexia-related symptoms, and chronic or recurrent systemic inflammatory responses. Refractory cachexia is observed in terminal stages of un-responsive and procatabolic disease, wherein patients have poor performance status and limited life expectancy.

Cancer cachexia is caused by a negative protein and energy balance stemming from reduced food intake and/or abnormal metabolism. Associated anorexia and reduced food intake in this syndrome can be altered directly by chemosensory disturbances, nausea, or decreased motility of the gastrointestinal tract. Hypercatabolism can be caused by tumor metabolism, systemic inflammation or neuro-hormonal alterations.

Counseling with a dietician and behavioral strategies (e.g., eating small and frequent meals, limiting liquid intake during meals) can help to alleviate loss of appetite, and can be targeted to specific nutrition impact symptoms (e.g., dry mouth). Exercise that is

appropriate for the patient's performance status and health status is recommended. Patients may be recommended to use commercially available oral nutrition supplements to increase lean body mass. In those who are malnourished and are unable to receive adequate nutrition orally, enteral or parenteral nutrition support may be indicated. In patients with moderate to severe decrease in functional status, pharmacological treatment may be prescribed. Megestrol acetate and medroxyprogesterone have been shown to increase appetite, food intake and weight gain in patients. Ghrelin, a gastric peptide hormone, can work to facilitate acid secretion and motility in the gastrointestinal tract. Randomized controlled trial evidence indicated improvement in body mass and arm strength in patients with cancer cachexia. Metoclopramide may be prescribed to decrease nausea and early satiety. Corticosteroids are widely used for limited periods to stimulate appetite. Polyunsaturated fatty acids (e.g., omega-3-fatty acids, eicosapentaenoic acid) are used in a lesser extent for treatment of cancer cachexia, however there is insufficient evidence to support their effectiveness.

Depression and Anxiety

Cancer-related distress is pervasive, and extends along a continuum ranging from sadness to debilitating feelings. It was defined by the National Comprehensive Cancer Network as "a multifactorial, unpleasant, emotional experience of a psychological (cognitive, behavioral, and emotional), social, and/or spiritual nature that may interfere with the ability to cope effectively with cancer, its physical symptoms, and its treatment". Given the difficult circumstances surrounding cancer diagnosis and treatment, it may be difficult to detect morbid anxiety. Patients may also experience heightened anxiety with "fear of recurrence" following treatment. Depression and anxiety may be complications of the disease itself (e.g., brain, parathyroid, lung cancers) or treatments. Depression is a recognized potential effect of certain chemotherapeutic agents (e.g., etoposide, cisplatin), molecular target agents, monoclonal antibodies, and hormonal treatments (e.g., letrozole, androgen deprivation). Radiation to certain sites such as the brain, head and neck, or pelvis can also cause depression through TSH, growth hormone, melatonin, and/or B12 deficiencies.

Depression should be considered in the presence of affect and prolonged impairment of function, with major depressive disorder being characterized by the presence of at least five of the following symptoms in a 2-week period: depressed mood most of the day; markedly diminished interest in activities; significant change in weight or appetite; insomnia or hypersomnia; psychomotor agitation or retardation; fatigue or loss of energy; feelings of worthlessness or guilt; diminished ability to think, make decisions or concentrate; recurrent thoughts of death or suicide, or plans or attempts to commit suicide. It is estimated that approximately 15%–25% of patients with cancer are affected by comorbid depression; however, lack of standardized methods in terms of instruments and diagnostic criteria make it challenging to estimate prevalence in this patient population. Possible medical causes for depressive symptoms include uncontrolled pain, metabolic abnormalities (e.g., anemia, hypercalcemia), endocrine abnormalities (e.g., hyperthyroidism), and certain medications. Anxiety can induce or heighten symptoms such as pain or nausea. Depression may negatively impact treatment compliance.

The American Society of Clinical Oncology guidelines provide an algorithm for assessment of depression in adults with cancer using the validated 9-item Personal Health Questionnaire (PHQ-9). Anhedonia and depressed mood require further investigation. In those who indicate moderate symptomology (score 8–14), low-intensity interventions are recommended such as individually-guided self-help based on cognitive behavioral therapy, group-based cognitive behavioral therapy for depression, group psychosocial interventions, structured physical activity programs or appropriate pharmacologic therapies. In those who indicate moderate-to-severe (score 15–19) or severe (score 20–27) symptomology, pharmacologic therapies and/or high-intensity interventions such as individual psychological cognitive behavioral therapy or interpersonal therapy. Also emphasized is support in the form of patient and family education on stress reduction strategies (e.g., progressive muscle relaxation), availability of supportive care services and financial support, information on sleep hygiene and self-management of fatigue, and signs and symptoms of worsening depression.

There is a similar algorithm for assessment of anxiety in adults with cancer using the 7-item generalized anxiety disorder questionnaire. It is recommended that patients with moderate symptomology (score 10–14) be offered low-intensity interventions such as education and active monitoring, non-facilitated or guided self-help based on cognitive behavioral therapy, group psychosocial interventions or appropriate pharmacologic therapies. Under this algorithm, patients with moderate-to-severe or severe symptomology should be offered high-intensity psychological (individual cognitive behavioral therapy or applied relaxation) and/or pharmacologic interventions.

Psychological interventions can be low- or high-intensity depending on symptom severity. They are delivered in a group or individual settings and provide psycho-education and training in coping skills and mindfulness based stress reduction. There is some evidence for the use of multi-component interventions including a structured physical program, visual imagination, and progressive relaxation techniques for acute stress or post traumatic disorder in this patient population. Antidepressants have demonstrated effectiveness in treating clinically significant depression in patients with cancer. However, routine use of antidepressants in patients with sub-threshold depressive symptoms or mild depression is discouraged. Major classes include selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), norepinephrine-dopamine reuptake inhibitors (NDRIs), atypical antidepressants, tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOs), and psychostimulants. Sertraline and citalopram are most commonly used in patients with cancer since they are generally well tolerated and have the least interactions with chemotherapy agents. SNRIs (e.g., desvenlafaxine) are often used in patients with comorbid pain syndromes. Buspirone is often used adjunctively to treat comorbid anxiety. Nonpharmacological interventions may include psychotherapies such as cognitive behavior therapy, which aims to change behavior by altering how patients think or feel about certain things.

Symptom Management in Advanced Cancer

The World Health Organization definition of palliative care describes it as focused on improving quality of life in patients and their families associated with life-threatening illness. A goal of palliative care is to prevent and provide relief of suffering from pain and other distressing symptoms. Complications of advanced cancer can produce varied symptomology. There is a range of options in palliative interventions, including surgery, minimally invasive procedures, radiation therapy, chemotherapy, and pharmacologic agents.

Bone metastases are the most common source of moderate-to-severe pain, with 75% of patients with advanced disease reporting the symptom. The clinical course of metastatic disease from the bone can be relatively long, spanning several years in patients with prostate or breast cancers. Patients can experience nociceptive and neuropathic pain from skeletal complications such as pathologic fractures, and spinal cord compression. External beam radiation, bone-targeting agents (e.g., bisphosphonates, calcitonin), and radiopharmaceuticals (e.g., radium-223) can provide analgesic relief while reducing risk of skeletal complications. Percutaneous vertebroplasty involves the injection of acrylic bone cement into the vertebral body to restore stability and/or height loss, and therefore relieves pain and functional impairment. Surgical stabilization of the long bones, and joint replacements may also reduce bony pain.

Metastatic spinal cord compression is an oncological emergency as it can lead to irreversible paralysis and neurological damage. While patients typically first experience back pain, progression may lead to limb weakness, decreased sensation and numbness of the toes and fingers, and autonomic dysfunction (e.g., incontinence, constipation). Corticosteroids and/or analgesics for the bony and neuropathic pain can be provided. Definitive treatment for spinal cord compression includes radiotherapy, surgery or chemotherapy. Surgery can decompress the spinal cord to reconstruct and stabilize the structure. Radiation can be used as an adjunct to surgery, or as primary treatment in patients who are not surgical candidates to prevent neurological deterioration, improve neurological function and provide pain relief. Chemotherapy may play a role in sensitive malignancies (e.g., lymphoma, small cell lung cancer).

Depending on the site of disease and degree of mass effect, brain metastases can cause various symptoms such as fatigue, impaired sight or hearing, nausea and vomiting, incontinence and limb immobility. Dexamethasone can provide rapid relief of symptoms due to swelling. Surgical resection may be used to treat solitary brain metastases. There is evidence of the ablative efficacy of stereotactic radiation therapy in the treatment of four or less metastases. In more extensive disease, whole brain radiation therapy can be used. Leptomeningeal carcinomatosis, or the spread of disease to the meninges of the brain and spinal cord, is a rare complication of advanced cancer. In patients with favorable risk status (e.g., high performance status, no major neurological deficits, minimal systemic disease), the MD Anderson Cancer Center suggests consideration of radiotherapy and systemic therapy, intraventricular catheters and/or a ventriculoperitoneal shunt for intrathecal chemotherapy. Although there is a concern for tumor seeding, ventriculoperitoneal shunts can provide palliation in patients with limited survival.

Malignant bowel obstruction occurs when there is a blockage of gastric or intestinal contents, and can cause abdominal pain, nausea and vomiting. Patients may be considered for palliative surgical resection (e.g., colostomy). In non-operable obstruction, procedures such as nasogastric tubes, percutaneous decompression gastronomy tubes or percutaneous endoscopic gastronomy may alleviate symptoms. Self-expanding metal stents can also be considered an option for patients with single points of obstruction. Although use of morphine as an analgesic is supported by clinical guidelines, lesser risk of constipation and availability as a transdermal formulation make fentanyl a convenient option for patients with malignant bowel obstruction. Nausea and vomiting due to malignant bowel obstruction can be managed with antisecretory drugs, octreotide, and antiemetics.

Patients with metastatic disease in the lung may produce dry cough, shortness of breath or chest pain due to a malignant blockage of the airway. Radiation therapy or laser ablative therapy through a bronchoscope may shrink pulmonary metastases to provide symptomatic relief. Oxygen supplementation may be recommended. Due to a high rate of recurrence, aspiration is only recommended in patients with limited life expectancy ($<$1 month). Pleural effusion can be treated with a thoracentesis or indwelling pleural catheters to drain fluid. The pleural space can be obliterated by pleurodesis to treat pleural effusion and prevent repeated fluid build-up. Similarly, fluid buildup in the pericardium, which can result in shortness of breath and tiredness, can be treated with pericardiocentesis and recurrence can be prevented with a pericardial window.

Patients with liver metastases may experience tiredness, loss of appetite, and abdominal pain. The inability of the liver to remove toxins may eventually cause hepatic encephalopathy, leading afflicted patients to feel confusion or fatigue. Patients with limited disease may undergo hepatic resections. Percutaneous radiofrequency ablation is a minimally invasive option. Blood supply to non-operative liver metastases can be obstructed with embolization therapy. Embolization can be combined with chemotherapy in trans-arterial chemoembolization, or with radiation in trans-arterial radioembolization. Hepatic artery infusion of chemotherapy allows direct delivery of treatment to the site of metastases to prolong drug exposure while minimizing toxicity to unaffected areas. Stereotactic radiation therapy to limited disease has been proven effective in symptom palliation.

Patient-Reported Outcome Measurement

In an effort to increase rigor and transparency in reporting of patient-reported outcomes, the Consolidated Standards of Reporting Trials (CONSORT) checklist underwent an extension in 2013 to include guidance on the reporting of these outcomes in randomized clinical trials. Item 6a stipulates use of an instrument with evidence of reliability and validity. A variety of instruments are

available for measurement of health-related quality of life in patients with cancer. Standardized pain assessment tools using visual analogue scales or verbal rating scales, which have been validated in patients with cancer, are recommended. Two widely used examples are the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire Core 30 (EORTC QLQ-C30) and the Functional Assessment of Cancer Therapy-General (FACT-G). Developed in the early 1990s, these two core questionnaires have since been revised and translated, and supplementary modules have been developed for subpopulations categorized by primary cancer site (e.g., lung cancer, bone metastases), symptoms (e.g., cancer related fatigue, neutropenia), and treatment (e.g., taxane therapy, bone marrow transplantation). The EORTC QLQ-30 is composed of 30 items, and evaluates five types of functioning (physical, role, emotional, social, cognitive) in addition to several independent symptoms and overall quality of life. The FACT-G is composed of 27 items, and evaluates symptoms classified under four types of well-being (physical, social/family, emotional, functional) and also includes an overall quality of life item.

Supplementary modules allow evaluation of other interrelated symptoms or influencing factors. The Functional Assessment of Anorexia/Cachexia Treatment (FAACT) for example assesses 12 items in domains of cancer cachexia (e.g., appetite, weight loss), and additional and/or interrelated symptoms (e.g., nausea, abdominal pain). It also assesses satiety, taste, difficulty eating certain foods, patient perception and concern regarding weight and food intake, and pressure from caregivers to eat. The FACIT-D is a diarrhea-specific module, with 11 items assessing frequency of bowel emptying, control of bowels, disruption of sleep, emotional impact, and limitations on physical, social, and sexual activity. The 13-item FACIT-F and the 12-item QLQ-FA12 are fatigue-specific assessments, and include items such as level of energy, weakness, sleepiness during the day, as well as ability to start tasks and complete usual activities.

Outside of the EORTC and FACIT, several multidimensional instruments are available to measure patient-reported outcomes. The 11-item Brief Pain Inventory was developed by the Pain Research Group of the World Health Organization Collaborating Centre for Symptom Evaluation in Cancer Care for assessment of pain intensity, functional interference and pain relief with analgesics. The Cancer Dyspnoea Scale (CDS) is a multidimensional measure of dyspnea, with three subscales assessing sense of effort, sense of anxiety and sense of discomfort. Originally developed to assess impact of chemotherapy-induced nausea and vomiting, the Functional Living Index-Emesis (FLIE) is an 18-item instrument assessing level of nausea and vomiting and their impact on various aspects of function and quality of life (e.g., appetite, making meals, social interactions). There are also validated questionnaires for use in the palliative setting. An example, EORTC QLQ C15-Palliative, is a brief questionnaire of 15 items assessing walking ability, independence in self-care and completing daily activities, overall quality of life as well as symptoms such as weakness, shortness of breath, pain, sleep disturbance, nausea, constipation, and feelings of tenseness and depression.

Alternatively, there are several brief symptom screening tools available for use in the clinical setting. The Edmonton Symptom Assessment System (ESAS) evaluates eight items of pain, tiredness, nausea, depression, anxiety, drowsiness, appetite and sensation of well-being on a scale from 0 to 10. Similarly, the validated MD Anderson Symptom Inventory (MDASI) assesses 13 core symptoms of pain, fatigue, nausea, sleep disturbance, distress, shortness of breath, difficulty remembering, lack of appetite, drowsiness, dry mouth, sadness, vomiting and numbness/tingling on a 0 to 10 scale.

Symptom Clusters

Due to common etiology or causal relationships, two or more symptoms may present concurrently in clinically significant "clusters." Clusters were originally defined as three or more concurrent, interrelated symptoms. Later definitions described symptom clusters as being composed of two or more related symptoms that form a stable group, and are independent of other clusters. The "partial mediation model" explains how two symptoms can influence each other indirectly through the effect of another common symptom (e.g., pain impacting fatigue through impairment of sleep). Frequency, severity and distress of symptoms have been reportedly higher in patients who experience clustered symptoms versus those who do not.

Methodology in symptom cluster research has yet to be standardized. Statistical methods include factor analyses, which are often used to identify groups of symptoms related through shared underlying causes; and cluster analyses, which can group symptoms exhibiting similar patterns.

Highlighted by the National Cancer Institute at the State-of-the-Science Conference in 2012, the symptom cluster of pain, depression and fatigue is highly prevalent in patients with cancer. The symptoms are thought to share common pathophysiological mechanism of systemic inflammation, and have been induced as "sickness behavior" in animal models following administration of inflammatory agents. This model of sickness behavior consists of physiological components (fever, pain, wasting) and behavioral components (e.g., decreased activity, cognitive impairment, somnolence, decreased social interaction) and has been hypothesized to be the common mechanism behind this symptom cluster. Other studies report emotional symptom clusters including distress, sadness, lack of appetite, sleep disturbances, anxiety and depression in both patients with early and advanced disease.

The gastrointestinal symptom cluster of nausea, vomiting and appetite loss has been identified in patients undergoing chemotherapy with consistency across the treatment trajectory. It has been recommended that interventions focus on nausea and vomiting as core symptoms in patients who present with this particular symptom cluster, with supplementary strategies appended to target associated symptoms (e.g., weight loss, gastrointestinal reflux, diarrhea, bloating).

Certain physical and psychological symptom clusters have consistently been identified in palliative cancer patient populations. Physical symptoms such as tiredness, pain, nausea, drowsiness, dyspnea and loss of appetite consistently cluster together; whereas,

psychological symptoms such as depression and anxiety often appear together. Smaller clusters of physical symptoms have also been highlighted in the literature, such as nausea and dyspnea.

Dodd et al. were among the first to demonstrate the clinical significance of this phenomenon as they provided early insights into the effects of symptom clusters on individual functional status. Analyses indicate compounding effects of clusters on quality of life exceeding effects observed with individual symptoms. Understanding of symptom clusters may help prepare clinicians and patients for increased burden and distress associated with particular symptoms, and allow for more strategic prophylaxis and management preceding, during and following interventions. Symptom clusters may help in the elucidation of common pathophysiology. Alternatively, information gained from this type of research may help to prioritize certain upstream symptoms, therein highlighting routes through which downstream symptoms can be relieved. Clinicians may also use clustering to identify sub-groups of patients at risk of experiencing higher symptom severity, thereby aiding in the targeting of high-risk individuals for particular interventions.

Due to the potential implications in targeting supportive and palliative oncological care, symptom cluster research in this patient population has recently been named a priority by the Oncological Nursing Society. More investigation is needed to clarify the composition of clinically significant clusters, and to support relationships between symptoms with underlying mechanisms in order to design and test appropriate interventions.

Conclusion

Patients with cancer can experience a constellation of symptoms preceding diagnosis, throughout the trajectory of treatment and into survivorship or end-of-life care. Symptom burden has profound impacts on patient function and quality of life, and its measurement has demonstrated prognostic value. Increased attentiveness to symptom experience, improved communication between patients and health care providers and adherence to clinical guidelines in symptom management may reduce reported rates of inadequate symptom control.

Further Reading

Anderson, B.L., DeRubeis, R.J., Berman, B.S., et al., 2015. Screening, assessment, and care of anxiety and depressive symptoms in adults with cancer: An American Society of Clinical Oncology guideline adaptation. Journal of Oncology Practice 11 (2), 133–134.

Andreyev, J., Ross, P., Donnellan, C., et al., 2014. Guidance on the management of diarrhoea during cancer chemotherapy. Lancet Oncology 15 (10), e447–e460.

Barsevick, A.M., Irwin, M.R., Hinds, P., et al., 2013. Recommendations for high-priority research on cancer-related fatigue in children and adults. Journal of the National Cancer Institute 105, 1432–1440.

Berger, A.M., Abernathy, A.P., Atkinson, A., et al., 2010. NCCN clinical practice guidelines cancer-related fatigue. Journal of the National Comprehensive Cancer Network 8 (8), 904–931.

Blum, B., Stene, G.B., Solheim, T.S., et al., 2014. Validation of the consensus-definition for cancer cachexia and evaluation of a classification model—A study based on data from an international multicenter project (EPCRC-CSA). Annals of Oncology 25 (8), 1635–1642.

Dodd, M.J., Miakowski, C., Paul, S.M., 2001. Symptom clusters and their effect on the functional status of patients with cancer. Oncology Nursing Forum 28 (3), 465–470.

Fearon, K., Strasser, F., Anker, S.D., et al., 2011. Definition and classification of cancer cachexia: An international consensus. Lancet Oncology 12 (5), 489–495.

Gotay, C.C., Kawamoto, C.T., Bottomley, A., Efficace, F., 2007. The prognostic significance of patient-reported outcomes in cancer clinical trials. Journal of Clinical Oncology. https://doi.org/10.1200/JCO.2007.13.3439.

Kloke, M., Cherny, N., On behalf of the ESMO Guidelines Committee, 2015. Treatment of dyspnea in advanced cancer patients: ESMO clinical practice guidelines. Annals of Oncology 26 (Suppl 5), v169–v173.

Martin, L., Senesse, P., Gioulbasanis, I., et al., 2015. Diagnostic criteria for the classification of cancer-associated weight loss. Journal of Clinical Oncology 33 (1), 90–99.

Roila, F., Molassiotis, A., Herrstedt, J., et al., 2016. 2016 MASCC and ESMO guideline update for the prevention of chemotherapy- and radiotherapy-induced nausea and vomiting in advanced cancer patients. Annals of Oncology 27 (suppl 5), v119–v133.

Solheim, T.S., Blum, D., Fayers, P.M., et al., 2013. Weight loss, appetite loss, and food intake in cancer patients with cancer cachexia: Three peas in a pod?—Analysis from a multicenter cross sectional study. Acta Oncologica 53 (4), 539–546.

Stephenson, J., Davies, A., 2006. An assessment of etiology-based guidelines for the management of nausea and vomiting in patients with advanced cancer. Supportive Care in Cancer 14, 348–353.

Tong, H., Isenring, E., Yates, P., 2009. The prevalence of nutrition impact symptoms and their relationship to quality of life and clinical outcomes in medical oncology patients. Supportive Care in Cancer 17, 83–90.

van den Beuken-van Everdingen, M.H.J., de Rijke, J.M., Kessels, A.G., et al. Prevalence of pain in patients with cancer: A systematic review of the past 40 years. Annals of Oncology 18, 1437–49.

Vargas-Schaffer, G., 2010. Is the WHO analgesic ladder still valid? Twenty-four years of experience. Canadian Family Physician 56 (6), 514–517.

Viola, R., Kiteley, C., Lloyd, N.S., et al., 2008. The management of dyspnea in cancer patients: A systematic review. Supportive Care in Cancer 16, 329–337.

Systems Biology Approach to Study Cancer Metabolism

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A Systems View on Metabolism

Cancer is a complex disease. In 2000 Hannahan and Weinberg specified the complexity by defining the six hallmarks of cancer—resisting cell death, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, inducing angiogenesis, and enabling replicative immortality (Hanahan and Weinberg, 2000). Eleven years later they expanded their concept by adding the features of avoiding immune destruction, deregulating cellular energetics, genome instability, and mutation- and tumor-promoting inflammation (Hanahan and Weinberg, 2011). It became clear that the tumor cell has to be considered an interactive network of biological functions. Cells randomly acquire mutations throughout life. These mutations can be either selective neutral or beneficial as they confer cell growth and survival advantages (Martincorena et al., 2017). Cancer develops as a consequence of clonal selection for cells that have sequentially accumulated advantageous mutations, so called driver mutations. A typical tumor exhibits two to eight driver mutations, while the selective neutral mutations, termed passenger mutations, occur more frequently (Vogelstein et al., 2013). Each small genetic alteration has extensive effects on the molecular characteristic of a cell by affecting levels of DNA, RNA, proteins, and metabolites (Werner et al., 2014). Considering the multitude of mutations that occur in a tumor cell, cancer is a complex adaptive system with emerging properties at various levels that cause multivariate dysregulations within the molecular network (Kreeger and Lauffenburger, 2009). Thus, cancer systems biology aims to understand the complexity of the disease via the combination of experimental, mathematical, and computational tools that enable the integrative analysis of networks (Werner et al., 2014).

Impact of Metabolism on Different Omics Layers

Traditionally, cancer studies focused on the examination of individual mutations with a distinct phenotype as guided by the central dogma of molecular biology (Crick, 1970). The concept implies the unidirectional flow of information from genes to proteins via transcripts. The linear logic was questioned by the discovery that proteins and metabolites, the functional units subsequent of the dogma, can loop back and regulate transcriptional as well as translational processes (**Fig. 1**) (Buescher and Driggers, 2016). On the one hand, metabolism provides building blocks in form of nucleotides and amino acids to build up DNA, RNA, and proteins. These processes are highly energy-consuming and thereby once more dependent on metabolism, which assures the sufficient supply of energy carriers such as ATP and GTP. On the other hand, metabolites allosterically control the activity of enzymes that act on distinct regulatory levels (Buescher and Driggers, 2016). For example, the activity of the 5′ adenosine monophosphate-activated protein kinase (AMPK) is dependent on the competitive binding of the metabolites AMP and ATP (Wegner et al., 2015). In case the cellular energy state is low, AMP binds to the regulatory domain of AMPK which leads to a conformational change of the protein resulting in its phosphorylation and thereby activation. The dominant presence of ATP reverts the conformational change and dephosphorylates the kinase leading to its inactivation (Wegner et al., 2015).

Furthermore, the translation of messenger RNA (mRNA) is regulated by metabolites. The RNA binding protein Musashi-1 is known to be allosterically inhibited by unsaturated fatty acids that eventually results in the transcriptional inhibition of stearoyl-CoA desaturase, a fatty acid desaturase (Wegner et al., 2015). Moreover, it was reported that metabolite-sensing RNA elements, namely riboswitches, which regulate the gene expression in bacteria, algae, fungi, and higher plants (Garst et al., 2011). In the presence of a metabolic ligand riboswitches form alternative secondary structures that interfere with the transcriptional and/or translational machinery. The transcriptional control via metabolites is either achieved by allosteric inhibition of transcription factors, for example, inhibition of carbohydrate-responsive element-binding protein by xylulose-5-phosphate, or by chromatin remodeling (Wegner et al., 2015). Wellen et al. (2009) provided evidence that histone acetylation is dependent on the enzyme ATP citrate lyase (ACLY) that catalyzes the conversion of citrate into acetyl coenzyme A, which is the major substrate of histone acetyltransferases (HATs). HATs transfer the acetyl group of acetyl-CoA to lysine residues of histones allowing the transcriptional access to the DNA and thereby epigenetic control of gene expression.

Time-Resolved Omics Studies

The expression of genes, transcripts, and proteins reflect mainly the genotype of a cell, while metabolites transmit the phenotype. The regulation of metabolism is dependent on control processes that occur on each regulatory level, starting with the gene that encodes for different enzyme isoforms, the transcriptional activation via transcription factors, followed by the control of alternative splicing, mRNA stability as well as translation and protein degradation processes (Wegner et al., 2015). These gene expression-dependent regulations are time- and energy-consuming processes that are not easily reversible and therefore realized upon chronic environmental alterations. In contrast, the response to acute environmental cues is a gene expression independent process and thereby flexible and fast since it is based on the allosteric regulation or posttranslational modification of enzymes (Wegner

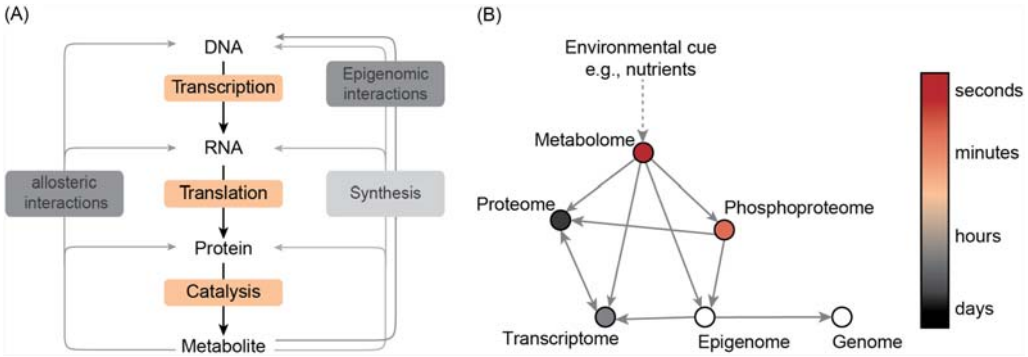

Fig. 1 Metabolomics in systems biology. (A) Regulation of omics levels (*gray arrows*) via metabolites beyond the central dogma of molecular biology. (B) Time-resolved trans-omics interactions in response to a nutritional cue. (A) Adapted and modified from Buescher, J. M. and Driggers, E. M. (2016). Integration of omics: More than the sum of its parts. *Cancer & Metabolism* **4**(1), 4; (B) Adapted and modified from Yugi, K. and Kuroda, S. (2018). Metabolism as a signal generator across trans-omic networks at distinct time scales. *Current Opinion in Systems Biology* **8**, 59–66.

et al., 2015; Yugi and Kuroda, 2018). Accordingly, the maintenance of the cellular homeostasis upon environmental stress is assured by a two-dimensional regulation: different omics levels which act on defined time scales (Yugi and Kuroda, 2018). The time-based delimitation is further discernible in the averaged half-lives of cellular metabolite (1 min), mRNA (10 h), and protein pools (24 h) estimated in HeLa cells (Yugi and Kuroda, 2018). The cellular function of each omics regime is defined by an inherent time scale allowing the dissection of sequential cellular processes in response to a stimulus. Upon a nutritional alteration the metabolome adapts first, within seconds, signaling reactions via kinases take seconds to minutes, whereas gene expression-dependent regulations are detectable after minutes, but rather hours (**Fig. 1**B) (Buescher and Driggers, 2016; Shaw et al., 2014; Yugi and Kuroda, 2018). Tahus, the recording of changing events on each omics layer over time provides information about the directionality of cellular processes that are induced upon a stimulus (Buescher and Driggers, 2016). Time-resolved experiments require frequent sampling in short intervals to monitor the response of the system. Each cellular response has a specific dynamic. The comparison of these inherent dynamics give information about the sequential interactions within the network (Buescher and Driggers, 2016). Especially in the field of cancer research the integrative multiomics approach provides the opportunity to: (i) uncover bypass reactions leading to drug resistance, (ii) identify biomarkers, (iii) and predict the most beneficial therapy for patients (Werner et al., 2014).

Required Tools for Metabolism-Centric Systems Biology

In order to conduct systems-level analyses, highly multivariate data sets are required. Advances in microarray and high-throughput methods enable the comprehensive analysis of all omics regimes. Genomics, transcriptomics, and epigenomics analyses are predominately based on DNA and RNA sequencing, whereas proteomics and metabolomics approaches rely on mass spectrometry (MS)-based methods that allow the direct identification of subjected molecules (Buescher and Driggers, 2016). Chromatographic methods, such as liquid and gas chromatography (LC and GC), are usually connected upstream of mass spectrometers performing the preseparation of biological compounds on the basis of their chemical properties. Proteins are usually subjected to LC–MS, while metabolites are analyzed with both methods. GC–MS is less sensitive than LC–MS, instead it enables the separation of structural isomers, such as glucose and fructose, and is thereby a suitable tool to examine the central carbon metabolism (Pietzke and Kempa, 2014).

Recently, metabolism-centric systems biology gained attention, because it links the biological phenotypes to environmental information. The metabolic adaptation in response to environmental cues might remain undetected due to interconnected, parallel, and circular pathways that balance the metabolic homeostasis (Pietzke et al., 2014). Differences in the abundance of metabolites are only detectable upon clear impairments of metabolic pathways as seen for knockdowns of key metabolic enzymes (Zasada, 2017). The application of stable isotope substrates overcomes this limitation as it allows to trace the isotope flow throughout the metabolic network (Lane et al., 2011; Wittmann and Heinzle, 1999; Metallo et al., 2009). A single GC–MS measurement provides the simultaneous analysis of metabolite abundance and isotope incorporation (Zasada, 2017). Usually stationary incorporation patterns are observed as a result of long labeling times that might reflect steady-state changes on the genome, transcriptome or proteome level. In contrast, the dynamic characterization of sequential metabolic events upon altered environmental conditions requires the application of stable isotopes in an instationary manner. Accurate kinetic studies require short sampling intervals and the uninterrupted supply of essential nutrients to minimize metabolic and mechanic stress that might affect nutrient conversion rates (Chokkathukalam et al., 2014). Accordingly, the experimental handling is technically challenging regarding the rapid replacement of carbon sources and the terminal quenching of metabolism. These vulnerabilities were overcome as Pietzke et al. (2014) developed an optimized work flow for stable-isotope incorporation in cell culture, termed pulsed stable isotope-resolved metabolomics (pSIRM). The method, optimized to reduce any kind of perturbation, allows a time- and atom-resolved tracing of the routing of ^{13}C- or ^{15}N-labeled nutrients within the central carbon metabolism (CCM) by GC–MS (**Fig. 2**).

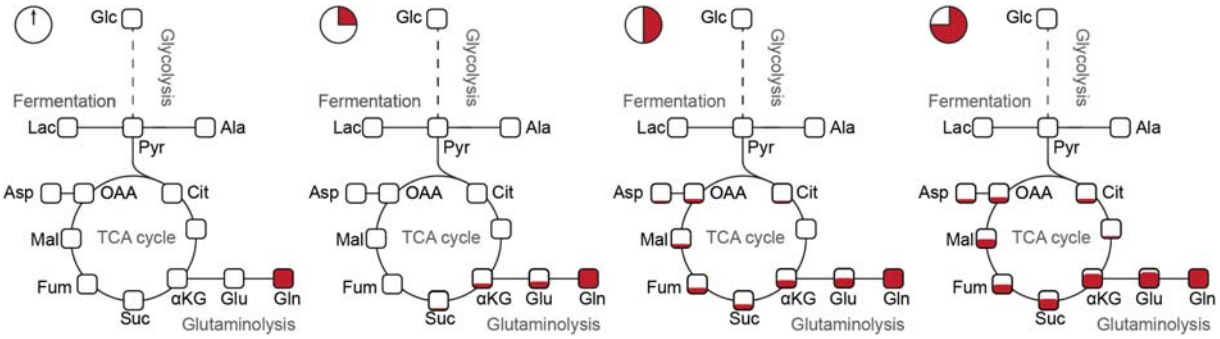

Fig. 2 Pulsed stable isotope-resolved metabolomics (pSIRM). Dynamics of ^{13}C incorporation (*red*) are shown for the application of u-^{13}C-labeled glutamine. Time-resolved pulsed labeling enables the monitoring of the turnover-dependent replacement of carbon-12 with carbon-13 (*red*) of each single metabolite pool. The comparison of these dynamics allows the estimation about the preferential routing of applied substrates in different conditions (*arrow*). Abbreviations: *Ala*, Alanine; *αKG*, α-Ketoglutarate; *Asp*, Aspartate; *Cit*, Citrate; *Fum*, Fumarate; *Gln*, Glutamine; *Glu*, Glutamate; *Lac*, Lactate; *Mal*, Malate; *OAA*, Oxaloacetate; *Pyr*, Pyruvate; *Suc*, Succinate.

See also: Glutamine Metabolism and Cancer. Inhibitors of Lactate Transport: A Promising Approach in Cancer Drug Discovery. Lipid Metabolism. Mevalonate Pathway. Pyruvate Kinase. TCA Cycle Aberrations and Cancer.

References

Buescher, J.M., Driggers, E.M., 2016. Integration of omics: More than the sum of its parts. Cancer & Metabolism 4 (1), 4.
Chokkathukalam, A., Kim, D., Barrett, M.P., Breitling, R., Creek, D.J., 2014. Stable isotope-labeling studies in metabolomics: New insights into structure and dynamics of metabolic networks. Bioanalysis 6 (4), 511–524.
Crick, F., 1970. Central dogma of molecular biology. Nature 227 (5258), 561–563.
Garst, A.D., Edwards, A.L., Batey, R.T., 2011. Riboswitches: Structures and mechanisms. Cold Spring Harbor Perspectives in Biology 3 (6), a003533.
Hanahan, D., Weinberg, R.A., 2000. The hallmarks of cancer. Cell 100 (1), 57–70.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: The next generation. Cell 144 (5), 646–674.
Kreeger, P.K., Lauffenburger, D.A., 2009. Cancer systems biology: A network modeling perspective. Carcinogenesis 31 (1), 2–8.
Lane, A.N., Fan, T.W.-M., Bousamra, M., Higashi, R.M., Yan, J., Miller, D.M., 2011. Stable isotope-resolved metabolomics (SIRM) in cancer research with clinical application to nonsmall cell lung cancer. OMICS 15 (3), 173–182.
Martincorena, I., Raine, K.M., Gerstung, M., Dawson, K.J., Haase, K., Van Loo, P., Davies, H., Stratton, M.R., Campbell, P.J., 2017. Universal patterns of selection in cancer and somatic tissues. Cell 171 (5), 1029–1041.e21.
Metallo, C.M., Walther, J.L., Stephanopoulos, G., 2009. Evaluation of 13C isotopic tracers for metabolic flux analysis in mammalian cells. Journal of Biotechnology 144 (3).
Pietzke, M., Kempa, S., 2014. Pulsed stable isotope-resolved metabolomic studies of cancer cells. Methods in Enzymology 543, 179–198.
Pietzke, M., Zasada, C., Mudrich, S., Kempa, S., 2014. Decoding the dynamics of cellular metabolism and the action of 3-bromopyruvate and 2-deoxyglucose using pulsed stable isotope-resolved metabolomics. Cancer & Metabolism 2 (1), 9.
Shaw, A.S., Kornev, A.P., Hu, J., Ahuja, L.G., Taylor, S.S., 2014. Kinases and pseudokinases: Lessons from RAF. Molecular and Cellular Biology 34 (9), 1538–1546.
Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz, L.A., Kinzler, K.W., 2013. Cancer genome lanscapes. Science 339 (6127), 1546–1558.
Wegner, A., Meiser, J., Weindl, D., Hiller, K., 2015. How metabolites modulate metabolic flux. Current Opinion in Biotechnology 34, 16–22.
Wellen, K.E., Hatzivassiliou, G., Sachdeva, U.M., Bui, T.V., Cross, J.R., Thompson, C.B., 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324 (5930), 1076–1080.
Werner, H.M.J., Mills, G.B., Ram, P.T., 2014. Cancer systems biology: A peak into the future of patient care? Nature Reviews. Clinical Oncology 11 (3), 167–176.
Wittmann, C., Heinzle, E., 1999. Mass spectrometry for metabolic flux analysis. Biotechnology and Bioengineering 62 (6), 739–750.
Yugi, K., Kuroda, S., 2018. Metabolism as a signal generator across trans-omic networks at distinct time scales. Current Opinion in Systems Biology 8, 59–66.
Zasada, C., 2017. Experimental and mathematical analysis of the central carbon metabolism in cancer and stem cells. Humbolt-Universität zu Berlin, Berlin.

Further Reading

Dejure, F.R., Royla, N., Herold, S., Kalb, J., Walz, S., Ade, C.P., Mastrobuoni, G., Vanselow, J.T., Schlosser, A., Wolf, E., Kempa, S., Eilers, M., 2017. The MYC mRNA 3UTR couples RNA polymerase II function to glutamine and ribonucleotide levels. The EMBO Journal 36 (13), 1854–1868.
Royla, N., 2018. Analysis of metabolic feedbacks regulating c-MYC. Freie Universität Berlin, Berlin.
Royla, N., Kempa, S., 2017. The answer's in the tail: MYC mRNA has a metabolic sensor that supports cancer chemoresistance. Molecular & Cellular Oncology 4 (4), e1338209.
Tarrado-Castellarnau, M., de Atauri, P., Cascante, M., 2016. Oncogenic regulation of tumor metabolic reprogramming. Oncotarget 7 (38), 62726–62753.
Vervoorts, J., Lüscher-Firzlaff, J., Lüscher, B., 2006. The ins and outs of MYC regulation by posttranslational mechanisms. Journal of Biological Chemistry 281 (46), 34725–34729.
Zasada, C., Kempa, S., 2016. Quantitative analysis of cancer metabolism: From pSIRM to MFA. In: Cramer, T., Schmitt, C.A. (Eds.), Metabolism in cancer. Springer, Cham, pp. 207–220.

TCA Cycle Aberrations and Cancer

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Glossary

Anabolic reactions Energy-requiring reactions involved in the production of macromolecules from smaller units.

Carboxylases Enzymes that catalyze the introduction of a carboxylic acid group to a substrate in an ATP-dependent manner. The opposite reaction is decarboxylation, with release of CO_2 and ATP.

Dehydrogenases Enzymes that couple the oxidation of a substrate with the reduction of an electron acceptor, usually NAD^+ or $NADP^+$, or a flavin coenzyme such as FAD.

Hydratases Enzymes that perform a reaction of hydration in which a substance combines with water.

Isoforms Proteins that can perform the same or similar biological roles. In the case of enzymes, they can perform the same enzymatic reaction.

Isomerization A chemical process through which a molecule is transformed into another with same atoms but different structural arrangement.

Oncometabolite A metabolite whose accumulation can promote the transformation of a cell or confer a cancer-associated phenotype.

Reactive oxygen species (ROS) Reactive chemical species deriving from partial reduction of molecular oxygen. They include superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$).

Introduction

Metabolic reorganization is a typical trait of cancer cells necessary to sustain the intensive proliferation rate and, more importantly, to face tumor environmental fluctuations in terms of nutrient limitations, changes in oxygen tension and response to therapeutic insults. The inherent intertwined nature of metabolic reactions actually renders difficult to foresee metabolic features of a specific tumor, which largely depend on the basal metabolic setting of the normal tissue and on the type of oncogenic signaling that has given rise to the tumorigenic event. Nevertheless, some specific characteristics of cancer metabolism have been traced; for instance, it is well established that some tumor types can favor glycolysis with respect to oxidative phosphorylation also in the presence of normal tension of oxygen, can have an intense utilization of glutamine, can activate de novo lipid biosynthesis. Many of these events rely on alterations of the tricarboxylic acid (TCA) cycle, also known as Krebs cycle or citric acid cycle. This is a multistep and central metabolic pathway occurring inside the mitochondrial matrix conveying carbohydrate, lipid, and protein metabolism for energetic or biosynthetic purposes.

Beginning from the canalization of carbohydrate-derived pyruvate into mitochondria and its conversion to acetyl-CoA by the pyruvate dehydrogenase complex, the conventional starting point of the TCA cycle is considered the reaction catalyzed by citrate synthase (CS), which combines an acetyl unit to oxaloacetate (OA) to produce citrate. This intermediate is isomerized by the hydratase activity of the mitochondrial aconitase or aconitase 2 (ACO2) into isocitrate, which becomes substrate for the oxidative decarboxylation reaction carried by the $NADP^+$-dependent IDH2 and NAD^+-dependent IDH3 isocitrate dehydrogenase enzymes, with release of α-ketoglutarate (α-KG). The activity of the α-KG dehydrogenase complex (α-KGDHC) further decarboxylates α-KG to form succinyl-CoA, an unstable intermediate subsequently converted in succinate by the succinyl-CoA synthetase. Succinate is then oxidized by the succinate dehydrogenase (SDH) complex in fumarate, to which a water molecule is added by the fumarate hydratase enzyme producing malate. Finally, OA is regenerated from malate by the activity of the malate dehydrogenase (MDH2) and it can be used for a further cycle in presence of acetyl-CoA (Fig. 1).

The overall yield of a single turn of TCA cycle starting from one acetyl-CoA unit consists of one molecule of GTP or ATP, three molecules of NADH and one of $FADH_2$. More importantly, the continuous production of NADH and $FADH_2$ in repeated TCA cycle turns and their re-oxidation in the electron transport chain (ETC) permit the generation of a mitochondrial intermembrane proton electrochemical gradient which is extinguished by the ATP synthase for ATP production.

The central position of the TCA cycle in cell metabolism is held by an intrinsic plasticity also determined by: (i) reversibility of most of TCA cycle reactions, apart from those of CS, IDH3 and α-KGDHC, (ii) the presence of two cytosolic proteins, namely aconitase 1 (ACO1) and isocitrate dehydrogenase 1 (IDH1), which recapitulate a portion of the TCA cycle outside mitochondria, (iii) participation of substrates in alternative reaction pathways. Consistently, most metabolites produced in the TCA cycle can function as precursors for biosynthesis of different types of macromolecules (cataplerotic reactions) which, in turn, can be used to replenish TCA cycle by anaplerotic reactions.

Among the others, the anabolic reactions based on TCA cycle intermediates and largely employed in tumorigenesis are lipid and aspartate biosynthetic pathways. De novo fatty acid biosynthesis, or lipogenesis, permits the production of new phospholipids for

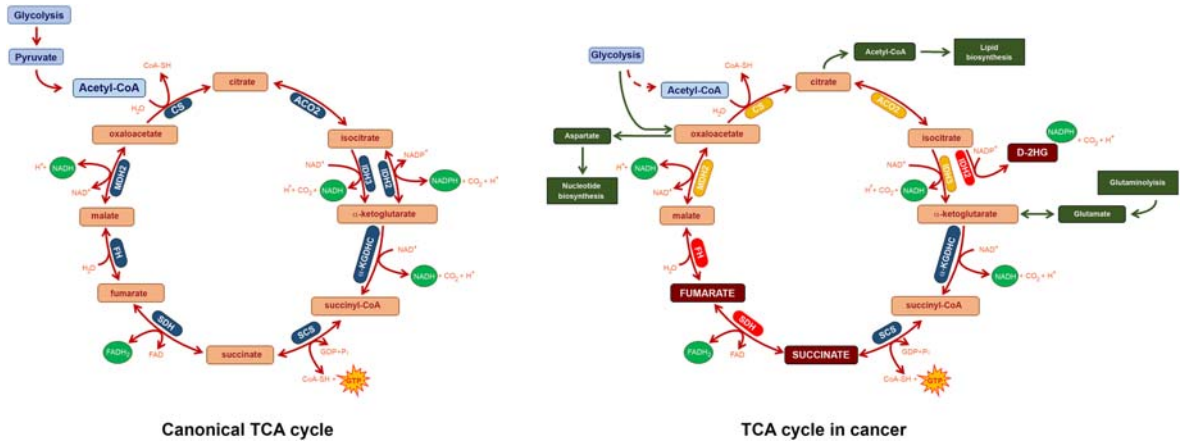

Fig. 1 *Canonical TCA cycle and aberrations in cancer.* Canonical TCA cycle (left) conventionally starts from glycolysis-derived acetyl-CoA condensation with a molecule of oxaloacetate (OA) to produce citrate. Seven further reactions (see text) are responsible for reconstitution of OA to permit the launch of another cycle in presence of acetyl-CoA. The overall yield of a cycle is three molecules of NADH (or two, plus one NADPH, depending on the IDH isoform used), one $FADH_2$ and one GTP. Most reactions are reversible, as shown by the *arrows*, except those of CS, IDH3 and α-KGDHC. Aberrations of the TCA cycle in cancer (right) can depend on either mutations (*red ellipses*) or altered gene expression (*orange ellipses*) of the enzymes. In particular, IDH2 mutants acquire neomorphic activity with production of D-2HG, while deficient SDH and FH activities lead to succinate and fumarate accumulation, respectively. A reduction of acetyl-CoA production from pyruvate (*dashed arrow*) can be compensated by anaplerotic reactions (in *green*), such as derivation of OA from pyruvate and of α-KG from glutaminolysis-derived glutamate. On the other hand, TCA cycle supports cancer-associated metabolic anabolism (in *green*) through citrate for lipid biosynthesis and OA for aspartate production, involved in nucleotide biosynthesis.

membranes. This is achieved through the translocation of citrate via the citrate transporter protein (CTP or SLC25A1) from mitochondria to cytosol, where it is cleaved back by the ATP citrate lyase (ACLY) to OA and acetyl-CoA, which becomes substrate for acetyl-CoA carboxylase (ACC) and then fatty acid synthase (FASN). Citrate for fatty acid biosynthesis can also be obtained from either pyruvate-derived acetyl-CoA or reductive carboxylation of α-KG by IDH2. Accumulation of aspartate can support proliferation by favoring nucleotide biosynthesis via activation of the trifunctional multidomain enzyme carbamoyl-phosphate synthase 2, aspartate transcarboxylase and dihydroorotase. Aspartate production from TCA cycle is mediated by the aspartate aminotransferase that catalyzes the reversible transamination of glutamate and OA to aspartate and α-KG. Aspartate biosynthesis can be sustained by pyruvate carboxylase, which replenishes OA from pyruvate and is one of the anaplerotic reactions largely up-regulated in cancer. Together with this, cancer cells depend on glutamine as main alternative fuel of the TCA cycle providing α-KG. This important anaplerotic reaction is supported in mitochondria by two consecutive deaminations into glutamate, via glutaminase 2, and then into α-KG by glutamate dehydrogenase 2 or by transaminases (Fig. 1).

TCA Cycle Alterations in Cancer

Defects in TCA cycle reactions in cancer have been attributed to altered activity of almost all enzymes, primarily as consequence of genetic mutations or aberrant gene expression. In many cases, oncogene activation or mutated tumor suppressors have been directly or indirectly linked to altered transcription of TCA cycle components or of genes involved in related pathways. Genetic mutations identified in TCA cycle genes can both disable enzymatic activity, inducing accumulation of specific metabolites, or bring to neomorphic enzymatic activity. Very interestingly, mutations of a specific gene of the TCA cycle are often responsible for predisposition to a particular type of tumor, indicating that some organs or tissues are more susceptible to alterations of certain TCA cycle reactions rather than others. Hereafter, information available for all TCA cycle enzymes in cancer will be provided with emphasis on the kind of alterations identified and on the associated metabolic and nonmetabolic outcomes.

Citrate Synthase

CS is a homodimeric protein conventionally designated as first enzyme of the TCA cycle, performing the irreversible condensation of acetyl-CoA with OA to form citrate. Indeed, the reaction is highly esoergonic and determines the whole TCA cycle rate. The number of reports showing alterations of CS in cancer are very limited and mainly demonstrating changes of gene expression rather than genetic mutations. Most data indicate that CS expression and/or activity is enhanced in cancer, as shown in ovarian malignant tumors and cell lines, pancreatic carcinoma and benign renal oncocytoma as well. Nevertheless, an antineoplastic role of CS was also shown as its decrease in cervical cancer cell lines contributed to the switch from oxidative to aerobic glycolytic metabolism,

a typical feature of many tumors also known as "Warburg effect". The different impact of CS in cancer may depend on the metabolic requirement of each cancer type. More specifically, upregulation of CS activity may be required by those cancer cells that rely on citrate to increase fatty acid biosynthesis or to burst TCA cycle rate for energetic or other anabolic purposes. On the contrary, cancer cells may exploit CS down-regulation to dampen oxidative metabolism in favor of aerobic glycolysis or to mainly rely on glutamine anaplerosis.

Aconitase

ACO2 represents the second enzyme of the TCA cycle, catalyzing the isomerization of citrate to isocitrate. The protein possesses a 4Fe-4S iron–sulfur cluster that directly interacts with the substrate. The regulation of citrate availability and of its utilization has been demonstrated as the only mechanism currently known for ACO2 in cancer metabolism. In particular, these events have been mainly characterized in prostate cancer due to the relevant role of ACO2 in normal prostate epithelium. This tissue physiologically relies on massive accumulation of citrate achieved through aconitase inhibition by high levels of zinc. Instead, prostate cancer cells restore aconitase activity thus permitting citrate isomerization at the expense of fatty acid synthesis. Notably, reduction of citrate levels is a suitable in vivo marker of transformed prostate epithelium. On the other hand, gastric cancer biopsies showed reduced expression of *ACO2* gene, which functions as prognostic factor for disease progression. Although no mechanisms have been proposed, disabling ACO2 activity or expression can be employed to truncate citrate flux permitting its extrusion outside mitochondria for lipogenesis.

Isocitrate Dehydrogenase

IDH is the third enzyme of the TCA cycle, performing a two-step reaction, starting with the oxidation of isocitrate to oxalosuccinate (a ketone) which is further decarboxylated, forming alpha-ketoglutarate. Pro-neoplastic mutations of IDH1 and IDH2 are typically observed in a large number of brain tumors, including astrocytomas, oligodendrogliomas and secondary glioblastomas, and are among the most frequent mutations (about 20%) in acute myeloid leukemia (AML). Although rare, IDH1/2 mutations also occur in osteosarcoma, intrahepatic cholangiocarcinoma, prostate and colon cancer. Instead, it has to be mentioned that no cancer-associated mutations in IDH3 subunits have been identified. Nevertheless, upregulation of IDH3 subunit α was shown to potentiate tumor growth, metabolic reprogramming and angiogenesis and was associated with reduced overall survival in patients with lung or breast cancer. Based on this evidence, it is conceivable that inactivation of NAD^+-dependent IDH3 would be detrimental for mitochondrial homeostasis and cancer survival, differently form mutations of $NADP^+$-dependent IDH1 and IDH2 enzymes. A potential explanation may lie in the key role of IDH3 in the TCA cycle, where it irreversibly catalyzes the first reaction deputed to the production of the electron carrier NADH. IDH3 critical relevance in the TCA cycle is also suggested by its tight regulation by substrate availability (i.e., isocitrate and NAD^+) and inhibition by ATP, α-KG and NADH. On the contrary, IDH2 and IDH1 contribution to mitochondrial energetic homeostasis is only partial as they produce α-KG, permitting TCA cycle to run, but in combination with NADPH instead of NADH. Moreover, IDH1/IDH2-catalyzed reaction is reversible allowing the reductive carboxylation of α-KG in isocitrate, which, once converted in citrate by ACO1/ACO2, can contribute to anabolic pathways together with NADPH.

IDH1/2 mutations mainly occur at substrate-binding amino acid residues, specifically Arg-132 of IDH1 and Arg-140 or Arg-172 of IDH2, and are somatically acquired. Moreover, the occurrence of IDH1 mutations in low grade gliomas is more frequent than IDH2 mutations, while both defects have a similar incidence in AML. The phenotypic outcome of IDH1/2 mutations is a neomorphic activity leading to the formation of D-2-hydroxyglutarate (D-2HG). Human cells normally produce low levels of D-2HG, mainly by the activity of D-3-phosphoglycerate dehydrogenase (PHGDH), but its accumulation is impeded by its conversion to α-KG through the FAD-dependent 2-hydroxyglutarate dehydrogenase (D2HGDH). In the case of IDH1/2 mutations, the accumulation of D-2HG is massive, reaching about a 50-fold increase and therefore it acts as an oncometabolite. Interestingly, the acquired mutation is heterozygous, permitting IDH enzymes to retain also wild-type activity. This mutational status is suitable for cancer cells, which can take advantage of D-2HG side effects in combination with canonical IDH1/2 activity highly useful for tumor-related anabolic reactions.

Experimental in vitro models based on either the use of cell permeable forms of D-2HG or overexpression of IDH mutants demonstrated their negative impact on cell differentiation in a wide panel of cell lines, including immortalized astrocytes or neuronal stem cells. This evidence, coupled with the early identification of IDH1 Arg-132 mutation in low-grade diffuse astrocytomas and oligodendrogliomas, actually supports a leading role of this TCA cycle aberration in glioma pathogenesis. However, the sole IDH1 mutation is not sufficient to trigger tumorigenesis in in vivo mouse models and it is frequently associated with secondary genetic alterations in human gliomas, particularly p53 and ATRX mutations or 1p/19q deletions. Similar evidence has been also collected for IDH2 mutations in leukemogenesis, as expression of mutant IDH in mouse myeloid progenitor and primary hematopoietic bone marrow cells was associated with a very low prevalence of myeloproliferative-like neoplasms or lymphoma. The long latency of these manifestations and the cooccurrence in AML patients of IDH2 aberrations with other frequent mutations in NPM1, FLT3, and DNMT3A also indicate a pivotal but synergistic effect of IDH2 mutants to tumor initiation.

Metabolic consequences arising from IDH1/2 mutations mainly consist in a preferential utilization of glutamine to feed the TCA cycle and in a reduction of NADPH production mining redox balance and antioxidant response. Beyond these events,

pro-tumorigenic properties of D-2HG largely rely on the competitive inhibition of several classes of α-KG–dependent dioxygenase enzymes, which impinge on epigenetic regulation, DNA damage response and hypoxia signaling.

Epigenetic modifications mediate a coordinated control of genome information regulating transcriptional programs in response to both extrinsic and intrinsic cues. Altered epigenetic regulation is a key feature of cancer cells that highly contributes to repression of tumor suppressor genes as well as activation of oncogenes. IDH1/2 neomorphic activity negatively impacts the Ten-eleven-translocation (TET) family enzymes, which act on DNA epigenetic modifications, and lysine demethylases (KDM) containing the Jumonji C domain, which instead act on histone lysine residues. The TET family members (TET1, TET2, and TET3) are α-KG/Fe (II) dioxygenases involved in DNA demethylation processes, driving the active removal of 5-methylcytosine (5mC), which generally mediates transcriptional silencing when present at gene promoters. This TET-dependent mechanism is accomplished through the sequential oxidation of 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine and 5-carboxylcytosine followed by active excision of modified base through DNA repair pathways. Therefore, the inhibition of TET enzymes due to accumulation of D-2HG actually induces DNA hypermethylation. In particular, a specific subset of gliomas exhibited conserved DNA methylation changes at specific loci, a phenomenon strongly associated with the presence of IDH1 mutations and termed as DNA hypermethylator phenotype. The same picture was also identifiable in AML patients bearing IDH1/2 mutations resulting in impaired differentiation of hematopoietic cells. This was largely ascribable to DNA methylation-mediated transcriptional repression of genes that favor expansion of progenitor cell. Consistently, the use of inhibitors affecting DNA methyltransferases induced cell differentiation reversing the DNA hypermethylated phenotype.

Concerning histone methylation, the transcriptional outcome of D-2HG-mediated inhibition on KDMs is not predictable as it depends on the specific lysine residue targeted. For instance, histone H3 lysine 9 trimethylation (H3K9me3), as well as histone H3 lysine 27 trimethylation (H3K27me3), are associated with inactive chromatin state while histone H3 lysine 4 trimethylation (H3K4me3) is a mark of permissive chromatin. In particular, a high affinity for D-2HG was demonstrated for the H3K9 demethylases KDM4A and 4C, the inhibition of which induced accumulation of H3K9me3 associated with defects in differentiation of lineage-specific progenitor cells into terminally differentiated cells. An indirect effect of KDM4A/B inhibition by D-2HG is persistence of DNA double-strand breaks due to impaired repair via homologous recombination. In fact, those demethylases contribute to chromatin remodeling necessary for the recruitment of DNA repair factors at sites of DNA damage. On the other side, a direct influence of IDH mutants on cell sensitivity to alkylating agents can be recognized due to inhibition of α-KG-dependent alkB homolog (ALKBH) DNA repair enzymes, which reverse alkylation of DNA bases including 1-methyladenine and 3-methylcytosine.

A complete different scenario in IDH1/2-mediated tumorigenesis involves the regulation of hypoxia signaling through the inhibition of important modulators of hypoxia-inducible factors (HIFs) 1 and 2, oxygen sensors transcriptionally involved in the regulation of multiple pathways. HIF signaling actually orchestrates angiogenesis, metabolism, proliferation and cell death pathways in order to restore oxygen homeostasis. Due to the limited availability of oxygen, especially in solid tumors, HIF proteins are frequently stabilized in cancer. HIF1 and HIF2 proteins are heterodimers composed of a common and stable subunit (HIF-1β) and a specific α subunit (HIF-1α and HIF-2α), the stability of which is highly regulated by hydroxylation catalyzed by two class of α-KG/Fe(II)-dependent dioxygenases. In normoxic environments, prolyl hydroxylase domain proteins (PHD1 and 2) and the asparaginyl hydroxylase activity of factor inhibiting HIF (FIH) modify HIF proteins targeting them for proteasomal degradation by means of the von Hippel-Lindau protein (pVHL) complex E3 ubiquitin ligase. Considering this, the accumulation of D-2HG can induce the inhibition of PHDs and/or FIH with subsequent stabilization of HIF proteins independently of oxygen availability, a condition referred to as "pseudohypoxia." However, the consequences of IDH1/2 mutations on hypoxia signaling seem more complex and controversial. In fact, a number of studies demonstrated a reduction of HIF stabilization, a dampened signaling following D-2HG-mediated inhibition of FIH and, even more, only a weak inhibitory effect on PHDs.

α-KG Dehydrogenase Complex

The α-KG dehydrogenase complex (α-KGDHC) is the fourth enzyme of the TCA cycle performing the decarboxylation of α-KG to succinyl-CoA. The α-KGDHC consists of three multirepeated enzymes, each one implicated in a specific reaction. The E1 enzyme is encoded by the *OGDH* gene and is the proper dehydrogenase catalyzing the decarboxylation of α-KG requiring thiamine pyrophosphate (TPP) as cofactor. The transfer to CoA and the final reduction of NAD^+ to NADH to generate the end product (succinyl-CoA) are respectively carried out by the E2 (dihydrolipoamide succinyl-transferase, DLST) and the E3 (dihydrolipoamide dehydrogenase, DLD) enzymes. As for CS, the reaction catalyzed by α-KGDHC is irreversible.

To our best knowledge, no report has described changes either in the expression or in the activity of α-KGDHC in cancer to date. However, a role for α-KGDHC in cancer can be conceived, mainly based on the evidence that α-KGDHC activity can be influenced by cancer-associated conditions. In fact, α-KGDHC activity strongly relies on thiamine (vitamin B1) availability. Thiamine is an essential water-soluble vitamin, critical for the activity of α-KGDHC, but also of pyruvate dehydrogenase, transketolase and branched chain α-keto acid dehydrogenase complex. Its conversion to the active coenzyme TPP is performed by thiamine pyrophosphokinase-1 (TPK1) upon intracellular transport. Thiamine uptake is performed by the thiamine transporter SLC19A3, whose decreased expression has been extensively demonstrated in breast, gastric and colon cancer as consequence of hypermethylated promoter. It is noteworthy mentioning that SLC19A3 expression results recovered upon hypoxic stimulation in breast cancer cells and this effect was ascribed to HIF-1α. Indeed, results obtained upon HIF-1α knockdown support the evidence that SLC19A3 could be an additional gene involved in the metabolic rewiring during hypoxic stress. In addition to this, a significant decrease of

TPP in patients with advanced stages of nonsmall cell lung cancer was observed in whole blood and a concomitant increased thiamine urinary excretion was also assessed in cancer patients. Thus, it is enticing to hypothesize that decreased thiamine levels in cancer could impair α-KGDHC activity.

α-KGDHC is both generator (by the E3 subunit) and target of reactive oxygen species (ROS). Notably, as many aspects of tumor initiation/progression implicate increased ROS production and/or signaling, it is tempting to speculate that α-KGDHC-mediated ROS production could contribute to the redox-dependent transduction pathways that control cell cycle progression and growth of cancer cells. On the other hand, excessive oxidative stress can impinge upon α-KGDHC reaction without a complete inactivation, contrarily to ACO2 activity, which is totally inhibited. In this scenario, ACO2 sensitivity to ROS impedes the first reactions of the cycle to occur and thus the anaplerotic derivation of α-KG from glutaminolysis becomes indispensable to guarantee TCA cycle flux. Based on this, α-KGDHC can be considered the driving force of the TCA cycle upon oxidative stress and a critical junction with glutaminolysis.

Importantly, a recent study uncovered a role for DLST in MYC-mediated leukemogenesis. Indeed, inactivation of the enzyme resulted in delayed tumor onset in a zebrafish model of MYC-mediated tumorigenesis and decreased cell viability and induction of apoptosis in human T-cell acute lymphoblastic leukemia cell lines. Moreover, recent insights have envisaged that OGDH activity could be used as marker for metabolic classification of cancer cell lines in subsets. In addition, α-KGDHC inhibition has been associated with altered autophagic signaling, an important process involved in the maintenance of cancer cell homeostasis. Altogether, these findings lend support for further investigation of α-KGDHC role in cancer.

Succinyl-CoA Synthetase

SCS, also known as succinyl CoA ligase (SUCL), is the fifth enzyme of the TCA cycle. The protein is a heterodimeric enzyme, the α subunit of which, encoded by the *SUCLG1* gene, couples the reversible conversion of succinyl-CoA to succinate bringing to the formation of a nucleoside triphosphate molecule (either GTP or ATP). The β subunit is encoded by either the *SUCLG2* or the *SUCLA2* gene, determining the specificity for GDP or ADP, respectively.

To date, no evidence about the presence of mutations in the genes coding for SCS subunits in cancer has arisen yet. However, a deleterious homozygous mutation in the *SUCLG1* gene has been detected and associated with a lethal neonatal disorder known as fatal infantile lactic acidosis (FILA). In particular, this metabolic disorder is characterized by lactic acidosis, ascribed to forced ATP production by glycolysis as effect of impaired TCA cycle. As this phenomenon is overlapping with the cancer-associated metabolic feature known as "Warburg effect", it is conceivable that *SUCLG1* mutation(s) -or at least its down-regulation- could be possible candidate(s) in the modulation of cancer metabolic rewiring. However, no data exist in this regard. In this scenario, down-regulation of *SUCLA2* in prostate malignancies has been related to altered metabolic flux and increased levels of succinate, whose role as oncometabolite will be discussed in the next sessions. In addition, though no mutation in *SUCGL2* has ever been described (and, for this reason, likely referred to as lethal), *SUCLG2* knockdown has been shown to markedly impair growth in fibroblasts. This could be an interesting starting point for unraveling putative *SUCGL2* role(s) in cancer. In this regard, the only evidence available concerns the diverse *SUCLG2* expression in thyroid follicular carcinoma and follicular adenoma as biomarker for diagnostic distinction of the two cancer types. However, no relevance for therapeutic treatments was disclosed.

Succinate Dehydrogenase

SDH complex, also designated as succinate: ubiquinone oxidoreductase or mitochondrial complex II, is the bridge enzyme between the TCA cycle and the ETC. SDH catalyzes the sixth step of TCA cycle, that is the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. The enzyme is heterotetrameric and composed of four subunits. The flavoprotein SDH-A and the iron'sulfur protein SDH-B are the two catalytic subunits, while SDH-C and SDH-D are the hydrophobic membrane-anchoring subunits also deputed to ubiquinone binding for ETC reactions.

Germinal *SDH* mutations have been implicated in increased occurrence of several neuroendocrine tumors, including hereditary paraganglioma (PGL) and pheochromocytoma (PCC). *SDH-B* mutations commonly cause type-4 PGLs (PGL4), including familial adrenal PCC. Malignant PGLs deriving from *SDH-B* mutations are commonly extra-adrenal (i.e., metastatic), such as abdominal and thoracic PGLs. Moreover, *SDH-B* mutations have been related to serous ovarian cancer, gastrointestinal stromal tumor (GIST), and familial renal cancer carcinoma (RCC). More than 30 mutations on the *SDH-C* gene have been found to increase the risk of type-3 PGL (PGL3). *SDH-D*-related tumors are typically benign type-1 PGLs (PGL1) and originate in head and neck (HNPGLs). Different *SDH-D* mutations have been found in people with the Cowden syndrome, which is characterized by multiple tumor-like lesions defined hamartomas and by predisposition to certain cancer types. Markedly, *SDH-D* gene is located in a hotspot locus on chromosome 11q23 which is commonly mutated in many types of cancer, including bladder, breast, lung, ovary and nasopharyngeal tumors. Furthermore, mutations in *SDH-D* have been found in individuals with GIST. The link between *SDH-A* mutation(s) and PGL/PCC is instead very weak as only one heterozygous germline *SDH-A* mutation has been identified in a rare malignancy known as catecholamine-secreting abdominal paraganglioma. Interestingly, germline loss-of-function mutations on the *SDHAF2*/*SDH5* gene, encoding for the mitochondrial protein needed for flavination of the SDH-A subunit, have been

implicated in hereditary type-2 PGL (PGL2). To date, it remains unclear whether the effect of *SDH* mutations are causative of papillary thyroid cancers, adrenal neuroblastoma or colon cancer.

The compromised -or completely lost- enzymatic activity driven by *SDH-x* mutations tightly affects the electron flow to ubiquinone, which is indispensable for electron transfer through the ETC. The main outcome of such alteration is the wrecked reduction of oxygen which promotes increased production of superoxide anion ($^{\bullet}O_2^-$), an oxygen reactive species, in mitochondria. Elevated $^{\bullet}O_2^-$ implicates augmented oxidative stress which results in protein carbonylation, DNA damage (by conversion of deoxyguanosine to 8-OH-deoxyguanosine), decreased mitochondrial membrane potential and structural changes in mitochondria. Consistently, a single Val-69 mutation in SDH-C has been suggested to promote a tumor-like phenotype in murine fibroblasts and to support tumorigenesis as consequence of $^{\bullet}O_2^-$-derived oxidative stress.

An additional consequence of mutations in *SDH* genes is the abnormal increase of succinate levels. Accumulation of succinate transforms it in an oncometabolite impinging on α-KG-dependent dioxygenase enzymes due to structural similarity with α-KG. As in the case of D-2HG produced by IDH mutants, the main target of succinate is the activity of epigenetic enzymes. Indeed, SDH-mutated PGL/PCCs exhibit low levels of 5hmC, giving rise to a hypermethylated DNA profile, and elevated histone H3K27 methylation, supporting the evidence that succinate inhibits both TET- and KDM-mediated demethylation. Coherently, chromaffin cells from *Sdhb* knock out (KO) mice have been demonstrated to possess higher 5mC/5hmC ratio with respect to wild-type cells, and this effect was reversed by exogenous addition of α-KG, revealing that succinate inhibition of α-KG-dependent dioxygenase occurs in competitive manner. Furthermore, reduction of 5hmC levels was also ascribed to nuclear exclusion of TET1 in *SDH*-deficient tumors. Interestingly, tumors harboring *SDH-B* mutations are characterized by a higher methylation profile across CpG islands than other *SDH*-mutated cancers. This could suggest that SDH-B activity is prevalent with respect to other SDH subunits fostering higher succinate accumulation and more relevant α-KG-dependent dioxygenases inhibition. Moreover, *SDH-B* mutations confer a peculiar more aggressive and metastatic phenotype. In this regard, SDH-B silencing has been also demonstrated to contribute to epithelial-to-mesenchymal transition (EMT) of mouse ovarian cancer cells by high histone methylation, in this case dependent on up-regulation of genes involved in S-adenosyl methionine (SAM) metabolism, the methyl donor of histone and DNA methyltransferase enzymes.

Similarly to *IDH*-mutated cancers, also *SDH*-deficient tumors display a HIF-dependent activation of a "pseudohypoxic" response. Indeed, as for TETs and KDMs, succinate can also inhibit the activity of PHD, resulting in HIF-1α stabilization. As the outcomes of HIF-1α stabilization by succinate in *SDH*-mutated cancers are very similar to those arising from fumarate accumulation in *FH*-related tumors, the concerning phenomena will be comprehensively described in the next session.

Fumarate Hydratase

FH, encoded by the *FH* gene, is the seventh enzyme of the TCA cycle performing the reversible hydration of fumarate to L-malate. Germline mutations can be found throughout the *FH* gene with consequent expression of inactive or even total loss of the enzyme. *FH* mutations strongly predispose to multiple cutaneous and uterine leiomyomas (MCUL) and to hereditary leiomyomatosis and renal cell cancer (HLRCC), including type-2 papillary renal cell cancer (PRCC2), with aggressive phenotype and untimely metastatization. Whole-exome sequencing has revealed, in an international cohort of patients, that *FH* mutations also bias for malignant PCC and PGL occurrence, with a SDH-like molecular phenotype. Moreover, some evidence supports that *FH* mutations might also be involved in the pathogenesis of breast, bladder and testis cancer. Importantly, beyond germline mutations, a decreased expression prolife of *FH* has been detected in some cancer types, including brain cancer, lung cancer, gastric cardia cancer (GCC) and sporadic renal cell cancer (RCC).

FH-deficient kidney cancers are characterized by a metabolic shift to aerobic glycolysis. Indeed, kidney cancer cell lines display enhanced glycolytic rate in order to overcome the impaired oxidative phosphorylation. In this context, increased expression levels of the glucose transporter *SLC2A1*/*GLUT1*, hexokinase 2 (*HK2*), pyruvate kinase (*PK*) and the vascular endothelial growth factor (*VEGF*) have been documented. In this scenario, glucose addiction of HLRCC may justify both the ATP production and the sustained proliferation rate of kidney cancer cells. The increased expression of the aforementioned genes in HLRCC can be explained by stabilization of HIF-1α. In fact, *FH*-mutated tumors, together with *SDH*-mutated ones, induce pseudohypoxic response due to accumulation of intracellular fumarate as the main outcome of FH deficiency. Consistently, high levels of fumarate, as D-2HG and succinate, can inhibit the activity of the α-KG-dependent dioxygenases due to structural similarity to α-KG. As a matter of fact, both fumarate and succinate contribute to competitive inhibition of PHDs resulting in HIF-1α stabilization. Over the years, more detailed models for HIF-1α stabilization in HLRCC tumors have been proposed. Indeed, the increased glucose uptake upon FH deficiency has been associated with depletion of intracellular Fe^{2+}, a PHD necessary cofactor, as consequence of boosted ROS production due to massive NADPH oxidase activation. The ROS-mediated inhibition of PHD activity has been validated by a reduction of tumor formation upon antioxidant treatment in HIF-driven models of tumorigenesis. Another mechanism proposed for increased HIF-1α stabilization in *FH*-deficient kidney tumors relies on the altered cytosolic iron concentration coming from reduced expression levels (and protein activation, i.e., phosphorylation) of both α and β1 subunits of the cellular energy sensor AMP-activated protein kinase (AMPK). Though the molecular mechanisms underlying this down-regulation in HLRCC are still elusive and controversial, AMPK reduction stabilizes HIF-1α upon iron depletion. Moreover, FH-mediated AMPK reduced expression/activation also results in increased activity of ACC, the enzyme that triggers the synthesis of fatty acids, likely promoting cell growth and proliferation of tumors due to increased lipid building blocks availability.

The pseudohypoxic response deriving from FH inactivation in mice kidney was identified as causative for renal cysts formation, plausibly by increased cell proliferation. However, as renal cysts formation still occured in *Fh* KO mice with depleted *Hif1a*, the involvement of further molecular mediator(s) in these processes was conceived. Remarkably, fumarate is a thiol-reactive compound capable to react with the sulphydryl moiety of cysteine residues to produce 2-succinyl-cysteine. This reaction is documented as succination and alters the activity of many cysteine-containing proteins, among which is Kelch-like ECH-associated protein 1 (KEAP1). In PRCC2, KEAP1 is highly succinated preventing ubiquitination and degradation of its molecular partner nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Coherently, Nrf2 targets are over-expressed in FH-inactivated tissues and its antioxidant signaling pathway was encouraged to promote renal cysts formation in FH-deficient mice, independently from HIF-1α-mediated signaling. Among the Nrf2 targets is *HMOX1*, the gene encoding for heme oxygenase, the enzyme that cleaves the heme ring to form biliverdin. *FH*-deficient murine kidney cells have been described to possess altered biosynthesis and degradation of heme, with high bilirubin excretion. This metabolic alteration is likely achieved through the accumulation of succinyl-CoA, the precursor for heme biosynthesis together with glycine, and is pivotal for maintaining mitochondrial functionality and mitochondrial NADH production. HMOX1 is a hot point target in HLRCC research since its inhibition has been demonstrated to be lethal for *FH*-inactive kidney cells.

It is noteworthy mentioning that there is no direct evidence indicating the dominancy either of Nrf2 or of HIF-1α pathway upon FH deficiency. Nevertheless, the activation of Nrf2 is a common feature of many cancer types, including PRCC2, lung, breast and colorectal cancers, and is mainly achieved by *Nrf2* gain-of-function mutations or *KEAP1* loss-of-function mutations. Importantly, the expression of constitutively active Nrf2 in embryonic renal cells showed severe and aggressive phenotype, thus revealing that Nrf2 activation could be oncogenic in kidney cancer.

Fumarate accumulation in *FH*-deficient tumors also implicates changes in the urea cycle. Indeed, the reaction of the argininosuccinate lyase (ASL) is reverted, promoting the production of argininosuccinate from fumarate and arginine. This metabolic alteration renders renal carcinoma cells auxotrophic for arginine and sensitive to arginine-depriving agents. On the basis of this, depletion of arginine has been proposed as novel therapeutic approach for renal carcinoma treatment.

Fumarate, similarly to succinate in *SDH*-deficient tumors, has been found to inhibit TET-catalyzed hydroxylation of 5mC in *FH*-deficient kidney cancer. Indeed, in vivo evidence supports the notion that fumarate accumulation markedly decreases 5hmC levels upon FH deficiency with outcome on transcriptional regulation. In this regard, lack of FH in both mouse and human cells has been linked to TET-mediated activation of EMT pathway via inhibition of the antimetastatic *mir-200ba429*. Overall, considering the well-characterized tumor suppressor function of TETs in several contexts, it is tempting to propose that fumarate- and succinate-mediated TET inhibition may contribute to the tumorigenesis of *FH*- and *SDH*-mutated cancers, respectively.

Interestingly, a methylome analysis performed in *IDH*-, *SDH*- and *FH*-mutated cancers has revealed that 17 genes are concordantly hyper-methylated and down-regulated in all tumors analyzed. This reveals a connection in the methylation profile of cancers carrying mutations in such genes, reflecting a similar inhibitory role for D-2HG, succinate and fumarate on epigenetic α-KG-dependent dioxygenases.

Malate Dehydrogenase

MDH2 is the eighth and last enzyme of the TCA cycle that reversibly couples the reduction of NAD^+ to NADH to the oxidation of L-malate to OA, thus permitting the launch of another cycle. Eukaryotic cells display two main isoforms of MDH. The first isoform, designated MDH2 and encoded by the *MDH2* gene, is located in the mitochondrial matrix and directly participates in the TCA cycle for L-malate oxidation. The second isoform, designated MDH1 and encoded by the *MDH1* gene, is cytosolic and coordinates the metabolic intersection between cytosol and mitochondria by the malate-aspartate shuttle.

Although limited information is available, a role for MDH2 in cancer has been envisaged. Indeed, *MDH2* has been proposed as new susceptible gene in PGL/PCC as a germline mutation in it, responsible for reduced gene expression, has been identified. Contrarily, elevated expression of MDH2 was depicted in different cancer types. High expression levels of MDH2 have been associated with resistance to chemotherapeutic agents both in prostate and in uterine cancer. Increased expression of both MDH2 and MDH1 was reported also in lung carcinomas. However, only high expression of MDH1 was associated with poor prognosis. In lung carcinomas and breast cancer, MDH1 displays a higher activity with respect to MDH2. This is aimed at supporting the increased glycolytic rate by NAD^+ regeneration, alternatively from the activity of the lactate dehydrogenase, the main enzyme involved in such process in glycolysis-addicted cancers.

Conclusions and Prospective Vision

The TCA cycle is a central metabolic hub funneling and redistributing metabolites with the ultimate aim of creating the electrochemical gradient necessary for ATP production in a normoxic environment. Beyond this classical standpoint, the TCA cycle has been demonstrated to be also engaged in hypoxic conditions. In this context, TCA cycle reactions produce reducing equivalents that can contribute to redox signaling instead of being used by respiratory complexes. Moreover, the generation of high-energy phosphates through substrate-level phosphorylation as well as of intermediates for macromolecule biosynthesis continues to be a prerogative of the TCA cycle. These features are largely managed by cancer cells to satisfy the metabolic demand necessary for proliferation and survival both in normal and hypoxic conditions. As described in this article, the induction of pseudohypoxic response is

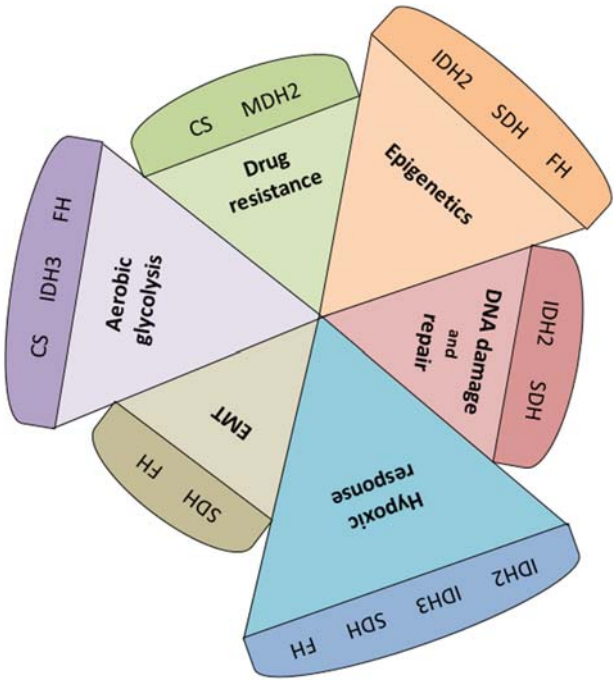

Fig. 2 *Effects of TCA cycle aberrations in cancer.* Each slide refers to the main events coming from the alterations of the enzymes of the TCA cycle in cancer. The different dimension of slides refers to the number of the enzymes involved in the described process.

a common trait of altered activity of several TCA cycle enzymes together with other cancer-related events, including epigenetic deregulation, drug resistance or metastatic potential (Fig. 2). Although outcomes of TCA cycle aberrations are shared among several enzymes, the complexity and dynamicity of the cycle do not simplify the selection of suitable enzymatic targets for cancer treatment. In this regard, the scientific community is now committed to the characterization and validation of metabolic antagonists to be employed in cancer therapeutics. Several approaches impinging on TCA cycle and its accessory pathways have been tested. An example is the use of glutaminolysis inhibitors in glutamine-addicted tumors. Interestingly, selective targetability of mutant metabolic enzymes has been recognized as a useful strategy for treatment of cancer patients. Promising results are being obtained in clinical trials for small molecule inhibitors targeting IDH1/IDH2 mutants in advanced solid tumors and hematological malignancy. In addition to the development of new efficient and safe molecules, future efforts should be addressed to the profiling of metabolic adaptations of each tumor in order to design personalized therapeutic plans based on metabolic inhibitors.

See also: Lipid Metabolism. Systems Biology Approach to Study Cancer Metabolism.

Further Reading

Anderson, N.M., Mucka, P., Kern, J.G., Feng, H., 2017. The emerging role and targetability of the TCA cycle in cancer metabolism. Protein & Cell. https://doi.org/10.1007/s13238-017-0451-1.

Bardella, C., Pollard, P.J., Tomlinson, I., 2011. SDH mutations in cancer. Biochimica et Biophysica Acta 1807, 1432–1443.

Casey, S.C., Amedei, A., Aquilano, K., Azmi, A.S., Benencia, F., Bhakta, D., Bilsland, A.E., Boosani, C.S., Chen, S., Ciriolo, M.R., Crawford, S., Fujii, H., Georgakilas, A.G., Guha, G., Halicka, D., Helferich, W.G., Heneberg, P., Honoki, K., Keith, W.N., Kerkar, S.P., Mohammed, S.I., Niccolai, E., Nowsheen, S., Vasantha Rupasinghe, H.P., Samadi, A., Singh, N., Talib, W.H., Venkateswaran, V., Whelan, R.L., Yang, X., Felsher, D.W., 2015. Cancer prevention and therapy through the modulation of the tumor microenvironment. Seminars in Cancer Biology 35, S199–S223.

Ciccarone, F., Vegliante, R., Di Leo, L., Ciriolo, M.R., 2017. The TCA cycle as a bridge between oncometabolism and DNA transactions in cancer. Seminars in Cancer Biology 47, 50–56.

Dang, L., Yen, K., Attar, E.C., 2016. IDH mutations in cancer and progress toward development of targeted therapeutics. Annals of Oncology 27, 599–608.

Lehtonen, H.J., Kiuru, M., Ylisaukko-Oja, S.K., Salovaara, R., Herva, R., Koivisto, P.A., Vierimaa, O., Aittomaki, K., Pukkala, E., Launonen, V., et al., 2006. Increased risk of cancer in patients with fumarate hydratase germline mutation. Journal of Medical Genetics 43, 523–526.

Pasini, B., Stratakis, C.A., 2009. SDH mutations in tumorigenesis and inherited endocrine tumors: Lesson from the phaeochromocytoma-paraganglioma syndromes. Journal of Internal Medicine 266, 19–42.

Sullivan, L.B., Gui, D.Y., Heiden, M.G.V., 2016. Altered metabolite levels in cancer: Implications for tumor biology and cancer therapy. Nature Reviews. Cancer 16, 680–693. https://doi.org/10.1016/j.semcancer.2015.02.007. Epub 2015 Apr 10. Review. PMID: 25865775 Free PMC Article.

Telomeres, Telomerase, and Cancer

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Glossary

Aneuploidy Abnormal and uneven number of chromosomes in a cell.
G-quadruplex Secondary structure made of four repeats that contains three guanines, forming three tandem G-tetrads.
Processivity Ability of polymerases to catalyze continuously on the substrate.
Senescence Cell status that is unable to re-enter cell division cycle.
Telomere crisis Activation of DNA damage response pathways caused by telomere shortening and loss of telomere protection.
Telomere length homeostasis Telomere maintenance achieved via stable equilibrium between telomere attrition and telomere addition by telomerase activity.
Telomeropathy Diseases associated with telomere shortening.

Nomenclature

ALT Alternative lengthening of telomeres
APB ALT-associated PML body
BFB Breakage-fusion-bridge
CTE C-terminal extension
DCs Dendritic Cells
DDR DNA-damage response
d-loop Displacement loop
DSBs Double-stranded breaks
FISH Fluorescence in situ hybridization
HDR Homology-directed repair
HR Homologous recombination
MMqPCR Monochrome Multiplex quantitative polymerase chain reaction
NHEJ Non-homologous end-joining
PARP Poly(ADP-ribose) polymerase
PML Promyelocytic leukemia
PNA peptide nuclei acid
ROS reactive oxygen species
RT Reverse-transcriptase
scaRNA Small Cajal body-specific RNA
STELA Single telomere length analysis
STORM Stochastic optical reconstruction microscopy
TEN Telomerase N-terminal
t-loop Telomere loop
TRAP Telomeric repeat amplification protocol
TRBD Telomerase RNA binding domain
TRF Telomere restriction fragment
T-SCE Telomere-sister chromatid exchange
t-stump Telomere stump
TWJ Three-way junction

Biology of Telomeres and Telomerase

Telomere maintenance is a hallmark of cancer and an essential factor for tumor propagation and immortality. This chapter will discuss how telomeres and telomerase function in cancer cells and the importance of telomeres as a potential target for cancer

therapies and a tool for cancer diagnostics. To understand the biology of telomeres and telomerase in cancer, it is first important to understand how they function in healthy cells. In this section, we introduce the concepts of chromosome replication and end protection, and summarize the structure and functions of telomeres and telomerase in humans.

The History of Telomere Discovery

In 1891, August Weismann suggested that: "Death takes place because worn out tissue cannot forever renew itself and because a capacity for increase by means of cell division is not everlasting but finite". This proved to be a remarkably prescient observation, made at a time before the concept of cellular aging existed. A number of milestone discoveries gave credence to this idea, beginning with cytogenetic studies in the 1930s. At this time, Hermann Muller began to reveal that the ends of linear chromosomes harbored special properties that distinguished them from DNA double-stranded breaks (DSBs). Using fruit flies, he examined how chromosomes responded to breakage induced by X-rays. He observed that, where DSBs were prone to rearrangement and fusions, the terminal ends of the chromosomes were exempt from these repair mechanisms. Although unsure of their significance, Muller termed these regions 'telomeres', from the Greek *telos* (end) and *meros* (component). At the same time, Barbara McClintock observed similar characteristic of chromosome ends in corn.

Moving into the 1960s, Leonard Hayflick described that normal somatic cells do not have an indefinite proliferative capacity. Rather, they divide approximately 50 times before undergoing a growth arrest, termed *replicative senescence*. In this state, cells are alive and can be maintained in culture yet remain irreversibly unable to divide. This is termed the 'Hayflick limit'; the number of times a cell can divide before undergoing senescence. Although the purpose of this growth limit was unknown at the time, it certainly echoed the idea of Weismann some 70 years earlier.

By the late 1970s, work was underway to sequence telomeric DNA, as Muller had initially predicted that a gene necessary for survival was located there, exempting telomeres from the repair mechanisms he had observed. Working in the ciliate *Tetrahymena thermophila*, Elizabeth Blackburn made use of their palindromic 'minichromosomes' which are relatively short and thus simpler to sequence. They discovered that they terminated in guanine-rich repeats with a 3′ end single-stranded overhang. Jack Szostak showed that *Tetrahymena* guanine-rich (G-rich) terminal sequence has an ability to protect linear DNA in yeast, proving Barbara and Muller's hypothesis. At a similar time, Alexey Olovnikov realized that chromosome ends had an issue when dividing; the end replication problem (**Fig. 1**). He described how a dead zone must exist at the ends of our DNA which cannot be replicated. He likened DNA to a train track with a train advancing, akin to the action of DNA polymerase. He suggested the enzyme must be unable to replicate the DNA beneath it at the very ends of chromosomes, analogous to the inability of a train to progress along the end of a track.

DNA is extended only in the 5′ to 3′ direction and thus cannot be copied where primers are laid down on the parental G-rich strand. Furthermore, both strands are processed to produce a sufficient length of the 3′ overhang to serve a structural purpose in end protection at chromosome ends (t-loop and shelterin formation, discussed below). Hence, each round of cell division results in the loss of 50–200 base pair from the ends of chromosomes (**Fig. 1**). Thus, rather than a crucial gene being situated at the chromosome ends, the repetitive telomeric sequence serves as an expendable source of DNA during replication. Consequently, telomeres get shorter with age. The Hayflick limit is therefore the number of divisions before telomeres become critically short. Hence, replicative senescence occurs to prevent the degradation of chromosomal DNA and telomeres act as a 'mitotic clock', defining the proliferative potential of a cell.

Telomeres must be replenished in certain cells, such as unicellular eukaryotes and stem and germ cells in plants and animals, in order to maintain cell division. Telomerase is a ribonucleoprotein enzyme capable of counteracting telomere shortening and was first identified by Elizabeth Blackburn and Carol Greider. Telomerase synthesizes the telomeric G-rich strand using its own RNA template. A sensitive telomerase detection assay, celled telomeric repeat amplification protocol (TRAP), was developed to determine the level of telomerase activity in different cell types. Using this method, the group of Jerry Shay and Wood Wright showed that telomerase activity was very low in most human cells. They screened a number of cancer cell types and found 85–90% of cancers expresses telomerase to maintain their telomeres. Hence, tumors are able to evade replicative senescence in order to grow indefinitely; a fundamental feature of cancer.

Telomere Structure and Chromosome End Protection

T-loops

Human telomeres are composed of the double-stranded 'TTAGGG' DNA repeats that span 5–10 kilobases (kb) in humans, and terminate at the 3′ end in a single-stranded G-rich overhang, or G-tail, which is approximately 130–210 bases in length. Telomeric DNA is folded and structured in such a way as to ensure chromosomes are fully protected and not inappropriately recognized as a broken fragment of DNA (**Fig. 2**A). The 3′ tail coils back on itself to form a 'telomeric loop', or *t-loop*. The end reaches the telomeric double-stranded DNA (dsDNA), imbedding itself to form a 'displacement loop' (or *d-loop*). This lariat-like structure effectively seals off the chromosome end in a protective manner and explains the differences Muller observed earlier, between DSBs in the DNA and the terminal ends. The t-loop configuration formed was first revealed in vitro and its existence later proved in cells using stochastic optical reconstruction microscopy (STORM).

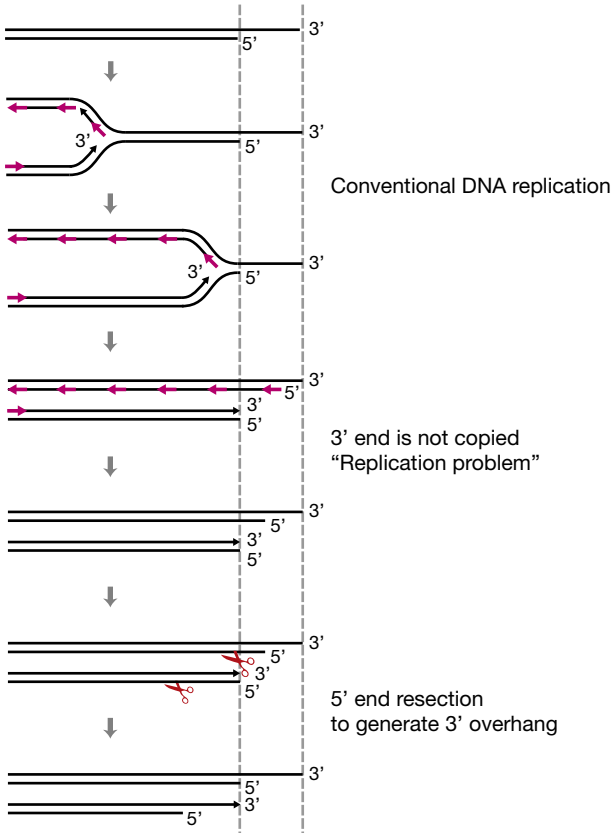

Fig. 1 Telomere replication and the end replication problem. Schematic representation of semi-conservative chromosome end replication. The DNA strands containing 3′ and 5′ ends are replicated via lagging strand synthesis and leading strand synthesis, respectively. Magenta represents RNA primer; black allow represents DNA polymerization. The leading strand is fully copied but the lagging strand is unable to replicate the 3′ end region (classical replication problem or lagging strand problem). Since the leading strand end is blunt, the 5′ end is digested to generate the 3′ end (resection). Both leading strand and lagging strand ends are subjected to resection to produce a sufficient length of overhang in mammalian genomes. The resulting sister chromatids are shorter than the mother strands (leading strand problem).

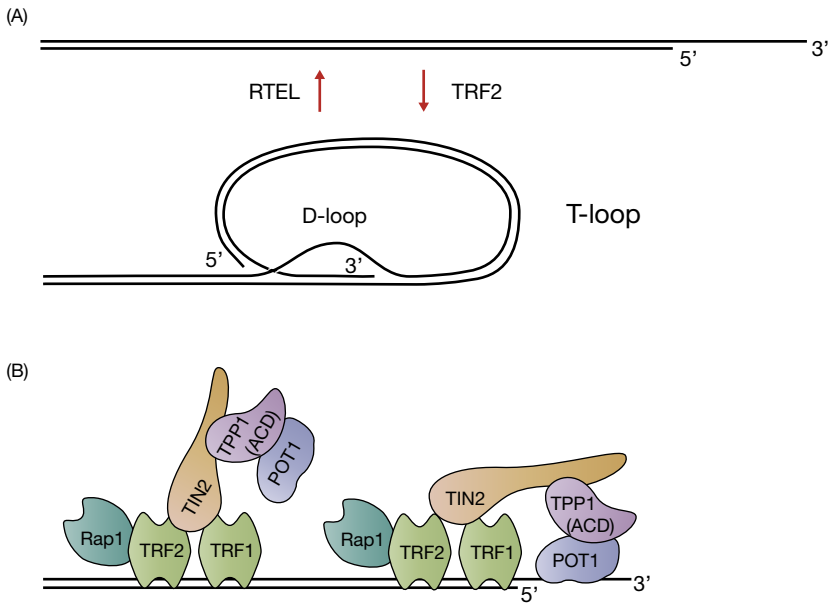

Fig. 2 Telomere structure. (A) Formation of t-loop. The telomeric dsDNA binding protein TRF2 promotes d-loop formation to form a lariat structure, termed telomere-loop or t-loop. The DNA helicase RTEL binds to TRF2 and dissolves the d-loop to expose the 3′ end overhang. (B) Telomeric proteins and shelterin formation. TRF1 and TRF2 bind to dsDNA and recruit TIN2. POT1 and TPP1 form a complex and are recruited to the telomere via TIN2 interaction. POT1 binds to telomeric ssDNA if available. Rap1 binds to TRF2.

The shelterin complex

The shelterin complex is made up of six family members that bind along the length of the telomere: TRF1 (*TERF1*), TRF2 (*TERF2*), RAP1 (*TERF2IP*), TIN2 (*TINF2*), TPP1 (*ACD*) and POT1 (*POT1*) (Fig. 2B). TRF1 and TRF2 bind to the telomeric dsDNA. They interact with TIN2, which recruits TPP1 and POT1. POT1 associates with the telomeric single-stranded DNA (ssDNA) at the 3′ overhang as well as at the d-loop within the t-loop structure. TRF2 controls the topology of telomeric DNA to aid t-loop formation. RAP1 binds TRF2 to support its function. Whereas TRF1 counteracts telomerase activity, the POT1-TPP1-TIN2 complex is essential for telomerase recruitment and activation. Thus, each shelterin protein has distinct roles in telomere maintenance.

If telomeres fail to interact with the shelterin complex and form t-loops, chromosome ends risk being recognized as damaged DNA ends. Interaction with shelterin serves to inhibit DNA damage response pathways to ensure senescent signaling pathways or apoptosis are not triggered. Furthermore, interaction with shelterin also facilitates DNA replication and prevents aberrant recombination events, which leads to genetic instability and aneuploidy, a key step in the development of cancer.

Regulation of DNA-Damage Response (DDR) Factors

Telomeric DNA is a repetitive sequence located at every end of chromosomes. Hence, DNA damage sensor proteins could potentially recognize telomere ends as DSBs. Activation of such DSB repair pathways must be avoided as it leads to chromosome end-to-end fusions or entanglements that cause chromosome segregation defects and catastrophic cell death during mitosis. The shelterin complex interacts with many DDR factors and serves to control the activation of DNA damage checkpoints as well as to suppress two major DNA DSB repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Fig. 3).

TRF2 directly binds to the kinase ATM (ataxia telangiectasia mutated) and a DNA repair complex called MRN (Mre11, Rad50 and Nbs1). By doing so, ATM and MRN are unable to activate p53 and H2AX (to generate γH2AX), which induce cell cycle arrest and trigger the DNA DSB repair pathways. TRF2 also binds to the Ku heterodimer complex, required for the canonical NHEJ (c-NHEJ) pathway. Interaction of TRF2 with Ku prevents downstream activation of ligase IV, crucial for the c-NHEJ pathway. TRF2-Ku also prevents activation of HR between sister chromatids (telomere sister chromatid exchange, T-SCE; described in the section: Alternative lengthening of telomeres/ALT). The shelterin protein RAP1 also plays a role in these inhibition activities. Shelterin formation itself and Ku redundantly suppress the alternative NHEJ pathway that utilizes micro-homology mediated DNA end-joining.

Like TRF2, the shelterin protein POT1 also has a role in repressing DNA damage response signaling. POT1 binding to telomeric ssDNA blocks the association of replication protein A (RPA), which recruits another DNA damage checkpoint kinase, ATR (ataxia telangiectasia and Rad3-related). The ssDNA-bound RPA can be replaced by RAD51, which plays a central role in HR. Therefore, POT1 inhibits the initiation of homology-directed repair (HDR). Importantly, recruitment of POT1 to the telomere requires the TRF2 analogue TRF1 and other shelterin components TIN2 and TPP1, highlighting the importance of the shelterin complex as a whole.

TRF1 binding to telomeric dsDNA is required for telomere replication. Lagging strand synthesis of telomeric dsDNA temporally exposes G-rich ssDNA, which can form a secondary structure termed a G-quadruplex. TRF1-bound BLM helicase removes such secondary structures to aid telomere replication. The stalled strand at the replication fork stall also recruit RPA and ATR, and ATR phosphorylates and temporary releases TRF1 from the telomere. Dissociation of TRF1 promotes recruitment of telomerase.

While telomeres are able to suppress DSB repair pathways, DNA single-strand breaks (SSBs), caused by factors such as reactive oxygen species (ROS), are repaired via the base excision repair pathway (BER). TRF1 interacts with tankyrase 1, a telomere-associated poly(ADP-ribose) polymerase (PARP). Enzymes of the PARP family covalently add polymers of ADP-ribose to modify

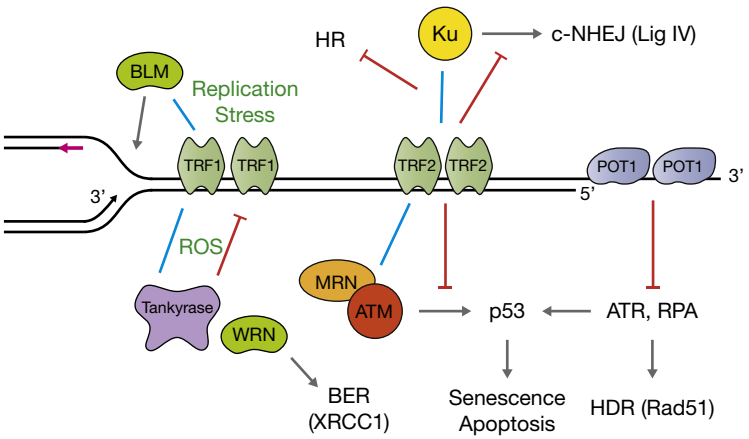

Fig. 3 Telomere protection. Schematic diagram of DDR control by telomeric proteins POT1, TRF2 and TRF1. POT1 binds to ssDNA and inhibits RPA and ATR, which activates p53 and Rad51 dependent HDR. TRF2 binds Ku to suppress HR and c-NHEJ. TRF2 also binds to MRN and ATM, which activate p53 and HR. TRF1 binding facilitates telomere replication by interacting with BLM. TRF1 also binds to tankyrase to efficiently repair telomeres damaged by ROS. PARylated TRF1 recruits WRN and BER factors including XRCC1.

proteins in a process called PARylation. Tankyrase 1 PARylates TRF1 and this stimulates the association of SSB repair proteins, including the Warner syndrome helicase WRN and XRCC1 to the telomere. PARylated TRF1 is then released from telomeric DNA and subjected to proteolytic degradation. Inhibition of tankyrase stabilizes TRF1, results in suppression of telomerase activity and telomere shortening. Mutation of WRN also accelerates shortening of telomeres. Thus, telomere protection mediated by TRF1 is crucial for telomere maintenance and protection from ROS.

When a telomere becomes critically short, it has fewer shelterin proteins bound along its length which may result in less efficient t-loop formation. In healthy cells, this situation can trigger the ATM/ATR pathways, leading to p53 activation and replicative senescence or apoptosis. This acts as a tumor suppressor mechanism as it prevents the cell from dividing further and leaves chromosome ends unprotected.

Telomerase Biogenesis and Telomere Addition

Telomerase assembly

The core component of telomerase is composed of a telomerase-specific RNA component, TERC, and a reverse transcriptase enzyme, TERT. Association of TERC and TERT alone is sufficient to extend telomeric DNA in vitro. However in vivo, telomerase requires several other components to be imported into the nucleus and recruited to the telomere to replenish telomeric repeats. TERC encodes a template sequence for telomeric DNA reverse transcription and recruits several RNA binding proteins to function in vivo.

In humans, TERC is 451 nucleotides in length, although this varies greatly between species. For example, the telomerase RNA molecule in fission yeast, TER1, is 1413 nucleotides long. TERC belongs to the family of small Cajal body-specific RNAs, termed scaRNAs. Although TERC is transcribed by RNA polymerase II, the 3′ poly-adenosine tail is enzymatically removed to produce a 'mature' molecule. Mature TERC has several domains, crucial for its stability and function (Fig. 4A). The 3′ end stem loop (the CR7-domain) contains a small motif called a 'Cajal body (CAB) box', which allows it to be recruited to small organelles called Cajal bodies. There is also an 'H/ACA box' within the scaRNA domain, which binds proteins to stabilize the RNA. The CR2/3 domain, also

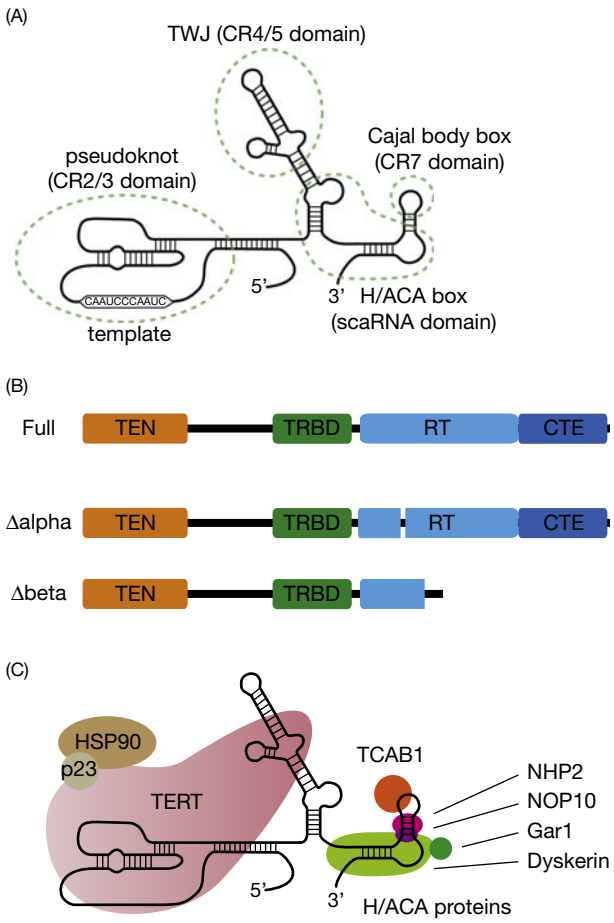

Fig. 4 Telomerase structure. (A) TERC contains 4 structural domains. CR = conserved region. (B) Schematic of the TERT protein with 4 domains and its major splice isoforms Δalpha and Δbeta. (C) Telomerase holoenzyme formation.

termed the *pseudoknot* domain, contains the telomeric C-strand template sequence 5′-CAAUCCCAAUC-3. TERT associates with TERC via the pseudoknot and the 'three-way junction (TWJ) domain' (or CR4/5). This assembly is required for its catalytic activity.

The telomerase catalytic subunit, TERT, contains four largely conserved domains; the N-terminal domain (TEN), the telomerase RNA binding domain (TRBD), the reverse-transcriptase domain (RT) and the C-terminal extension domain (CTE) (Fig. 4B). The RT and CTE domains together hold template RNA and substrate DNA hybrid duplex and are enzymatically essential for the reverse-transcription. The TRBD domain is required for stable association with TERC. The TEN domain is dispensable for enzymatic activity, but is required for telomerase recruitment and its processivity. Further, TERT associates with the chaperon proteins HSP90 and p23 at the TEN domain for its stability and telomerase activity in vivo. The TEN domain also contains a nuclear localization signal sequence and the mitochondrial targeting signal sequence, making it crucial for the correct localization of telomerase in the cell.

In order for the active telomerase complex to form, TERC must join together with TERT (Fig. 4C). For this to happen, mature TERC associates with four so-called 'H/ACA proteins' comprised of dyskerin, NOP10, NHP2 and NAF1, and traffics to the nucleolus. Here, TERC replaces NAF1 with GAR1 to become a stable and 'mature' form. TERT associates with p23 and HSP90 molecular chaperons and traffics to the nucleus where is assembles with TERC, forming the complete telomerase RNP. A mammalian conserved protein TEP1 associates with telomerase to aid telomerase activity. The pontin and reptin ATPases bind to both TERT and dyskerin during S-phase in order to aid the assembly of telomeres. However, they are excluded from the complex when telomerase becomes active. Finally, TCAB1 interacts with the TERC CAB-box motif when TERC has complexed with TERT. This facilitates its trafficking to the Cajal bodies where active telomerase can be stored.

Telomerase recruitment and processivity

Telomerase activity is controlled at multiple levels (Fig. 5). Telomerase localization to the telomere and telomere extension are observed only during S-phase. Outside of S-phase, chromosome ends are hidden from telomerase access and telomerase components are also modified or destroyed in a cell cycle-dependent manner. A helicase enzyme, RTEL1 (Regulator of Telomere Length 1), interacts with TRF2 only in S-phase. This recruitment specifically unfolds t-loop structures and exposes the telomeric 3′ end that serves as a telomerase substrate. During this time, Cajal bodies localize to the telomeres, facilitating the recruitment of mature telomerase where it can act.

Telomerase does not directly bind to telomeric DNA in vivo, instead binding to the shelterin protein TPP1 in order to dock at the chromosome end. TIN2 also mediates this recruitment of telomerase. Further association of TIN2-TPP1 with TRF1/2 or Pot1 is required for their telomere localization. Recruitment of telomerase is telomere-length dependent and is stimulated by local activation of ATM or ATR, which destabilize TRF1; telomerase preferentially extends shorter telomeres thereby maintaining an appropriate mean length.

Human telomerase is processive. This means that during telomere extension, TERT copies the template provided by TERC and, before dissociating from the telomere, repositions towards the 3′ end and proceeds to add additional repeats. This is a more efficient means of extending telomeres, and the rate at which it occurs is termed *processivity*. The telomeric ssDNA-bound TPP1 and POT1 complex aids this processivity by assisting in its repositioning to improve the efficiency of telomere elongation.

Telomerase activity must also be terminated. This is achieved by the CST complex (formed of CTC1, STN1 and TEN1). The CST complex associates with the polymerase α-primase complex to aid the restarting of the fork-stalled replisome as well as G-rich lagging-strand synthesis. CST interacts with POT1-TPP1 in late S/G2-phase, and is thought to replace telomerase and initiate lagging strand synthesis of the newly synthesized G-rich strand. This prevents telomerase from being recruited to the telomere and ensures an appropriate level of extension occurs.

Function of Telomeric Proteins and Telomerase outside the Telomere

Telomere function is not limited to chromosome ends (Fig. 6). Telomeric proteins locally establish a heterochromatic state. This means that the internal telomeric repeats are able to repress or modulate transcription. The shelterin protein RAP1 (repressor/activator protein 1) was first identified in budding yeast where it is known as a transcriptional regulator. Budding yeast Rap1 directly binds the telomeric DNA sequence and controls gene expression. Similarly, human telomeric proteins TRF2 and RAP1 appear to be involved in transcriptional regulation via their binding to internal telomeric repeats or interstitial telomeric sequence. TRF2 regulates neuronal gene expression during development and RAP1 controls transcription of metabolic genes in some tissues including the liver. In the cytoplasm, mammalian RAP1 is also an activator of NFκB signaling, by binding to IKKs activator kinases. NFκB signaling is responsive to stimuli including infections, cytokines and ROS, and has a crucial role in cancer development. Further, TIN2 can localize to mitochondria when it is not complexed with TPP1. Here it represses oxidative phosphorylation for ATP synthesis to reduce levels of ROS. Thus, telomeric DNA can store these signaling regulators, and their shortening may result in aberrant changes in metabolism, signaling and transcriptional regulation.

The function of telomerase in telomere lengthening is limited to a short time frame of the cell cycle. Away from telomeres, telomerase is also involved in regulation of RNA transcription. During development, the Wnt pathway activates the β-catenin coactivator to initiate transcription of Wnt response genes. TERT is predominantly expressed during embryonic development. Interestingly, TERT can co-localize with β-catenin and BRG1, a SWI/SNF-related chromatin remodeling factor, on promoters to modulate transcription. Similarly, TERT also promotes transcription of the NFκB signaling target genes by interacting with RelA, a p65 subunit of the NFκB complex. The significance of the ability of TERT to contribute towards transcription still needs to be validated.

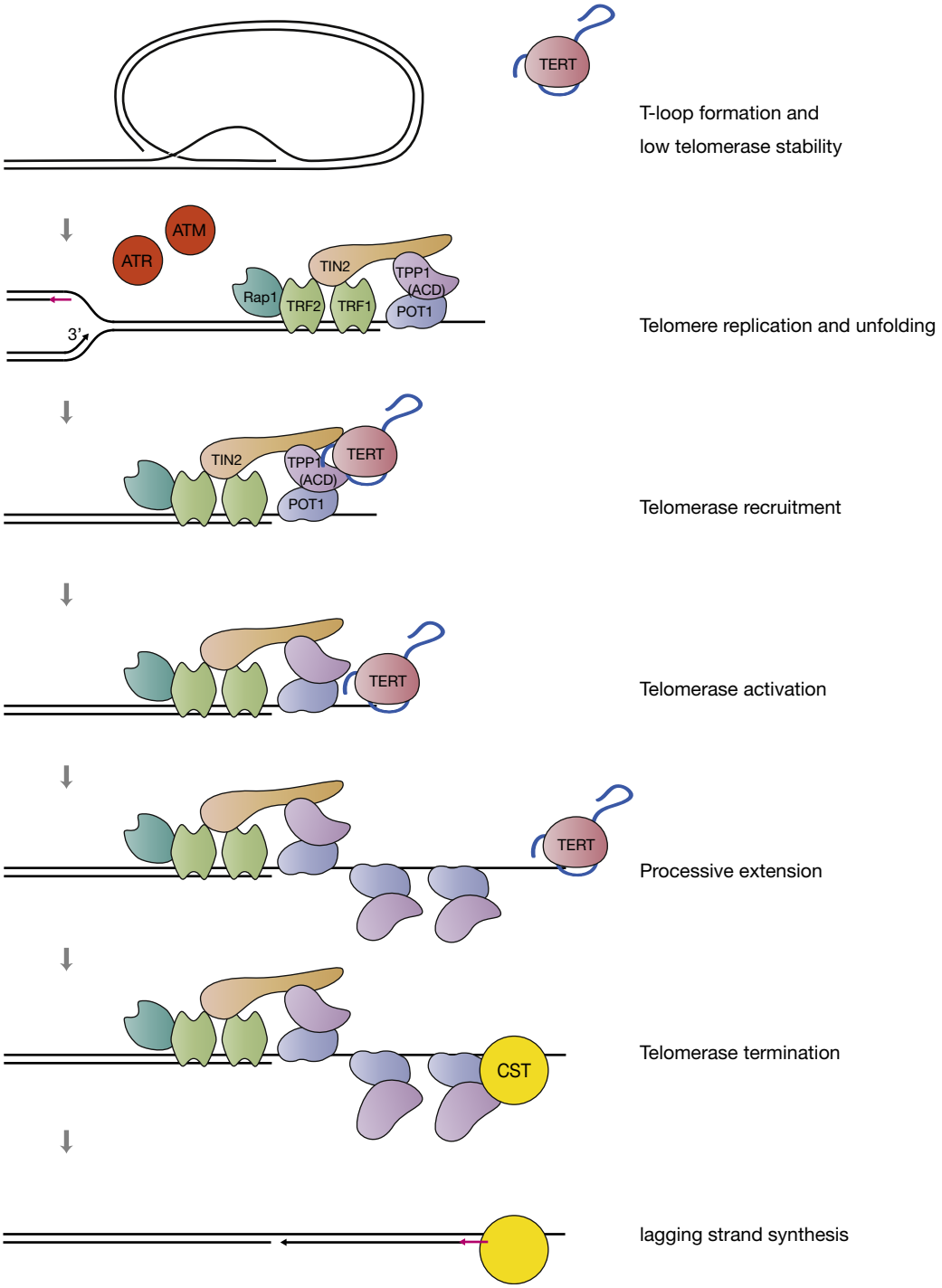

Fig. 5 Telomerase action. Schematic representation of telomerase action. Outside S-phase, telomeres are not extendible, presumably by t-loop formation. Telomeric 3′ ends are exposed during S-phase and bound by Pot1. Local and transient activation of the DDR checkpoint proteins, ATR and ATM, mark short telomeres that promote telomerase recruitment. TERT binds to TPP1 and is translocated to the tip of the telomere ssDNA via annealing of the RNA template region within TERC. Processive telomere G-strand synthesis is supported by the POT1-TPP1 complex. At later stages of S-phase, the CST complex is recruited to POT-TPP1 and terminates telomerase activity. CST recruits the polymerase α-primase complex to complete telomere extension.

TERT also travels to mitochondria. Upon oxidative stress, TERT is excluded from the nucleus and re-localizes to mitochondria. Mitochondrial TERT is involved in reducing ROS and the integrity of mitochondria. Here, TERT complexes but mitochondrial tRNA, and localizes to the mitochondrial matrix and binds to mitochondrial DNA. The functional significance of these activities at the mitochondria remains to be establish. Another intriguing function is the silencing of mitochondrial gene expression. TERT also

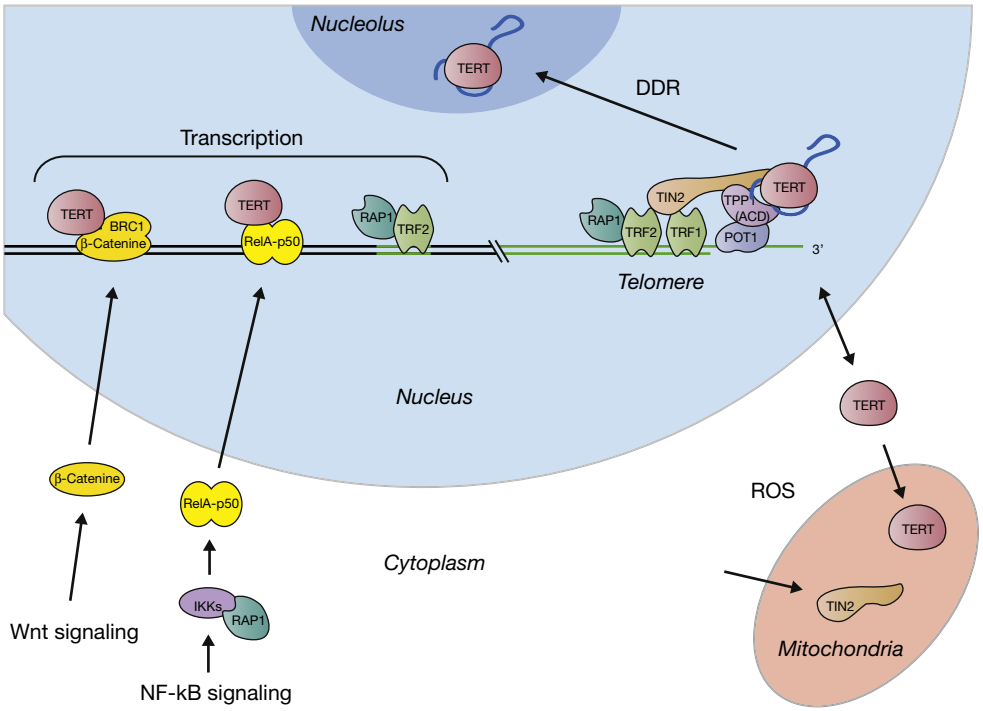

Fig. 6 Non-canonical functions of telomeric proteins and telomerase. Schematic diagram showing trafficking and non-canonical functions of telomeric proteins and telomerase. TERT is shuttled between the nucleus and the cytoplasm via its post-translational modifications. Upon DDR, telomerase accumulates in the nucleolus. TERT and TIN2 are trafficked to the mitochondria under ROS stimuli. Cytoplasmic RAP1 binds to IKKs to promote NFκB signaling. TERT associates with the NFκB (RelA and p50) and the β-Catenin complexes to facilitate gene expression. TRF2 and RAP1 localize to internal telomeric sequence loci and control local transcription.

complexes with the non-coding RNA subunit of mitochondrial RNA processing endoribonuclease, and functions as a RNA-dependent RNA polymerase, generating dsRNA from mitochondrial mRNA resulting in their degradation through an RNA interference pathway. All of TERT's functions in the mitochondria require its enzymatic activity but they are independent of TERC. These extra-nuclear activities are also observed in cancer cells.

Telomere Maintenance in Cancer

In this section, we summarize our current knowledge of telomere biology in cancer and how telomeres and telomerase can contribute to tumorigenesis and its evolution. Telomere maintenance is essential for cancer cells in order to sustain proliferation. However, paradoxically telomerase-expressing cancer cells often maintain very short telomeres. This can cause end-protection defects that activate DDR pathways, promoting chromosomal rearrangements and genetic instability, aiding cancer progression. The ability of telomeric proteins and telomerase to function as transcriptional modulators can also potentially contribute to tumorigenesis.

Maintenance of Short Telomeres in Cancer

Despite that fact that cancer cells express telomerase, many cancer cells, including glioma, colorectal, lung, breast and prostate cancers, maintain their telomeres shorter than normal healthy somatic cells (see the section: telomere length and cancer risk). The molecular mechanisms behind this phenomenon have not been fully elucidated. In normal cells, telomere length homeostasis is established by the balance between the degree of telomere erosion and the level of active telomerase expressed. Adult stem cells and progenitor cells can express TERT, but the level is insufficient to fully extend telomeres, so their telomeres still gradually shorten (Fig. 7). Only spermatocytes express high levels of telomerase and therefore maintain long telomeres. Hence, the regulation of telomerase in cancer, resulting in stably maintained short telomeres, must be different from telomerase regulation in normal cells.

Telomerase action in cancer

In cancer cells, telomerase is still carefully regulated and is only active during S-phase. Curiously, during S-phase, just 5–7% of telomeres associate with telomeres at any one time, however all telomeres get extended during cell division. Analysis of synchronized cancer cells suggested that telomerase can extend telomeres from the beginning of the S-phase, independently of the replisome and

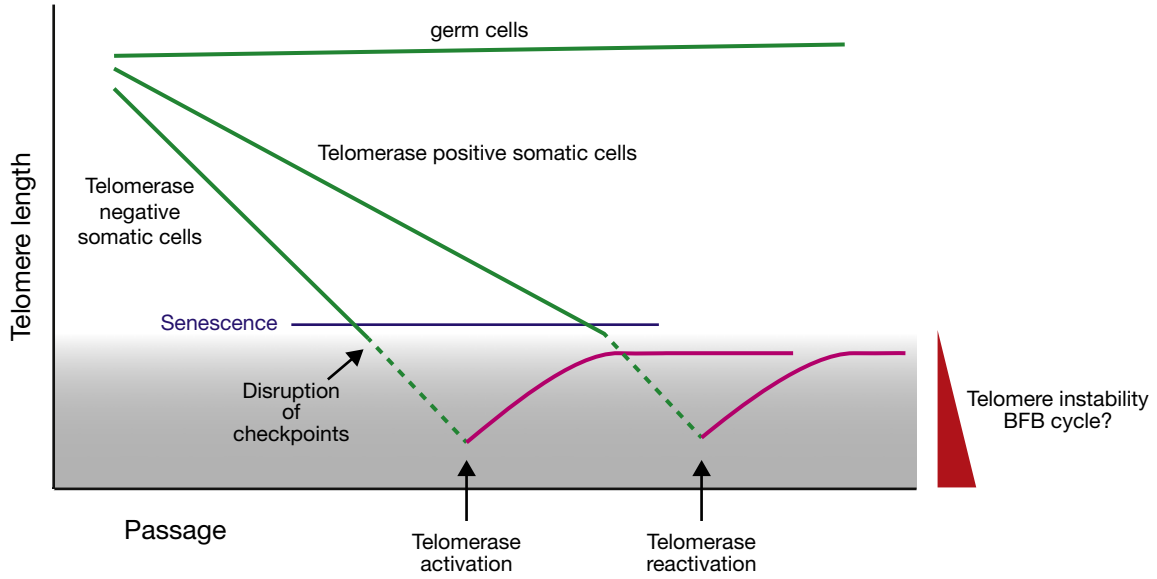

Fig. 7 Telomere shortening and crisis. Graph representing cell division-associated telomere loss and eventual telomere crisis and immortalisation. Y- and X-axis indicate length of telomeres and passage of cells, respectively. Telomeres are progressively shortened through generations until cells activate senescence checkpoint (*green line*). TERT positive cells display slower telomere shortening but eventually reach replicative senescence. Only germ cells maintain telomere length homeostasis. Cells that escape this cell cycle arrest system further lose telomere repeats (*green dotted line*). Shortened telomeres elicit DDR pathways, leading to BFB cycling (telomere crisis: *gray area*). Cells that gain the ability to maintain chromosome ends (by activation of telomerase) dominate and establish their lineage. Survivors retain short telomeres.

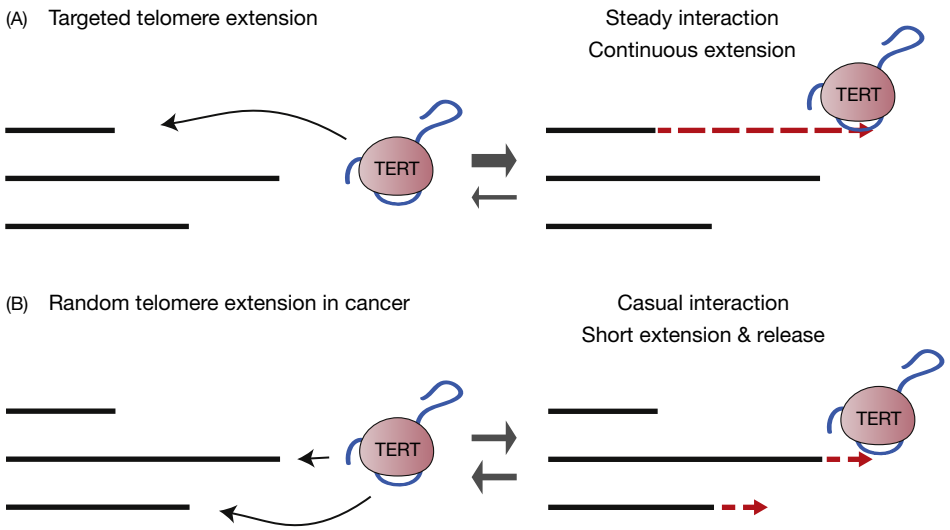

Fig. 8 Telomerase action in cancer. Schematic models depicting two modes of telomere length homeostasis in (A) normal and (B) cancer cells. (A) Telomerase is recruited to the shortest telomere and it is progressively extended until it is elongated. (B) Telomerase probes every telomere and randomly extend. Telomerase activity seems transient at the telomere. Images are adapted from Armstrong, C. A. and Tomita, K. (2017). Fundamental mechanisms of telomerase action in yeasts and mammals: understanding telomeres and telomerase in cancer cells. *Open Biol 7*.

the lagging strand synthesis complex. However, the number of telomeric repeats added is minimal, resulting in short telomere extension (Fig. 8). This is different from canonical telomerase activity, in which telomerase typically targets chromosomes with the shortest telomere. Nevertheless, telomerase appeared to repeatedly probe every telomere, thus achieving overall extension.

Some chromosomes in cancer cells have very short telomeres termed *t-stumps*, that are rarely found in normal cells. It is common in cancer to lose cell-cycle checkpoint proteins and this likely contributes to the failure of t-stumps to trigger replicative senescence. T-stumps appear to be capable of binding shelterin proteins and, to a certain degree, protecting the chromosome ends, allowing the cell to continue dividing. However, retention of critically short telomeres in cancer cells suggests that the ability of telomerase to selectively elongate only the shortest telomeres, is disrupted.

Telomere instability and the chromosome breakage-fusion-bridge cycle

Retention of t-stumps is likely beneficial for tumor growth due to the associated genetic instability driving cancer development. Failure to extend t-stumps leads to loss of protection of chromosome ends, a state termed 'telomere crisis'. This elicits activation of DDR pathways in an attempt to protect genomic integrity (Fig. 9). The cell cycle checkpoint is activated with remaining shelterin complex helping prevent chromosome fusions. However, chromosome end-to-end fusion (between sister chromatids or different chromosomes) can still occur, and the resulting fused dicentric chromosomes cause chromosome segregation defects. Breakage-fusion-bridge (BFB) cycles are initiated, whereby dicentric chromosomes break and undergo subsequent fusion events in a daughter cell. This can have several outcomes, including non-reciprocal translocations. This happens when a broken chromosome that has not undergone telomere repair, invades and copies part of another in a one-way transfer. Loss of heterozygosity can also occur when a dicentric chromosome breaks and lacks a large terminal region, potentially encompassing whole genes. Reactivated telomerase can seal broken chromosome ends and, when inherited by a daughter cell, lost chromosomal fragments result in gene deletion. This is a common occurrence in cancer and is especially damaging when tumor suppressor genes are lost. Thus, short telomeres in cancer cells provide sufficient chromosome stability for their propagation whilst generating diversity for selective pressure to allow further development of the tumor. Prolonged mitotic cell cycle arrest often triggers apoptosis or senescence, but can bypass the chromosome segregation phase and re-enter S-phase; this leads duplication of chromosomes.

Telomerase Expression and Carcinogenesis

The *TERT* gene is silenced in most cells, meaning that telomerase is not expressed and telomeres are programmed to shorten with age. Cancer progenitor cancer cells must gain the ability to maintain chromosome ends in order for them to propagate indefinitely. Although dependent on the type and subtype of cancer, most cancer cells express telomerase. Therefore, expression of TERT is a hallmark of cancer. However, it remains to be established how cancer precursor cells reactivate telomerase.

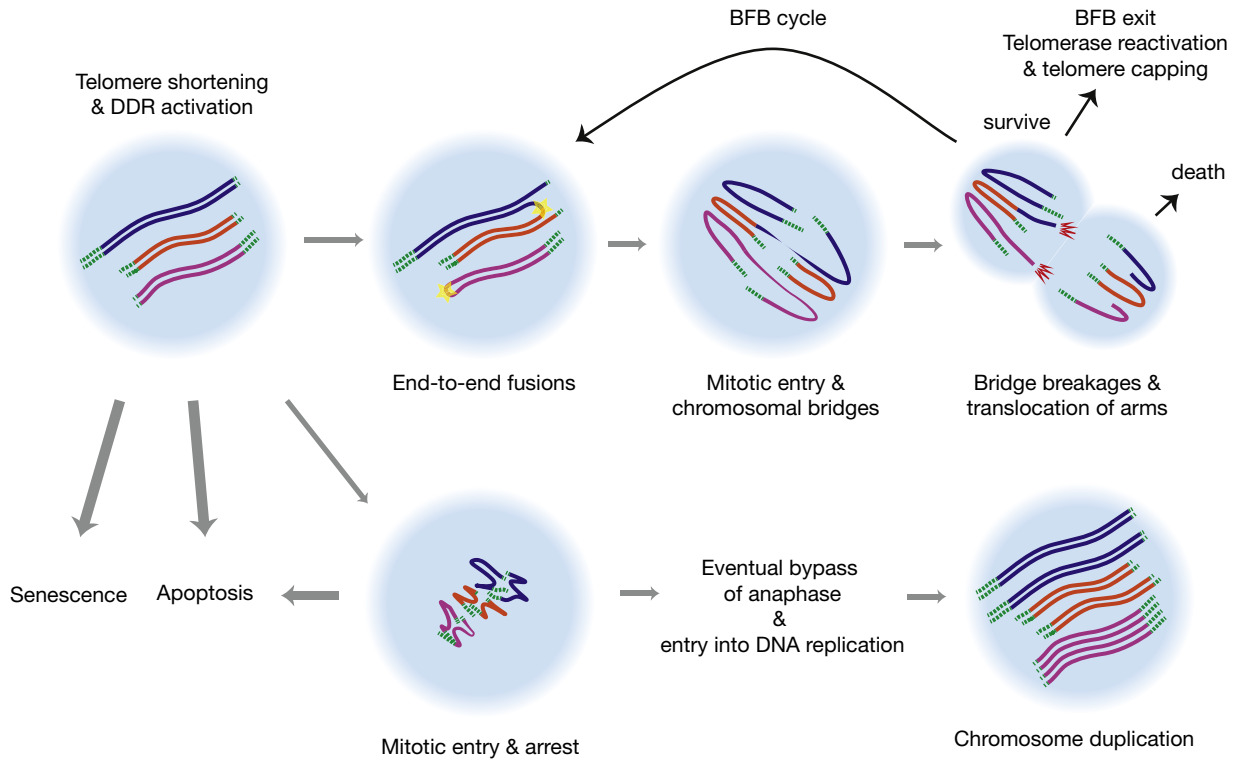

Fig. 9 Telomere-induced chromosome instability. Short telomeres activate persistent DDR and the senescence checkpoint to arrest cell cycle, leading to cellular senescence or apoptosis. Loss of telomere function allows chromosome end-to-end fusions. Chromosome segregation at mitosis can be stalled by catenation of two chromosomes during mitosis. Nucleases digest chromosome bridges to complete mitosis. This causes translocation of chromosomes and the generation of new telomere-free ends. A cell fuses the broken ends and moves to the next round of cell cycle. A cell that has lost a chromosome arm is likely to die. This, so-called "Breakage-fusion-bridge cycle" continues until the broken ends are capped by telomeres (by telomerase reactivation). Alternatively, cells that arrest in mitosis for extended periods can eventually evade the chromosome segregation phase. *Re*-entry of S-phase results in chromosome duplication.

Telomerase reactivation

The stage at which telomerase reactivation occurs in cancer remains unknown and is likely variable. One possibility is that it occurs at the time or following telomere crisis (Fig. 8). Early in tumorigenesis, the loss of tumor suppressor genes such as p53/p21 or the Retinoblastoma protein (Rb) allows the evasion of replicative senescence resulting in continued cell division. Telomeres become progressively shorter until the state of telomere crisis. Loss of telomere protection triggers genetic alterations (via constant BFB cycles). This results in the acquisition of spontaneous mutations and chromosomal rearrangements, including aneuploidy (another hallmark of cancer). As tumors develop, telomeres must be re-stabilized and recover from telomere crisis. Such selective pressure is believed to reactivate the *TERT* gene, permitting the continued growth of these aberrant, selected cancer cells. Hence, inactivation of the senescence checkpoints and consequent telomerase reactivation are crucial for cancer immortality. Most tumors require 4–6 critical mutations in order to become malignant. In the normal lifespan of a cell, this is unlikely to occur as pre-malignant cells undergo senescence before having the opportunity to develop into a cancerous tumor. Thus, the events of telomere crisis and telomerase reactivation result in the evasion of senescence and both allow the cell to continue dividing and enhance the rate of mutation.

TERT expression is tightly controlled through the action of transcriptional activation and repressor complexes. The *TERT* promoter is GC-rich and contains binding sites for various transcription factors, including two canonical *E*-boxes for the transcription factors, c-Myc and USF, and consensus sequences for ETS (E26-transcription-specific) family transcription factors. Importantly, NFκB and β-Catenin, which associate with TERT, are known to localize and promote transcription. c-Myc is also activated by β-Catenin. Hence, reactivated TERT protein may further enhance its transcription via NFκB and Wnt/β-Catenin pathways. Conversely, the tumor suppressor proteins p53 and p21 can negatively regulate *TERT* expression. Collectively, combination of transcriptional activator and repressor complexes appears to tightly control telomerase expression.

Cancer associated mutations within the TERT promoter

Analysis of the *TERT* promoter region in tumors from patients with different cancers has revealed some common mutations that correlate with *TERT* expression. The frequency of mutations within the promoter is considerably high in some cancer types. Common mutations are found upstream of the start codon at the −124 position and −146 positions, which in both cases represent cytosine to thymine changes (−124C/T and −146C/T respectively). These are seen in various cancers, including melanomas, glioblastomas, thyroid cancers, hepatocellular carcinomas and bladder and urothelial carcinomas. Mechanistically, both mutations generate additional ETS binding sequence. In addition, the -146A/C mutation also creates binding site for the NFκB complex. Another common mutation has been found at the −57 position, resulting in a change from adenine to cytosine (−57A/C). Site-directed mutagenesis studies using embryonic stem cells, whereby mutated stem cells were differentiated into fibroblast, suggested that the introduction of these mutations increased transcription 4–12 fold in differentiated mutant fibroblasts, compared to non-mutated fibroblasts. Among these mutations, −124C/T resulted in the highest transcription efficiency, where activity levels were comparable to those observed in cancer cells.

It is worth mentioning that mutations at the *TERT* promoter that enhance its expression have been observed at all stages in a number of cancers. These mutations can trigger the reactivation of telomerase expression and may do so at an earlier stage than telomere crisis; however, *TERT* expression alone is insufficient to maintain telomeres and does not promote carcinogenesis. Hence, additional oncogenic mutations that permit the evasion of cell cycle arrest/senescence pathways and accelerate the cell cycle must also occur in progenitor cancer cells during tumorigenesis.

TERT as an oncogene

Many scientists have attempted to create 'model' cancer cell lines from normal epithelial cells and fibroblasts. These stepwise cancer transformation studies have demonstrated that co-expression of three factors, an oncogenic mutant form of H-Ras (G12 V), the simian virus 40 Large T-antigen and TERT, is sufficient for malignant transformation of various differentiated cells. Induction of oncogenic H-Ras expression induces a replication defect at telomeric regions, leading to the induction of DDR pathways and cellular senescence (so called 'telomere dysfunction-induced senescence', TDIS). Large T-antigen in turn suppress p53 and RB activation. Enforced *TERT* expression then allows cells to evade senescence by facilitating telomere replication. Hence, TERT is able to support oncogenic H-Ras signaling to establish tumors.

Activation of the NFκB and Wnt/β-catenin pathways is known to promote tumor development. As TERT complexes with NFκB and β-catenin for transcription, contribution of TERT in tumorigenesis is anticipated. Moreover, TERT associates with the RNA polymerase III complex and promotes expression of tRNAs. This enhances translational capacity, enabling efficient cell division. Cancer cells are exposed to elevated levels of ROS and TERT function in mitochondria may help to mitigate this. Therefore, TERT functions not only in telomere maintenance but also may contribute to transcription and metabolisms that assists tumor development.

Cancer-Specific Isoforms of Telomerase

The aberrant regulation of telomere length homeostasis in cancer suggests that the regulation of telomerase activity differs somehow to that of healthy cells. Mounting evidence suggests that there is only a weak correlation between the level of *TERT* expression and telomerase activity in cancer cells, i.e. it is not simply a case of the amount of telomerase present in the cell.

This raises additional questions regarding mechanisms that might regulate TERT function. Two interesting areas of investigation involve studies on the expression of different TERT splice-isoforms and the existence and function of post-translational modification on TERT protein.

TERT is a large gene, spanning 42 kb and containing 15 introns. Alternative splicing of *TERT* mRNA can result in exon skipping, trimming of exons and insertion of intronic regions. While approximately 22 splice variants have been reported, many of these are degraded via mRNA surveillance mechanisms including the non-sense mediated decay pathway. However, although some splice variants may be translated, only full length TERT exhibits reverse transcriptase activity. Two alternative *TERT* isoforms are particularly well documented (Fig. 4B). One carries a 36 bp truncation in exon 6, resulting in the loss of 12 amino acids within the RT domain (termed the '△ alpha-isoform'). Another lacks exons 7 and 8, resulting in a frame-shift after exon 6 generating a stop codon leading to a short isoform containing only the TEN and the telomerase RNA binding domain (termed the '△ beta-isoform'). Over expression studies suggest that the dysfunctional △ alpha-isoform can compete with endogenous TERT and impair telomerase activity. Although the △ beta-isoform is recessive to endogenous telomerase activity, it retains the ability to bind TERC and localizes to the nucleus and the mitochondria. Over expression of the isoform in breast cancer confers resistance to the anti-cancer drug cisplatin, implying retention of non-canonical functions. Although determination of the ratio for these translated splice isoforms remains challenging, they may contribute to tumorigenesis and the modulation of telomerase activity.

Phosphorylation of TERT is known to have a pivotal role in regulating its localization, which is tightly controlled during the cell cycle. Translocation of TERT from the nucleus to the cytoplasm or to the nucleolus prevents telomerase activity at the telomere. In many cancer cells, kinase and phosphatase activity is modified resulting in aberrant phosphorylation of TERT leading to its accumulate within the nucleoplasm. This change in TERT trafficking might contribute to differences in the regulation of telomerase activity in cancer.

Alternative Lengthening of Telomeres (ALT)

Although the majority of cancers express telomerase, ~10% maintain telomeres via an alternative telomerase-independent mechanisms; this phenomenon is termed alternative lengthening of telomeres or ALT. As well as a lack of detectable telomerase expression, a distinctive hallmark of cancers active in ALT is an increase in DNA DSBs at the telomere. Rather than telomeric repeats being added processively, ALT-positive cells use a method of HR, whereby DNA is copied from telomere to telomere. The ALT pathway is particularly common in osteosarcomas, soft tissue sarcomas and astrocytic brain tumors.

Various recombination pathways

There are a few pathways by which telomeres can potentially clone their repeats (Fig. 10). The template may be *inter-telomeric* in fashion (occurring between sister chromatids or different chromosomes), or i*ntra-telomeric*, where the template derives from within an existing d-loop at the same telomere. As a result, the telomeres in ALT cells are highly heterogeneous in length and can range 2-50 kb. ALT cells also carry abundant extrachromosomal telomeric repeat circles (t-circles), which again range from 1 to 50 kb in length.

Since telomere terminate in a 3′ overhang, which resembles to the processed broken DNA end for the double-strand break repair, shortened telomere ends can directly invade homologous telomeric dsDNA to form a d-loop. The complementary template strand of the recipient telomere is then copied to extend the invading telomere 3′ end via elongation of the d-loop (Fig. 10A). Displacement of the elongated telomere strand results in cloning of the telomere. As telomeres can form a t-loop, the 3′ end can therefore be directly replicated through the loop (Fig. 10B).

Telomeres are fragile and can generate G-quadruplex structures. Replication fork stalling or collapse at the telomere can also activate HR (Fig. 10C). In this case, the broken telomere is most likely to restart DNA replication by displacing telomeric DNA from the sister chromatid (a process called break-induced replication). The d-loop can be resolved by the Holiday junction nucleases. Cross-over resolution of telomere recombination intermediates between the sister chromatids leads to taking over of the telomere duplex (telomere-sister chromatid exchanges, or T-SCE) (Fig. 10D). T-circles are generated by cross-over resolution of a d-loop at intra-telomere recombination or t-loop (Fig. 10E). Such telomere trimming results in immediate shortening of telomeres. Conversely, crossover recombination between a chromosome end and a t-circle results in incorporation of telomere stretches. ALT cells contain t-circles and exhibit T-SCE in many chromosomes, implying that telomere exchanges are a common occurrence.

Biology of ALT

Strand invasion requires the recruitment of the RAD51 protein complex. The 3′ single-strand of the telomere is usually bound to the shelterin protein POT1, which outcompetes HR factors RPA and RAD51. TRF1 facilitate telomere replication and TRF2 suppress HR. However, ALT telomeres appear to have mutations (e.g. TTAGGG > TCAGGG), which impairs shelterin binding and promotes the recruitment of HR factors required for ALT.

Telomeres of ALT cells cluster together and are often found at promyelocytic leukemia (PML) nuclear bodies. Hence, telomere recombination is thought to take place in PMLs. Specifically, these are termed ALT-associated PML bodies, or APBs and are suborganelles found predominantly in the nucleus. The PML complexes dramatically change with the cell cycle and stress responses,

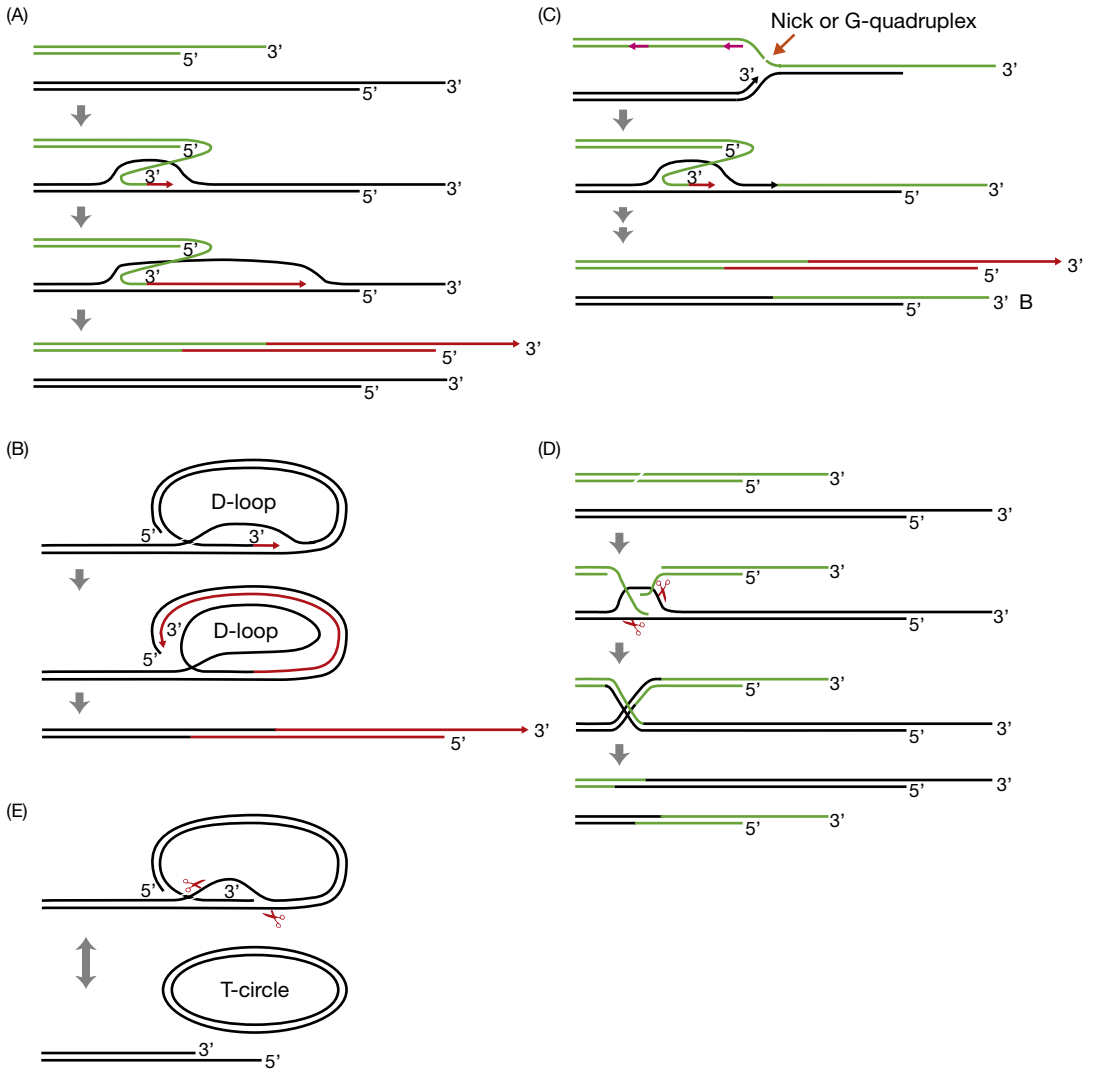

Fig. 10 Alternative lengthening of telomere. Models for telomere recombination and replication in ALT. (A) Pirate telomere copy model—shortened telomere G-tails invade long telomeres to form d-loops. The invading 3′ end can extend by unwinding the double strand. After dissolution, the lagging strand is filled. (B) 3′ ends within t-loops can be extended. (C) Replication fork collapse caused by SSB (nick) or G-quadruplex formation induces break-induced repair pathway. Broken 3′ end invades (newly synthesized) sister chromatid telomere. (D) Robbing telomeric repeat model—The damaged telomere recombines with sister chromatid telomere. d-loop can be resolved by nucleases. Crossover resolution of the junction results in T-SCE. (E) Crossover resolution at t-loop results in telomere trimming and generation t-circles. T-circles can be used as a template for the strand invasion and recombination/replication.

and accommodate many transcription regulators, DNA replication and DNA damage response proteins, including p53, ATRX and the histone chaperon DAXX.

De-repression of ALT

Mutations in *ATRX* and *DAXX* has been observed in many ALT cancer types. ATRX is a chromatin remodeling protein that binds to tandem repeat sequences and G-quadruplex and localizes to telomeric and peri-centromeric heterochromatin regions. ATRX binding to DAXX is necessary for the loading of Histone 3.3 and telomere cohesion. Although the mechanism remains to be established, loss of ATRX-DAXX interaction alters chromatin characteristics of telomeric region, leading to telomere replication defects. This is likely to induce replicative stress and the break-induced replication pathway. In fact, re-installation of ATRX by ectopic over expression can suppress telomere recombination in ALT cells, implicating ATRX as a key ALT repressor. However, mutation of ATRX itself does not activate ALT in normal or telomerase-positive cancer cells. Hence, further alterations such as evasion of shelterin function may be required to de-repress ALT. Since the mutations in either ATRX or DAXX are common in ALT, downstream functions of the ATRX-DAXX complex, such as histone H3.3 deposition and further its post-translational modifications, may be crucial in facilitating telomere replication. Given their frequent occurrence, mutations in *ATRX* and *DAXX* are also considered a hallmark of ALT type cancers.

Telomere Length and Cancer Risk

Cancer is an aging-associated disease. Epidemiological studies support this notion and that many cancers may arise from aged cells with shortened telomeres. The overall telomere length in the body is defined/established at the time of embryonic development (except for spermatocytes). Telomeres shorten throughout an individual's life, and highly propagating tissues are more likely to have shorter telomeres (Fig. 11A, B). With age, cells may accumulate oncogenic mutations, particularly in those that have short telomeres. As discussed, they are active in the mechanisms that give rise to increased genomic instability. In some cases, short telomeres can be detected from progenitors of cancers in rapidly regenerating tissues including the gastrointestinal tract, and cancer cells often harbor shorter telomeres than found in healthy blood cells (Fig. 11B). These facts reveal a strong correlation between telomere shortening and carcinogenesis. Short telomeres also induce cellular senescence to counteract carcinogenesis, but this in turn impairs tissue regenerative ability. For this reason, cancer is considered to be a disease of aging.

Leukocyte Telomere Length and General Cancer Risk

Since blood cells are one of the most heavily dividing, the length of telomeres in blood cells can be used as a surrogate to represent the overall telomere length of every tissue and thereby serve as an indicator of tissue aging. Among blood cells, leukocytes are crucial immune cells that protect the body from infection. The immune system also identifies and rejects irregular cells, cancer precursors and neoplastic cells. This means that telomere shortening in leukocyte is predicted to impair the ability of immune response against cancers.

Telomere length of peripheral blood mononuclear cells has been assessed from patients with different cancers at various stages. A correlation between telomere length and tumor progression was found in many cancer types, including glioma, lung, pancreatic, kidney, esophageal, gastric, colorectal, bladder, breast and ovarian cancers. Hence, it is likely that telomere shortening generally increases risk of carcinogenesis. Furthermore, patients with shorter telomeres are more likely to have poor prognosis and lower overall survival.

In some cancer types, such as melanoma, leukemia and kidney and lung cancer, long rather than short telomeres are associated with disease. Indeed, studies in healthy individuals have indicated that those with either extremely short or long telomeres have between a 1.3 to >2 fold higher chance of developing cancer within 20 years (Fig. 11C). Hence, mounting data suggests a correlation between telomere length and cancer risk. Possible explanation of these phenomena would be that short telomeres promote cellular senescence and tissue aging, increasing the possibility of telomere crisis. Longer telomeres might trap telomeric protein such as TRF2 and RAP1 that serve as transcriptional modulators. Instances of telomere replication defects and oxidative damages of telomeric DNA might be associated with alterations in their length. Although the scientific significance that supports the rationale of this relationship needs to be validated, leukocyte telomere length may have prognostic value in cancer development and progression.

However, it should be noted that epidemiological studies of telomere length are not always consistent and are often unable to take into account the different environmental variables existing between individuals. Categorical variables of the telomere length are not universal but are grouped among its distribution within samples in each study. Further developments in the standardization of methods/procedures and more universally defined ways to measure telomeres would be required to improve the utility of telomere length-based diagnostics in cancer prediction and prognosis.

Inherited Telomere Mutations and Cancer Risk

A number of inherited disorders exist caused by mutations in genes encoding telomerase or telomerase regulatory proteins. These cause short telomeres in patients which can manifest in a broad range of phenotypes, referred to as telomeropathies. Dyskeratosis congenita (DC) is an inherited bone marrow failure syndrome caused by short telomeres that result in the premature death of rapidly dividing blood progenitor cells. DC patients have an enhanced risk of developing cancer as a result of their short telomeres, as a state of telomere crisis is reached early on which promotes further genetic instability.

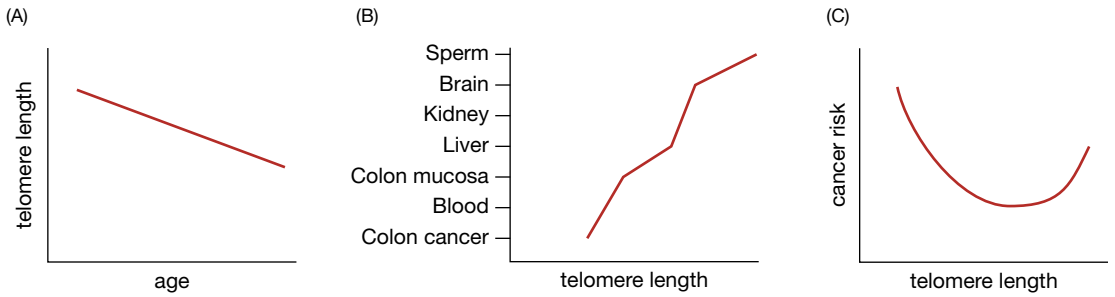

Fig. 11 Telomere length and cancer risk. (A) Graph showing telomere shortening with age. (B) Graph representing telomere length of each organ and cancer. (C) Graph showing telomere length in leukocyte and cancer risk in general.

Several germline mutations in the *POT1* and *ACD* (encoding TPP1) genes have been reported in cases of familial melanoma, glioma and chronic lymphocytic leukemia. These mutations either impair protein stability or impact the interaction of POT1 with TPP1 or the telomeric ssDNA. Mutations in other shelterin components such as *TE2IP* (encoding telomeric RAP1) have also been reported in patients with these cancers. These mutations destabilize shelterin formation, and lead to genetic instability associated with cancer progression. This therefore emphasizes the importance of the shelterin complex in protecting and regulating telomeres, as its disruption can result in cancer.

Telomeres and Cancer Therapeutics

Telomere maintenance is a key feature of cancer biology, as all cancers must sustain their telomeres in order to proliferate. In 85–90% of cancers, this is achieved through the reactivation of telomerase making this as ideal molecular diagnostic for cancer. Furthermore, telomerase is a highly attractive target for cancer therapeutics, providing an excellent opportunity to eliminate a wide range of cancers without damaging normal tissues. In this section, we introduce experimental methods applied to clinical diagnostics and discuss current strategies in telomere targeted cancer therapeutics.

Detection of Telomerase Activity

Typically, telomerase is expressed at low levels in most cells. In addition, TERT expression does not necessarily reflect the level of active telomerase. Hence, direct detection of telomerase activity is a better indicator when determining telomerase reactivation. The telomerase repeat amplification protocol (TRAP, developed by the groups of Shay and Wright) is a biochemical assay that detects reverse-transcriptase activity of telomerase. A useful method for detecting/measuring telomerase activity simply involved the mixing of a telomere substrate oligo (telomere seed) with cell lysate in the presence of dNTPs. Telomerase present can then incorporate a radioisotope-labeled dNTP as it extends the telomere seed. The level of extension can then be visualized. Alternatively, the TRAP assay use a unique non-telomeric oligo as a telomerase substrate. The product of telomerase extension can be amplified by quantitative polymerase chain reaction (qPCR) to measure the activity, instead of direct detection of telomere extension. This assay is robust and can be used to quantify the level of telomerase activity with material from only 100–1000 cells. As telomerase activity is below detectable levels in most somatic cells, the TRAP assay therefore makes for a highly sensitive test to detect telomerase activity in cancer biopsies when the number of cells in limiting.

Telomere Length Diagnostics

As we discussed in the previous section, measuring patient telomere length can give an indication of either cancer risk or the status of disease progression. A number of methods exists for measuring telomere length, and these have distinct advantages and disadvantages.

Telomere southern blot: TRF analysis

The gold standard to measure telomere length is terminal restriction fragment (TRF) analysis using a Southern blotting method. Here, genomic DNA is digested into fragments using restriction endonucleases that do not cut within telomere sequence. Isolated telomeric DNA fragments are resolved through an agarose gel and the DNA is transferred onto a membrane which can then be detected using a labeled telomere probe. This assay is non-biased and robust and allows for the range of telomere lengths in the cell population tested to be visualized. However, this method requires a large quantity (at least 1–4 μg) of DNA which is often not possible to acquire from patient biopsies or fixed specimens.

Single telomere length analysis (STELA)

Shorter telomeres are more difficult to detect. Duncan Baird developed the method to measure telomere length of a specific chromosome arm, using a PCR-based method. This was achieved by ligation of a specific probe to the telomere 3′ overhang and identification of unique sequence on the subtelomeric region (i.e. telomere-adjusted region). PCR may then be used, which preferably amplifies shorter telomeres. The variation in PCR products may then be resolved through an agarose gel, and the telomeric products detected by Southern blot. Universal STELA adapted this method to amplify every chromosome arm, allowing for detection of any short telomeres. Long telomeres are not favorable for PCR thus this method cannot determine average and variation of telomere length. Nevertheless, since one short telomere can elicit replicative senescence, this method is well suited for detection of critically short telomeres such as t-stumps.

Telomere monochrome multiplex qPCR (telomere MMqPCR)

In this method, developed by Richard Cawthon, both telomeric DNA (T) and a reference gene (S) are amplified by multiplex qPCR and the amount of T relative to S is expressed ratio metrically. Using synthetic DNA oligos as a standard curve, the absolute total telomere length can be calculated. As this is a PCR-based method, only a small sample of genomic DNA is required, making this system more feasible than Southern blotting. This assay is widely used to measure relative telomere length from patients' blood or biopsy samples. However, as the result reveals only the relative telomere quantity, variations in telomere length cannot be assessed.

Telomere qFISH

Fluorescent in situ hybridization (FISH) of telomeric DNA has been developed to quantify signal intensity and assess telomere length of each chromosome ends. Metaphase chromosome spreads are prepared and telomeric DNA is hybridized with a stable oligonucleotide telomere C-strand probe (three tandem 5′-CCCTTA-3′, synthesized as peptide nucleic acids, PNA). As PNA/DNA hybrids are more stable than DNA duplexes, all telomeric DNA can be labeled with PNA probes. As each telomere end is observed as a concentrated dot, variations in telomere length within a cell can be assessed. Furthermore, this is the only assay capable of detecting telomere loss as well as telomere damage, fusions and recombination on each chromosome. For post-mitotic cells, although assessment of each telomere is not possible, interphase qFISH can still provide the relative telomere length when compared to centromere qFISH. In this case, because of the simplified manner in sample preparation, it is well-suited for use in high throughput and automated screens.

Flow-FISH

By combining qFISH and Flow cytometry, Flow-FISH allows the separation of different cell types. Telomere length is provided as an average length for the cell population. Therefore, this method is suitable with hematopoietic cell subtyping, and is often used in clinical studies. As this method does not use microscope, non-specific signal needs to be taken in account.

Telomerase as an Anti-Cancer Drug Target

Since telomerase is expressed in the majority of cancer cells and rarely in normal tissue, telomerase has been considered as one of the best targets to uniquely kill cancer cells. However, telomere attrition, leading to cells death, takes time, which provides a window for further potentially oncogenic mutations to accumulate. In addition, hematopoietic progenitor cells have a high proliferative capacity and themselves express telomerase, meaning side-effects must also be considered. Nevertheless, different characteristics of telomere maintenance in cancer cells provide an opportunity for the development of targeted drugs. Combination chemotherapy with telomerase inhibition and TERT-targeted cancer immunotherapy might also help improve therapeutic strategies.

Telomerase targeting drugs

Telomerase activity requires the complexing of both TERC and TERT. BIBR1532 is a non-competitive inhibitor that specifically binds to the CTE domain of TERT and inhibits association with TERC via the CR4/5 domain (Fig. 12A). Treatment of cells with BIBR1532 causes telomere shortening and induces replicative senescence or apoptosis in telomerase-positive cancer cells. However, it remains unknown if it also inhibits non-canonical telomerase activities.

Telomerase copies the template sequence within TERC onto chromosome ends in order to extend telomeric DNA. Imetelstat, formally named GRN163L, is an oligonucleotide-based compound that is complementary to the template sequence within TERC (Fig. 12B). It binds with a high affinity, blocking the interaction between TERC and the 3′ telomere overhang, thereby preventing template annealing and polymerization activity. To conquer issues relating to nucleotide stability, aqueous solubility and cellular uptake, this template antisense oligonucleotide is fused to a palmitoyl lipid moiety. To date, Imetelstat is the best evaluated telomerase inhibitor. It has demonstrated high specificity against many telomerase-positive cancer types, including glioblastoma, esophageal, lung and pancreas cancers in in vitro and pre-clinical models. Imetelstat has proven effective when used with other anti-cancer drugs (combination chemotherapy) or radiotherapy (radiosensitization).

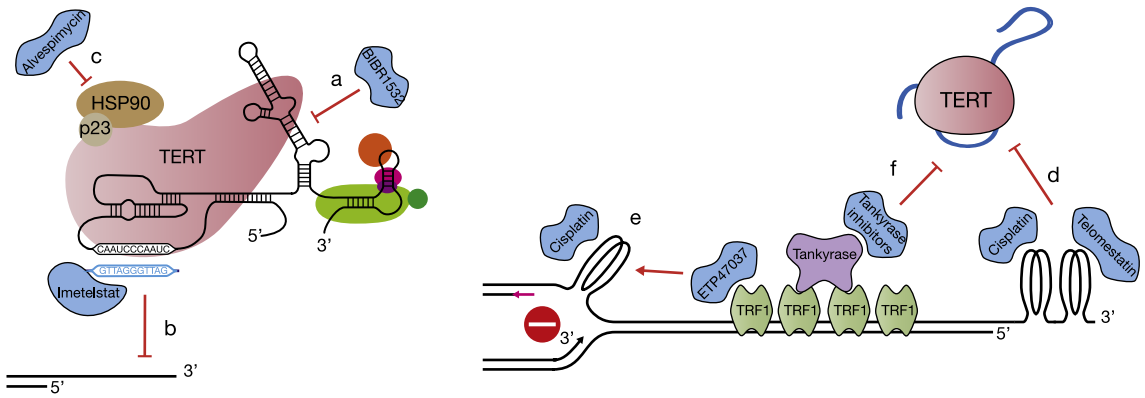

Fig. 12 Drugs that target telomerase and telomere. Schematic overview of telomere and telomerase targeting drugs. (A) BIBR1532 binds to CTE domain and inhibit TERC (CR4/5) interaction. (B) Imetelstat nucleotide complements RNA template and blocks telomere 3′ end binding. (C) HSP90 inhibitors such as Alvespimycin destabilize TERT. (D) Telomestatin and Cisplatin stabilize G-quadruplex at the 3′ end to block telomerase interaction. (E) G-quadruplex formation during lagging strand synthesis and TRF1 destabilization by ETP47037 accumulate telomere replication damage. (F) Tankyrase inhibitors stabilize TRF1 binding and suppress telomerase recruitment.

HSP90 is an essential chaperon for telomerase assembly and stability. HSP90 is often upregulated in cancer and contributes to the stabilization of client (substrate) proteins, including oncogenic proteins, to promote tumor development and heterogeneity. HSP90 inhibitors geldanamycine analogues, tanespimycin (17-AAG) and the more advanced alvespimycin (17-DAMG) reduce telomerase activity and block oncogenic signaling pathways (Fig. 11C). Telomere shortening can be synergized with Imetelstat.

Telomeres targeting drugs

Telomeres themselves are also potential targets for anti-cancer therapeutics. Cisplatin is a drug that binds preferentially to guanine bases, causing intra-strand crosslinks in dsDNA and G-quadruplex formation in ssDNA. Telomeric DNA is G-rich, making it an ideal target for Cisplatin, which can act to both impair telomerase activity as well as limit telomeric DNA replication (Fig. 12D, E). A natural compound, telomestatin, specifically stabilizes telomeric G-quadruplex structures with minimal effect on duplexed DNA found in non-telomeric regions. With further development, telomestatin could prove useful for the targeting of telomeric DNA in cancer.

Alternate telomeric nucleotides can also be exploited to disrupt telomere protection. The GTP analogue, 6-thio-2′-deoxyguanosine can also be used to take advantage of the G-rich state of telomeric DNA. An analog of 6-thio-guanosine, which is used for leukemia treatment, 6-thio-2′-deoxyguanosine also mimics GTP and has high affinity towards telomerase. Hence, it can be incorporated into the telomere by telomerase. This causes telomere de-protection and rapid cell death. Unlike 6-thio-guanosine, damaging to healthy telomerase negative cells seems low. However, further development is necessary to specifically target telomerase and an important issue remains the emergence of alternative methods of telomere lengthening, caused by destabilizing telomeric proteins such as TRF2.

Telomeric proteins can also be anti-cancer drugs targets. Telomerase activity is repressed by the presence of TRF1, which in turn is stabilized by tankyrase 1. Inhibition of tankyrases leads to impaired telomere maintenance and shortening of telomeres in vitro (Fig. 12F). Notably, tankyrases also control Wnt/β-catenin signaling, Hippo/YAP signaling and mitotic progression, which contribute tumor development, making them all potential targets for cancer therapy. A number of PARP inhibitors have been developed but selectivity and efficacy on other superfamily members remain an issue. A novel TRF1 inhibitor ETP-47037 that has been tested on lung carcinoma leads to proliferation defects via telomere replication catastrophe and apoptosis (Fig. 12E). Although effects on normal tissues must be validated, altering shelterin function at fragile telomeres in cancer cells represents an attractive strategy in the development of cancer therapy.

TERT Targeting Cancer Immunotherapy

Immunotherapy is a promising new approach for targeting cancer. Many cancer cells express TERT and therefore present TERT peptides on their surface via MHC Class I. Given that most normal cells express low levels of this protein, this could serve as an effective cancer-targeting antigen. TERT peptide-based vaccines such as GV1001 and Vx-001 have been developed to stimulate immune responses against TERT-expressing tumor cells. As an alternative approach, Dendritic Cells (DCs) have also been genetically modified to express TERT-peptide antigens. Such engineered DCs (GRNVAC1/2) efficiently stimulate immune responses. These vaccines seem to be well- tolerated in patients indicating this as a promising approach for cancer therapy. However, further trials and larger studies to prove significant curative effects are necessary to determine the long-term effects and potential toxicity.

Prospective Vision

The telomere biology field holds some exciting prospects for improving our understanding of cancer and for the development of new anti-cancer therapies. Further research aims to provide a greater understanding of how and when telomerase becomes reactivated during oncogenesis. In addition, understanding how telomerase regulation differs in cancer cells, resulting in the ability to maintain short telomeres that promote chromosomal damage, will be a crucial step in improving our knowledge of cancer progression and identifying new potential therapeutic targets.

Although telomerase itself is not oncogenic, its non-canonical functions may contribute to tumor progression. Recent studies have started to unveil relationships between telomere/telomerase and Wnt/β-catenin and NFκB signaling pathways. These pathways promote TERT expression and TERT facilitates their transcription activities. Establishing the function of their interaction in the context of cancer biology is anticipated.

Epidemiological studies will continue to reveal correlations between telomere length and risk of carcinogenesis and may well provide new insights into cellular aging. An exciting area for the future is the use of telomerase as an antigen to target cancer. With telomerase expression occurring in the majority of cancers, strategies to target this and telomeres promise the development of novel, broadly applicable anti-cancer therapies.

See also: Senescence and Cellular Immortality.

Further Reading

Armstrong, C.A., Tomita, K., 2017. Fundamental mechanisms of telomerase action in yeasts and mammals: understanding telomeres and telomerase in cancer cells. Open Biology 7, 160338.
Barrett, J.H., Iles, M.M., Dunning, A.M., Pooley, K.A., 2015. Telomere length and common disease: study design and analytical challenges. Human Genetics 134, 679–689.
Basu, N., Skinner, H.G., Litzelman, K., Vanderboom, R., Baichoo, E., Boardman, L.A., 2013. Telomeres and telomere dynamics: relevance to cancers of the GI tract. Expert Review of Gastroenterology & Hepatology 7, 733–748.
Blackburn, E.H., Epel, E.S., Lin, J., 2015. Human telomere biology: a contributory and interactive factor in aging, disease risks, and protection. Science 350, 1193–1198.
Heidenreich, B., Kumar, R., 2017. TERT promoter mutations in telomere biology. Mutation Research 771, 15–31.
Jafri, M.A., Ansari, S.A., Alqahtani, M.H., Shay, J.W., 2016. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. Genome Medicine 8, 69.
Lazzerini-Denchi, E., Sfeir, A., 2016. Stop pulling my strings—what telomeres taught us about the DNA damage response. Nature Reviews Molecular Cell Biology 17, 364–378.
Li, Y., Tergaonkar, V., 2014. Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. Cancer Research 74, 1639–1644.
Maciejowski, J., de Lange, T., 2017. Telomeres in cancer: tumor suppression and genome instability. Nature Reviews Molecular Cell Biology 18, 175–186.
Martinez, P., Blasco, M.A., 2017. Telomere-driven diseases and telomere-targeting therapies. The Journal of Cell Biology 216, 875–887.
Pickett, H.A., Reddel, R.R., 2015. Molecular mechanisms of activity and derepression of alternative lengthening of telomeres. Nature Structural & Molecular Biology 22, 875–880.
Ruden, M., Puri, N., 2013. Novel anticancer therapeutics targeting telomerase. Cancer Treatment Reviews 39, 444–456.
Schmidt, J.C., Cech, T.R., 2015. Human telomerase: biogenesis, trafficking, recruitment, and activation. Genes & Development 29, 1095–1105.
Wu, R.A., Upton, H.E., Vogan, J.M., Collins, K., 2017. Telomerase mechanism of telomere synthesis. Annual Review of Biochemistry 86, 439–460.
Ye, J., Renault, V.M., Jamet, K., Gilson, E., 2014. Transcriptional outcome of telomere signaling. Nature Reviews. Genetics 15, 491–503.

TGF-β in Cancer Progression: From Tumor Suppressor to Tumor Promotor

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Glossary

Angiogenesis A process in which new blood vessels arise from pre-existing vessels.

Anti-sense oligonucleotides Short single-stranded RNAs that are complementary to transcribed messenger RNAs within a cell.

Cyclin-dependent kinases A family of serine/threonine protein kinases that phosphorylate various proteins involved in cell cycle progression. CDKs form complexes with cyclins to become functionally active.

Deubiquitinating enzymes A family of proteases that cleave the peptide or isopeptide bond between ubiquitin and substrate proteins.

Epithelial to mesenchymal transition A process in which epithelial cells lose their cell polarity and cell–cell adhesion, demonstrate a decrease in the expression of epithelial markers such E-Cadherin and zonula occludens-1, and acquire spindle cell morphology and mesenchymal traits (showing a decrease in the expression of mesenchymal markers such as N-cadherin and a-smooth muscle actin). Upon EMT, the cells become more migratory and invasive.

E3 ubiquitin ligases A family of enzymes that mediate the attachment of ubiquitin molecule(s) onto lysine residues of proteins, thereby regulating their function, for example, stability, cellular localization, protein–protein interactions and activity.

Metastasis The process in which cancer cells spread from the initial or primary site to a different or secondary site.

Phosphatases A family of enzymes that remove phosphate groups from substrates; their function opposes that of kinases, which catalyze the ATP-dependent transfer of phosphate groups onto substrates.

Smad transcription factors A family of proteins that act as intracellular effectors of TGF-β family members. The proteins in this family were named for their similarity to the *Caenorhabditis elegans* SMA protein and the *Drosophila* gene product mothers against decapentaplegic (MAD).

Nomenclature

ALK Activin receptor-like kinase
ANGPT4 Angiopoietin-like 4
AON Anti-sense oligonucleotide
CAR Coxsackie and adenovirus receptor
CDK Cyclin dependent kinase
CSC Cancer stem cell
CTGF Connective tissue growth factor
DAPK Death associated protein kinase
ECM Extracellular matrix
EMT Epithelial to mesenchymal transition
ER Estrogen receptor
ERK Extracellular regulated kinase
GRB2 Growth factor receptor-bound protein 2
HGF Hepatocyte growth factor
HIF-1 Hypoxia inducible factor-1
IL Interleukin
JNK Jun amino-terminal kinase
LLC Large latent complex
MAPK Mitogen activated protein kinase
MEG3 Maternally expressed gene 3

*Both these authors contributed equally.

miR Micro RNA
MMP Matrixmetalloproteases
mTOR Mammalian target of rapamycin
NEDD4L Neural precursor cell expressed, developmentally down-regulated 4-like
NK Natural killer
PAK2 p21-activated kinase
PDP Pyruvate dehydrogenase phosphatase
PI3K Phosphatidylinositol-3-kinase
PP Protein phosphatase
PTHrP Parathyroid hormone-related protein
RANKL Receptor activator of nuclear factor κB ligand
ROS Reactive oxygen species
S6K S6 kinase
SCL Small latent complex
SDF-1 Stromal cell-derived factor 1
SHC SH2 containing protein
SHIP SH2-containing inositol phosphatase
SMA Smooth muscle actin
Smad Sma and Mad related protein
SMURF Smad ubiquitin regulatory factor
SOS Son of sevenless
TAB1 TAK1 binding protein
TAK1 TGF-β activated kinase-1
TGF-β Transforming growth factor-β
TIEG TGF-β-inducible early-response gene
TIMΠ Tissue inhibitor of MMP
TRAF Tumor necrosis factor receptor associated factor
TβR TGF-β receptor
USP Ubiquitin specific protease
VEGF Vascular endothelial growth factor
XIAP X chromosome-linked inhibitor of apoptosis
ZO-1 Zonala occludence-1

Introduction

Transforming growth factor (TGF)-β is a multifunctional secreted cytokine that exerts highly context dependent effects on many different cell types, including growth inhibition, extracellular matrix (ECM) production, apoptosis and differentiation (Massagué, 2012; Ayyaz et al., 2017). TGF-β1 is the prototype of a large family of evolutionarily conserved structurally and functionally related dimeric proteins that includes TGF-βs, activins and bone morphogenetic proteins (BMPs). Signaling occurs via transmembrane serine/threonine kinase type I and type II receptors (Feng and Derynck, 2005). TGF-β induces the formation of a complex of type I and type II receptors, upon which the type II kinase phosphorylates type I, thereby transmitting the signal across the membrane. Inside the cell, the activated type I receptor phosphorylates specific down-stream effector molecules, among which are canonical Smad and non-Smad signaling components. Smads can act as transcription factors and thus relay the signal from the membrane into the nucleus (Hill, 2016). Each step of the signaling pathway is intricately regulated to fine tune the potent cellular responses of TGF-β (Xu et al., 2016).

Misregulation of TGF-β signaling has been causally associated with many diseases, including cancer, fibrosis and cardiovascular diseases (Zhang et al., 2017; Kim et al., 2017a; Goumans and ten Dijke, 2017). In this review, we focus on its dual role in cancer. Moreover, because TGF-β stimulates invasion and metastasis, this pathway has been subject to therapeutic targeting by academic and industrial laboratories. We provide an update on the latest clinical developments of TGF-β targeting agents for the treatment of cancer (Colak and ten Dijke, 2017; Akhurst, 2017).

TGF-β Signaling

Ligands and Their Receptors

Shortly after the discovery of TGF-β1 in 1985, the structurally and functionally related TGF-β2 and TGF-β3 were identified (Moses et al., 2016). In this review, we indicate specific TGF-β isoforms when relevant, for example, when they have distinct functional properties; otherwise we refer to them as TGF-β. TGF-β is a conserved 12.5-kDa polypeptide that forms a disulphide-linked dimer (Galat, 2011). While predominantly present as homodimers, heterodimers between different TGF-β isoforms have been described (Cheifetz et al., 1987). Of note, TGF-β may exert diverse, sometimes even opposing, regulatory roles depending on the various cell types and different development stages (Massagué, 2012; Ayyaz et al., 2017). The three TGF-β isoforms are differentially expressed. TGF-β1 is highly abundant in platelets and bone and is widely expressed and synthesized among diverse tissues. TGF-β is secreted in an inactive form in which the amino-terminal pro-peptide (also termed the latency-associated peptide) is non-covalently associated with the carboxy-terminal mature peptide (Robertson and Rifkin, 2016). Activation can be mediated via specific proteases and cell surface-associated integrins that liberate the mature peptide, which can then interact with cell surface receptors (Khan and Marshall, 2016; Jenkins, 2008). This activation step is an important control mechanism that regulates the local bioavailability of TGF-β.

Activated TGF-β initiates cellular responses by binding to cell surface single transmembrane TGF-β type I and type II serine/threonine kinase receptors, that is, TβR1 and TβRII, respectively (Wrana et al., 1994). TGF-β induces the formation of a heterotetrameric complex containing two TβRIIs and two TβRIs (Yamashita et al., 1994). Initially, TGF-β1 and TGF-β3 (but not TGF-β2) bind to TβRII, and thereafter, TβRI is recruited. The TGF-β type III coreceptor (also termed betaglycan), which lacks intercellular enzymatic activity, can facilitate the interaction between TβRI and TβRII (López-Casillas et al., 1993). In particular, TGF-β2 requires TβRIII for efficient binding to signaling receptors (Villarreal et al., 2016). Upon the ligand-induced TβRI/TβRII complex formation, TβRI is phosphorylated by TβRII kinase on specific serine and threonine residues in the glycine/serine-rich (GS) domain. The extracellular ligand signal is thereby transduced across the membrane, and the activated TβR complex is then ready to initiate intracellular responses by phosphorylating intracellular effector proteins (ten Dijke and Hill, 2004; Shi and Massagué, 2003; Heldin et al., 1997) (**Fig. 1**).

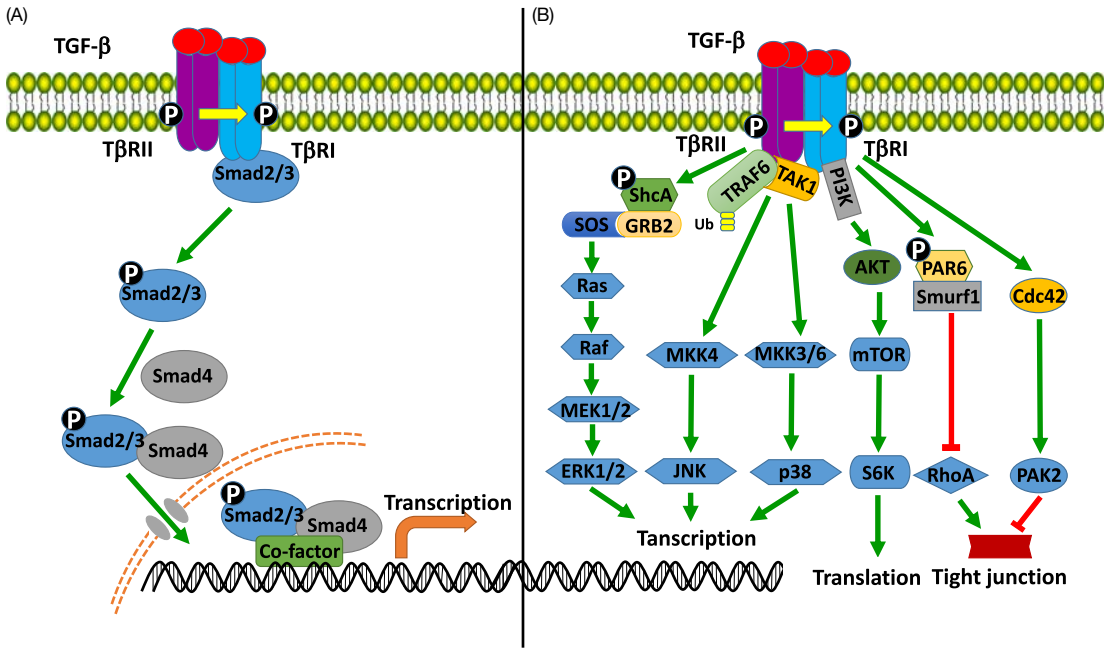

Fig. 1 TGF-β/Smad and non-Smad signaling. (A) In the Smad-dependent pathway, binding of active TGF-β induces the assembly of TβRI and TβRII into a complex in which TβRI is phosphorylated by TβRII kinase. Activated TβRI subsequently signals by recruiting and phosphorylating Smad2 and 3, which form heteromeric complexes with Smad4. The Smad complexes then translocate into the nucleus and regulate target gene transcription by cooperating with other cofactors. (B) In the non-Smad signaling pathways, TGF-β receptors activate other pathways including MAPKs (such as ERKs, p38 and JNK) and PI3K-AKT signaling to modulate transcription and translation events and regulate Rho-like GTPase activity for tight junction dissolution. Abbreviations: ERK, extracellular regulated kinase; GRB2, growth factor receptor-bound protein 2; mTOR, mammalian target of rapamycin; PI3K, phosphatidyl inositol-3-kinase; S6K, S6 kianse; Smurf, Smad ubiquitin regulatory factor; SOS, son of sevenless; TAK1, TGF-β activated kinase; TβR, TGF-β receptor; TGF-β, transforming growth factor-β; TRAF, TNF associated factor; Ub, ubiquitin.

TGF-β/Smad and Non-Smad Signaling

With the help of genetic approaches in worms and fruit flies, Sma- and Mad-related proteins, termed Smads, were identified in vertebrates as unique and pivotal intracellular effectors of TGF-β (Derynck et al., 1996). Smads are classified into three groups: the receptor-regulated Smads (R-Smads), the common Smads (Co-Smads) and the inhibitory Smads (I-Smads) (Nakao et al., 1997a, 1997b; Hayashi et al., 1997). R- and Co-Smads share two conserved domains, termed an amino-terminal Mad Homology 1 (MH1) and C-terminal MH2 domain. Both domains are separated by a proline-rich linker region. I-Smads also have an MH2 domain.

Upon activation, TβRI recruits and phosphorylates R-Smad family members, Smad2 and Smad3, at their carboxy-terminal regions at two serine residues. Activated Smad2 and 3 form heteromeric complexes with Smad4 that subsequently translocate into the nucleus. Activated Smad complexes can form transcriptional complexes in conjunction with a large variety of DNA binding cofactors and thereby gain high affinity and specificity for DNA. The intrinsic binding activity of Smad3 and Smad4 (via their MH1 domain) is weak, and their ability to bind directly with DNA is lacking in the predominantly expressed splice variant of Smad2. These Smad-containing transcription factor complexes interact with coactivators, corepressors and chromatin remodeling factors to regulate the transcription of target genes in a cell type-dependent manner (**Fig. 1**A) (Shi and Massagué, 2003; Nakao et al., 1997a) (Derynck and Zhang, 2003).

In addition to the canonical Smad-dependent pathway, non-Smad signaling pathways can be initiated by activated TGF-β receptor complexes in specific cell types (**Fig. 1**B). These pathways can also modulate Smad pathways (Zhang, 2017). Via phosphorylation or direct interaction with signaling modules, TGF-β receptors can activate pathways such as the mitogen-activated protein kinase (MAPK) signaling cascade, which includes extracellular signal-regulated kinases (ERKs), p38 and c-Jun amino terminal kinase (JNK), phosphatidylinositol-3 kinase (PI3K)-AKT signaling and Rho-like GTPase activity (Zhang, 2017; Mu et al., 2012; Lee et al., 2007; Arsura et al., 2003). The ERK MAPK pathway is initiated when tyrosine residues of the adaptor ShcA are phosphorylated by TβRI. Tyrosine phosphorylated ShcA provides a docking site for adaptor GRB2 that interacts with the exchange factor SOS, which activates the pro-oncogenic Ras-Raf-MEK1/2-ERK1/2 signaling pathway (Lee et al., 2007). Phosphorylated ERK1/2 then translocates into the nucleus and regulates gene transcription by phosphorylating target transcription factors (Zavadil et al., 2001). TGF-β activated kinase 1 (TAK1), a MAP kinase kinase kinase (MAPKKK), which is recruited on the TGF-β receptor complex by polyubiquitylated TRAF6, phosphorylates specific MAP kinase kinases (MKKs), leading to the further phosphorylation of JNK and p38 (Wang et al., 2001). In addition, TGF-β stimulation triggers the interaction between TβRI and the PI3K subunit p85, leading to AKT phosphorylation and the activation of downstream effectors (e.g., mTOR, P70S6K and 4E-BP1) (Yi et al., 2005; Lamouille and Derynck, 2007). PAR6 can also be phosphorylated by TβRI and recruits Smurf1 to degrade RhoA, which regulates cell–cell interactions via tight conjunctions (Ozdamar et al., 2005). Cdc42, another GTPase, can be recruited to the TGF-β receptor complex and mediate the activation of p21-activated kinase 2 (PAK2), which stimulates tight conjunction disassociation (**Fig. 1**B) (Wilkes et al., 2003; Barrios-Rodiles et al., 2005).

Regulation of TGF-β/Smad Signaling

As a pivotal cytokine in cell homeostasis, TGF-β signaling activity is under precise regulatory control, from ligand bioavailability to receptor and Smad activation (**Fig. 2**). After synthesis and intracellular furin-mediated cleavage of the precursor protein (removal of the signal peptide), the bioactive growth-factor domain (mature TGF-β) and prodomain, also termed the latency-associated peptide (LAP), are secreted in a small latent complex (SLC) form. The binding of TGF-β to its receptors is prevented by LAP. The large latent complex (LLC), a more commonly deposited complex, contains the SLC and latent TGF binding protein (LTBP), which is covalently bound to LAP (Robertson and Rifkin, 2016; Shi et al., 2011; Costanza et al., 2017; Robertson et al., 2015). LTBP has an important function in facilitating TGF-β secretion. Stromal-derived molecules including proteases and reactive oxygen species (ROS) substantially contribute to the increase in active TGF-β levels by interacting with the latent TGF-β complex (Costanza et al., 2017; Mu et al., 2002; Yu and Stamenkovic, 2000; Jobling et al., 2006). In addition, contractile forces exerted by integrins across the LLC play a vital role in the release of mature TGF-β (Shi et al., 2011; Annes et al., 2004; Dong et al., 2017; Munger and Sheppard, 2011). Fibronectin that is deposited in the ECM prior to the formation of the LLC impairs TGF-β1 bioactivity by interacting with LTBP (Dallas et al., 2005). Decorin, a member of the proteoglycan family, also exerts a suppressive role on TGF-β activity via binding to all isoforms of soluble TGF-β (Merline et al., 2011).

Apart from the ECM level, TGF-β responsiveness is tightly controlled at the cell membrane. Glycosylation of the extracellular domain of TβRII inhibits its transportation to the cell membrane and lowers the affinity for TGF-β binding (Goetschy et al., 1996; Kim et al., 2012). E3 ubiquitin ligases such as Smad-specific E3 ubiquitin protein ligase 1/2 (Smurf1/2) cooperate with inhibitory Smad7 to regulate the availability of TβRI receptors on the cell surface by ubiquitylation and degradation (Ebisawa et al., 2001; Kavsak et al., 2000). Deubiquitinating enzymes ubiquitin-specific protease (USP) 4, 11 and 15 inhibit the degradative ubiquitination of TβRI and stabilize this receptor (Liu et al., 2016). Two phosphatases, protein phosphatase (PP)1c and PP2A, impair receptor activation by targeting TβRI for dephosphorylation (Shi et al., 2004; Batut et al., 2008). Akin to ubiquitylation, sumoylation and neddylation have also been implicated in the regulation of TGF-β receptor stability. The interaction between TGF-β receptors and the coreceptors located on the cell membrane is another determinant for signaling strength (ten Dijke and Hill, 2004; Budi et al., 2017). The coreceptor betaglycan stabilizes the receptor complex between TβRI and TβRII and propagates the signaling transduction initiated by TGF-β2 (Stenvers et al., 2003). Endoglin, another accessory protein at the plasma membrane that is structurally

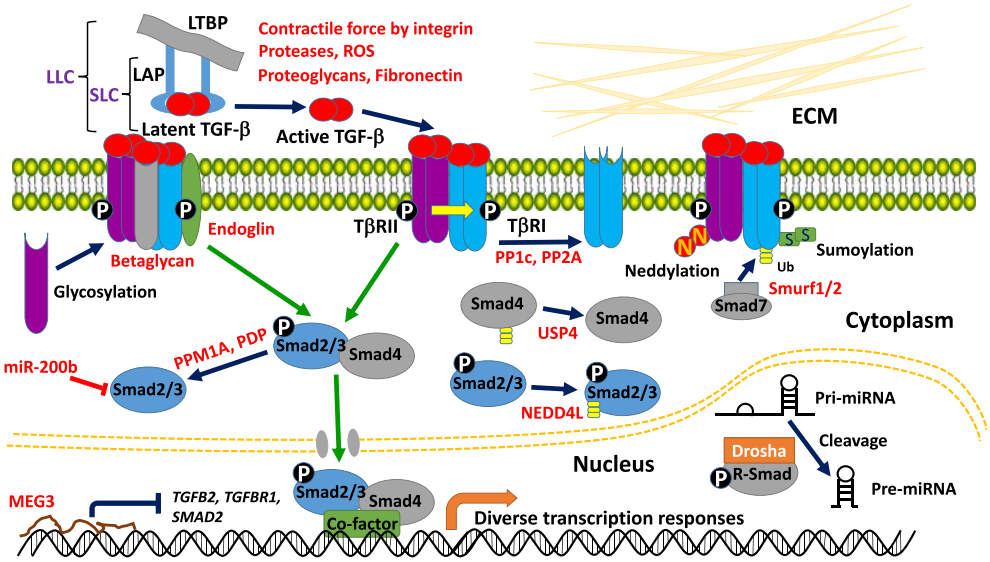

Fig. 2 Regulation of TGF-β/Smad signaling. Proteases, ROS, integrin-mediated contractile forces and stromal-derived factors modulate the bioavailability of TGF-β and accessibility to its receptors. On the cell membrane level, the activity of TβRs is modified by glycosylation, phosphorylation, ubiquitylation, deubiquitylation, sumoylation and neddylation, as well as the interaction with coreceptors and other accessory proteins. At the cytoplasmic level, Smad proteins are under tight control of phosphatases, ubiquitylating enzymes, deubiquitylating enzymes and miRNAs. In the nucleus, the Smad complex affects different transcriptional responses in combination with diverse cofactors. Smad proteins are also required for the maturation process of miRNAs. Moreover, transcription levels of TGF-β pathway components are regulated by modulators such as lncRNAs.

related to betaglycan, inhibits the TGF-β/ALK5-mediated Smad2/3 pathway but promotes TGF-β/ALK1-induced Smad1/5/8 signaling in endothelial cells (Lebrin et al., 2004; Scherner et al., 2007).

At the cytoplasmic level, phosphorylated Smad proteins can be deactivated by phosphatases such as PPM1A and PDP, leading to signal termination (Chen et al., 2006; Sapkota et al., 2006; Lin et al., 2006). Similar to the TGF-β receptors, Smads are under the intricate regulation of multiple ubiquitylating enzymes such as Smurf1/2 and NEDD4L, which inhibit Smad2/3 stability (Lo and Massagué, 1999; Gao et al., 2009). Conversely, USP4 promotes Smad4 activity by removing the suppressive monoubiquitination triggered by Smurf2 (Zhou et al., 2017). MicroRNAs (miRNAs) also modulate TGF-β signaling by inhibiting various signaling components of this pathway (Gulei et al., 2017). MiR-200b, one miRNA that is downregulated by TGF-β1, attenuates the TGF-β signaling response by targeting Smad2 at the post-transcriptional level, thereby forming a negative feedback loop (Chen et al., 2013).

Upon activation, phosphorylated Smad2/3/4 translocate into the nucleus and form a transcription complex with other cofactors. In combination with different sequence-specific transcription factors, the Smad complex generates a vast variety of transcriptional responses in a context and cell type-dependent manner (Massagué, 2012; Itoh et al., 2000; Piek et al., 1999; Mullen et al., 2011). In addition, activated Smad proteins participate in the maturation of microRNAs (miRNAs) by recruiting the RNA helicase p68 (DDX5) to the Drosha complex (Davis et al., 2008). MEG3, an intranuclear long noncoding RNA (lncRNA), can bind to the distal regulatory elements of genes encoding TGF-β signaling components, including *TGFB2*, *TGFBR1* and *SMAD*, and inhibit their transcription (Mondal et al., 2015).

TGF-β as a Tumor Suppressor

TGF-β-Induced Growth Inhibition

TGF-β-induces growth inhibition (**Fig. 3**) and the induction of apoptosis (**Fig. 4**) of normal epithelial (and certain premalignant) cells; these properties are associated with its function as a tumor suppressor (Schuster and Krieglstein, 2002). The molecular mechanisms by which TGF-β elicits these processes involve multiple intracellular pathways (Macias et al., 2015; Fabregat et al., 2014; Chandrasinghe et al., 2017). Numerous studies support the notion that TGF-β inhibits the proliferation of cells by arresting cells in the G1 phase of the cell cycle (**Fig. 3**). Smad-containing protein and transcriptional coactivator complexes can activate the transcription of two major cell cycle inhibitors, CDK inhibitors (CKIs), p15 and p21 (ten Dijke et al., 2012; Liu, 2006). In keratinocytes, TGF-β/Smad signaling induces the expression of cyclin-dependent kinase inhibitors p15INK4b and p21CIP/WAF1, which inhibit the CDK4/6-cyclin D complex (Massagué et al., 2000). These cyclin-dependent kinase inhibitors inhibit CDK activities associated with the G1 to S phase progression, prevent phosphorylation of Rb by cyclin-dependent kinases, and arrest cells in G1 (Tarasewicz and Jeruss, 2012). The activated Smad proteins target the promoters of the c-Myc and CDK genes and repress their transcription in cooperation with nuclear corepressors (Chen et al., 2002). Non-Smad signaling TGF-β receptor-initiated signaling can also be implicated in an antiproliferative effect in some cell types (Siegel and Massagué, 2003).

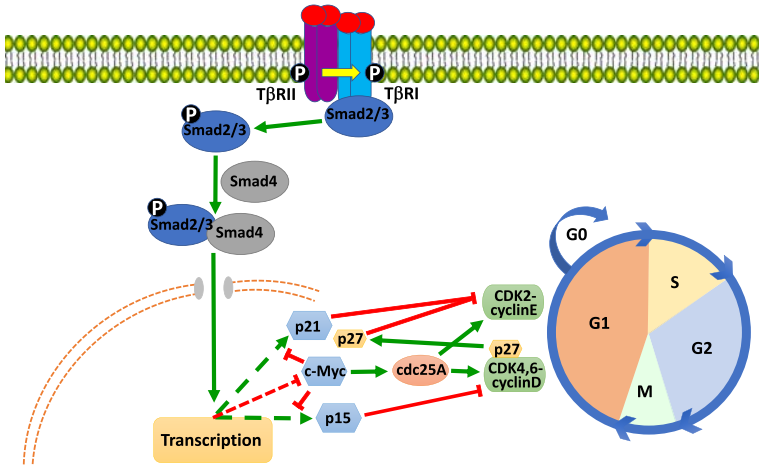

Fig. 3 Gene regulation in TGF-β-induced cell cycle arrest. TGF-β receptor activation leads to Smad2/3 phosphorylation. Phosphorylated Smad2/3 then binds Smad4, and the Smad2/3–Smad4 complex translocates to the nucleus to modulate transcription. c-Myc and cdc25A genes are repressed, while p15INK4[b] and p21CIP/WAF1 expression can be induced by TGF-β and cause a G1 arrest of the cell cycle. Abbreviations: CDK, cyclin dependent protein kinase; TβR, TGF-β receptor kinase; TGF-β, transforming growth factor-β.

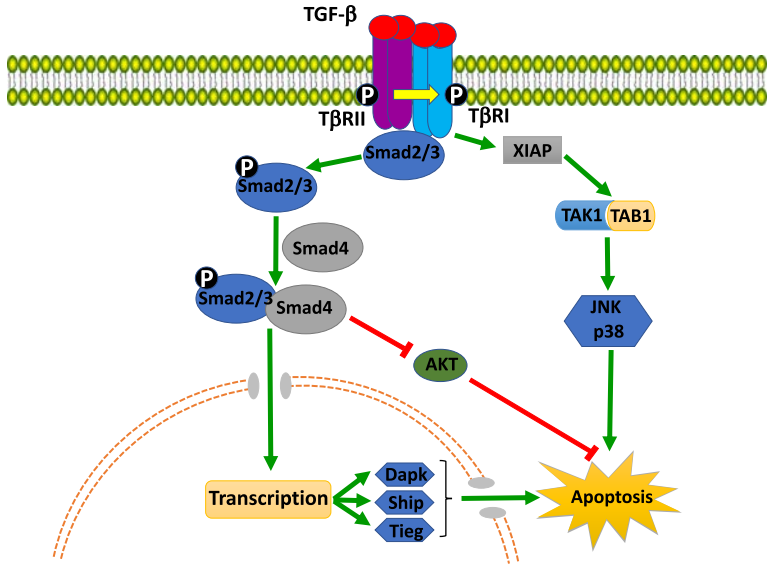

Fig. 4 TGF-β-induced cell apoptosis. TGF-β promotes the activation of Smads and the expression of pro-apoptotic genes such as Dapk, Ship and Tieg. Smads also bind and inactivate the survival kinase AKT, thereby inducing apoptosis. TGF-β-induced activation of the JNK and p38 pathways can also lead to apoptosis. TGF-β can also induce, via the adaptor XIAP, the activation of the TAK1-TAB complex, leading to JNK or p38 activation, both of which can lead to apoptosis. Abbreviations: Dapk, death associated protein kinase; Ship, SH2-containing inositol phosphatase; TAB1, TAK1 binding protein; TAK1, TGF-β activating kinase; TGF-β, transforming growth factor-β; Tieg, TGF-β-inducible early-response gene; XIAP, X-chromosome-linked inhibitor of apoptosis.

TGF-β-Induced Apoptosis

TGF-β can induce cell apoptosis in normal epithelial (and some premalignant) cells (Fig. 4). Several apoptotic regulators have been implicated downstream of the TGF-β signaling pathway, often in a cell- or tissue-specific manner (Ozaki et al., 2011). Stimulation and inhibition of expression of pro-apoptotic genes such as Ship and Tieg have been implicated in TGF-β-induced apoptosis (Moustakas and Heldin, 2005). In liver cancer cells, the Daxx adaptor protein couples the TGF-β signaling pathway to cell death machinery through its interaction with the type II TGF-β receptor (TβRII) (Perlman et al., 2001). In liver cancer cells, TGF-β can induce the expression of the death-associated protein kinase DAPK, which promotes cell death (Jang et al., 2002). In addition, TGF-β-induced activation of TGF-β-activated kinase-1 (TAK-1), a protein of the MAPKKK family, which activates p38 and JNK signaling, has been shown to be involved in TGF-β-induced apoptosis (Kokkinakis et al., 2004). TGF-β can also induce apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells (Wang et al., 2008) (Fig. 4).

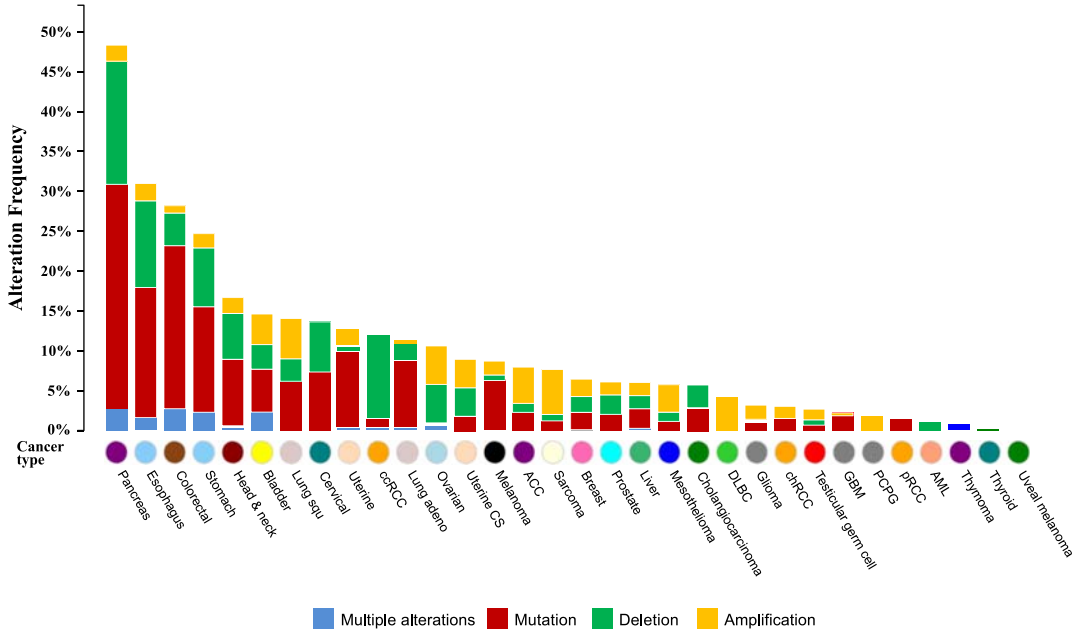

Fig. 5 Frequency of genetic alterations in *TGFBR1*, *TGFBR2*, *Smad2*, *Smad3* and *Smad4* by cancer type. The graph displays the frequency of genetic alterations (point mutations, deletions, amplifications, or multiple alterations) in *TGFBR1*, *TGFBR2*, *Smad2*, *Smad3* and *Smad4* in different types of cancer. Data were derived from TCGA datasets (The Cancer Genome Atlas, cancergenome.nih.gov/) at the time of this writing. Analysis was done using cBioPortal (www.cbioportal.org/).

Mutation in TGF-β Signaling Components in Cancer

Analysis from clinical tumor samples reveals that TGF-mediated signaling is indeed strongly implicated in the regulation of cancer (Wels et al., 2008). Recent studies have shown that in various human tumor types, components of the TGF-β signaling pathway, namely, *TGFBR2*, *TGFBR1*, *SMAD2*, *SMAD3* and *SMAD4*, are commonly inactivated through mutation (Macias et al., 2015; Massagué, 2008). Multiple genetic alterations in genes encoding central components in the TGF-β signaling pathway are found in human cancers of various origin, in particular in pancreatic, esophagus, colorectal and head and neck cancer (**Fig. 5**) (Derynck et al., 2001). Indeed, *TGFBR2*-inactivating mutations in its poly A gene tract are frequently found in cancers associated with microsatellite instability (MSI) (Alvi et al., 2001). Smad point mutations associated with cancer are loss-of-function mutations that either target functional elements or affect the overall stability of the protein. Studies in cultured cells have shown that these inactivating mutations mediate an escape from TGF-β-induced growth arrest and apoptosis. In addition to the known mutations in the TGF-β receptors and Smad pathway, other types of (epi)genetic alterations may also affect TGF-β signaling and tumor formation (Siegel and Massagué, 2003). For example, oncogenic activation of the Ras-Raf-MAPK pathway and c-Jun NH2-terminal kinase in hepatocellular carcinoma has been reported to induce phosphorylation of the Smad3 linker domain by MAPK, further preventing C-terminal phosphorylation of Smad by the TβRI kinase domain and thereby inhibiting the TGF-β cytostatic effects (Nagata et al., 2009).

TGF-β as a Tumor Promotor

TGF-β-Induced EMT and Invasion

In the late stage of tumor progression, TGF-β switches from a tumor suppressor to a tumor promotor by inducing EMT, tumor invasion, distant dissemination, angiogenesis and immune evasion (Meulmeester and ten Dijke, 2011; Seoane and Gomis, 2017; Drabsch and ten Dijke, 2012; Huang and Blobe, 2016). During EMT, the tumor cells dedifferentiate from an epithelial phenotype into a mesenchymal phenotype, upon which they gain higher migratory and invasive abilities. Moreover, they acquire cancer stem cell (CSC) properties and become more resistant to detachment-induced apoptosis (Thiery et al., 2009). During EMT, tumor cells downregulate genes encoding epithelial markers, for example, Epithelial (E)-cadherin, Occludin and Zonula occludens-1 (ZO-1), upregulate genes encoding mesenchymal markers, for example, N-cadherin, Vimentin and α-smooth muscle actin (SMA), and dissolve tight junctions. These EMT changes greatly facilitate tumor cell invasion (**Fig. 6**A) (Nieto et al., 2016; Lamouille et al., 2014). In response to TGF-β, the Smad complex directly increases the expression of multiple EMT-transcription factors including Zeb, Twist and Snail family proteins by activating their promotors. In addition, in combination with Zeb2 or Snail1, the Smad complex suppresses the transcription of genes encoding E-cadherin and Occludin, conferring a mesenchymal trait to tumor cells (Comijn et al., 2001; Vincent et al., 2009). In addition, Smad4 binding enhances the promoter activity of miR-155. Increased

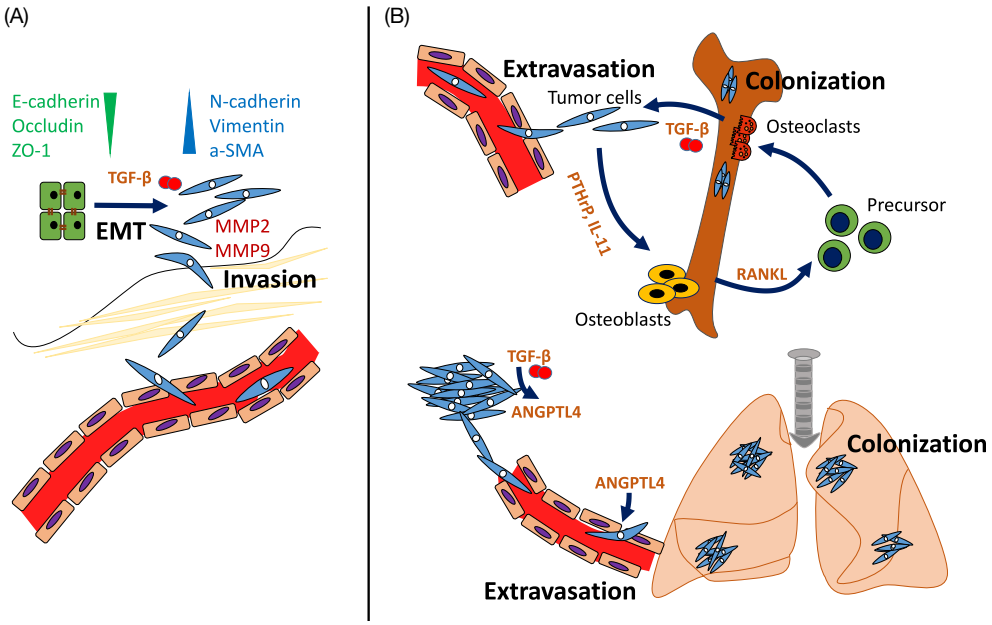

Fig. 6 TGF-β-induced EMT, invasion and metastasis. (A) TGF-β induces the EMT of epithelial cells by decreasing levels of epithelial makers (in *green*) and increasing mesenchymal markers (in *blue*). TGF-β also promotes the secretion of MMP2 and MMP9, thereby further conferring an invasive ability to tumor cells. (B) Bone-derived TGF-β increases the secretion of PTHrP, which activates osteoclast activity through interacting with RANKL, thereby promoting osteolytic metastasis. IL-11 and CTGF are also key modulators induced by TGF-β in this process. Osteolysis leads to more local TGF-β release, leading to the formation of a positive feedback loop. Moreover, TGF-β-driven ANGPTL4 secretion plays a vital role in disrupting junctions between pulmonary endothelial cells and contributes to seeding metastases to the lung. Abbreviations: ANGPTL4, angiopoietin-like 4; EMT, epithelial to mesenchymal transition; MMP, matrixmetalloproteinase; SMA, smooth muscle actin; RANKL, receptor activator of nuclear factor κB ligand; TGF-β, transforming growth factor-β.

expression of miR-155 dissolves tight junctions by targeting RhoA mRNA and downregulates E-cadherin expression by inhibiting the transcriptional activator C/EBPβ (Kong et al., 2008; Johansson et al., 2013). LncRNA-ATB, a long noncoding RNA activated by TGF-β, serves as a sponge for the miR-200 family, which restrains protein levels of Zeb1/2, thereby promoting EMT and hepatocellular carcinoma progression (Yuan et al., 2014). In combination with the Smad-dependent pathway, Smad-independent pathways also serve as promoters in TGF-β-induced EMT (Zhang, 2017; Lamouille et al., 2014; Moustakas and Heldin, 2016). Activation of proto-oncogenes such as Ras and receptor tyrosine kinase pathways cooperate with the TGF-β pathway to promote EMT (Janda et al., 2002; Zhang et al., 2013a). By directly modulating the activity of AP1 transcription factors that can partner with Smads through their phosphorylation or by phosphorylation of R-Smads in their linker region, the ERK, p38 and JNK MAPK pathways play key roles in TGF-β-induced EMT and tumor invasion (Bakin et al., 2002; Yamashita et al., 2006; Zhang et al., 2013b; Kretzschmar et al., 1999). In addition, PI3K/AKT signaling participates in TGF-β-triggered EMT by activating mTOR and EMT-related transcription factors such as Snail and Twist1 (Lamouille and Derynck, 2007; Zhou et al., 2004; Julien et al., 2007; Xue et al., 2012). Activation of Rho family GTPases including RhoA, Rac1, and Cdc42 by TGF-β receptors can contribute to cell–cell junction dissolution and cytoskeletal reorganization, which are important determinants for EMT (Ozdamar et al., 2005; Bhowmick et al., 2001; Menezes et al., 2016).

Local invasion through the surrounding ECM and stromal cell layers is the first step of the invasion-metastasis cascade (Hanahan and Weinberg, 2011). Evidence from human cancer specimens suggests that coexpression of Smad3/4 and Snail is correlated with loss of E-cadherin and coxsackie and adenovirus receptor (CAR), a tight junction-associated cell adhesion molecule, at the invasive front (Vincent et al., 2009). Apart from conferring EMT properties to cancer cells, TGF-β induces the expression and secretion of matrix metalloproteinases 2/9 (MMP2/9) in tumor cells or/and stromal cells (e.g., myofibroblasts). These two proteinases promote the proteolysis of ECM and collagens, leading to the invasion of tumor cells into their stromal compartment (**Fig. 6**A) (Wiercinska et al., 2011; Stuelten et al., 2005). In addition, TGF-β employs miR-181b to inhibit the protein level of TIMP3, an inhibitor of metalloprotease. The latter results in an enhancement of the activity of MMP2 and MMP9 and promotes the invasion of hepatocellular carcinoma cells (Wang et al., 2010).

TGF-β-Induced Metastasis to Bone, Lung and Other Organs

The metastasis of cancer cells from the primary site to distant organs contributes to the death of most cancer patients (Valastyan and Weinberg, 2011). Bone metastasis is a common event in specific cancer types, including breast, lung and prostate cancers. The interaction between disseminated tumor cells and resident skeletal cells disrupts bone integrity, conferring a receptive microenvironment

for the outgrowth of metastatic tumor cells (Mundy, 2002; Suva et al., 2011). Bone-derived TGF-β can act on breast cancer cells and promotes the activation of the Smad-dependent pathway, which increases the secretion of parathyroid hormone-related protein (PTHrP), a major osteoclastogenic factor. PTHrP subsequently activates osteoclast activity by interacting with receptor activator of nuclear factor κB ligand (RANKL), thereby promoting bone metastasis (**Fig. 6**B) (Suva et al., 2011; Yin et al., 1999; Kakonen et al., 2002; Lacey et al., 1998). By employing *in vivo* selection of highly metastatic cell lines and functional imaging, Kang et al. identified a bone metastasis gene set signature that includes *chemokine receptor CXCR4*, *interleukin 11* (*IL-11*) and *connective tissue growth factor* (*CTGF*), which contribute to metastasis by mediating the homing of breast cancer cells to bone, osteolysis and angiogenesis, respectively (Kang et al., 2003; Kang et al., 2005). IL-11 and CTGF are induced by TGF-β. Bone degraded by cancer cells in turn increases the secretion of stored factors including TGF-β, thus forming a positive feedback loop called a "vicious cycle" (Zheng et al., 2016).

Apart from contributing to the spreading to bone, TGF-β signaling also contributes to seeding metastases to the lung. A TGF-β-induced gene expression signature in estrogen receptor (ER)-negative breast cancer cells was found to correlate with the potential to form lung metastases. Blockade of TGF-β signaling impaired the extravasation of ER-negative breast cancer cells through the tight endothelial junctions in lung capillaries, while prior and transient treatment of TGF-β increased the metastatic abilities of tumor cells (Padua et al., 2008). The expression of the TGF-β-driven adipokine *angiopoietin-like 4* (*ANGPTL4*) was identified to play a vital role in the disruption of junctions between the pulmonary endothelial cells (**Fig. 6**B). However, bone metastasis was not affected by TGF-β preincubation or ANGPTL4 knockdown, which can be explained by the difference in the microvasculature of these two organs (Welm, 2008).

TGF-β also participates in the metastatic growth of tumor cells to the liver (Kang et al., 2011; Tu et al., 2015). Upon extravasating into liver parenchyma, TGF-β released by colorectal cancer cells acts on surrounding hepatic satellite cells (HSCs) and promotes the transformation of HSCs into myofibroblasts. Tumor-associated myofibroblasts in turn increase the expression of SDF-1 and hepatic growth factor (HGF), which triggers the metastatic growth of tumor cells (Liu et al., 2013).

Stimulation of Angiogenesis and Immune Evasion by TGF-β

Angiogenesis, the formation of new blood vessels, is indispensable for solid tumors larger than 2–3 mm^3 to obtain oxygen and nutrients, remove waste products and spread through the circulatory system (Carmeliet and Jain, 2000). An elevated level of TGF-β in plasma is correlated with an increase in tumor angiogenesis and poor clinical outcome in many cancer types (Ito et al., 1995; Ivanovic et al., 1995; Wikstrom et al., 1998; Hasegawa et al., 2001). TGF-β can directly activate endothelial cells by activating the TGF-β/ALK1 signaling pathway (de Vinuesa et al., 2016). The coreceptor endoglin, which is highly expressed on activated endothelial cells, can promote this signaling response (Paauwe et al., 2013). Moreover, in the tumor niche with low oxygen, hypoxia and TGF-β signaling can cooperate to initiate an angiogenic program in tumor cells. Mechanistically, hypoxia-induced HIF-1, in cooperation with Smad3, enhances the transcription of *vascular endothelial growth factor* (*VEGF*), which is of pivotal importance in capillary formation and the migration of endothelial cells, thereby promoting tumor angiogenesis (Pertovaara et al., 1994; Sanchez-Elsner et al., 2001). Silencing Smad2 (in contrast to Smad3 depletion) in the MDA-MB-231 breast cancer cell line enhanced TGF-β-induced VEGF secretion *in vitro* and promoted the formation of bone metastases *in vivo* (Petersen et al., 2010). TGF-β also enhances the transcription of *CTGF*, another key angiogenic factor, in breast cancer cells with high bone metastatic potential (Kang et al., 2003).

In addition to supporting EMT, invasion, metastasis and tumor angiogenesis, TGF-β also contributes to tumor progression by stimulating tumor evasion from immune surveillance. CD8+ cytotoxic T cells are a cell population that can induce cancer cell apoptosis. TGF-β represses the transcription of *granzyme*, *perforin* and *interferon-γ* through Smad and ATF1 in CD8+ T cells, thereby inhibiting its cytotoxic activity (Thomas and Massagué, 2005; Ahmadzadeh and Rosenberg, 2005). TGF-β can also induce the differentiation of regulatory T-cells (Tregs), which further suppress the proliferation and activation of CD8+ cytotoxic T-cells, resulting in immunosuppression and a decrease in immunosurveillance (Fu et al., 2004; Liu et al., 2008; Somasundaram et al., 2002). The activation of natural killer (NK) cells, another cytotoxic cell type, is attenuated by TGF-β-induced downregulation of IL-15 and NKG2D, an activating receptor of NK cells (Wilson et al., 2011; Crane et al., 2010). In addition, TGF-β-triggered miR-183 represses the DAP12 protein level, leading to the destabilization of the NK receptor and inhibition of cytotoxicity (Donatelli et al., 2014). In addition, TGF-β is a driver for the transition of the tumor-suppressive M1 macrophage phenotype into the tumor-promoting M2 phenotype, thereby promoting the production of tumor-promoting factors and inhibiting the activity of T cells (Sica et al., 2006; Gong et al., 2012).

Targeting the TGF-β Signaling Pathway in Cancer

Due to the strong pro-oncogenic effects of TGF-β, TGF-β inhibitory targeting agents have been developed, including antisense oligonucleotides (AON), small molecule receptor kinase inhibitors and neutralizing antibodies. The mechanisms of these inhibitors involve inhibition of TGF-β and receptor expression, interference in receptor kinase signaling, and blockade of TGF-β ligand and receptor binding (**Fig. 7**, **Table 1**). These agents have been tested in the preclinical and clinical stages. While inhibiting tumor progression by blocking the TGF-β signaling pathway is a promising approach, the biphasic action of TGF-β in cancer progression and its multifunctionality make it a challenging target.

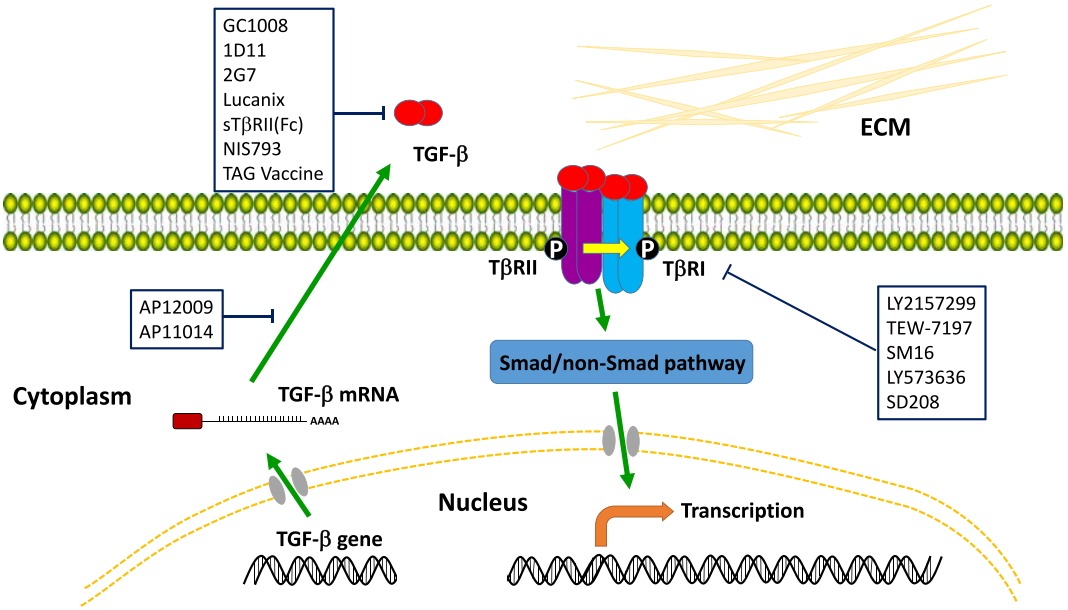

Fig. 7 Targeting TGF-β in cancer. The TGF-β signaling pathway has an important effect on tumor progression and provides a new approach for tumor targeting therapy. Many inhibitors of the TGF-β pathway (including kinase inhibitors, AON, and antibodies) have already been applied in preclinical and clinical trials (see **Table 1**). Abbreviations: ECM, extracellular matrix; TβR, TGF-β receptor; TGF-β, transforming growth factor-β.

Table 1 Overview of clinical trials with TGF-β targeting agents

Stage	*Drug*	*Type*	*Target*	*Disease*	*Clinical trial identifier*
Phase 1	AP 12009	AON	TGF-β2	Multiple cancers	NCT00844064
	TAG Vaccine	Vaccine therapy	TGF-β2 and immune	Carcinoma/advanced metastatic	NCT00684294
	TEW-7197	Kinase inhibitor	TβRI	Advanced stage solid tumors	NCT02160106
	NIS793	Antibody	TGF-β	Multiple cancers	NCT02947165
	Paclitaxel/Carboplatin + Galunisertib	Kinase inhibitor	TβRI	Carcinosarcoma, ovarian	NCT03206177
	TEW-7197	Kinase inhibitor	TβRI	Multiple myeloma	NCT03143985
	LY2157299	Kinase inhibitor	TβRI	Multiple myeloma	NCT00689507
	LY573636	Kinase inhibitor	TβRI	Hematopoietic malignancies	NCT00718159
	Fresolimumab(CG1008)	Antibody	TGF-β2	Multiple cancers	NCT00356460
Phase 1| Phase 2	Fresolimumab(CG1008)	Antibody	TGF-β2	Multiple cancers	NCT02581787
	LY2157299	Kinase inhibitor	TβRI	Multiple cancers	NCT02423343
	LY2157299	Kinase inhibitor	TβRI	Glioma	NCT01220271
Phase 2	LY2157299	Kinase inhibitor	TβRI	Prostate cancer	NCT02452008
	Lucanix (belagen-pumatucel)	Vaccine therapy	TGF-β2 and immune	Multiple cancers	NCT01058785
	PF03446962	Antibody	ALK1	Transitional cell carcinoma of bladder	NCT01620970
	Fresolimumab(CG1008)	Antibody	TGF-β2	Primary brain tumors	NCT01472731
	LY2157299	Kinase inhibitor	TβRI	Metastatic breast cancer	NCT02538471
	LY2157299	Kinase inhibitor	TβRI	Rectal adenocarcinoma	NCT02688712
	AP 12009	AON	TGF-β2	Glioblastoma/anaplastic astrocytoma	NCT00431561
	Fresolimumab(CG1008)	Antibody	TGF-β2	Pleural malignant mesothelioma	NCT01112293
	Fresolimumab(CG1008)	Antibody	TGF-β2	Renal cell carcinoma	NCT00923169
	LY2157299	Kinase inhibitor	TβRI	Hepatocellular carcinoma	NCT02178358
	Fresolimumab(CG1008)	Antibody	TGF-β2	Metastatic breast cancer	NCT01401062
	LY2157299	Kinase inhibitor	TβRI	Hepatocellular carcinoma	NCT01246986
	LY573636	Kinase inhibitor	TβRI	Melanoma	NCT00383292
	LY573636	Kinase inhibitor	TβRI	Non-small cell lung carcinoma	NCT00363766

Table 1 Overview of clinical trials with TGF-β targeting agents—cont'd

Stage	*Drug*	*Type*	*Target*	*Disease*	*Clinical trial identifier*
	Fresolimumab(CG1008)	Antibody	TGF-β2 and immune response	Kidney cancer	NCT00899444
Phase 3	AP 12009	AON	TGF-β2	Anaplastic Astrocytoma| glioblastoma	NCT00761280
	Lucanix	Vaccine therapy	TGF-β2 and immune	Non-small cell lung cancer	NCT00676507
Phase 4	Vitamin D3		TGF-β1	Multiple cancers	NCT02460380
Preclinical					
	LY2109761	Kinase inhibitor	TβRI/TβRII	Pancreatic cancer	
	SD208	Kinase inhibitor	TβRI	Melanoma	
	SM16	Kinase inhibitor	TβRI	Multiple cancers	
	TGFBRII Antibody	Antibody	TβRII	Multiple cancers	
	sTβRII (Fc)	Ligand Trap	TβRII	Multiple cancers	
	sBetaglycan	Ligand Trap	Betaglycan	Multiple cancers	
	1D11	Antibody	TGF-β1/2/3	Multiple cancers	
	2G7	Antibody	TGF-β1/2/3	Multiple cancers	

Data from www.clinicaltrials.gov

Antisense Oligonucleotides

The binding of ligands and receptors is the first step in activating the TGF-β signaling pathway; antisense oligonucleotides (AON) that promote TGF-β mRNA degradation and lead to a decreased biosynthesis of TGF-β have been developed (**Fig. 7**) (Crooke, 1999). The antisense RNA drugs AP12009 and AP11014 targeting TGF-β2 and TGF-β1, respectively, have been used in (pre)clinical cancer treatment studies. AP12009 has been reported to inhibit neovascularization and tumor invasion and has been used to treat high-grade glioma and anaplastic astrocytoma patients (Bogdahn et al., 2008; Hau et al., 2007; Nagaraj and Datta, 2010). In addition, AP11014 has been reported to display an anti-tumor effect in animal models for colon cancer, prostate cancer and lung cancer and is currently being studied in preclinical research (Bogdahn et al., 2010; Schlingensiepen et al., 2004).

TGF-β Receptor Kinase Inhibitors

Small ATP-mimetic compounds have been identified that selectively inhibit TβRI (and TβRII) kinase activity (**Fig. 7**). These compounds have been tested in preclinical and clinical studies of a wide range of cancer types. Systemic administration of the TβRI kinase inhibitor SD208 can increase the median survival of mice with malignant glioma vaccination (Mohammad et al., 2011) and reduce tumor metastasis in pancreatic cancer and breast cancer (Gaspar et al., 2007; Ge et al., 2006). LY2157299 is the first TGF-β kinase inhibitor that has been reported to inhibit primary tumor growth in breast and lung cancer cell lines (Bueno et al., 2008; Herbertz et al., 2015). To optimize the applicability of LY2157299 to tumor therapy, a first-in-human dose evaluation found that LY2157299 administration at 300 mg per day is safe (Rodon et al., 2015). Another kinase inhibitor, LY2109761, inhibits both the activity of AKL5 and TβRII. A large number of studies have indicated that LY2109761 exhibited great potential in the prevention of tumor metastasis, including colon (Zhang et al., 2010) and pancreatic cancer (Melisi et al., 2008), glioblastoma (Zhang et al., 2011) and ovarian cancer (Alsina-Sanchis et al., 2016; Gao et al., 2015). TEW-7197 is an orally administered small molecule that targets TβRI kinase activity. It stimulated apoptosis and suppressed TGF-β-induced activation of Smad2/3 in human and murine MM cells *in vitro*, leading to the inhibition of MM cell growth and viability (Kim et al., 2017b). While the preclinical results of these studies are encouraging, the clinical translation has been difficult. On-target side effects on the cardiovascular system have halted clinical advancement. By using an intermittent dosing strategy, these adverse side effects may be overcome (Colak and ten Dijke, 2017; Herbertz et al., 2015).

Antibodies Against TGF-β Ligands and Extracellular Domains of TGF-β Receptors

1D11, an antibody that recognizes all three TGF-β isoforms, interferes with TGF-β-receptor binding and, thus, neutralizes (**Fig. 7**) TGF-β activity. This antibody has been used in (pre)clinical studies. 1D11 significantly increases NK cell and nuclear T cell invasion, as well as NKG2D expression and cytotoxic perforin and granzyme B release in breast cancer, thereby enhancing the anti-tumor effect of CD8+ T cells and NK cells (Nam et al., 2008). Additionally, 1D11 has also been found to suppress bone metastasis in prostate cancer (Sturge et al., 2011). Like 1D11, 2G7 also inhibited the invasion of MDA-MB-231 cells (a human breast cancer cell line). Additionally, the combination of dendritic cell (DC)-based vaccines and 2G7 potently inhibited the development of established murine mammary tumors (Kobie et al., 2003; Nam et al., 2006). A clinical trial of another monoclonal antibody, fresolimumab (CG1008), which inhibits all three TGF-β isoforms, demonstrated its safety and efficacy in improving metastatic melanoma and renal cell carcinoma (Morris et al., 2014). Fresolimumab may help to stabilize the condition of patients during malignant

pleural mesothelioma therapy. Importantly, adverse effects, such as skin toxicity (including formation of cutaneous squamous-cell carcinomas and basal cell carcinoma), have been reported in cancer patients after treatment with fresolimumab (Morris et al., 2014).

Similar to neutralizing antibodies, soluble TβRII and TβRIII ligand traps are also used to block the TGF-β signaling pathway. These molecules are expressed in the extracellular domain of the receptor, which prevent ligands from binding to TGF-β receptors (Drabsch and ten Dijke, 2012). The ligand trap TβRII:Fc (a fusion of the extracellular TGF-β-binding domain of TβRII with IgG1 Fc domain) showed anti-tumor effects on multiple cancers, including inhibition of mesothelioma growth and suppression of breast cancer cell viability and migration (Muraoka et al., 2002; Suzuki et al., 2004). The expression of soluble TβRIII (sBetaglycan) could effectively suppress tumor growth in human breast carcinoma MDA-MB-231 xenograft-bearing athymic nude mice (Bandyopadhyay et al., 2002) and inhibit glioma and non-small cell lung cancer progression in other mouse models (Finger et al., 2008; Naumann et al., 2008). Due to the risk of tumor development caused by the TGF-β soluble receptor (Huntley et al., 2004; Tang et al., 2003), these receptors have not yet entered clinical research.

Targeting the TGF-β signaling pathway provides a new approach and opportunity for tumor therapy. Since the TGF-β pathway is also involved in many normal biological functions, the exact mechanism of action in the patients and the adverse reactions caused by systemic inhibition of TGF-β are still not clear. A further understanding of the dual roles of TGF-β will be beneficial to the development of therapeutics specifically targeting TGF-β in tumor progression. Sole treatment with TGF-β-targeting agents will likely not be successful in curing cancer patients, and a combination of TGF-β targeting therapies with chemo- and radiotherapy or other forms of targeted therapy should be explored.

Concluding Remarks

TGF-β has a dual action in cancer by acting as a tumor suppressor in early stages and a tumor promotor in the late phases of tumor progression. Cancer cells are insensitive to the cytostatic effects of TGF-β through the activation of proto-oncogenes and inactivation of tumor suppressor genes. The latter (epi)genetic changes also cooperate with TGF-β to mediate EMT, thereby facilitating invasion and metastasis. Moreover, TGF-β promotes tumorigenesis by stimulating immune evasion and promoting angiogenesis. The biphasic role in cancer and its multifunctional properties in the maintenance of tissue homeostasis make TGF-β a challenging pathway to target for treatment of cancer patients. A more detailed understanding of the mechanism of action in cancer patients, careful dosing and the selection of patients who will most benefit from the TGF-β targeting agents will be important for their clinical implementation.

References

Ahmadzadeh, M., Rosenberg, S.A., 2005. TGF-β1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. Journal of Immunology 174, 5215–5223.

Akhurst, R.J., 2017. Targeting TGF-β Signaling for therapeutic gain. Cold Spring Harbor Perspectives in Biology 9, a022301.

Alsina-Sanchis, E., Figueras, A., Lahiguera, Á., Vidal, A., Casanovas, O., Graupera, M., Villanueva, A., Viñals, F., 2016. The TGF-β pathway stimulates ovarian cancer cell proliferation by increasing IGF1R levels. International Journal of Cancer 139, 1894–1903.

Alvi, A.J., Rader, J.S., Broggini, M., Latif, F., Maher, E.R., 2001. Microsatellite instability and mutational analysis of transforming growth factor β receptor type II gene (TGFBR2) in sporadic ovarian cancer. Molecular Pathology 54, 240–243.

Annes, J.P., Chen, Y., Munger, J.S., Rifkin, D.B., 2004. Integrin αVβ6-mediated activation of latent TGF-β requires the latent TGF-β binding protein-1. The Journal of Cell Biology 165, 723–734.

Arsura, M., Panta, G.R., Bilyeu, J.D., Cavin, L.G., Sovak, M.A., Oliver, A.A., Factor, V., Heuchel, R., Mercurio, F., Thorgeirsson, S.S., Sonenshein, G.E., 2003. Transient activation of NF-κB through a TAK1/IKK kinase pathway by TGF-β1 inhibits AP-1/SMAD signaling and apoptosis: Implications in liver tumor formation. Oncogene 22, 412–425.

Ayyaz, A., Attisano, L., Wrana, J.L., 2017. Recent advances in understanding contextual TGFβ signaling. F1000Research 6.

Bakin A.V., Rinehart C., Tomlinson A.K., Arteaga C.L. (2002). p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration. Journal of Cell Science 115, 3193-206.

Bandyopadhyay, A., López-Casillas, F., Malik, S.N., Montiel, J.L., Mendoza, V., Yang, J., Sun, L., 2002. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. Cancer Research 62, 4690–4695.

Barrios-Rodiles, M., Brown, K.R., Ozdamar, B., Bose, R., Liu, Z., Donovan, R.S., Shinjo, F., Liu, Y., Dembowy, J., Taylor, I.W., Luga, V., Przulj, N., Robinson, M., et al., 2005. High-throughput mapping of a dynamic signaling network in mammalian cells. Science 307, 1621–1625.

Batut, J., Schmierer, B., Cao, J., Raftery, L.A., Hill, C.S., Howell, M., 2008. Two highly related regulatory subunits of PP2A exert opposite effects on TGF-β/Activin/Nodal signalling. Development 135, 2927–2937.

Bhowmick, N.A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C.A., Engel, M.E., Arteaga, C.L., Moses, H.L., 2001. Transforming growth factor-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Molecular Biology of the Cell 12, 27–36.

Bogdahn, U., Mahapatra, A., Olyushin, V., Parfenov, V., Stockhammer, G., Ludwig, S., Wuerth, G., Heinrichs, H., Schlingensiepen, K., 2008. Results of a phase IIb active-controlled study with AP 12009 for patients with recurrent or refractory anaplastic astrocytoma. Journal of Clinical Oncology 26, 2076.

Bogdahn, U., Hau, P., Stockhammer, G., Venkataramana, N., Mahapatra, A., Suri, A., Balasubramaniam, A., Nair, S., Oliushine, V., Parfenov, V., 2010. Targeted therapy for high-grade glioma with the TGF-β2 inhibitor trabedersen: Results of a randomized and controlled phase IIb study. Neuro-Oncology 13, 132–142.

Budi, E.H., Duan, D., Derynck, R., 2017. Transforming growth factor-β receptors and Smads: Regulatory cmplexity and functional versatility. Trends in Cell Biology 27, 658–672.

Bueno, L., de Alwis, D.P., Pitou, C., Yingling, J., Lahn, M., Glatt, S., Trocóniz, I.F., 2008. Semi-mechanistic modelling of the tumour growth inhibitory effects of LY2157299, a new type I receptor TGF-β kinase antagonist, in mice. European Journal of Cancer 44, 142–150.

Carmeliet, P., Jain, R.K., 2000. Angiogenesis in cancer and other diseases. Nature 407, 249–257.

Chandrasinghe, P., Cereser, B., Moorghen, M., Al Bakir, I., Tabassum, N., Hart, A., Stebbing, J., Warusavitarne, J., 2017. Role of SMAD proteins in colitis-associated cancer: From known to the unknown. Oncogene in press.

Cheifetz, S., Weatherbee, J.A., Tsang, M.L.-S., Anderson, J.K., Mole, J.E., Lucas, R., Massagué, J., 1987. The transforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. Cell 48, 409–415.
Chen, C.-R., Kang, Y., Siegel, P.M., Massagué, J., 2002. E2F4/5 and p107 as Smad cofactors linking the TGFβ receptor to c-myc repression. Cell 110, 19–32.
Chen, H.B., Shen, J., Ip, Y.T., Xu, L., 2006. Identification of phosphatases for Smad in the BMP/DPP pathway. Genes & Development 20, 648–653.
Chen, Y., Xiao, Y., Ge, W., Zhou, K., Wen, J., Yan, W., Wang, Y., Wang, B., Qu, C., Wu, J., Xu, L., Cai, W., 2013. miR-200b inhibits TGF-β1-induced epithelial-mesenchymal transition and promotes growth of intestinal epithelial cells. Cell Death & Disease 4, e541.
Colak, S., ten Dijke, P., 2017. Targeting TGF-β signaling in cancer. Trends Cancer 3, 56–71.
Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huylebroeck, D., van Roy, F., 2001. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Molecular Cell 7, 1267–1278.
Costanza, B., Umelo, I.A., Bellier, J., Castronovo, V., Turtoi, A., 2017. Stromal modulators of TGF-β in cancer. Journal of Clinical Medicine 6, 7.
Crane, C.A., Han, S.J., Barry, J.J., Ahn, B.J., Lanier, L.L., Parsa, A.T., 2010. TGF-β downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients. Neuro-Oncology 12, 7–13.
Crooke, S.T., 1999. Molecular mechanisms of action of antisense drugs. Biochimica et Biophysica Acta 1489, 31–43.
Dallas, S.L., Sivakumar, P., Jones, C.J., Chen, Q., Peters, D.M., Mosher, D.F., Humphries, M.J., Kielty, C.M., 2005. Fibronectin regulates latent transforming growth factor-β (TGF-β) by controlling matrix assembly of latent TGFβ-binding protein-1. The Journal of Biological Chemistry 2280, 18871–18880.
Davis, B.N., Hilyard, A.C., Lagna, G., Hata, A., 2008. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 454, 56–61.
Derynck, R., Zhang, Y.E., 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature 425, 577–584.
Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C.-H., Kern, S.E., Massagué, J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B., 1996. Nomenclature: Vertebrate mediators of TGFβ family signals. Cell 87, 173.
Derynck, R., Akhurst, R.J., Balmain, A., 2001. TGF-β signaling in tumor suppression and cancer progression. Nature Genetics 29, 117–129.
ten Dijke, P., Hill, C.S., 2004. New insights into TGF-β-Smad signalling. Trends in Biochemical Sciences 29, 265–273.
ten Dijke, P., Goumans, M.J., Itoh, F., Itoh, S., 2012. Regulation of cell proliferation by Smad proteins. Journal of Cellular Physiology 19, 1–16.
Donatelli, S.S., Zhou, J.M., Gilvary, D.L., Eksioglu, E.A., Chen, X., Cress, W.D., Haura, E.B., Schabath, M.B., Coppola, D., Wei, S., Djeu, J.Y., 2014. TGF-β-inducible microRNA-183 silences tumor-associated natural killer cells. Proceedings of the National Academy of Sciences of the United States of America 111, 4203–4208.
Dong, X., Zhao, B., Iacob, R.E., Zhu, J., Koksal, A.C., Lu, C., Engen, J.R., Springer, T.A., 2017. Force interacts with macromolecular structure in activation of TGF-β. Nature 542, 55–59.
Drabsch, Y., ten Dijke, P., 2012. TGF-β signalling and its role in cancer progression and metastasis. Cancer Metastasis Reviews 31, 553–568.
Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., Miyazono, K., 2001. Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. The Journal of Biological Chemistry 276, 12477–12480.
Fabregat, I., Fernando, J., Mainez, J., Sancho, P., 2014. TGF-β signaling in cancer treatment. Current Pharmaceutical Design 20, 2934–2947.
Feng, X.H., Derynck, R., 2005. Specificity and versatility in TGF-β signaling through Smads. Annual Review of Cell and Developmental Biology 21, 659–693.
Finger, E.C., Turley, R.S., Dong, M., How, T., Fields, T.A., Blobe, G.C., 2008. TβRIII suppresses non-small cell lung cancer invasiveness and tumorigenicity. Carcinogenesis 29, 528–535.
Fu, S., Zhang, N., Yopp, A.C., Chen, D., Mao, M., Chen, D., Zhang, H., Ding, Y., Bromberg, J.S., 2004. TGF-β induces Foxp3 + T-regulatory cells from CD4+ CD25− precursors. American Journal of Transplantation 4, 1614–1627.
Galat, A., 2011. Common structural traits for cystine knot domain of the TGFβ superfamily of proteins and three-fingered ectodomain of their cellular receptors. Cellular and Molecular Life Sciences 68, 3437–3451.
Gao, S., Alarcon, C., Sapkota, G., Rahman, S., Chen, P.Y., Goerner, N., Macias, M.J., Erdjument-Bromage, H., Tempst, P., Massagué, J., 2009. Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-β signaling. Molecular Cell 36, 457–468.
Gao, Y., Shan, N., Zhao, C., Wang, Y., Xu, F., Li, J., Yu, X., Gao, L., Yi, Z., 2015. LY2109761 enhances cisplatin antitumor activity in ovarian cancer cells. International Journal of Clinical and Experimental Pathology 8, 4923.
Gaspar, N.J., Li, L., Kapoun, A.M., Medicherla, S., Reddy, M., Li, G., O'Young, G., Quon, D., Henson, M., Damm, D.L., 2007. Inhibition of transforming growth factor β signaling reduces pancreatic adenocarcinoma growth and invasiveness. Molecular Pharmacology 72, 152–161.
Ge, R., Rajeev, V., Ray, P., Lattime, E., Rittling, S., Medicherla, S., Protter, A., Murphy, A., Chakravarty, J., Dugar, S., 2006. Inhibition of growth and metastasis of mouse mammary carcinoma by selective inhibitor of transforming growth factor-β type I receptor kinase in vivo. Clinical Cancer Research 12, 4315–4330.
Goetschy, J.F., Letourneur, O., Cerletti, N., Horisberger, M.A., 1996. The unglycosylated extracellular domain of type-II receptor for transforming growth factor-β. A novel assay for characterizing ligand affinity and specificity. European Journal of Biochemistry 241, 355–362.
Gong, D., Shi, W., Yi, S.J., Chen, H., Groffen, J., Heisterkamp, N., 2012. TGFβ signaling plays a critical role in promoting alternative macrophage activation. BMC Immunology 13, 31.
Goumans, M.J., ten Dijke, P., 2017. TGF-β Signaling in control of cardiovascular function. Cold Spring Harbor Perspectives in Biology 9, a022210.
Gulei, D., Mehterov, N., Ling, H., Stanta, G., Braicu, C., Berindan-Neagoe, I., 2017. The "good-cop bad-cop" TGF-β role in breast cancer modulated by non-coding RNAs. Biochimica et Biophysica Acta 186, 1661–1675.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: The next generation. Cell 144, 646–674.
Hasegawa, Y., Takanashi, S., Kanehira, Y., Tsushima, T., Imai, T., Okumura, K., 2001. Transforming growth factor-β1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. Cancer 91, 964–971.
Hau, P., Jachimczak, P., Schlingensiepen, R., Schulmeyer, F., Jauch, T., Steinbrecher, A., Brawanski, A., Proescholdt, M., Schlaier, J., Buchroithner, J., 2007. Inhibition of TGF-β2 with ap 12009 in recurrent malignant gliomas: From preclinical to phase I/II studies. Oligonucleotides 17, 201–212.
Hayashi H., Abdollah S., Qiu Y., Cai J., Xu Y.Y., Grinnell B.W., Richardson M.A., Topper J.N., Gimbrone M.A., Jr., Wrana J.L., Falb D. (1997). The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. Cell 89, 1165-73.
Heldin, C.H., Miyazono, K., ten Dijke, P., 1997. TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390, 465–471.
Herbertz, S., Sawyer, J.S., Stauber, A.J., Gueorguieva, I., Driscoll, K.E., Estrem, S.T., Cleverly, A.L., Desaiah, D., Guba, S.C., Benhadji, K.A., 2015. Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-β signaling pathway. Drug Design, Development and Therapy 9, 4479.
Hill, C.S., 2016. Transcriptional control by the SMADs. Cold Spring Harbor Perspectives in Biology 8, a022079.
Huang, J.J., Blobe, G.C., 2016. Dichotomous roles of TGF-β in human cancer. Biochemical Society Transactions 44, 1441–1454.
Huntley, S.P., Davies, M., Matthews, J.B., Thomas, G., Marshall, J., Robinson, C.M., Eveson, J.W., Paterson, I.C., Prime, S.S., 2004. Attenuated type II TGF-β receptor signalling in human malignant oral keratinocytes induces a less differentiated and more aggressive phenotype that is associated with metastatic dissemination. International Journal of Cancer 110, 170–176.
Ito, N., Kawata, S., Tamura, S., Shirai, Y., Kiso, S., Tsushima, H., Matsuzawa, Y., 1995. Positive correlation of plasma transforming growth factor-β1 levels with tumor vascularity in hepatocellular carcinoma. Cancer Letters 89, 45–48.
Itoh, S., Itoh, F., Goumans, M.J., ten Dijke, P., 2000. Signaling of transforming growth factor-β family members through Smad proteins. European Journal of Biochemistry 267, 6954–6967.
Ivanovic, V., Melman, A., Davis-Joseph, B., Valcic, M., Geliebter, J., 1995. Elevated plasma levels of TGF-β1 in patients with invasive prostate cancer. Nature Medicine 1 (4), 282.

Janda, E., Lehmann, K., Killisch, I., Jechlinger, M., Herzig, M., Downward, J., Beug, H., Grünert, S., 2002. Ras and TGFβ cooperatively regulate epithelial cell plasticity and metastasis. The Journal of Cell Biology 156, 299–314.
Jang, C.W., Chen, C.H., Chen, C.C., Chen, J.Y., Su, Y.H., Chen, R.H., 2002. TGF-β induces apoptosis through Smad-mediated expression of DAP-kinase. Nature Cell Biology 4, 51–58.
Jenkins, G., 2008. The role of proteases in transforming growth factor-β activation. The International Journal of Biochemistry & Cell Biology 40, 1068–1078.
Jobling, M.F., Mott, J.D., Finnegan, M.T., Jurukovski, V., Erickson, A.C., Walian, P.J., Taylor, S.E., Ledbetter, S., Lawrence, C.M., Rifkin, D.B., Barcellos-Hoff, M.H., 2006. Isoform-specific activation of latent transforming growth factor β (LTGF-β) by reactive oxygen species. Radiation Research 166, 839–848.
Johansson, J., Berg, T., Kurzejamska, E., Pang, M.F., Tabor, V., Jansson, M., Roswall, P., Pietras, K., Sund, M., Religa, P., Fuxe, J., 2013. MiR-155-mediated loss of C/EBPβ shifts the TGF-β response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer. Oncogene 32, 5614–5624.
Julien, S., Puig, I., Caretti, E., Bonaventure, J., Nelles, L., van Roy, F., Dargemont, C., de Herreros, A.G., Bellacosa, A., Larue, L., 2007. Activation of NF-κB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. Oncogene 26, 7445–7456.
Kakonen, S.M., Selander, K.S., Chirgwin, J.M., Yin, J.J., Burns, S., Rankin, W.A., Grubbs, B.G., Dallas, M., Cui, Y., Guise, T.A., 2002. Transforming growth factor-β stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. The Journal of Biological Chemistry 277, 24571–24578.
Kang, N., Gores, G.J., Shah, V.H., 2011. Hepatic stellate cells: Partners in crime for liver metastases? Hepatology 54, 707–713.
Kang, Y., Siegel, P.M., Shu, W., Drobnjak, M., Kakonen, S.M., Cordón-Cardo, C., Guise, T.A., Massagué, J., 2003. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3, 537–549.
Kang, Y., He, W., Tulley, S., Gupta, G.P., Serganova, I., Chen, C.R., Manova-Todorova, K., Blasberg, R., Gerald, W.L., Massagué, J., 2005. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proceedings of the National Academy of Sciences of the United States of America 102, 13909–13914.
Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., Wrana, J.L., 2000. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation. Molecular Cell 6, 1365–1375.
Khan, Z., Marshall, J.F., 2016. The role of integrins in TGFβ activation in the tumour stroma. Cell and Tissue Research 365, 657–673.
Kim, B.-G., Sergeeva, O., Luo, G., Choi, S.H., Lee, Z., Kim, S.-J., Letterio, J., Malek, E., 2017b. TGF-β type I receptor inhibitor (TEW-7197) diminishes myeloma progression by multiple immunomodulatory mechanisms in combination with ixazomib. Cancer Research 77 (13 Suppl.), 2647.
Kim, K.K., Sheppard, D., Chapman, H.A., 2017a. TGF-β1 Signaling and tissue fibrosis. Cold Spring Harbor Perspectives in Biology 9, a022293.
Kim, Y.W., Park, J., Lee, H.J., Lee, S.Y., Kim, S.J., 2012. TGF-β sensitivity is determined by N-linked glycosylation of the type II TGF-β receptor. The Biochemical Journal 445, 403–411.
Kobie, J.J., Wu, R.S., Kurt, R.A., Lou, S., Adelman, M.K., Whitesell, L.J., Ramanathapuram, L.V., Arteaga, C.L., Akporiaye, E.T., 2003. Transforming growth factor β inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. Cancer Research 63, 1860–1864.
Kokkinakis, D.M., Liu, X., Chada, S., Ahmed, M.M., Shareef, M.M., Singha, U.K., Yang, S., Luo, J., 2004. Modulation of gene expression in human central nervous system tumors under methionine deprivation-induced stress. Cancer Research 64, 7513–7525.
Kong, W., Yang, H., He, L., Zhao, J.J., Coppola, D., Dalton, W.S., Cheng, J.Q., 2008. MicroRNA-155 is regulated by the transforming growth factor β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Molecular and Cellular Biology 28, 6773–6784.
Kretzschmar, M., Doody, J., Timokhina, I., Massagué, J., 1999. A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras. Genes & Development 13, 804–816.
Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., et al., 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93, 165–176.
Lamouille, S., Derynck, R., 2007. Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. The Journal of Cell Biology 178, 437–451.
Lamouille, S., Xu, J., Derynck, R., 2014. Molecular mechanisms of epithelial-mesenchymal transition. Nature Reviews. Molecular Cell Biology 15, 178–196.
Lebrin, F., Goumans, M.J., Jonker, L., Carvalho, R.L., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H.M., ten Dijke, P., 2004. Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. The EMBO Journal 23, 4018–4028.
Lee, M.K., Pardoux, C., Hall, M.C., Lee, P.S., Warburton, D., Qing, J., Smith, S.M., Derynck, R., 2007. TGF-β activates Erk MAP kinase signalling through direct phosphorylation of ShcA. The EMBO Journal 26, 3957–3967.
Lin, X., Duan, X., Liang, Y.Y., Su, Y., Wrighton, K.H., Long, J., Hu, M., Davis, C.M., Wang, J., Brunicardi, F.C., Shi, Y., Chen, Y.G., Meng, A., et al., 2006. PPM1A functions as a Smad phosphatase to terminate TGFβ signaling. Cell 125, 915–928.
Liu, C., Billadeau, D.D., Abdelhakim, H., Leof, E., Kaibuchi, K., Bernabeu, C., Bloom, G.S., Yang, L., Boardman, L., Shah, V.H., Kang, N., 2013. IQGAP1 suppresses TβRII-mediated myofibroblastic activation and metastatic growth in liver. The Journal of Clinical Investigation 123, 1138–1156.
Liu, F., 2006. Delineating the TGF-β/Smad-induced cytostatic response. In: ten Dijke, P., Heldin, C.-H. (Eds.), Smad Signal Transduction. Springer, pp. 75–91.
Liu, S., de Boeck, M., van Dam, H., ten Dijke, P., 2016. Regulation of the TGF-β pathway by deubiquitinases in cancer. The International Journal of Biochemistry & Cell Biology 76, 135–145.
Liu, Y., Zhang, P., Li, J., Kulkarni, A.B., Perruche, S., Chen, W., 2008. A critical function for TGF-β signaling in the development of natural CD4+ CD25+ Foxp3+ regulatory T cells. Nature Immunology 9, 632–640.
Lo, R.S., Massagué, J., 1999. Ubiquitin-dependent degradation of TGF-β-activated Smad2. Nature Cell Biology 1 (8), 472.
López-Casillas, F., Wrana, J.L., Massagué, J., 1993. Betaglycan presents ligand to the TGFβ signaling receptor. Cell 73, 1435–1444.
Macias, M.J., Martin-Malpartida, P., Massagué, J., 2015. Structural determinants of Smad function in TGF-β signaling. Trends in Biochemical Sciences 40, 296–308.
Massagué, J., 2008. TGFβ in Cancer. Cell 134, 215–230.
Massagué, J., 2012. TGFβ signalling in context. Nature Reviews. Molecular Cell Biology 13, 616–630.
Massagué, J., Blain, S.W., Lo, R.S., 2000. TGFβ signaling in growth control, cancer, and heritable disorders. Cell 103, 295–309.
Melisi, D., Ishiyama, S., Sclabas, G.M., Fleming, J.B., Xia, Q., Tortora, G., Abbruzzese, J.L., Chiao, P.J., 2008. LY2109761, a novel transforming growth factor β receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. Molecular Cancer Therapeutics 7, 829–840.
Menezes, M.E., Shen, X.N., Das, S.K., Emdad, L., Sarkar, D., Fisher, P.B., 2016. MDA-9/Syntenin (SDCBP) modulates small GTPases RhoA and Cdc42 via transforming growth factor β1 to enhance epithelial-mesenchymal transition in breast cancer. Oncotarget 7, 80175–80189.
Merline, R., Moreth, K., Beckmann, J., Nastase, M.V., Zeng-Brouwers, J., Tralhao, J.G., Lemarchand, P., Pfeilschifter, J., Schaefer, R.M., Iozzo, R.V., Schaefer, L., 2011. Signaling by the matrix proteoglycan decorin controls inflammation and cancer through PDCD4 and MicroRNA-21. Science Signaling 4, ra75.
Meulmeester, E., ten Dijke, P., 2011. The dynamic roles of TGF-β in cancer. The Journal of Pathology 223, 205–218.
Mohammad, K.S., Javelaud, D., Fournier, P.G., Niewolna, M., McKenna, C.R., Peng, X.H., Duong, V., Dunn, L.K., Mauviel, A., Guise, T.A., 2011. TGF-β-RI kinase inhibitor SD-208 reduces the development and progression of melanoma bone metastases. Cancer Research 71, 175–184.
Mondal, T., Subhash, S., Vaid, R., Enroth, S., Uday, S., Reinius, B., Mitra, S., Mohammed, A., James, A.R., Hoberg, E., Moustakas, A., Gyllensten, U., Jones, S.J., et al., 2015. MEG3 long noncoding RNA regulates the TGF-β pathway genes through formation of RNA–DNA triplex structures. Nature Communications 6, 7743.
Morris, J.C., Tan, A.R., Olencki, T.E., Shapiro, G.I., Dezube, B.J., Reiss, M., Hsu, F.J., Berzofsky, J.A., Lawrence, D.P., 2014. Phase I study of GC1008 (fresolimumab): A human anti-transforming growth factor-β (TGFβ) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. PLoS One 9, e90353.
Moses, H.L., Roberts, A.B., Derynck, R., 2016. The discovery and early days of TGF-β: A historical perspective. Cold Spring Harbor Perspectives in Biology 8, a021865.
Moustakas, A., Heldin, C.H., 2005. Non-Smad TGF-β signals. Journal of Cell Science 118, 3573–3584.

Moustakas, A., Heldin, C.H., 2016. Mechanisms of TGFβ-induced epithelial-mesenchymal transition. Journal of Clinical Medicine 5, 63.
Mu, D., Cambier, S., Fjellbirkeland, L., Baron, J.L., Munger, J.S., Kawakatsu, H., Sheppard, D., Broaddus, V.C., Nishimura, S.L., 2002. The integrin αvβ8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. The Journal of Cell Biology 157, 493–507.
Mu, Y., Gudey, S.K., Landström, M., 2012. Non-Smad signaling pathways. Cell and Tissue Research 347, 11–20.
Mullen, A.C., Orlando, D.A., Newman, J.J., Loven, J., Kumar, R.M., Bilodeau, S., Reddy, J., Guenther, M.G., DeKoter, R.P., Young, R.A., 2011. transcription factors determine cell-type-specific responses to TGF-β signaling. Cell 147, 565–576.
Mundy, G.R., 2002. Metastasis to bone: Causes, consequences and therapeutic opportunities. Nature Reviews. Cancer 2, 584–593.
Munger, J.S., Sheppard, D., 2011. Cross talk among TGF-β signaling pathways, integrins, and the extracellular matrix. Cold Spring Harbor Perspectives in Biology 3, a005017.
Muraoka, R.S., Dumont, N., Ritter, C.A., Dugger, T.C., Brantley, D.M., Chen, J., Easterly, E., Roebuck, L.R., Ryan, S., Gotwals, P.J., 2002. Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases. The Journal of Clinical Investigation 109, 1551.
Nagaraj, N.S., Datta, P.K., 2010. Targeting the transforming growth factor-β signaling pathway in human cancer. Expert Opinion on Investigational Drugs 19, 77–91.
Nagata, H., Hatano, E., Tada, M., Murata, M., Kitamura, K., Asechi, H., Narita, M., Yanagida, A., Tamaki, N., Yagi, S., Ikai, I., Matsuzaki, K., Uemoto, S., 2009. Inhibition of c-Jun NH2-terminal kinase switches Smad3 signaling from oncogenesis to tumor-suppression in rat hepatocellular carcinoma. Hepatology 49, 1944–1953.
Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C.H., Miyazono, K., ten Dijke, P., 1997a. TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4. The EMBO Journal 16, 5353–5362.
Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.E., Heldin, C.H., ten Dijke, P., 1997b. Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. Nature 389, 631–635.
Nam, J.-S., Suchar, A.M., Kang, M.-J., Stuelten, C.H., Tang, B., Michalowska, A.M., Fisher, L.W., Fedarko, N.S., Jain, A., Pinkas, J., 2006. Bone sialoprotein mediates the tumor cell–targeted prometastatic activity of transforming growth factor β in a mouse model of breast cancer. Cancer Research 66, 6327–6335.
Nam, J.-S., Terabe, M., Mamura, M., Kang, M.-J., Chae, H., Stuelten, C., Kohn, E., Tang, B., Sabzevari, H., Anver, M.R., 2008. An anti–transforming growth factor β antibody suppresses metastasis via cooperative effects on multiple cell compartments. Cancer Research 68, 3835–3843.
Naumann, U., Maass, P., Gleske, A.-K., Aulwurm, S., Weller, M., Eisele, G., 2008. Glioma gene therapy with soluble transforming growth factor-β receptors II and III. International Journal of Oncology 33, 759–765.
Nieto, M.A., Huang, R.Y., Jackson, R.A., Thiery, J.P., 2016. Emt: 2016. Cell 166, 21–45.
Ozaki, I., Hamajima, H., Matsuhashi, S., Mizuta, T., 2011. Regulation of TGF-β1-induced pro-apoptotic signaling by growth factor receptors and extracellular matrix receptor integrins in the liver. Frontiers in Physiology 2 (78).
Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.R., Zhang, Y., Wrana, J.L., 2005. Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science 307, 1603–1609.
Paauwe, M., ten Dijke, P., Hawinkels, L.J., 2013. Endoglin for tumor imaging and targeted cancer therapy. Expert Opinion on Therapeutic Targets 17, 421–435.
Padua, D., Zhang, X.H., Wang, Q., Nadal, C., Gerald, W.L., Gomis, R.R., Massagué, J., 2008. TGFβ primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 133, 66–77.
Perlman, R., Schiemann, W.P., Brooks, M.W., Lodish, H.F., Weinberg, R.A., 2001. TGF-β-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nature Cell Biology 3, 708–714.
Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., Alitalo, K., 1994. endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells. The Journal of Biological Chemistry 269, 71–74.
Petersen, M., Pardali, E., van der Horst, G., Cheung, H., van den Hoogen, C., van der Pluijm, G., ten Dijke, P., 2010. Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. Oncogene 29, 1351–1361.
Piek, E., Heldin, C.H., ten Dijke, P., 1999. Specificity, diversity, and regulation in TGF-β superfamily signaling. The FASEB Journal 13, 2105–2124.
Robertson, I.B., Rifkin, D.B., 2016. Regulation of the bioavailability of TGF-β and TGF-β-related proteins. Cold Spring Harbor Perspectives in Biology 8, a021907.
Robertson, I.B., Horiguchi, M., Zilberberg, L., Dabovic, B., Hadjiolova, K., Rifkin, D.B., 2015. Latent TGF-β-binding proteins. Matrix Biology 47, 44–53.
Rodon, J., Carducci, M.A., Sepulveda-Sánchez, J.M., Azaro, A., Calvo, E., Seoane, J., Brana, I., Sicart, E., Gueorguieva, I., Cleverly, A.L., 2015. First-in-human dose study of the novel transforming growth factor-β receptor I kinase inhibitor LY2157299 monohydrate in patients with advanced cancer and glioma. Clinical Cancer Research 21, 553–560.
Sanchez-Elsner, T., Botella, L.M., Velasco, B., Corbi, A., Attisano, L., Bernabeu, C., 2001. Synergistic cooperation between hypoxia and transforming growth factor-β pathways on human vascular endothelial growth factor gene expression. The Journal of Biological Chemistry 276, 38527–38535.
Sapkota, G., Knockaert, M., Alarcon, C., Montalvo, E., Brivanlou, A.H., Massagué, J., 2006. Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-β pathways. The Journal of Biological Chemistry 281, 40412–40419.
Scherner, O., Meurer, S.K., Tihaa, L., Gressner, A.M., Weiskirchen, R., 2007. Endoglin differentially modulates antagonistic transforming growth factor-β1 and BMP-7 signaling. The Journal of Biological Chemistry 282, 13934–13943.
Schlingensiepen, K.-H., Bischof, A., Egger, T., Hafner, M., Herrmuth, H., Jachimczak, P., Kielmanowicz, M., Niewel, M., Zavadova, E., Stauder, G., 2004. The TGF-β1 antisense oligonucleotide AP 11014 for the treatment of non-small cell lung, colorectal and prostate cancer: Preclinical studies. Journal of Clinical Oncology 22, 3132.
Schuster, N., Krieglstein, K., 2002. Mechanisms of TGF-β-mediated apoptosis. Cell and Tissue Research 307, 1–14.
Seoane, J., Gomis, R.R., 2017. TGF-β family signaling in tumor suppression and cancer progression. Cold Spring Harbor Perspectives in Biology 9, a022277.
Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., Springer, T.A., 2011. Latent TGF-β structure and activation. Nature 474, 343–349.
Shi, W., Sun, C., He, B., Xiong, W., Shi, X., Yao, D., Cao, X., 2004. GADD34-PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor. The Journal of Cell Biology 164, 291–300.
Shi, Y., Massagué, J., 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113, 685–700.
Sica, A., Schioppa, T., Mantovani, A., Allavena, P., 2006. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. European Journal of Cancer 42, 717–727.
Siegel, P.M., Massagué, J., 2003. Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nature Reviews. Cancer 3, 807–821.
Somasundaram, R., Jacob, L., Swoboda, R., Caputo, L., Song, H., Basak, S., Monos, D., Peritt, D., Marincola, F., Cai, D., Birebent, B., Bloome, E., Kim, J., et al., 2002. Inhibition of cytolytic T lymphocyte proliferation by autologous CD4+/CD25+ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-β. Cancer Research 62, 5267–5272.
Stenvers, K.L., Tursky, M.L., Harder, K.W., Kountouri, N., Amatayakul-Chantler, S., Grail, D., Small, C., Weinberg, R.A., Sizeland, A.M., Zhu, H.J., 2003. Heart and liver defects and reduced transforming growth factor β2 sensitivity in transforming growth factor β type III receptor-deficient embryos. Molecular and Cellular Biology 23, 4371–4385.
Stuelten, C.H., DaCosta Byfield, S., Arany, P.R., Karpova, T.S., Stetler-Stevenson, W.G., Roberts, A.B., 2005. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-α and TGF-β. Journal of Cell Science 118, 2143–2153.
Sturge, J., Caley, M.P., Waxman, J., 2011. Bone metastasis in prostate cancer: Emerging therapeutic strategies. Nature Reviews. Clinical Oncology 8, 357–368.
Suva, L.J., Washam, C., Nicholas, R.W., Griffin, R.J., 2011. Bone metastasis: Mechanisms and therapeutic opportunities. Nature Reviews. Endocrinology 7, 208–218.
Suzuki, E., Kapoor, V., Cheung, H.-K., Ling, L.E., DeLong, P.A., Kaiser, L.R., Albelda, S.M., 2004. Soluble type II transforming growth factor-β receptor inhibits established murine malignant mesothelioma tumor growth by augmenting host antitumor immunity. Clinical Cancer Research 10, 5907–5918.
Tang, B., Vu, M., Booker, T., Santner, S.J., Miller, F.R., Anver, M.R., Wakefield, L.M., 2003. TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. The Journal of Clinical Investigation 112, 1116.

Tarasewicz, E., Jeruss, J.S., 2012. Phospho-specific Smad3 signaling: Impact on breast oncogenesis. Cell Cycle 11, 2443–2451.
Thiery, J.P., Acloque, H., Huang, R.Y., Nieto, M.A., 2009. Epithelial-mesenchymal transitions in development and disease. Cell 139, 871–890.
Thomas, D.A., Massagué, J., 2005. TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 8, 369–380.
Tu, K., Li, J., Verma, V.K., Liu, C., Billadeau, D.D., Lamprecht, G., Xiang, X., Guo, L., Dhanasekaran, R., Roberts, L.R., Shah, V.H., Kang, N., 2015. Vasodilator-stimulated phosphoprotein promotes activation of hepatic stellate cells by regulating Rab11-dependent plasma membrane targeting of transforming growth factor β receptors. Hepatology 61, 361–374.
Valastyan, S., Weinberg, R.A., 2011. Tumor metastasis: Molecular insights and evolving paradigms. Cell 147, 275–292.
Villarreal, M.M., Kim, S.K., Barron, L., Kodali, R., Baardsnes, J., Hinck, C.S., Krzysiak, T.C., Henen, M.A., Pakhomova, O., Mendoza, V., 2016. Binding properties of the transforming growth factor-β coreceptor betaglycan: Proposed mechanism for potentiation of receptor complex assembly and signaling. Biochemistry 55, 6880–6896.
Vincent, T., Neve, E.P., Johnson, J.R., Kukalev, A., Rojo, F., Albanell, J., Pietras, K., Virtanen, I., Philipson, L., Leopold, P.L., Crystal, R.G., de Herreros, A.G., Moustakas, A., et al., 2009. A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-β mediated epithelial-mesenchymal transition. Nature Cell Biology 11, 943–950.
de Vinuesa, A.G., Bocci, M., Pietras, K., ten Dijke, P., 2016. Targeting tumour vasculature by inhibiting activin receptor-like kinase (ALK)1 function. Biochemical Society Transactions 44, 1142–1149.
Wang, B., Hsu, S.H., Majumder, S., Kutay, H., Huang, W., Jacob, S.T., Ghoshal, K., 2010. TGFβ-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. Oncogene 29, 1787–1797.
Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., Chen, Z.J., 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412, 346–351.
Wang, J., Yang, L., Yang, J., Kuropatwinski, K., Wang, W., Liu, X.Q., Hauser, J., Brattain, M.G., 2008. Transforming growth factor β induces apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells. Cancer Research 68, 3152–3160.
Welm, A.L., 2008. TGFβ primes breast tumor cells for metastasis. Cell 133, 27–28.
Wels, J., Kaplan, R.N., Rafii, S., Lyden, D., 2008. Migratory neighbors and distant invaders: Tumor-associated niche cells. Genes & Development 22, 559–574.
Wiercinska, E., Naber, H.P., Pardali, E., van der Pluijm, G., van Dam, H., ten Dijke, P., 2011. The TGF-β/Smad pathway induces breast cancer cell invasion through the up-regulation of matrix metalloproteinase 2 and 9 in a spheroid invasion model system. Breast Cancer Research and Treatment 128, 657–666.
Wikstrom, P., Stattin, P., Franck-Lissbrant, I., Damber, J.E., Bergh, A., 1998. Transforming growth factor β1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. The Prostate 37, 19–29.
Wilkes, M.C., Murphy, S.J., Garamszegi, N., Leof, E.B., 2003. Cell-type-specific activation of PAK2 by transforming growth factor β independent of Smad2 and Smad3. Molecular and Cellular Biology 23, 8878–8889.
Wilson, E.B., El-Jawhari, J.J., Neilson, A.L., Hall, G.D., Melcher, A.A., Meade, J.L., Cook, G.P., 2011. Human tumour immune evasion via TGF-β blocks NK cell activation but not survival allowing therapeutic restoration of anti-tumour activity. PLoS One 6, e22842.
Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., Massagué, J., 1994. Mechanism of activation of the TGF-β receptor. Nature 370, 341–347.
Xu, P., Lin, X., Feng, X.-H., 2016. Posttranslational regulation of Smads. Cold Spring Harbor Perspectives in Biology 8, a022087.
Xue, G., Restuccia, D.F., Lan, Q., Hynx, D., Dirnhofer, S., Hess, D., Ruegg, C., Hemmings, B.A., 2012. Akt/PKB-mediated phosphorylation of Twist1 promotes tumor metastasis via mediating cross-talk between PI3K/Akt and TGF-β signaling axes. Cancer Discovery 2, 248–259.
Yamashita, H., ten Dijke, P., Franzen, P., Miyazono, K., Heldin, C.-H., 1994. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-β. The Journal of Biological Chemistry 269, 20172–20178.
Yamashita, M., Fatyol, K., Jin, C., Wang, X., Liu, Z., Zhang, Y.E., 2006. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Molecular Cell 31, 918–924.
Yi, J.Y., Shin, I., Arteaga, C.L., 2005. Type I transforming growth factor β receptor binds to and activates phosphatidylinositol 3-kinase. The Journal of Biological Chemistry 280, 10870–10876.
Yin, J.J., Selander, K., Chirgwin, J.M., Dallas, M., Grubbs, B.G., Wieser, R., Massagué, J., Mundy, G.R., Guise, T.A., 1999. TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. The Journal of Clinical Investigation 103, 197–206.
Yu, Q., Stamenkovic, I., 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. Genes & Development 14, 163–176.
Yuan, J.H., Yang, F., Wang, F., Ma, J.Z., Guo, Y.J., Tao, Q.F., Liu, F., Pan, W., Wang, T.T., Zhou, C.C., Wang, S.B., Wang, Y.Z., Yang, Y., et al., 2014. A long noncoding RNA activated by TGF-β promotes the invasion-metastasis cascade in hepatocellular carcinoma. Cancer Cell 25, 666–681.
Zavadil, J., Bitzer, M., Liang, D., Yang, Y.C., Massimi, A., Kneitz, S., Piek, E., Bottinger, E.P., 2001. Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. Proceedings of the National Academy of Sciences of the United States of America 98, 6686–6691.
Zhang, B., Halder, S.K., Kashikar, N.D., Cho, Y.J., Datta, A., Gorden, D.L., Datta, P.K., 2010. Antimetastatic role of Smad4 signaling in colorectal cancer. Gastroenterology 138, 969–980.e3.
Zhang, L., Zhou, F., ten Dijke, P., 2013a. Signaling interplay between transforming growth factor-β receptor and PI3K/AKT pathways in cancer. Trends in Biochemical Sciences 38, 612–620.
Zhang, L., Zhou, F., Garcia de Vinuesa, A., de Kruijf, E.M., Mesker, W.E., Hui, L., Drabsch, Y., Li, Y., Bauer, A., Rousseau, A., Sheppard, K.A., Mickanin, C., Kuppen, P.J., et al., 2013b. TRAF4 promotes TGF-β receptor signaling and drives breast cancer metastasis. Molecular Cell 51, 559–572.
Zhang, M., Kleber, S., Röhrich, M., Timke, C., Han, N., Tuettenberg, J., Martin-Villalba, A., Debus, J., Peschke, P., Wirkner, U., 2011. Blockade of TGF-β signaling by the TGFβR-I kinase inhibitor LY2109761 enhances radiation response and prolongs survival in glioblastoma. Cancer Research 71, 7155–7167.
Zhang, Y., Alexander, P.B., Wang, X.F., 2017. TGF-β family signaling in the control of cell proliferation and survival. Cold Spring Harbor Perspectives in Biology 9, a022145.
Zhang, Y.E., 2017. Non-Smad signaling pathways of the TGF-β family. Cold Spring Harbor Perspectives in Biology 9, a022129.
Zheng, H., Li, W., Kang, Y., 2016. Tumor-stroma interactions in bone metastasis: Molecular mechanisms and therapeutic implications. Cold Spring Harbor Symposia on Quantitative Biology 81, 151–161.
Zhou, B.P., Deng, J., Xia, W., Xu, J., Li, Y.M., Gunduz, M., Hung, M.C., 2004. Dual regulation of Snail by GSK-3β-mediated phosphorylation in control of epithelial-mesenchymal transition. Nature Cell Biology 6, 931–940.
Zhou, F., Xie, F., Jin, K., Zhang, Z., Clerici, M., Gao, R., van Dinther, M., Sixma, T.K., Huang, H., Zhang, L., ten Dijke, P., 2017. USP4 inhibits SMAD4 monoubiquitination and promotes activin and BMP signaling. The EMBO Journal 36, 1623–1639.

Further Reading

ten Dijke, P., Heldin, C.-H. (Eds.), 2006. Smad signal transduction. Proteins and cell regulation, vol. 5. Springer, Dordrecht, The Netherlands.
Derynck, R., Miyazono, K. (Eds.), 2017. The biology of the TGF-β family. Cold Spring Harbor Laboratory Press, New York.

Thyroid Cancer: Pathology, Genetics, Diagnosis, and Treatment

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Abbreviations

ATC Anaplastic thyroid carcinoma
CEA Carcinoembryonic antigen
DTC Differentiated thyroid carcinoma
FAP Familial adenomatous polyposis
FNA Fine-needle aspiration/aspirate
FMTC Familial medullary thyroid carcinoma
FNMTC Familial non-medullary thyroid carcinoma/familial follicular cell-derived carcinoma
FTC Follicular thyroid carcinoma
ICD-O International Classification of Diseases for Oncology
MEN Multiple endocrine endoplasia
MTC Medullary thyroid carcinoma
PDTC Poorly differentiated thyroid carcinoma
PTC Papillary thyroid carcinoma
RAI Radioactive iodine
RT Radiotherapy
rhTSH Recombinant human thyroid-stimulating hormone
Tg Thyroglobulin
TSH Thyroid-stimulating hormone

Definition and Classification

Cancer of the thyroid is a group of diseases comprising a variety of tumor types differing in morphology, etiology, biological behavior, and molecular characteristics. They include the differentiated papillary and follicular carcinomas, carcinomas lacking characteristic differentiation which include undifferentiated carcinomas and anaplastic carcinomas, and medullary carcinoma as a separate category. The 2017 WHO classification also describes a variety of subtypes and rare tumor types which are not discussed in this entry.

Over 95% of thyroid cancers derive from follicular cells, with a vast majority being well-differentiated tumors (differentiated thyroid carcinomas, DTCs). The classification of thyroid cancers has been a subject of debate and several changes in recent years, in particular concerning controversial variants rendering differential diagnosis challenging. Recent genomic and transcriptomic data suggest that further reclassifications of some of the variants based on their molecular profiles combined with histological features may soon be worth considering.

The predominant form of thyroid cancer, in both adults and children, is papillary thyroid carcinoma (PTC; ICD-O code: 8260/3), also called papillary thyroid adenocarcinoma, which accounts for up to 90% or even 95% of all thyroid carcinomas. PTC is a malignant epithelial tumor with follicular cell differentiation, distinctive nuclear features and a papillary growth pattern. A number of PTC histological variants exist, including follicular variant (ICD-O code: 8340/3), encapsulated variant (8343/3), papillary microcarcinoma (8341/3), columnar cell variant (8344/3), and oncocytic variant (8342/3).

The second most common thyroid cancer is follicular thyroid carcinoma (FTC), also referred to as follicular thyroid adenocarcinoma or follicular carcinoma. It accounts for 5%–15% of all thyroid cancer cases. FTC is defined as a thyroid malignancy derived from follicular cells in which the diagnostic nuclear characteristics of PTC are absent. It includes three variants: minimally invasive FTC (ICD-O code: 8335/3), encapsulated angioinvasive FTC (8339/3) and widely invasive variant (8330/3).

Medullary thyroid carcinoma (MTC; ICD-O code: 8345/3), a malignant tumor of the thyroid gland composed of cells with evidence of C-cell differentiation, accounts for no more than 2%–3% of cases and is mainly associated with familial cancer syndromes.

The most aggressive primary thyroid malignancy is anaplastic thyroid carcinoma (ATC; ICD-O code: 8020/3) which is composed of undifferentiated follicular cells. Also this thyroid cancer is very rare (0.8%–5% of cases).

Epidemiology ad Risk Factors

Thyroid Cancer Burden

Thyroid cancer accounts for about 90% of all endocrine malignancies. With nearly 300,000 new cases diagnosed in 2012, it was the 16th most common cancer when considering both sexes together and the 7th most common in women worldwide. The incidence rates are two to four times higher in women than in men (Fig. 1). The peak incidence is at about 50 years of age. Even though thyroid cancer is rare in children, it is the third most common solid malignancy and the most frequent endocrine childhood malignancy. There has been a big increase in the reported thyroid cancer and in particular PTC incidence since the introduction of new high-resolution techniques into clinical practice. However, overall mortality from thyroid cancer is very low. PTC, the most common form of the disease, tends to be biologically indolent, giving rise to the 5-year survival rates of 96% and 20-year survival rates still over 90%. Follicular and medullary carcinoma have a relatively good prognosis as well, with 5-year survival rates of 50%–90% for FTC (for widely invasive and minimally invasive FTC, respectively), and 80% for MTC. The thyroid carcinoma-associated mortality is mainly due to ATC which gives median 1-year survival rates of only 10%–20%, and 5-year survival rates below 1%.

Etiology and Risk Factors

As thyroid cancer is more frequent in women, female sex has been suggested as a risk factor. However, the disparity in incidence between the two sexes decreases with increasing patient age. Some studies also suggest that Asian race is associated with a higher risk of thyroid cancer.

Since the mortality associated with thyroid cancer is very low, the etiology of this cancer type has been less studied than that of cancers arising at other sites. The major and best documented cause of thyroid cancer is exposure to radiation. Environmental exposure to ionizing radiation is a well-established risk factor for both papillary (PTC) and follicular thyroid carcinoma (FTC), although it presents a much lower risk for developing the latter. In contrast, the risk of medullary thyroid carcinoma (MTC) is not associated with ionizing radiation exposure at all.

Radiation therapy administered in infancy or childhood for benign conditions of the head and neck increases the risk of thyroid cancer, with diagnosis occurring in as few as 5 years after exposure. Radiation exposure as a consequence of nuclear fallout has also been associated with a high risk of thyroid cancer, especially in children. Insufficient dietary iodine intake is an important risk factor for follicular carcinoma (Table 1). Introducing iodine supplementation in iodine-insufficient areas results in a decreased FTC to PTC ratio. However, it is unclear whether it affects the overall rates of thyroid carcinoma.

Other factors, such as obesity, diabetes, reproductive factors, smoking, alcohol consumption, and dietary nitrates have been identified as putative risk factors for PTC. However, their exact link with the disease is unclear.

The causative factors of anaplastic thyroid carcinoma (ATC), the rare and highly aggressive form of the disease, remain unknown. However, the existence of differentiated areas in these tumors suggests that they may evolve from differentiated tumors, like PTC or FTC. MTC, in contrast, seems to be mostly associated with familial cancer syndromes.

Most thyroid cancers are sporadic. However, a family history of thyroid disease (including cancer) is associated with a higher risk of thyroid cancer. Moreover, there are two groups of familial thyroid cancers which are associated with familial cancer syndromes: familial non-medullary thyroid carcinoma (FNMTC, also called familial follicular cell-derived carcinoma) arising from follicular cells and familial medullary thyroid carcinoma (FMTC) which arises from calcitonin-producing C cells.

Overall, approximately 30% of MTCs are associated with germline mutations in the *RET* proto-oncogene manifesting as the multiple endocrine neoplasia (MEN) type 2A (which includes familial MTC as a variant) or type 2B. The genetic susceptibility background of FNMTC is less straightforward. Hereditary cancer syndromes underlying the development of this disease include among others familial adenomatous polyposis, Carney complex and Werner syndrome. Among PTC patients, approximately 5% of cases are familial, developing in the context of the Familial adenomatous polyposis (FAP) syndrome. This is caused by a mutation in the *APC* gene. About 1%–2% of FAP patients develop PTC.

Familial FTC occurs in the context of the PTEN hamartoma tumor syndrome, a rare syndrome associated with mutations in the *PTEN* gene. Patients with a germline *PTEN* mutation have a 35% lifetime risk for the development of FTC.

Presentation and Diagnosis

Both PTC and FTC can occur in any location of the thyroid gland and anywhere where ectopic thyroid tissue may be present, whereas MTCs are usually located at the junction of the upper and mid portions of the thyroid lobe where the C cells (parafollicular cells) are typically concentrated.

PTC usually presents as an asymptomatic painless mass in the thyroid with or without enlarged cervical lymph nodes. Later symptoms may include hoarseness and dysphagia or dysphonia.

The clinical presentation of FTC is as an asymptomatic painless single thyroid nodule. Larger tumors may compress cervical structures leading to dyspnea or dysphagia. Hoarseness may also appear. Rarely, the tumor may be hyperfunctional, associated with clinical signs and symptoms of hyperthyroidism.

Most patients with sporadic MTC present with a painless single thyroid nodule. In an iodine scan, this will be a cold nodule. Regional lymph node enlargement is frequent as a large proportion of patients have lymph node metastases at initial presentation.

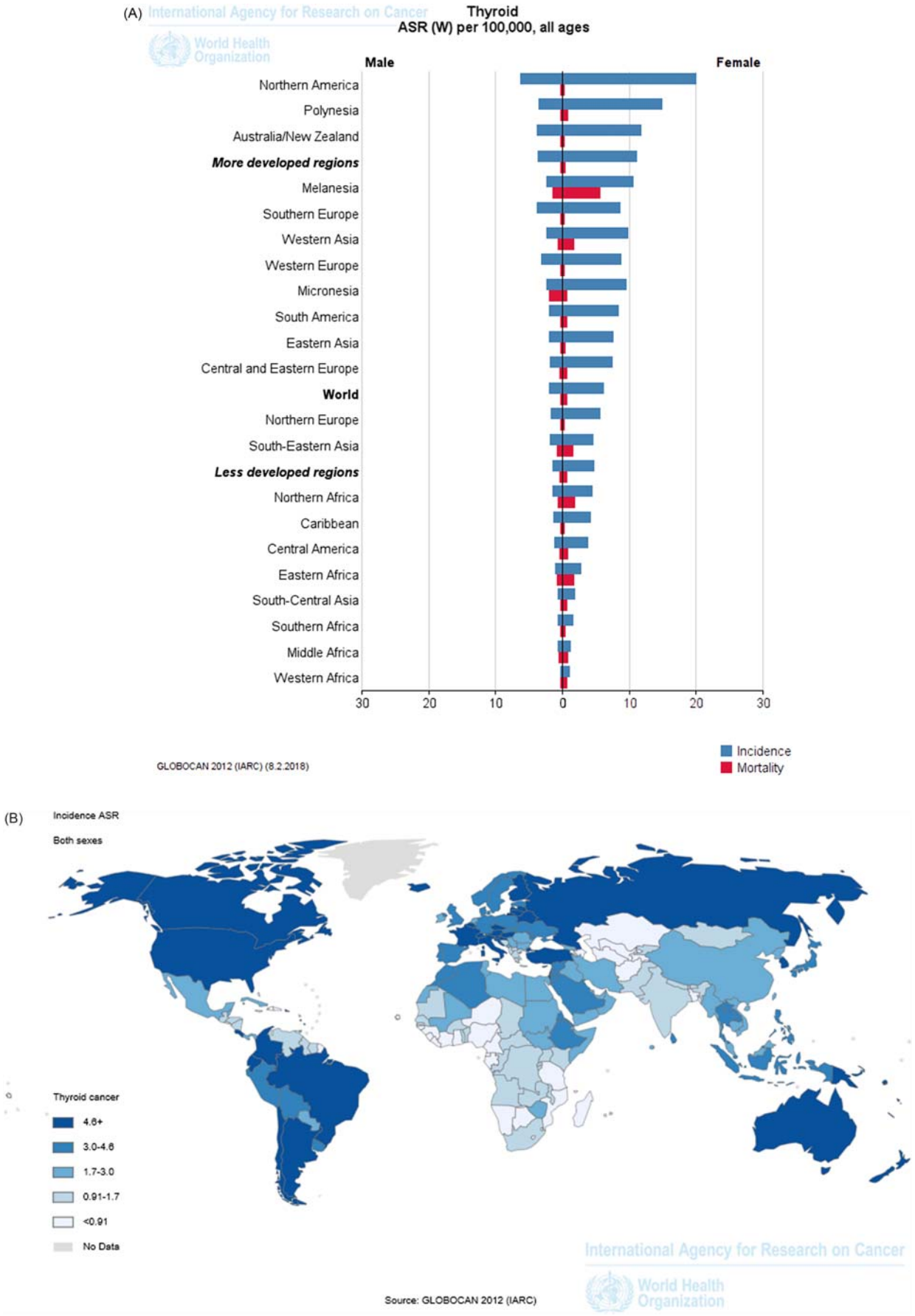

Fig. 1 Incidence and mortality of thyroid cancer worldwide. (A) Age-standardized incidence and mortality rates (ASR) by gender and geographical area. (B) Incidence distribution worldwide. From Ferlay, J., et al. (2013). *GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet].* Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr, accessed on February 10, 2018.

Table 1 Risk factors for thyroid cancer

Carcinogenic agents classified by IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (volumes 1–120)
Agents of sufficient evidence for kidney carcinogenicity in humans
Radioiodines, including iodine-131
X-radiation and gamma-radiation
Other carcinogenic agents and lifestyle risk factors
Nuclear fallout

Later symptoms may include hoarseness and dysphagia or dysphonia. MTC produces carcinoembryonic antigen (CEA) and this is a much used test for initial diagnosis and follow-up. Tests for calcitonin secretion are also positive. As far as familial MTC is concerned, screening for production of calcitonin in MEN2 families allows early diagnosis even at a stage of (premalignant) C-cell hyperplasia.

Poorly differentiated thyroid carcinomas present as large thyroid masses. In about 15% of cases, patients present with metastases. The tumor spreads by local invasion into perithyroidal tissues, and by distant metastases to the lungs, bone, and other organs.

ATC patients typically present with a rapidly enlarging, firm and widely infiltrative neck mass associated with pain, hoarseness, difficulty breathing, and dysphagia. Less common symptoms include vocal cord paralysis, cervical pain, and dysphonia. ATCs present with considerable local invasion, and frequently with local and distant metastases (one third of patients have distant metastases at presentation).

As thyroid nodules are very common and most of them are relatively inoffensive, differentiating between benign and malignant thyroid nodules in the initial workup is crucial for proper management choices. A combination of serum thyroid-stimulating hormone (TSH) testing, imaging techniques (usually ultrasound) of the thyroid and neck, and clinical features is used to determine whether there is a suspicion of a malignant potential. If it is the case, a fine-needle aspiration (FNA) biopsy is taken to allow for a cytological and immunohistochemical evaluation. Some clinicians, especially in Europe, recommend measuring serum calcitonin levels to evaluate for MTC, however the cost-effectiveness of this procedure remains controversial. Molecular testing to detect individual mutations (e.g., in the *BRAF* or *RET* gene) or specific patterns may be useful to evaluate biopsy samples that are indeterminate and thus guide management decisions (see "**Biomarkers**" section).

Pathology and Genetics

The thyroid hosts two different organ systems. The most conspicuous is the follicular compartment, responsible for the production of thyroid hormones and origin of neoplasms with follicular differentiation. Less conspicuous are the parafollicular or C-cells, responsible for the biosynthesis of calcitonin. Parafollicular cells are of neural crest origin and arrive in the thyroid by migration. Therefore, they represent a lineage which is entirely distinct from that of follicular epithelium. They are considered part of the diffuse neuro-endocrine system.

Papillary Thyroid Carcinoma

Pathology

PTC may arise anywhere in the thyroid or in the isthmus and occasionally in ectopic thyroid tissue in the neck region. At gross examination, it presents as a poorly circumscribed solid mass, with a granular white cut surface. Calcification may be found. Occasionally the tumor is encapsulated. Usual size is 2–3 cm. In about 20% of cases, multifocal PTC is found.

The name of this entity comes from characteristic architectural features of a papillary carcinoma: the presence of papillae (long, straight or arborizing stromal stalks covered with neoplastic epithelium). These architectural features or characteristic nuclear features (irregular nuclear contour, nuclear pseudo-inclusions responsible for the "ground glass" appearance of the nucleoplasm, prominent nuclear grooves) are essential for a diagnosis of a papillary carcinoma (**Fig. 2**). Some thyroid carcinomas have a follicular architecture but nuclear features characteristic of a papillary carcinoma—these are classified as a follicular variant of papillary carcinoma (**Fig. 3**). PTCs are often surrounded by a dense fibrous capsule, representing the encapsulated variant. Some show diffuse dense fibrous stroma, representing the diffuse sclerosing variant. Microscopic structures classically associated with PTC are psammoma bodies. Invasion of lymphatic vessels is not unusual and is associated with a tendency for lymphogenous metastasis. Immunohistochemically, PTCs are positive for thyroglobulin, TTF-1, PAX8, and cytokeratin (pancytokeratin and CK7).

Progression of PTC consists primarily of metastasis to cervical lymph nodes. Distant metastases are rare. The relative indolence of PTC is reflected in its excellent prognosis, with 5-year survival rates of about 95% and overall mortality rates of 1%–5%.

Genetics

PTC has a more stable genome with few chromosomal rearrangements and a lower number of mutations than carcinomas arising in other organs. *BRAF V600E* is the most common driver mutation found in PTC (found in up to 95% of *BRAF*-mutant tumors). The follicular variant has a much lower frequency of *BRAF* mutations. Other, less frequent *BRAF* alterations include *K601E* mutations

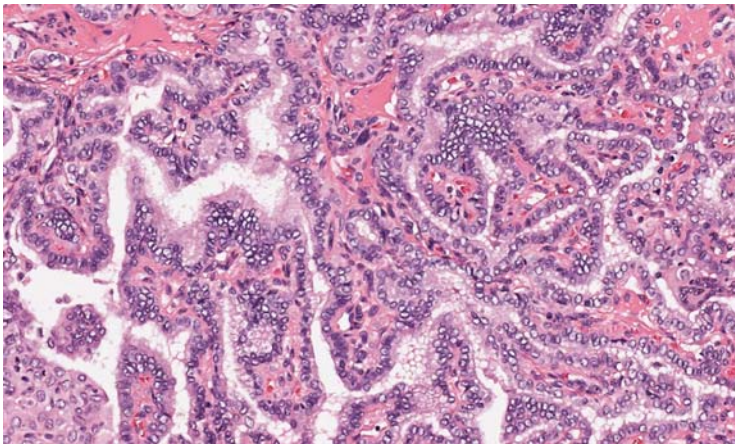

Fig. 2 Papillary carcinoma. Note the papillary architecture with fibrovascular stalks covered with a single layer of cuboid epithelial cells. Nuclei show irregular contour, pseudo-inclusions responsible for the "ground glass" appearance of the nucleoplasm, and prominent nuclear grooves, characteristic of papillary carcinoma.

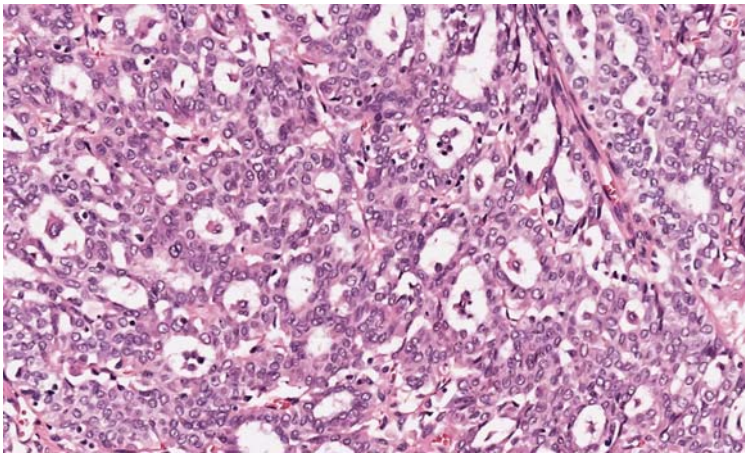

Fig. 3 Papillary carcinoma, follicular variant. Note the follicular architecture but nuclear features typical of papillary carcinoma.

and small insertions or deletions surrounding codon 600 as well as chromosomal rearrangements, such as *AKAP9-BRAF* fusions. Mutations in the *RAS* genes (mostly *HRAS* and *NRAS*) are also common (up to 30% of cases) and often mutually exclusive with *BRAF* alterations. The most frequent chromosomal rearrangements leading to gene fusions involve the *RET* gene, the most common being *RET*/PTC rearrangements, followed by those involving *NTRK3*, *NTRK1*, *ALK*, and other genes. Rearrangements are typical for radiation-related PTC and for younger patients, including children. PTC progression is associated with accumulation of mutations in other genes, such as *TP53*, *PIK3CA*, and *AKT1*. Mutations in the promoter of the telomerase reverse transcriptase (*TERT*; *C228T* and *C250T*) are found in more advanced PTCs and are associated with a higher risk of recurrence, distant metastasis, and PTC-related mortality (**Table 2**). The Cancer Genome Atlas Research Network has identified two major subgroups of PTCs based on their gene expression profile: *BRAF V600E*-driven tumors and *RAS* mutation-driven tumors (**Fig. 9**), with striking molecular signaling differences and also distinct histological features between the two subtypes. However, the prognostic utility of *BRAF V600E* remains controversial, with conflicting literature reports. The mutation profile of PTC has been changing over the past decades, with a sharp increase in the prevalence of *RAS* mutations and a steady decrease in the prevalence of *RET*/PTC rearrangements, while the proportion of tumors carrying the *V600E BRAF* mutation has remained stable or even increased. Familial PTC (about 5% of cases) is associated with mutations in the *APC* gene, typically proximal to the mutation cluster region located centrally in the open reading frame and overlapping with the region encoding the 20 amino acid repeats which bind with β-catenin.

Follicular Carcinoma

Pathology

FTC cannot be adequately covered without dedicating a few words to follicular adenoma. The term "follicular" refers to the typical architecture of the tumor, which mimics follicles in the normal thyroid. By definition, follicular adenoma is a benign epithelial

Table 2 Gene loci that are most frequently altered in thyroid carcinoma

Pathway	*Gene (locus)*	*Alteration type*	*Estimate*
MAPK signaling	*BRAF* (7q34)	Point mutations (most frequent: *V600E*), small insertions or deletions, chromosomal rearrangements	30%–90% of PTCs; *BRAF V600E* identified as a driver mutation in a specific PTC subset and also present in about 20% of ATCs
	RET (10q11.21)	Rearrangements (most common: *RET/PTC*)	5%–35% of sporadic PTCs; characteristic of radiation-induced tumors and young patients
		Point mutations	40%–60% of sporadic MTCs
	RAS family (*HRAS, NRAS*)	Point mutations	0%–35% of PTCs, 30%–50% of FTCs; 20% of ATCs
Telomere maintenance	*TERT* (5p15.33)	Promoter mutations	5%–25% of PTCs and about 20% of FTCs; associated with more aggressive tumor behavior and worse prognosis
Fatty acid storage and glucose metabolism	*PPARG* (3p25.2)	Rearrangements/fusions frequently involving the thyroid transcription factor *PAX8*	20%–50% FTCs; *PAX8-PARG* fusions associated with younger patient age and tumor vascular invasion
PI3K/PTEN/Akt: control of cell metabolism	*PTEN* (10q23)	Inactivating mutations	Up to 10% of FTCs; variable proportions of ATCs
	PIKCA3 (3q26)	Mutations and copy number gains	Mutations in up to 10% of FTCs; variable proportions of ATCs
p53 signaling	*TP53* (17p13.1)	Mutations	Late events, associated with tumor progression, particularly frequent in ATCs (40%–80% of cases)

ATC, anaplastic thyroid carcinoma; *FTC*, follicular thyroid carcinoma; *PTC*, papillary thyroid carcinoma.

tumor in the thyroid with follicular architecture. Epidemiological data are not very reliable because follicular adenomas occur most frequently in females in the 5th or 6th decade of life, when nodular goiter is relatively frequent, in particular in iodine-deficient regions. In a multinodular thyroid, it is rather difficult to distinguish between a (polyclonal) hyperplastic nodule and a (monoclonal) follicular adenoma. Molecular analysis would be necessary to make the distinction and in this context many pathologists will not make this diagnosis. Large autopsy series suggest an incidence of about 5%. In iodine-sufficient regions, palpable single thyroid nodules have been reported in 3%–5% of the population, consistent with these autopsy data.

At gross examination, a follicular adenoma presents as a regular round encapsulated solid gray-white to yellow or tan mass. The yellow/tan color is indicative of the presence of well-developed follicles containing colloid. Microscopically, the tumor shows a follicular architecture. The follicles are composed of cuboidal epithelial cells surrounding a lumen that may contain colloid (Fig. 4). Nuclear features are bland. Mitotic activity is rare. In a relatively normal thyroid, the presence of an encapsulated lesion is sufficient for a diagnosis of adenoma. In a nodular goiter, the presence of fibrosis complicates this criterion. It is often impossible to distinguish a hyperplastic nodule from an adenoma as their cytonuclear features can be indistinguishable. When a diagnosis of follicular adenoma is considered, a careful examination of the tumor capsule becomes crucial as the only distinction between a follicular adenoma and a follicular carcinoma may be the presence of capsular or (preferably) vascular invasion.

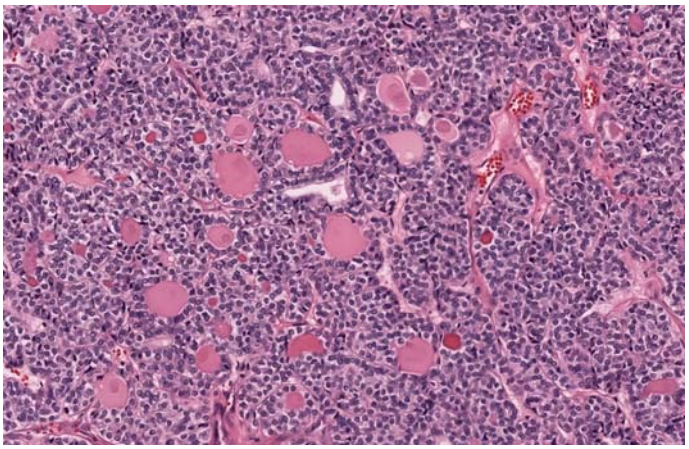

Fig. 4 Follicular adenoma. The lesion is composed of small follicles, occasionally with a lumen filled with colloid. The follicular cells show bland nuclear features, little pleomorphism, and no mitotic activity.

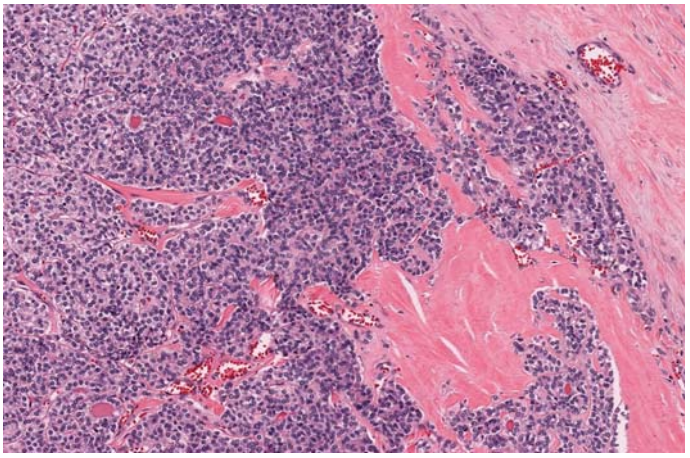

Fig. 5 Minimally invasive follicular carcinoma. Architectural and cytonuclear features are almost identical to those of follicular adenoma but the capsule shows areas of unequivocal capsular invasion.

FTC is an epithelial malignancy arising from follicular cells in the absence of nuclear features of papillary carcinoma. This somewhat odd definition "by exclusion" is due to the fact that nuclear features determine the diagnosis more than architecture, which has been corroborated by molecular genetic findings. Like PTC, it can occur anywhere in the thyroid and even in ectopic thyroid tissue in the neck region.

Grossly, FTCs are usually encapsulated solid gray-whitish lesions. Occasionally, macroscopic invasion into surrounding thyroid tissue can be found. Microscopically, the tumor is characterized by epithelial cells in follicular architecture, surrounded by a delicate fibrovascular stroma. Often, cytonuclear features are bland, complicating the distinction between adenoma and carcinoma. As stated above, in such cases the diagnosis relies entirely on the evidence of capsular or (preferably) vascular invasion (Fig. 5). Based on this criterion, FTCs have been subdivided into minimally invasive FTC (exclusively capsular invasion), encapsulated angioinvasive FTC, and widely invasive FTC. Vascular invasion is typically in veins. This explains the strong tendency for hematogenous metastasis of FTC. Lymphovascular invasion is rather uncommon. FTCs express the lineage-specific markers thyroglobulin, TTF-1, and PAX8.

Progression of FTC is primarily by invasive growth into adjacent organs and by hematogenous metastasis, with bone, lung, brain, and liver being the most frequently affected organs. Prognosis depends on the extent of invasion. Minimally invasive carcinomas have an excellent prognosis, while in carcinomas with vascular invasion, prognosis depends on the extent of vascular invasion (the more vessels involved, the higher the likelihood of distant metastasis).

FTC has a higher number of numerical chromosomal aberrations as well as gains and losses of chromosomal regions than PTC, with cytogenetic changes found in about 65% of the tumors. Hyperploidy with extra copies of chromosome 7 is particularly frequent. The most common somatic mutations are *RAS* point mutations (in particular in codon 61 of *NRAS* and codon 61 of *HRAS*) and gene fusions involving the *PPARG* gene, a member of a nuclear receptor superfamily. *PAX8-PPARG*-positive tumors tend to present in younger patients and more often in tumors with vascular invasion. Mutations in the PI3K/PTEN/Akt pathway genes are also found in FTC, however with a lower prevalence. *TERT* promoter mutations are found in about 20% of FTC cases and have been shown to be associated with a more aggressive tumor behavior (more advanced stage at diagnosis, higher rates of tumor recurrence and tumor-related mortality; Table 2). Recent data suggest that they may be an early event in FTC tumorigenesis, occurring before tumor invasion. Familial FTC occurs in the context of germline *PTEN* mutations associated with the PTEN hamartoma tumor syndrome.

Poorly Differentiated Thyroid Carcinoma

Pathology

Some tumors arising from follicular epithelial cells show limited evidence of follicular differentiation with a solid or trabecular growth pattern. These are called poorly differentiated thyroid carcinomas (PDTCs) and display a behavior which is in between that of FTC and that of anaplastic carcinoma. PDTC is distinctly rare, its proportion among thyroid cancers varying between less than 1 to about 7%. An association with longstanding goiter has been substantiated.

At gross examination, the tumors are usually large (> 5 cm), often partially encapsulated, with extension into adjacent parenchyma or perithyroidal structures.

As stated above, PDTC is histologically characterized by neoplastic follicular cells growing in solid masses (so-called insular) or trabecular structures. Colloid is minimally present or altogether absent. The tumor cells tend to show hyperchromatic pleomorphic nuclei with easily demonstrable mitotic activity (Fig. 3A). Necrosis may be extensive. Immunohistochemically, PDTC shows expression of thyroglobulin, although usually less intense than well differentiated tumors and with a microfollicular or perinuclear distribution, as well as of TTF-1 and PAX8, providing evidence of follicular differentiation.

Genetics

Genome aberrations in PDTC are in part comparable to those found in FTC. The karyotype is unstable with complex karyotypic abnormalities. *RAS* mutations occur with the same frequency as in FTC. Also mutations in the *TERT* promoter are relatively frequent. *BRAF* mutations are distinctly less frequent (5%–15%) than in FTC. *TP53* is mutated in up to 30% of cases, while *ALK* and *PTEN* mutations are found in 5%–10% (Table 2).

Anaplastic Thyroid Carcinoma

Pathology

Anaplastic thyroid carcinoma (ATC) is a highly aggressive malignancy composed of undifferentiated cells, presumably of follicular origin. The latter is supported by cases in which areas of follicular cell differentiation are still found in the tumor.

ATC often presents as a large bulky mass in the region of the thyroid, with invasion into adjacent structures. Gross examination will show a firm, solid, whitish mass, often with areas of necrosis and hemorrhage, extending into perithyroidal structures.

Histology of ATC is complex and highly variable. The sarcomatous variant shows pleomorphic spindle cells reminiscent of a high-grade sarcoma. The giant cell variant additionally shows the presence of numerous multinuclear pleomorphic giant cells. The epithelial variant shows masses of epithelial-like cells with abundant eosinophilic cytoplasm, occasionally with signs of squamous differentiation (Fig. 6). In all the three variants, a high mitotic rate, striking infiltrative growth with evidence of vascular invasion, and necrosis are found. Markers for thyroid lineage, for example thyroglobulin, TTF1 and PAX8, are negative. Evidence of epithelial differentiation defined by cytokeratin expression may be found but its absence does not favor a diagnosis of sarcoma.

Genetics

ATC is characterized by a wide variety of molecular alterations resulting in a dysregulation of multiple pathways, the most common being *TP53* mutations which are found in up to 80% of ATC cases while relatively rare in PTCs and FTCs without a differentiated component. Other alterations include many of those found in PTC and FTC, like *BRAF V600E* mutation as well as mutations involving *RAS*, *ALK*, and members of the PI3K/PTEN/Akt pathway (Table 2), which is consistent with the hypothesis that ATC evolves from differentiated thyroid tumors (Fig. 9).

Medullary Thyroid Carcinoma

Pathology

At gross examination, sporadic MTC presents as a mass of on average 2–3 cm but the lesions may also be very small or involve an entire thyroid lobe. In familial cases, the lesions may be small, often microscopic, multiple and in both thyroid lobes. Microscopically, MTCs show various histological patterns. Tumor cells can be polygonal, plasmacytoid or spindle-shaped and can be arranged in solid, lobular, trabecular or insular structures (Fig. 7). These tend to be surrounded by a varyingly voluminous stroma. Characteristically, the stroma contains appreciable amounts of amyloid. In familial cases, the tumors may be small (<5 mm, called microcarcinomas) and in some cases only C-cell hyperplasia is found (Fig. 8).

Familial MTCs are a heterogeneous group of tumors comprising MTC with parathyroid hyperplasia and pheochromocytoma/paraganglioma which are associated with MEN2A, as well as MTC with parathyroid hyperplasia and pheochromocytoma/paraganglioma, and ganglioneuromatosis, which are associated with MEN2B.

Immunohistochemically, MTCs usually express calcitonin but some express only calcitonin-related peptide. A whole range of neurohormonal peptides have been reported in MTC, including ACTH, somatostatin, gastrin-releasing peptide, and neurotensin.

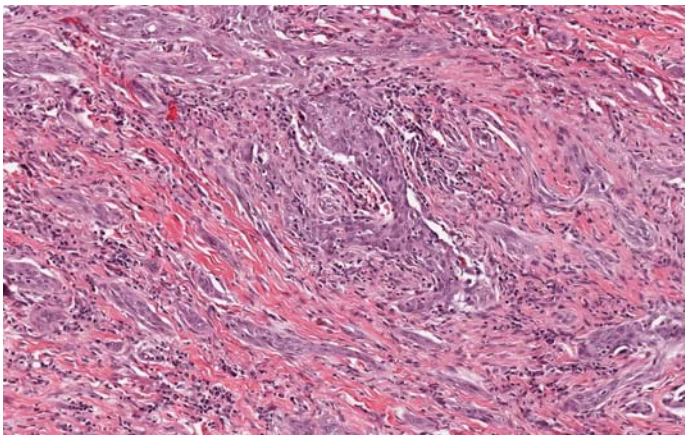

Fig. 6 Anaplastic carcinoma, epithelial type. Nests and strands of highly atypical epithelial cells are embedded in a dense fibrous stroma. There is a hint of squamous differentiation.

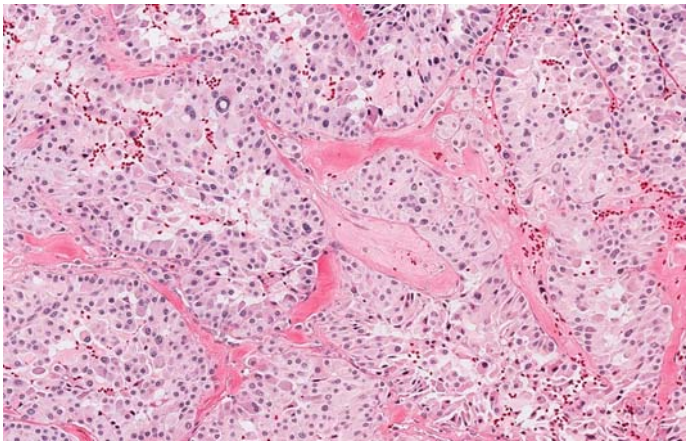

Fig. 7 Medullary carcinoma. Large nests and fields of epithelial cells are surrounded by strands of fibrous stroma. This is focally amorphous and hypereosinophilic, evidence of amyloid deposition. The tumor cells show voluminous slightly granular eosinophilic cytoplasm and moderately pleomorphic nuclei with finely granular chromatin. Some nuclear inclusions are seen.

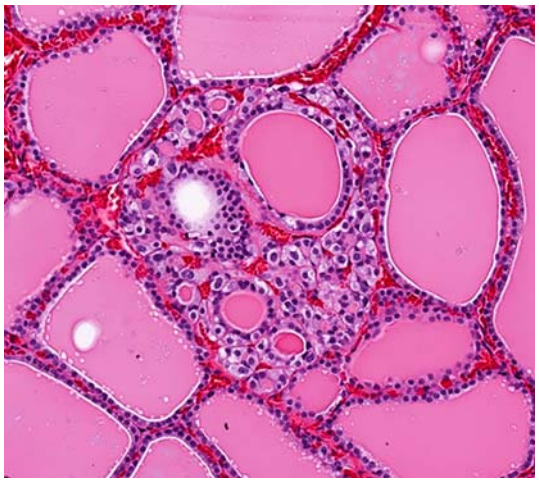

Fig. 8 C-cell hyperplasia in a MEN2 patient. In the center, small follicles show partial replacement of follicular cells by C-cells with clear cytoplasm, which have also diffusely proliferated around the follicles.

For basic diagnostic purposes, generic neuroendocrine markers chromogranin A and synaptophysin can be used. CEA is usually positive. TTF1 can be positive but PAX8 expression is often weak and focal.

Prognosis of MTC is stage-dependent. In young patients with early stage disease, 5-year survival rates of 90% have been reported, while in aged patients with advanced disease, these have been reported to be as low as 65%. Early diagnosis is therefore of paramount importance. As for other neuroendocrine neoplasms, Ki67 labeling index reflecting proliferative activity has been reported as prognostic.

Genetics

Mutations in the *RET* proto-oncogene are the major genetic alteration associated with MTC, both inherited and sporadic. *The M918T RET* (exon 16) is present in a vast majority of MEN2B-associated MTC cases and is also the most frequent somatic mutation in sporadic tumors. The *M918T* mutation confers the highly aggressive capacity to MTC tumors and only few other molecular alterations have been reported. These were mainly *RAS* mutations and *ALK* fusions, at a relatively low prevalence. Complex genotype–phenotype relations in familial MTC are still under study.

Thyroid Carcinoma Molecular Pathogenesis: Conclusion

Overall, somatic rearrangements of *RET* and *TRK* are almost exclusively found in PTC and may be found at early stages. The most distinctive molecular features of FTC are the prominence of aneuploidy as well as the high prevalence of *RAS* mutations and *PAX8-PPARG* rearrangements. *TP53* alterations seem to play a crucial role in the dedifferentiation process of thyroid carcinoma and its

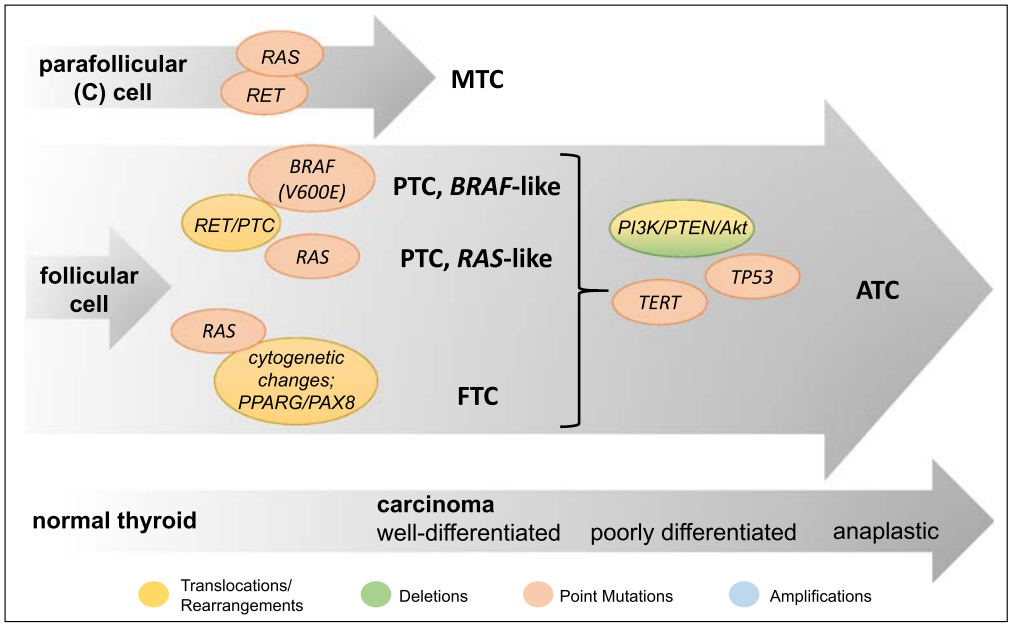

Fig. 9 A model of molecular progression of normal thyroid cells to thyroid carcinoma. Medullary thyroid carcinoma (MTC) develops from thyroid parafollicular (C) cells and is mainly associated with mutations in the *RAS* and *RET* genes, the latter being also affected by germline mutations in familial MTC. Papillary and follicular thyroid carcinomas (PTC and FTC, respectively) develop from follicular thyroid cells through distinct pathways. Moreover, molecular analyses have identified two molecular subtypes of PTC, driven by *BRAF* or *RAS* mutations. Accumulation of genetic alterations in other genes, such as *TERT* or *TP53* in either PTC or FTC may result in tumor dedifferentiation and their progression to anaplastic thyroid carcinoma (ATC), the highly aggressive form of thyroid cancer. Modified from Acquaviva, G. et al. (2018). Molecular pathology of thyroid tumours of follicular cells: A review of genetic alterations and their clinicopathological relevance. *Histopathology* **72**, 6–31.

progression to more aggressive variants. MTC develops through a distinct molecular progression pathway, with *RET* mutations being the most prevalent events (Fig. 9).

Biomarkers

Diagnostic Biomarkers

As thyroid nodules are extremely common, the key to effective screening for thyroid cancer is differentiating between benign nodules and those with a malignant potential. Elevated serum TSH levels found at the initial evaluation of a thyroid nodule are a biomarker of possible malignancy, indicating the need for further examination. In patients with a family history of medullary thyroid cancer or other features of the MEN2 syndrome, elevated serum calcitonin and/or carcinoembryonic antigen (CEA) levels are associated with MTC diagnosis.

The panel of markers determining the thyroid lineage has been discussed above: thyroglobulin, TTF1 (which is not entirely specific for the thyroid as it may be expressed also by other lineages) and PAX8. As a rule, this panel will be used to establish thyroid origin of a neoplasm. Ki67 can be useful to establish proliferative activity. Staining for p53 is negative in PTC and FTC but can be positive in PDTC and is usually positive in ATC. MTC expresses neuroendocrine markers (chromogranin A and synaptophysin) as well as calcitonin.

Molecular testing may be useful in diagnostic evaluation of fine-needle thyroid aspirates which have been classified as indeterminate (about 25% of cases). This may include individual mutation analysis and multigene assays. The presence of the *BRAF V600E* mutation alone is highly specific for thyroid cancer diagnosis. Testing for this mutation is particularly useful to confirm PTC diagnosis in patients with higher-risk fine-needle aspirates with suspected nuclear features of papillary carcinoma but uncertain cytological diagnosis. Several multiple gene diagnostic assays have also been proposed as either rule-in or rule-out tests for classifying thyroid nodules before surgery, the most used being the Afirma® Gene Classifier (Table 3). However, their clinical usefulness and accuracy remain to be confirmed and none of them has yet been approved for routine clinical practice.

Prognostic Biomarkers

A majority of thyroid cancers have excellent prognosis, with the clinicopathological features being reliable prognostic markers. Measuring serum thyroglobulin (Tg) levels has the highest sensitivity (95%–100%) to detect persistent or recurrent disease in a post-surgical evaluation of DTC patients. Additionally, *TERT* promoter mutations have been reported to be associated with a worse

Table 3 Biomarkers for the detection and management of thyroid carcinoma

Biomarker	*Characteristics and clinical utility*
Early detection (screening) and diagnostic biomarkers	
Serum TSH	Elevated levels in patients with thyroid nodules suggest a possibility of a malignancy
Serum calcitonin and CEA	Recommended for patients with a family history of thyroid cancer, the MEN syndrome or a suspicion of MTC; elevated levels are suggestive of MTC
Thyroglobulin	Immunohistochemical marker specific for thyroid cancer; less expressed in poorly differentiated cancers
HBME-1	Immunohistochemical marker relatively specific for thyroid cancer, with a membranous staining pattern; used in combination with Galectin-3 and CK19
Galectin-3	Immunohistochemical marker relatively specific for thyroid cancer, with a nuclear and cytoplasmic staining pattern; used in combination with HBME-1 and CK19
Cytokeratin 19	Immunohistochemical marker expressed in thyroid cancer but with limited specificity; used in combination with HBME-1 and Galectin-3
TTF-1 and PAX8	Immunohistochemical marker combination to establish thyroid origin of a cancer metastasis; TTF-1 is also expressed in lung cancer, while PAX8 is not expressed in lung cancer; both show a nuclear staining pattern
BRAF V600E mutation	Used to confirm PTC diagnosis in suspected PTC aspirates with uncertain cytological diagnosis; over 99% specific for thyroid carcinoma
Afirma® Gene Expression Classifier (GEC; Veracyte, United States)	mRNA profiling for indeterminate thyroid nodules; used in the US (not FDA-approved) as a rule-out test, in particular in cases when surgery is not desirable
Thyroseq2® v.2 (CBLPath, Inc., United States)	Next-generation sequencing-based test simultaneously detecting over 400 mutations and gene fusions in overs 60 genes associated with thyroid cancer to identify malignant (diagnostic marker) and more aggressive tumors (prognostic marker)
Prognostic and monitoring biomarkers	
Serum thyroglobulin (Tg) levels/anti-Tg antibodies	High sensitivity for detecting persistent or recurrent DTC
Serum calcitonin and CEA	Elevated levels in post-surgical MTC patients indicate relapsing or metastatic disease
TERT promoter mutations	Independent markers of adverse prognosis in PTC and FTC patients
Ki-67 staining	Increased staining indicating higher proliferation rates is associated with aggressive tumor behavior in MTC patients
RET mutations	the *RET M918T* mutation associated with poor clinical outcome in sporadic MTC and with more aggressive behavior in MEN-associated MTC
Thyroseq2® v.2 (CBLPath, Inc., United States)	*See above*
Predictive biomarkers	
BRAF mutations	Used to select patients for targeted treatment with BRAF inhibitors in DTC
EGFR expression and *VEGFR* alterations	Allows to select ATC patients for therapies targeting these molecules

ATC, anaplastic thyroid carcinoma; *CEA*, carcinoembryonic antigen; *DTC*, differentiated thyroid carcinoma; *FDA*, US Food and Drug Administration; *MEN*, multiple endocrine endoplasia: *MTC*, medullary thyroid carcinoma; *TSH*, thyroid-stimulating hormone.

prognosis in PTC and FTC patients. It has also been suggested that the *BRAF V600E* mutation may be associated with an increased risk of recurrence but the data on its usefulness as an independent prognostic factor remain controversial. Prognosis of PDTC is distinctly less favorable than that of PTC or FTC, with 5-year survival rates of 60%–70%. Extensive tumor necrosis, tumor size (>5 cm), extrathyroidal extension and distant metastases are unfavorable prognostic factors. Postoperative radio-iodine treatment is less effective as less differentiated tumor cells take up iodine less effectively. No markers predicting clinical behavior of ATC exist. These tumors, however, are generally very aggressive and have a very poor prognosis.

In MTC patients, regular elevated serum calcitonin and CEA levels following surgery are markers of relapsing or metastatic disease. Higher proliferation rates as assessed by Ki-67 staining have been associated with aggressive tumor behavior, while vascular invasion and stromal desmoplasia have been suggested as indicative of a relapsing disease and a metastatic potential, respectively. Mutations of the *RET* gene have a high prognostic value in MTC patients. Tumors in patients with the MEN2B syndrome (carrying the *M918T RET* mutation) are commonly more aggressive than those in MEN2A patients (*RET C634R*). The *RET M918T* mutation has also been associated with poor clinical outcome in sporadic MTC. Moreover, it has been suggested that patients with *RAS*-mutated sporadic MTC may have a less aggressive clinical course than those with non-*RAS*-mutant tumors but this remains to be validated.

Predictive Biomarkers

With the development of molecular testing, targeted therapeutic approaches are being developed for different thyroid cancer types, in particular for advanced or metastatic disease which is refractory to standard surgery and radioactive iodine (RAI) treatment. *BRAF* mutations in DTC are an indication for treatment with BRAF inhibitors (e.g., vemurafenib in RAI-refractory PTC). In ATCs, the epidermal growth factor (EGFR) is expressed and has been used to guide targeted therapy decisions in selected patients. Alterations in the vascular endothelial growth factor receptor (*VEGFR*) may also be considered as markers for therapeutic patient stratification, with several targeted drugs being already in use or under clinical trials.

Management and Therapy

Surgery is the treatment of choice for all types of thyroid cancer. For the two most common follicular cell-derived differentiated thyroid cancers (PTC and FTC), lobectomy is recommended for small and low-risk tumors, and total or nearly total thyroidectomy for higher-risk tumors. The stratification of patients so as to the risk of recurrence following surgery is essential for determining further management choices. It is mainly based on patient characteristics (e.g., age at diagnosis) and on clinicopathological features of the tumor. Several scoring systems taking into account these variables have been proposed.

For many years, it has been standard practice to give radioactive iodine (^{131}I; RAI) to all thyroid cancer patients after surgery. However, its benefits are hard to evaluate and it is currently recommended only for patients at an intermediate and high risk of recurrence, at different doses. RAI may be accompanied with administration of recombinant human thyrotropin (rhTSH) or with thyroid hormone withdrawal in order to stimulate thyroid-stimulating hormone (TSH/thyrotropin). Compared with thyroid hormone withdrawal, administering rhTSH maintains quality of life and reduces the radiation dose delivered to the body while stimulating ^{131}I uptake in thyroid remnants in patients after thyroidectomy. For patients with low recurrence risk, observation is recommended, with monitoring serum thyroglobulin (Tg) levels and relevant imaging biomarkers.

For medullary thyroid carcinoma, total thyroidectomy is recommended in all cases, with measurements of serum calcitonin and CEA levels as baseline tumor markers being part of the preoperative planning. Screening for *RET* mutations is also recommended. In case of known metastatic nodal disease, central and lateral neck dissections are performed simultaneously with the thyroidectomy. Whether these dissections should be performed in patients without known nodal disease, remains controversial. Thyroxine is indicated to replace thyroid function following surgery. However, it does not suppress tumor growth in MTC patients as is the case with follicular-derived tumors (PTC and FTC). Similarly, there is no role for radioiodine. Regular monitoring of serum calcitonin and CEA as markers of relapsing or metastatic disease is done following surgery.

Relapsing PTC and FTC that are not treatable by a combination of surgery, thyroxine therapy, and repeat doses of ^{131}I respond poorly to external beam RT and systemic therapy. Tyrosine kinase inhibitors, such as lenvatinib and sorafenib, showed efficacy in metastatic disease. Vemurafenib or dabrafenib can be used in *BRAF*-mutant PTC. Persistent MTC disease is managed with a combination of surgical resection, external beam RT, local ablation, and—in some cases—observation, depending upon bulk of metastatic foci and tumor marker doubling time. Tyrosine kinase inhibitors (vandetanib, cabozantinib) have shown some efficacy in prolongation of disease-free survival.

Anaplastic thyroid cancer has a mortality that approaches 100%. Therefore, the treatments are mainly palliative. Surgery is done for smaller tumors that are limited to the thyroid but most cases are detected after extrathyroidal invasion has occurred. External beam radiotherapy and chemotherapy are used both as primary and adjuvant therapy, and there is some evidence of benefit in the combination of the two.

Further Reading

Acquaviva, G., et al., 2018. Molecular pathology of thyroid tumors of follicular cells: A review of genetic alterations and their clinicopathological relevance. Histopathology 72, 6–31.
Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.
Cancer Genome Atlas Research Network, 2014. Integrated genomic characterization of papillary thyroid carcinoma. Cell 159 (3), 676–690.
Guilmette, J., Nosé, V., 2018. Hereditary and familial thyroid tumors. Histopathology 72, 70–81.
Lloyd, R.V., Osamura, R.Y., Klöppel, G., Rosai, J. (Eds.), 2017. WHO classification of tumors of endocrine organs, 4th edn. International Agency for Research on Cancer, Lyon, France.
National Comprehensive Cancer Guidelines (NCCN). NCCN clinical practice guidelines in oncology. Thyroid carcinoma. Version 2.2017 – May 17, 2017: https://www.nccn.org/professionals/physician_gls/default.aspx#site
Poller, D.N., Glaysher, S., 2017. Molecular pathology and thyroid FNA. Cytopathology 28 (6), 475–481.

Relevant Websites

https://www.thyroid.org/—American Thyroid Association.
http://www.eurothyroid.com/—European Thyroid Association.

TP53

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Glossary

p53 Response element (p53RE) The short DNA sequence motif that is specifically recognized by the p53 transcription factor.
Transactivation domain (TAD) A transcription factor domain which contains binding sites to recruit cofactors that promote transcription.
Posttranslational modification (PTM) An enzymatic modification of amino acids following protein biosynthesis.
Li-Fraumeni syndrome (LFS) An autosomal dominant, hereditary disorder that is linked to germline mutations of p53.
Neomorph A dominant gain of function that is different from the normal function.
Missense mutation A single nucleotide change in a codon that results in a different amino acid code.
Nonsense mutation A single nucleotide change that forms a premature stop codon.
Alternative splicing A process that turns transcripts into different processed mRNAs through inclusion or exclusion of particular exons.
The cancer genome atlas (TCGA) A project that generates, stores and analyzes high-throughput genome and transcriptome data from cancers.

Abbreviations

TAD transactivation domain
CTD c-terminal domain
PRD proline-rich domain
DBD DNA-binding domain
p53RE p53 response element
PTM post-translational modification
HDAC histone deacetylase
HGSOC high-grade serous ovarian cancer
LOH loss of heterozygosity
DREAM DP, RB-like, E2F4 and MuvB complex
MuvB multivulva class B
RB retinoblastoma protein
E2F adenovirus early gene 2 binding factor
CDK cyclin-dependent kinase
LFS Li-Fraumeni syndrome
IARC International Agency for Research on Cancer

Introduction

The recent explosion of sequencing of cancer genomes unequivocally identifies p53 as the most frequently mutated gene across the spectrum of human cancers. It is therefore unsurprising that the p53 protein and its encoding gene *TP53* (OMIM 191170) are the most frequently cited protein/gene in the literature (approaching 90,000 entries in PubMed), positioning this "guardian of the genome" as the most important tumor suppressor.

The p53 transcription factor is activated in response to a range of stress to affect diverse transcriptional programs. Canonically activation by acute DNA damage results in growth suppression by inducing cell cycle arrest to enable DNA repair or if damage is insurmountable induces senescence or cell death (Fig. 1). Despite functional genomics rapidly advancing our understanding of the mechanisms and diversity of downstream signaling networks regulated by p53 transcriptional activity, it remains incompletely understood how differential cell fates are affected and their relative contribution to tumor suppression. This article provides an

*Both authors contributed equally.

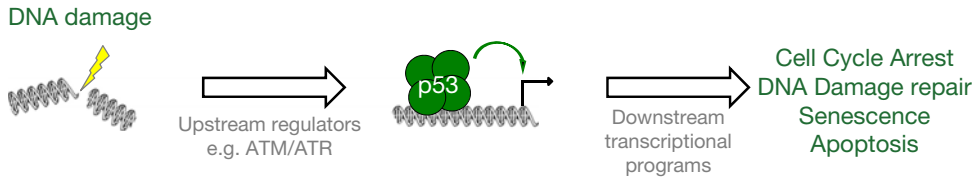

Fig. 1 *Canonical activation of p53.* In response to DNA damage stabilizes p53 and activates the p53 tetramer to bind to and activate p53 response elements associated with target genes to induce cell cycle arrest, DNA damage repair and if damage is sustained convert this signal to cell death or senescence.

overview of p53 structure and function, the diverse mechanisms through which cancers circumvent p53 activation, and highlights how functional genomic approaches have increased our understanding of the direct and indirect mechanisms through which p53 regulates diverse transcriptional programs. Finally, we discuss the burgeoning range of strategies aimed at exploiting p53 status therapeutically and highlight the importance of emerging roles for p53 in regulating the interaction with the stromal, immune and microbial microenvironment.

Discovery of the p53 Tumor Suppressor

The p53 protein was discovered more than 30 years ago as a 53 kDa binding partner of simian virus 40 (SV40) Large T antigen oncoprotein at the height of breakthroughs in the identification of viral oncogenes. Paradoxically this resulted in its initial classification as an oncogene, a presumption that was supported by the oncogenic properties exhibited by tumor derived forms of p53, which were in fact missense mutant p53 neomorphs. This misnomer was eventually rectified upon the cloning of Wild-type p53 and discovery that it exhibited tumor suppressive properties in vitro; that p53 knockout (KO) mice were highly cancer prone and the identification of germ-line autosomal dominant p53 mutations as the causal variants associated with highly cancer prone Li-Fraumeni Syndrome (LFS) families. Together establishing p53 as a *bona fide* tumor suppressor.

p53 Structure and Function

While a number of nontranscriptional activities of p53 in the nucleus, cytoplasm and mitochondria with potential tumor suppressive functions have been identified, the majority of p53s' tumor suppressive functions are thought to be exerted through its role as a sequence specific transcription factor. This is supported by observations that mutations identified sporadically in tumors and in germ-lines of LFS families predominantly affect the DNA-binding domain with the exception of the R337H mutation, which is found at high frequency in Brazil (Fig. 2).

Canonically in response to a variety of stress signals post-translational modification and stabilization of p53 results in binding of a p53 tetramer to internal or upstream p53 response elements (p53RE), enabling p53 to coordinate transcription of an expanding repertoire of genes affecting diverse biological processes. This is facilitated by the activities of the two N-terminal transcription activatory domains (TAD1 and TAD2), adjacent to a proline rich domain (PRD), the central DNA-binding domain (DBD) and a multifunctional carboxy terminus encoding oligomerization domain (OD), nuclear localization signals (NLS) and a flexible lysine-rich C-terminal domain (CTD) (Fig. 2). Understanding p53 function is complicated by the existence of at least 12 p53 protein isoforms, as a result of extensive alternative splicing, internal initiation and alternative promoter usage (Fig. 3). This is perhaps unsurprising given that the two additional p53 family members p63 and p73 discovered in the late 1990s, which function in development stratifying epithelia and neuronal derived tissues respectively, are known to undergo extensive alternative splicing; resulting in both

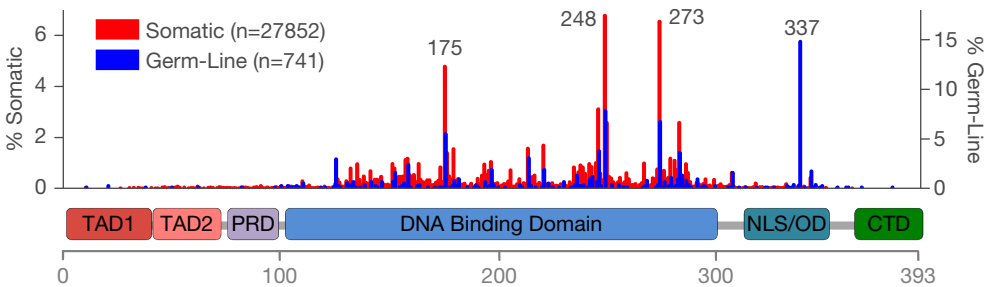

Fig. 2 *Schematic overview of p53 domain structure and mapping of frequency of somatic and germ-line mutations.* p53 protein domain structure includes two N-terminal transcription activatory domains (TAD1 and TAD2), proline rich domain (PRD), central DNA-binding domain (DBD), multifunctional Carboxy terminus encoding oligomerisation domain (OD), nuclear localization signals (NLS) and a flexible lysine rich-domain C-terminal domain (CTD). Frequency plot of p53 mutations detected in 27,852 somatic tumors (*red*) and 741 germ-line Li-Fraumeni patients (*blue*) for each of 393 amino acids in full length p53 from IARC p53 mutation database.

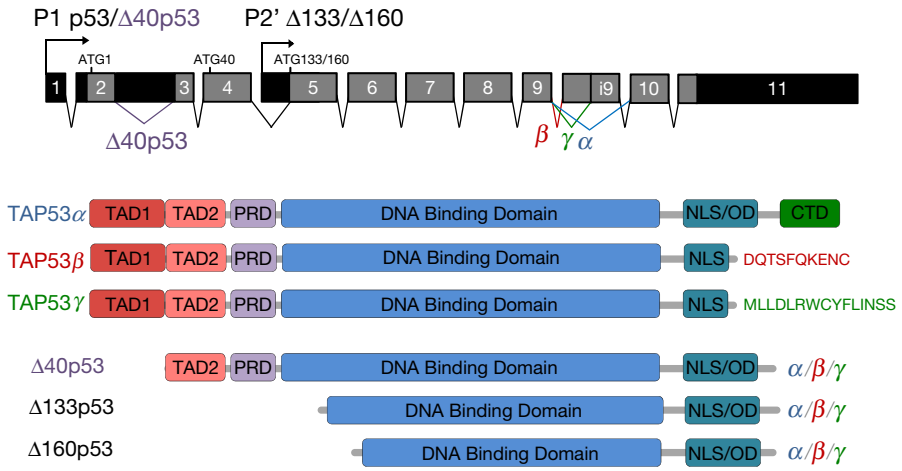

Fig. 3 *Schematic representation of TP53 Gene structure, mRNA splicing and resulting protein isoforms.* (A) Canonical full–length pTAP53α, originates from P1, containing both transcriptional activatory domains (TADs), while the Δ40 forms initiate from an internal ATG as result of alternative splicing or an internal ribosome entry site (IRES). truncated ΔN forms Δ133 and Δ160 originate from the P2 promoter, which along with the TA and Δ40 can be combined with alternative C-terminal splicing resulting in α, β and γ variants to generate a total of 12 protein isoforms.

tumor suppressive and oncogenic roles depending on the specific isoforms expressed. Similar to *TP63* and *TP73*, *TP53* encodes for several isoforms through conserved mechanisms resulting in combinations of alternative N- and C-termini. Canonical full–length p53, or TAP53α, originates from P1, containing both TADs and the longest C-term, and the Δ40 forms initiate from an internal ATG as result of alternative splicing or an internal ribosome entry site (IRES). Similarly, truncated ΔN forms Δ133 and Δ160 originate from the P2 promoter, which like the TA and Δ40 can be combined with alternative splicing of the C-term α, β and γ variants to generate 12 protein isoforms. Importantly, like p63 and p73, there is emerging evidence indicating that these different isoforms exhibit strikingly different expression patterns in different tissues, stages of development and differential activities. However, precise roles remain poorly understood. For example, expression of Δ133 has been shown to exhibit dominant negative activity over full-length TAP53α and elevated expression is associated with poor prognosis in certain cancer types.

Loss of p53 Tumor Suppressive Function in Cancer

Loss of the tumor suppressive functions of p53 is thought to be a prerequisite for the development of most tumors. This occurs through mutation in greater than 50% of tumors or in tumors retaining wild-type p53, its functions are often suppressed as a consequence of other oncogenic events that circumvent p53 tumor suppressive functions.

The vast majority of p53 mutations in both sporadic tumor and germ-line LFS occur in the DBD, highlighting the importance of DNA-binding and transcriptional function for tumor suppression. In the majority of sporadic and germ-line related tumor p53 mutation is accompanied by loss-of-heterozygosity (LOH) of the wild-type allele, however unlike canonical tumor suppressors such as RB or BRCA1 the majority of p53 mutations encode missense mutations (Fig. 4), with the most frequent somatic mutations R175H, Y220C, R249S, R248Q/W, R273C/H and R282W. The prevalence of missense mutations relative to nonsense, frameshift

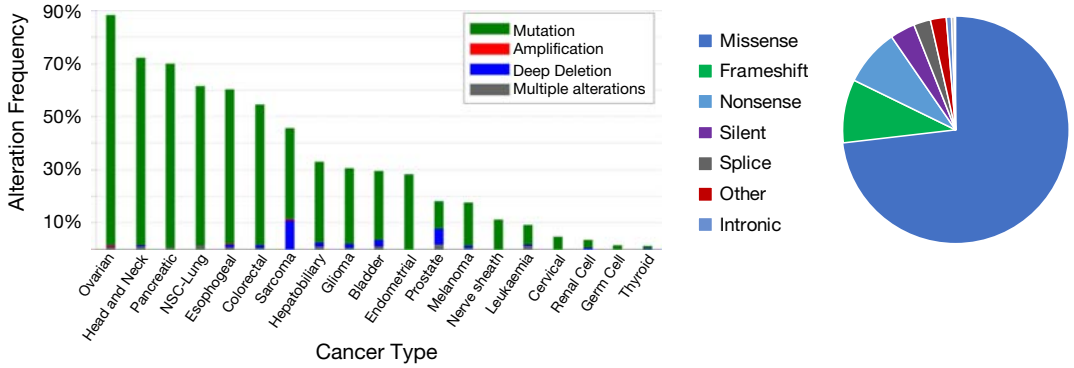

Fig. 4 *Frequency of p53 mutation and impact of mutations*: (A) Frequency of p53 mutation, copy number alterations, deep deletions and multiple aberrations in TCGA provisional datasets with >150 patients. (B) Frequency of different types of p53 mutations in IARC p53 mutation database.

and deletions suggest selection for expression of mutant p53. Missense mutations cluster in hot-spots in the DNA-binding domain (Fig. 2), result in expression of full length mutant forms of p53 with a dramatically reduced capacity to bind to p53REs and activate canonical target genes. Importantly, these neomorphic p53 proteins are expressed at high levels in tumors and have been shown to exhibit oncogenic gains-of-functions as well as dominant negative effects on residual wild-type p53 in tumors retaining a wild-type p53 allele. Missense mutations can be broadly separated into two groups: *Contact mutants* affect key arginine residues 248, 273 and 280 which are required for p53 to directly contact DNA and these generally retain wild-type confirmation and p53's ability to form tetramers. The effect of *conformation mutants* depends on the location and type of mutation, but in general they significantly alter p53 secondary structure and/or zinc binding, which affects the capacity to form tetramers, and they are prone to aggregate. The ability to form aggregates is an emergent property of mutant p53, which occurs due to an aggregation prone region surrounding Ile254 in the DBD that nucleates aggregates that are thought to play important roles in mutant p53 GoF. Interestingly, recent data further suggests that the frequent hot-spot nonsense mutation R213* results in expression of a truncated form of p53 with *de-novo* oncogenic functions. The emerging paradigm is that p53 mutant GOFs are likely context dependent in terms of the nature of the selection for different mutations, the tumor type, its microenvironment and the availability of binding partners through which a mutant exerts its GOF activity. It is also important to note that the role of mutant p53 isoforms remains largely unexplored.

What is evident from burgeoning studies of cancer genomes is that *TP53* mutational profiles differ in frequency and nature depending on tissue, molecular subtype and disease stage, suggesting specific underlying selection for different *TP53*-genotypes. The frequency of mutation is highly variable between cancer types: *TP53* is mutated in more than 95% of primary high grade serous ovarian cancer (HGSOC) where TP53 mutation has been shown to be a signature genetic event, whereas mutation frequencies are $<5\%$ in primary renal, germ cell and thyroid cancers (Fig. 4). While homozygous deletion of p53 is a rare event, distribution of these events also differs between disease site, being most frequently observed in primary Sarcomas and Prostate cancer. Genomic analyses are also revealing that most cancer types comprise several molecular subtypes that go beyond tissue/organ or histological classification. Dramatic differences in both frequency and nature of *TP53* mutation are observed in different subtypes of disease; for example, mutation rates in breast cancers range from 12% in Luminal A tumors to greater than 75% in triple negative basal-like tumors, which exhibit higher rates of nonsense and frameshift mutations than other subtypes. In colon cancers *TP53* mutations are very infrequently observed in microsatellite stable or BRAF mutant cancers despite being mutated in $>50\%$ of all primary colorectal cancers. Importantly, mutation rates for *TP53* show a strong association with late stage and metastatic tumors of various cancers and as increasing numbers of drug resistance tumor recurrences are sequenced it is evident that there is often strong selection of *TP53* mutation even in diseases where *TP53* mutation frequency in primary tumors is low. This is perhaps best exemplified by prostate cancer where *TP53* mutation is observed in only 12% of primary resected prostatectomies (Fig. 4), whereas *TP53* mutations are detected in $\sim 50\%$ of metastatic castrate resistant prostate cancers and selected for in vivo studies of resistance to antiandrogen therapies. *TP53* mutation rate and mutation type are also influenced by carcinogen exposure, where for example both Lung Adeno- and Squamous carcinoma exhibit high p53 mutation rate and similar mutation types and hepatocellular carcinomas have high levels of R249S driven by G-T transversions linked to aflatoxin exposure. Notably, very low *TP53* mutation rates are detected in cervical cancers in which the initiating event is high-risk human papilloma virus infection in greater than 95% of tumors, which inactivates p53 through the activities of the oncoprotein E6 that targets p53 for degradation. Similarly, *TP53* mutations are infrequently observed in the $\sim 50\%$ of Head and Neck Squamous Cell Carcinomas of the oropharynx associated with high-risk HPV infection.

Regulation of p53 Activity and Its Suppression in Cancer

As highlighted by the absence of p53 mutations in HPV-driven cervical and head and neck squamous cell carcinomas, the majority of the $\sim 50\%$ of tumors retaining wild-type p53 are presumed to circumvent or suppress p53 tumor suppressive functions through aberrations in the many regulatory pathways that impact on p53 function.

In homeostasis p53 is actively suppressed by a range of negative regulators, the best described of which is the E3 ligase mouse double minute 2 (MDM2). MDM2 binds to the p53 N-terminus and suppresses its transcriptional activity, exposing the p53 nuclear export signal (NES), and also acting as a specific E3 ubiquitin ligase that mediates poly-ubiquitination of multiple lysines in the p53 C-terminus leading to degradation by the proteasome (Fig. 5). This is further regulated by MDM4 and USP7, which augment MDM2 activity through inhibiting or removing MDM2 auto-ubiquitination. Canonically this is best understood in response to DNA damage when the ATM and/or ATR kinases directly and indirectly through CHK1/CHK2 kinases, sequentially phosphorylate a number of serine residues in the p53 N-terminus to block its interaction with MDM2. This leads to reduced of ubiquitination of the C-terminus and stabilization and decreasing nuclear export and alleviating repression of TAD to activate p53. These phosphorylation events can be removed by competitive activity of PPM1D phosphatase, affording an additional level of negative regulation. Consequently, this results in oscillating cycles of p53 activity in response to DNA damage, the amplitude and frequency of which is regulated by nature and intensity of upstream signal and is thought to ultimately impact transcriptional output and cell fate. Oncogene activation induced stress leads to up-regulation of the tumor suppressor p14-ARF, which through an alternative mechanism sequesters MDM2 in the nucleolus also leading to p53 stabilization. The central importance of this axis is further highlighted by observations that embryonic lethality observed in MDM2 and MDM4 knockout mice are rescued by p53 inactivation, and predisposition to early onset cancer in human patients with an SNP in MDM2 associated with elevated MDM2 levels.

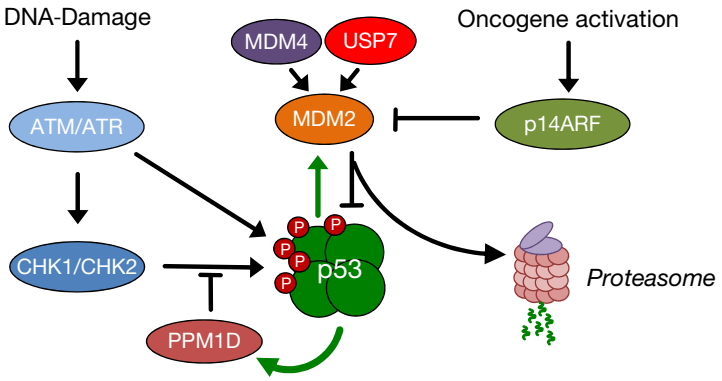

Fig. 5 *Core p53-MDM2 regulatory loop*: MDM2 binds to the p53 N-terminus suppressing transcriptional activity and also targets the p53 C-terminus for polyubiquitination leading to degradation by the proteasome. MDM4 and USP7 augment MDM2 activity through inhibiting or removing MDM2 auto-ubiquitination. This is inhibited by phosphorylation of N-terminus of p53 in response to DNA damage activated by ATM, ATR, CHK1 and CHK2, a subset of which can be competitively removed by activity of PPM1D phoshphatase. Oncogene activation induces p53 activation through inducing p14ARF which sequesters MDM2 in the nucleolus.

Unsurprisingly, tumors hijack this core p53 regulatory apparatus to suppress p53 function through amplification and overexpression of negative regulators including MDM2, MDM4, USP7, PPM1D and many other; mutation or deletion of upstream activators ATM/ATR and INK4A-ARF or oncogene-induced inhibition of p53 activation through activation of MDM2 or suppression of p53 activity.

Differential p53 activities are regulated through coordinated posttranslational modification (PTM) of amino acids scattered throughout the modular structure of p53, which collectively function to alter p53 stability, localization and transcriptional activity. This is affected through changes in affinity of p53 for DNA-binding and cofactor recruitment for transcriptional initiation as well as modulation of local chromatin environment. Large regions of flexible disorder between domains provide conformational flexibility to enable interaction with a large range of binding cofactors extensively regulated by competitive PTM. While important PTMs are observed throughout the p53 protein, these are particularly rich within intrinsically disordered N- and C-terminal regions.

The N-terminal TAD is involved in important negative regulatory interactions with MDM2/MDMX as discussed above, which not only enables targeting for degradation but also competes for binding coactivators p300 and CREB-binding protein (CBP). This competition is regulated by phosphorylation of multiple residues in the TAD (including S15, S20, T18), which block interaction with MDM2 stabilizing p53 and incrementally increasing affinity for coactivators enabling a stepwise response to genotoxic stress. Specific events such as phosphorylation of S46 by HIPK2 and other stress induced kinases have been suggested to enhance activation of cell death in part through breaking interaction with inhibitor iASPP (PPP1R13L).

The flexible C-terminal domain also plays a critical role in regulating p53 activity through PTM of the same six lysines residues (K370, 372, 373, 381, 382, 386) that are targeted for polyubiquitination by MDM2. Upon activation, recruitment of p300 to the phosphorylated N-terminus induces activatory acetylation of these and other key lysine residue (K120, 164, 320) in p53's DNA-binding and oligomerization domains. These residues are all are subject to extensive competitive PTM by acetyl-transferases (p300, PCAF, CBP, TIP60 and others) and de-acetylases (including HDAC1, 2 and SIRT1). This provides perhaps the best studied paradigm for p53 PTM and target selectivity and cofactor recruitment to differentially affect transactivation of pro-apoptotic target genes. This can be further influenced by phosphorylation of S366/T387 or S378/T377 or methylation (regulated by methyltransferease and demethylases) of the same lysine residues. Moreover, recent data has shown that loco-regional changes in charge of the acidic lysine rich C-terminal domain which forms a strong interaction with negative regulator SET that is abrogated upon neutralization of charge through acetylation of six lysine residues.

Specific acetylation of K373 and K120 has been suggested to favor apoptosis and K320 antagonizes such activity in cancer cells; however findings in terms of individual residues vary significantly between cell and tissue type, genotype and different stress responses. For example, paradoxically K373 acetylation induced by HDAC inhibitors in combination with DNA damage enhances death of cancer cells and survival of normal neuronal cells.

Importantly, our understanding of the inter-dependence and temporal interaction of even these relatively well described p53 PTMs remains limited and is further complicated by additional sites and moieties including mono- and di-methylation, sumoylation, neddylation, O-GlcNAcylation and ADP-ribosylation. The myriad of posttranslational modifications of p53 are regulated downstream of ever increasing diversity of stresses including DNA damage, oncogene activation, replication stress, ribosomal stress, hypoxia, telomere erosion, nucleotide depletion, oxidative stress, hypoxia, nutrient starvation and mechanical stress. The complex interaction of PTM and the burgeoning number of p53 direct targets (see later) likely underlies our limited understanding of how differential p53 activation results in a diversity of cell fates and their relative contribution to tumor suppression which almost certainly extends far beyond the classical view of cell cycle arrest, apoptosis and senescence (Fig. 6).

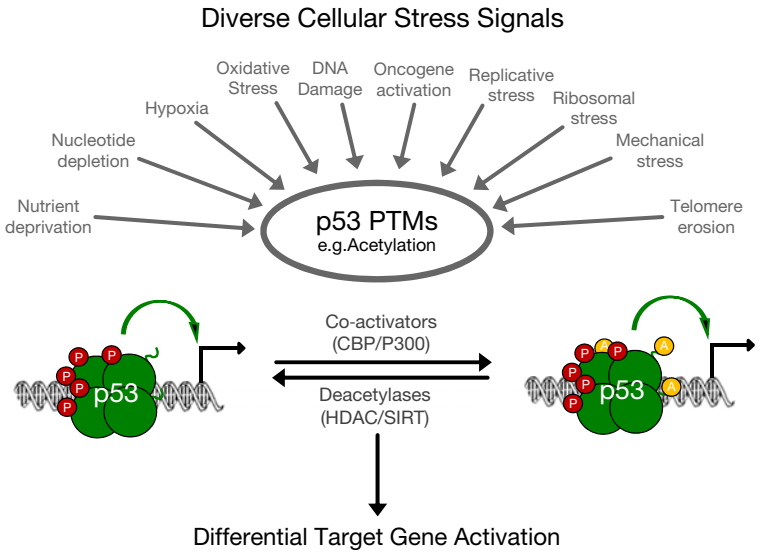

Fig. 6 *Overview of p53 regulatory signals.* Activation of p53 is mediated by a range of post-translational modification (PTM) downstream of sensors of highly diverse range of stress signals. These modifications are added or removed by competitive activity of enzymes with opposing functions. For example P300/CBP mediated acetylation of p53 C-term and other residues in the DNA-binding domain are removed by opposing activities of Histone- and Sirtuin de-acetylases. Combined effects of PTM of p53 tetramer influences the range of diverse transcriptional targets induced.

Transcription Regulation by p53

As illustrated above the prevailing function through which p53 exerts its tumor suppressive activities is transcriptional control of target genes that in turn regulate numerous downstream cellular processes. While it is clear that p53 activation leads to both the up- and down-regulation of a plethora of genes, it has long been disputed how p53 discriminates between activation and repression with numerous models for p53-dependent gene down-regulation being suggested over the years. Recent genome-wide studies, however, provide evidence that in the vast majority of cases, p53 itself functions solely as an activator of gene expression and that transcriptional down-regulation by p53 is mediated indirectly and largely dependents on the up-regulation of the best described and most robust p53 target gene the cyclin dependent kinase inhibitor p21 (CDKN1A) (Fig. 7).

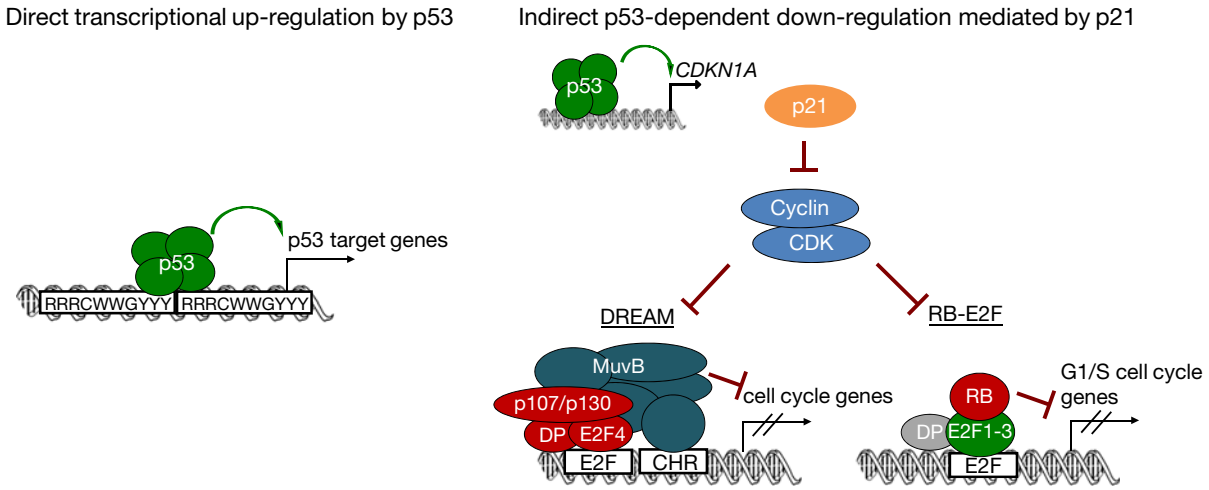

Fig. 7 *Mechanisms of p53-mediated transcription control.* Mechanisms involving direct target gene up-regulation by p53 and indirect down-regulation through p53-p21-DREAM/RB are supported by genome-wide data. When activated, p53 accumulates and binds target genes as a tetramer, which comprises two dimers that each binds a decameric half site with the consensus sequence RRRCWWGYYY. The highly responsive p53 target p21 inhibits CDK1 and CDK2 to re-activate RB family proteins that block E2F1–3 activity and form the DREAM repressor complex that down-regulates most cell cycle genes.

Activation of p53 Target Genes

As described earlier, in unstressed conditions, the transcriptional activity of p53 is masked by the ubiquitin ligase MDM2, which also promotes the degradation of p53. When activated, p53 accumulates and binds target genes as a tetramer, which comprises two dimers that each binds a decameric half site with the consensus sequence RRRCWWGYYY (R = A/G, W = A/T, Y = C/T). The two decameric half sites that form the p53RE can be separated by a spacer of 0–13 base pairs. Most functional p53REs contain no spacer and an increasing spacer length makes it less likely that the motif is functional and bound by p53. Notably, also decameric half sites without a nearby second decamer, have been identified to bind p53 and to mediate target gene activation. Unlike most transcription factors, p53 has pioneer like functions and can bind to DNA regardless of open or closed chromatin, however, only in some cases, p53 can open the closed chromatin to activate gene expression. In that regard p53 is lacking full pioneer function as it is unable to open most of the closed chromatin it can bind to and it likely requires other accessory proteins to induce transcription. Consequently, p53 can bind to DNA harboring its consensus binding sequence regardless of the local context such as chromatin state, cell type or binding of cofactors. However, the context is relevant for enabling p53-mediated recruitment of RNA polymerase II and to initiate target gene expression following DNA binding. To bind DNA, p53 functions as a dimer of dimers and it employs two transactivation domains (TADs) to recruit RNA polymerase II and to drive target gene transcription. Most p53 activated target genes recruit p53 in proximity (~2.5 kilo bases) to their transcription start site (TSS). In some cases, however, p53 has been shown to bind to an alternative TSS or enhancer elements located at intronic regions. When p53 binds to intronic regions, it can induce expression of an isoform that is shorter than the longest isoform and misses, for example, the 5′UTR or the first exon. The transactivation potential of p53 tends to decline with increasing distance to a gene's TSS.

The discovery of the first p53 target genes, including *CDKN1A* (*p21*) and *MDM2*, inspired numerous researchers to identify additional genes that mediate p53's tumor suppressor function. Today several hundred genes are known to be directly bound and upregulated by p53, which has been rapidly advanced by the use of next generation-based genomic approaches such as ChIP-seq and RNA-seq.

Indirect Gene Down-Regulation Mediated by the p53 Target p21

Numerous mechanisms have been proposed for mediating gene down-regulation and transcriptional repression in response to p53 activation, including sequestration of transcriptional activators by p53 or recruitment of p53 to specialized p53 response elements. However, multiple studies integrating ChIP-seq with global transcriptional profiling over the past few years provide evidence that p53 binding to promoters almost exclusively is associated with activation of transcription. While the vast majority of genes down-regulated in a p53-dependent manner are not associated with direct binding of p53 to promoter associated p53 REs, the data shows rather that suppression is largely mediated indirect through the highly responsive p53 target p21. The cyclin-dependent kinase (CDK) inhibitor p21 (also known as CDKN1A) performs important function in cell cycle regulation: it inhibits CDK1 and CDK2 that are essential for a cell to progress through the cell cycle. The cell cycle is divided into four phases that include mitosis (M), DNA synthesis (S), and the gap phases G1 and G2. Cell cycle entry and progression require that specific proteins are expressed at well-defined time points. Progression through the cell division cycle is driven by CDKs, which are bound and activated by cyclins. A critical step in the cell cycle is passage through the restriction point, which is also known as G1/S checkpoint. Cyclin D-CDK4/CDK6 and cyclin E-CDK2 complexes control the RB protein family that consists of RB, p107 and p130. During quiescence in G0, when cells have exited the cell cycle, and early during G1 phase, the RB family proteins are un-phosphorylated. In this state, hypo-phosphorylated RB binds to and sequesters the activating E2F transcription factors E2F1, E2F2 and E2F3 (E2F1–3), and RB's siblings p107 and p130 bind to E2F4 and to the MuvB complex to form the transcription repressor complex DREAM. When bound to RB, the activating E2F1–3 are incapable of activating their target genes, which comprise cell cycle genes critically required for entry to and progression through S phase. The DREAM complex represses transcription of both major classes of cell cycle genes: those with important function during S phase (G1/S genes) and those that function during mitosis (G2/M genes). G1/S cell cycle genes are largely controlled through an E2F (adenovirus early gene 2 binding factor) promoter element that recruits E2F transcription factors, and G2/M cell cycle genes are largely controlled through a CHR (cell cycle genes homology region) promoter element that recruits the MuvB complex. CDK-mediated phosphorylation of the RB family proteins leads to their inactivation and release of the activating E2F1–3. When the CDKs are inhibited by p21, RB family proteins become re-activated to block E2F1–3 activity and to form the DREAM repressor complex that down-regulates most cell cycle genes (Fig. 7). Thus, genes down-regulated in response to p53 activation are usually cell cycle genes. In addition to p21, several noncoding RNAs, including miRNAs and long noncoding RNAs, were reported to support gene down-regulation as downstream effectors of p53.

Cellular Responses

The tumor suppressor p53 functions primarily as a transcription factor that exerts its tumor suppressor function through many target genes (Table 1). These targets function in multiple processes that include, but are not limited to, programmed cell death, cell cycle arrest, senescence, DNA repair, autophagy, senescence, angiogenesis, stem cell renewal, stromal and immune interactions, metabolism, translation control and feedback mechanisms. Notably, knockout studies in mice and shRNA screens in human cell

Table 1 Examples of diversity of direct p53 target genes and associated biological processes

Cell Cycle Arrest	DNA Repair	Apoptosis	Sensescence
CDKN1A *SFN* *BTG2* *GADD45A* *FBXW7* *mir-34a*	*GADD45A* *RRM2B* *DDB2* *XPC* *POLH*	*BBC3* *PMAIP1* *BAX* *FAS* *TNFRSF10A-D* *APAF1*	*SERPINE1* *PML* *CDKN1A*
Autophagy	**Metabolism Antioxidant**	**Stem cell biology**	***Envrionment Immune***
PRKAB1 *SESN1* *SESN2*	*TIGAR* *PANK1* *PRKAB1* *GLS2* *SESN1/2* *FDXR*	*CDKN1A* *mir-34a* *mir-145* *NOTCH1*	*SERPINB5* *TLR3* *mir-34a*

lines revealed that there is no key p53 target gene that confers p53's tumor suppressor function but p53 distributes this function across numerous of its targets.

Programmed Cell Death: Apoptosis and Autophagy

Programmed cell death is important for multicellular species to maintain tissue homeostasis and to eliminate individual cells that may be harmful for the organism as a whole. Several different types of an ever-increasing number of cell death types including apoptosis, autophagy, necrosis and ferroptosis have been linked to p53.

Apoptosis in particular is the predominant p53 function that is conserved among p53's ancestral homologs. Both types of apoptosis, intrinsic and extrinsic, are regulated by direct p53 target genes (Fig. 8). The extrinsic apoptosis signaling pathway is largely controlled by the tumor necrosis factor (TNF) receptor family. These death receptors can be activated by external stimuli such as binding with FAS ligand, TRAIL or TNF-α, thereby leading to direct caspase activation that effects the destruction of the cell. The TNF receptor family encoding genes *FAS*, *TNFRSF10A*, *TNFRSF10B*, *TNFRSF10C* and *TNFRSF10D* are direct p53 target genes that become up-regulated in response to p53 signaling. Thus, p53 induces the expression of death receptors that can sense external stimuli to trigger apoptosis. On the other hand, the intrinsic apoptosis pathway is activated cell-autonomously and involves mitochondria. The BCL-2 family of proteins controls the release of cytochrome *c* from mitochondria. Pro-apoptotic BCL-2 family proteins, such as BAX and BAK, can homo-oligomerize at the outer mitochondria membrane to form pores that cause permeabilization of the membrane and release of cytochrome *c*. Antiapoptotic BCL-2 family members, such as BCL-2 and BCL-X, block interaction between pro-apoptotic BCL-2 proteins. BCL-2 family proteins characterized by a BH3-only region, including BBC3 (also known as PUMA) and PMAIP1 (also known as NOXA), can either activate the pro-apoptotic BCL-2 proteins or block the antiapoptotic BCL-2 proteins. Upon stress signaling, p53 directly up-regulates the expression of *BAX*, *BBC3* and *PMAIP1* to trigger the release of cytochrome *c* from mitochondria. Additionally, p53 up-regulates expression of the ceramide synthase CERS5 leading to the de novo production of C16-ceramide, which supports BAX translocation from the cytoplasm to the mitochondria. When released from mitochondria, cytochrome *c* binds to APAF1 and procaspase 9 to form the apoptosome. The apoptosome activates effector caspases to effect the destruction of the cell. Through direct up-regulation of *APAF1*, p53 further supports the intrinsic apoptosis pathway. Additionally, p53 induces the apoptosis enhancing nuclease *AEN* that supports apoptosis through digestion of double stranded DNA.

Autophagic cell death is another type of programmed cell death and autophagy represents a catabolic process engaged under metabolic stress. Autophagy starts with the activation of a preinitiation complex containing the Unc-51-like kinase 1 (ULK1) and autophagy related (ATG) proteins ATG13 and ATG17. This preinitiation complex is controlled by the metabolic checkpoint complexes AMP-activated protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1). Under metabolic stress, when AMP and ADP accumulate in the cell, AMPK activates the preinitiation complex and suppresses the activity of mTORC1. In contrast, mTORC1 inhibits the preinitiation complex in unstressed cells. When activated, the preinitiation complex recruits and activates the initiation complex containing the scaffold Beclin 1 and the kinases VPS34 and VPS15 that produce phosphatidylinositol 3-phosphate (PI3P) and promote formation of the autophagosome. The autophagosome can fuse with a lysosome to form an

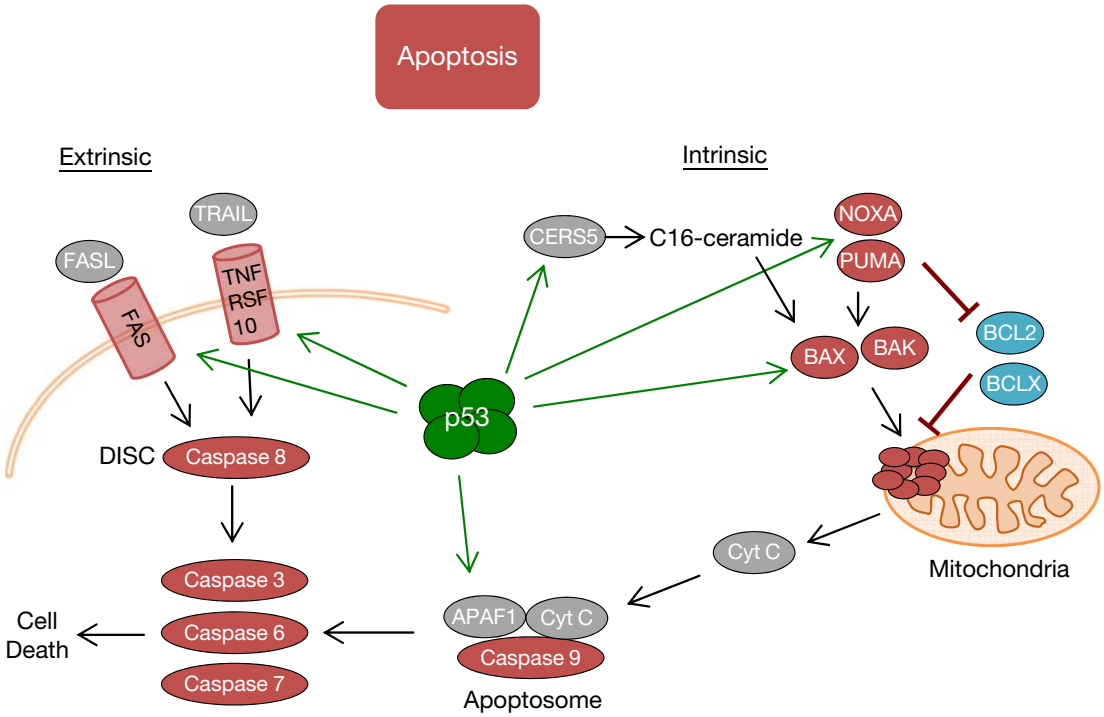

Fig. 8 *p53 can induce apoptosis through multiple direct target genes.* Genes directly up-regulated by p53 control the extrinsic and intrinsic apoptosis pathways. p53 up-regulates the expression of death receptors that can sense external stimuli to trigger apoptosis. A complex interplay of BCL-2 family proteins including BAX, BAK, NOXA, PUMA, BCL2 and BCLX regulates integrity of the outer mitochondrial membrane and release of cytochrome C (Cyt C). The initiator caspases (Caspase 8 and 9) can activate the effector caspases (Caspase 3, 6 and 7) that affect cell death. (*Green arrows* indicate direct transcriptional up-regulation by p53).

autolysosome that degrades any engulfed material. While autophagy, in general, is a survival process to eliminate protein aggregates and damaged organelles, it accompanies and often is required for autophagic cell death. Activation of p53 in response to nutrient deprivation, hypoxia or endoplasmic reticulum stress can induce the autophagy pathway through direct up-regulation of *PRKAB1*, which encodes for the catalytic subunit of AMPK and through up-regulation of *SESN1* and *SESN2*, which encode for proteins that activate AMPK. Together p53 can induce multiple programmed cell death types through direct up-regulation of various target genes.

Cell Cycle Arrest and Senescence

To maintain genomic integrity, it is important for cells to monitor and repair DNA damage. Cell cycle arrest is a critical means employed by a cell to allow for DNA damage repair before continuing the cell division cycle. To understand DNA damage-induced cell cycle arrest, it is critical to appreciate what drives the cell cycle in the first place and the checkpoints that are in place to monitor successful completion of different stages of division (Fig. 9). Cells resting in G0 or dividing cells are stimulated to enter the cell cycle by a mitogenic signal that activates cyclin D-CDK4/CDK6 complexes to drive G1 entry and progression. In late G1 phase, cyclin E-CDK2 complexes hyper-phosphorylate and inactivate RB, leading to the release of the activating transcription factors E2F1, E2F2 and E2F3 (E2F1–3) and passage through the restriction point. Once the G1/S boundary is passed, cells are usually committed to progress though one full cell division cycle. E2F1–3 induce the expression of cell cycle genes that are important for DNA replication during S phase. Cyclin A-CDK2, cyclin A-CDK1 and cyclin B-CDK1 complexes then enable progression through S, G2 and M phases. The transcription factors B-MYB and FOXM1 together with the MuvB complex are important for the expression of proteins that function in mitosis. These cell cycle checkpoints can be "activated" by a variety of stress signals to halt cell cycle progression. In the case of DNA damage this is largely affected through the activation of the checkpoint kinases ATM and ATR directly and indirectly through CHK1 and CHK2 kinases, which together can activate both p53-dependent and -independent cell cycle checkpoints. A rapid, p53-independent, cell cycle arrest involves inactivation of CDC25 phosphatases by CHK1 and CHK2 that are required for activation of CDK2 and CDK1 complexes. p53, however, is critical for a slower but sustained cell cycle arrest. p53 up-regulates the expression of the CDK inhibitor p21, which blocks CDK2 and CDK1 activity. Moreover, abrogated CDK2 and CDK1 activity leads to re-activation of the RB protein family that blocks the transcription factors E2F1–3 and re-shapes the MuvB complex to form the transcriptional repressor complex DREAM. Most, if not all, cell cycle genes become down-regulated through the DREAM complex. Moreover, p21 can bind to and block PCNA, a protein critical for DNA replication. Thus, by activating p21, p53 can block cell cycle progression through abrogation of CDK activity, inhibiting DNA replication and through depletion of hundreds

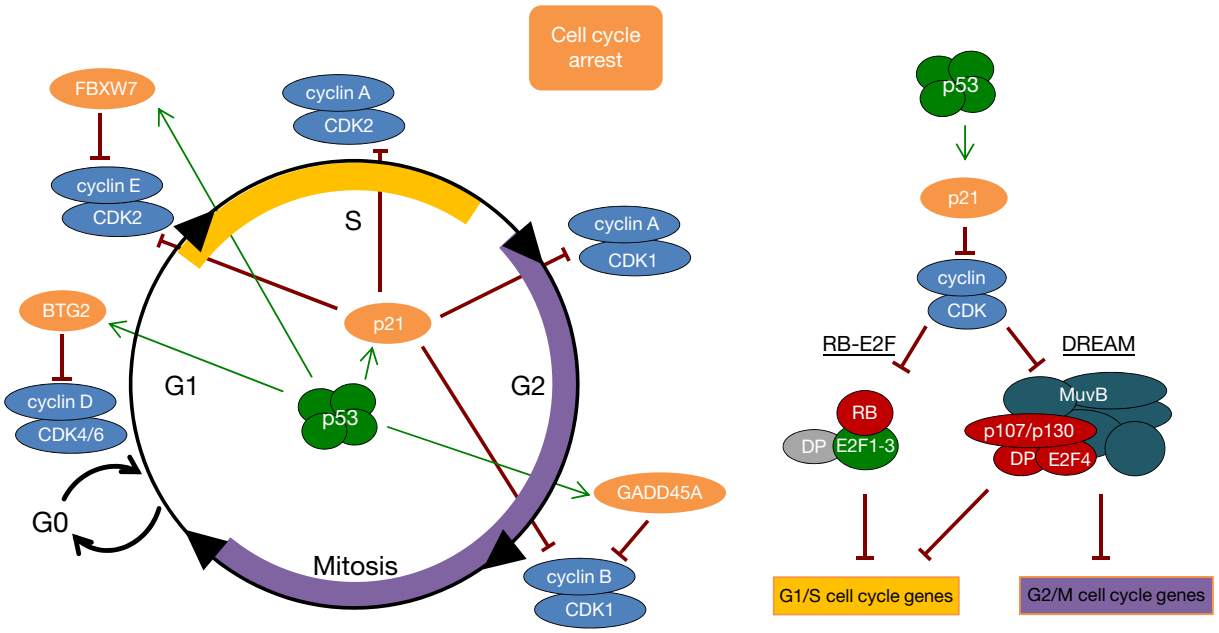

Fig. 9 *p53 controls the cell cycle.* Direct p53 target genes function in the regulation of the cell cycle. Through direct up-regulation of *CDKN1A* (*p21*), *BTG2*, *GADD45A* and *FBXW7* p53 can halt the cell cycle at multiple check points. The highly responsive p53 target p21 inhibits CDK1 and CDK2 complexes to re-activate RB family proteins that block E2F1–3 activity and form the DREAM repressor complex to down-regulate most cell cycle genes. (Green arrows indicate direct transcriptional up-regulation by p53.)

of cell cycle proteins. Notably, induction of p21 is sufficient to induce cell cycle arrest and both, p21 and RB, are required for cell cycle arrest. Additional p53 targets that contribute to cell cycle arrest include BTG2, which inhibits cyclin D expression and leads to G1/S arrest, and GADD45A, which dissociates cyclin B-CDK1 complex and leads to G2/M arrest (Fig. 9). Moreover, the p53 target SFN (also known as 14–3-3σ) translocates cell cycle proteins from the nucleus to the cytoplasm and the p53 target FBXW7 mediates degradation of the cell cycle proteins cyclin E and c-MYC through the SCF ubiquitin ligase complex.

Cell cycle arrest triggered by DNA damage is reversible after the damage is repaired and p53 becomes inactive. In contrast, excessive DNA damage, telomere shortening through too many cell divisions or oncogene-activation can trigger cellular senescence. A senescent cell displays larger cell size, autophagy and high lysosomal β-gal activity, and is usually incapable of re-entering the cell cycle. Senescence is largely controlled by the CDK inhibitors p21 and p16. Sustained expression of p21 or p16 is sufficient to induce cellular senescence. Excessive or un-repaired DNA damage or telomere shortening, which activates the DNA damage response, can continuously activate p53, which in turn sustains p21 expression. The senescent effect of p21 is largely mediated by RB. Oncogene-activation or excessive mitogenic signals can activate p16, which blocks CDK4 and activates RB independent of p53. Notably, oncogene-activation or excessive mitogenic signals also induce ARF, which in turn activates the p53 pathway. Thus, p21 is often activated in addition to p16. Abrogation of p21, p16 or RB impairs the senescence phenotype.

DNA Repair

As a key signal transducer in the DNA damage response, it is not surprising that p53 can activate DNA repair processes. Although it is still actively debated whether p53-mediated DNA repair is part of p53's function as tumor suppressor, it clearly supports cell viability. There are different types of DNA repair. The p53 target XPC, for examples, binds to RAD23B and is the initial DNA damage recognition factor in the global genomic nucleotide excision repair pathway that repairs thermodynamically destabilized DNA duplexes. Similarly, the p53 target DDB2 binds to DDB1 to form the UV-damage DNA binding complex that specifically recognizes UV-induced cyclopyrimidine dimers and recruits proteins for nucleotide excision repair. The cell cycle protein and p53 target PCNA is used in nucleotide excision repair to allow for incision of a new nucleotide by a polymerase after the erroneous nucleotide has been removed. Another process in response to DNA damage is translesion DNA synthesis (TLS), which allows insertion of a base opposite a lesion and bypass of the damaged DNA during replication. The p53 target POLH constitutes such a TLS polymerase that improves bypass of DNA damage and enhances cell survival. Importantly, p53 employs its target RRM2B, a ribonucleotide reductase, to fuel DNA repair by supplying precursors.

Most DNA repair genes, however, are also cell cycle-regulated genes that are critical to solve DNA damage that occurs during DNA replication, and as cell cycle genes these DNA repair genes become down-regulated in response to p53 activation through p21 and the DREAM complex. Thus, only a few DNA repair proteins become up-regulated in response to p53 activation and most of them are involved in nucleotide excision repair, which is particularly important in response to UV-induced DNA damage.

Interestingly, response to UV-induced DNA damage has been suggested to be one of the key drivers for the evolution of the p53 response in multicellular organisms.

Metabolism and Translation Control

Nutrient stress caused by nutrient deprivation can trigger a p53 response through AMPK activation and p53 targets participate in multiple metabolic pathways. Tumor cells often utilize aerobic glycolysis to fuel ATP levels, which is particularly important during hypoxic conditions when a tumor outgrows the blood supply. The p53 target TIGAR (TP53-induced glycolysis and apoptosis regulator) degrades fructose-2,6-bisphosphate leading to decreased activity of phosphofructokinase 1, which catalyzes an essential step in the glycolysis pathway (Fig. 10). Thus, through induction of TIGAR, p53 can impair aerobic glycolysis to decrease energy levels in tumor cells. P53 is also involved in regulating the synthesis and processing of coenzyme A (CoA) (Fig. 10). The p53 target *PANK1* encodes for a pantothenate kinase that catalyzes the initial rate-limiting step in the synthesis of CoA. At the same time, p53 up-regulates AMPK's catalytic subunit PRKAB1 and AMPK deactivates acetyl-CoA carboxylase (ACC) and beta-hydroxy beta-methylglutaryl-CoA reductase (HMGCR). Inactivation of ACC and HMGCR leads to reduced production of lipids and further increases the pool of acetyl-CoA that is available for entering the tricarboxylic acid (TCA) cycle. Additional metabolic enzymes among p53 targets include the fucosidase FUCA1 that degrades the carbohydrate fucose and is required for complete breakdown of glycolipids and glycoproteins; the mitochondrial flavoprotein FDXR that initiates electron transport for cytochromes P450 that also function to metabolize potentially toxic compounds; and GLS2 that catalyzes the hydrolysis of glutamine to glutamate and ammonia. Together, p53 employs a variety of targets to regulate metabolic pathways in the cell.

Protein biosynthesis and mRNA translation are linked to metabolism and p53. The mTOR protein can form the TORC1 complex that controls cell growth and protein biosynthesis. TORC1 is controlled by a TSC1-TSC2 complex, which inhibits TORC1 function.

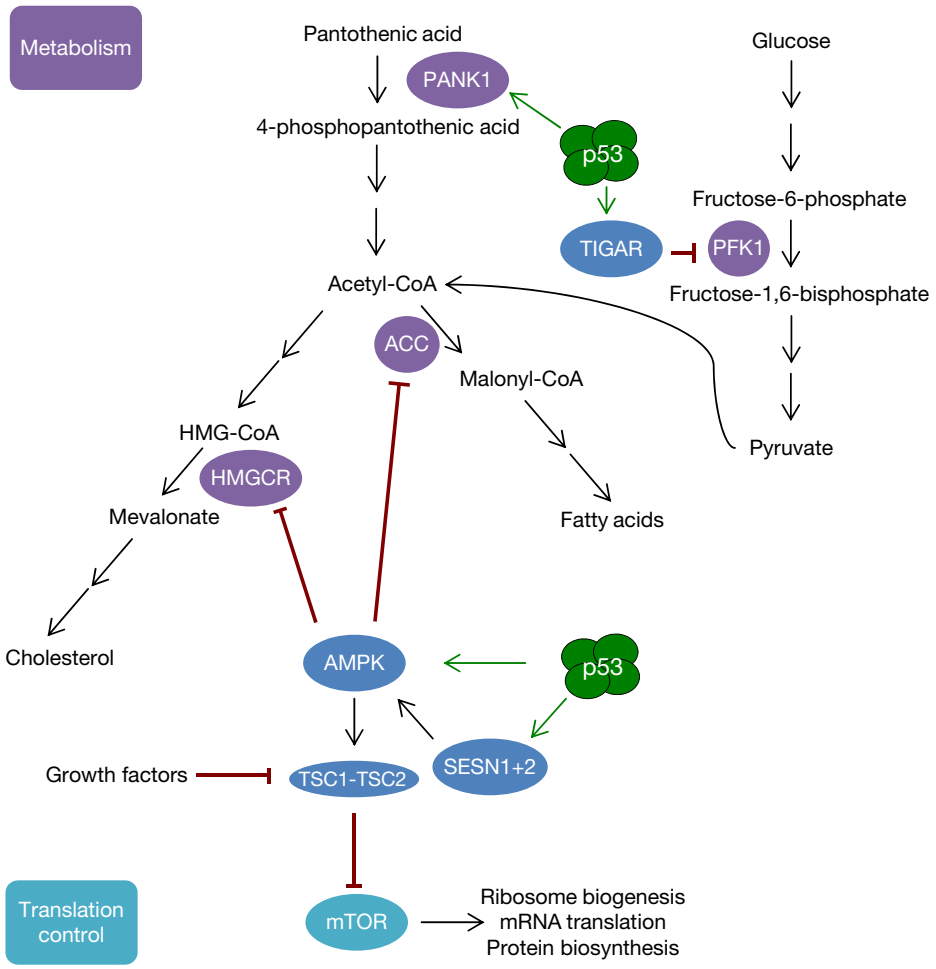

Fig. 10 *Metabolic pathways influenced by p53.* Cellular metabolism and translation is regulated by p53 through direct p53 target genes such as *PANK1*, *TIGAR*, *PRKAB1*, *SESN1* and *SESN2*. Through its direct targets, p53 inhibits glycolysis, reduces production of fatty acids and increases coenzyme A (CoA) levels. Moreover, p53 target genes negatively regulate mTOR to decrease protein biosynthesis. (Green arrows indicate direct transcriptional up-regulation by p53.)

TSC2 is negatively regulated by growth factors but activated by AMPK. The p53 targets SESN1 and SESN2 activate AMPK to positively regulate TSC2 leading to inhibition of mTOR (Fig. 10). Inhibition of TORC1 activity decreases protein biosynthesis, mRNA translation and cell growth. Moreover, p53 signaling leads to down-regulation of ribosomal RNA (rRNA) that is required for mRNA translation. Thus, p53 employs multiple means to impair protein biosynthesis and cell growth.

Feedback Regulation

Through activation of its target genes, p53 activates several feedback loops, both positive and negative. The best-known feedback loop employs the p53 target MDM2, a ubiquitin ligase, which mediates degradation of p53 (Fig. 5). Additionally, the p53 target cyclin G1 activates MDM2 through dephosphorylation. Thus, while phosphorylated active p53 cannot be bound by MDM2, it increases levels of active MDM2. At the same time, the p53 target PPM1D (also known as WIP1) accumulates and can dephosphorylate p53 leading to its MDM2-mediated degradation. Together, p53 signaling becomes abrogated through its own targets. Moreover, multiple p53 targets dampen lethal programs induced by p53. The p53 target TRIAP1, for example, is an inhibitor of apoptosis and the p53 targets PGF, TGFA and KITLG are growth factors that can stimulate cell proliferation. Thanks to these negative feedback circuits, cells that are able to resolve stress conditions can survive instead of rapidly committing to lethal processes such as programmed cell death or senescence.

Exploiting p53 Status

The near ubiquitous nature of de-regulation of p53 function in cancer has led to significant effort in therapeutic strategies to exploit the different underlying mechanisms and consequences of p53 inactivation.

Activating Wild-Type p53

Re-activation or hyper-activation of the tumor suppressive functions of p53 in the ~50% of tumors which harbor wild-type p53 represents an attractive therapeutic target particularly since genetic reactivation is highly effective at inducing tumor regression in mouse models. The central regulatory importance of MDM2-p53 has received significant attention and led to the development of the Nutlins. These small molecule protein-protein interaction inhibitors block the interaction between MDM2 and p53 N-terminus, mimick the effects of phosphorylation and are highly effective at stabilizing p53. However, these first-generation agents promote variable fates depending on cell type in all but MDM2-amplified cells, in which they induce cell death and while tolerated in vivo, toxicities including neutropenia and thrombocytopenia have proven to be dose-limiting for first generation MDM2 inhibitors (MDM2i) in the clinic. Nonetheless, there are significant ongoing efforts to develop next-generation small molecule and peptide inhibitors of MDM2 which overcome these potentially on-target toxicities. Moreover, an increased recognition that inhibition of both MDM2 and MDMX may be required for full p53 activation has led to the development of dual targeting inhibitors and stapled peptides and most recently novel agents targeting MDM2 stability through inhibition of USP7.

Direct effectors of oncogenic repressive post-translational modifications of p53 also potentially provide a rich source of potential targets. For example, PPM1D inhibitors that block removal of Ser15 and block p53-MDM2 interactions have been developed to target PPM1D amplified tumors. p53 activity has also be shown to be increased by inhibitors of sirtuins and histone deacetylases. Such p53 activatory strategies along with those targeting a burgeoning number of interactions associated with chromatin-modifying enzymes which impact on p53 function are targets for drug-development. These represent tractable targets alone or in combination with conventional or targeted agents (including MDM2i) or indeed with agents that restore wild-type p53 conformation.

Targeting Mutant p53

A number of strategies have been developed to re-activate abundantly expressed missense mutant p53 and one such molecule, PRIMA-met, is already in clinical trials in HGSOC. The use of suppressors of premature termination codons and resulting nonsense mediated decay for mutations such as R213* have also shown some promise. While the mechanism and partners responsible for mutant p53 GOF are diverse and context dependent, recent evidence suggests that the transcriptional mechanisms endowed through interaction or sequestration of transcription factors may alter expression of core set of target pathways or processes (e.g., chromatin modification). These may be clinically targetable; for example targeting activation of SREBP targets in the mevalonate pathway in mutant p53 expressing tumors with statins. While alternative strategies exploiting addiction to mutant p53 stabilization indirectly with inhibitors of HDACs and heat shock proteins or peptide inhibitors of aggregation exhibit preclinical efficacy.

Exploiting p53 Deficiency

A number of strategies aimed at exploiting synthetic lethal liabilities induced by homeostatic or therapy induced DNA-damage in p53 deficient cells have also been identified. These are mostly related to induction of DNA damage induced cell cycle checkpoints. Strategies involving inhibitors of ATM, CHK2, ATR, CHK1, PLK1, CDC7 and WEE1 are actively being investigated alone or in combination with genotoxic agents. p53 mutant tumors are also being targeted with oncoloytic tumor viruses capable of replicating only

in p53 deficient cells and viral and mRNA re-introduction of chimeric hyperactive wild-type p53. Inhibitors of POL2RA that exploit collateral effect of p53 deletion resulting in haploinsufficiency of POL2RA open a therapeutic window for POL2RA inhibitors. Moreover, MDM2 inhibitors are also being explored for their use in cyclotherapy wherein the transient induced arrest induced in normal cells protects cells from the cytotoxic side effects of chemotherapies that require cell division and enables the use of higher drug doses.

Prospective Vision

As highlighted above, genomic technologies are rapidly increasing our understanding of the spectrum of genes and pathways regulated directly and indirectly downstream of p53 activation and how they are differentially affected by the various mechanisms of p53 inactivation. Deployment of such technologies to profile responses across 1000's of single cells in the lab or across 1000's of cancer patients will significantly increase our understanding of the contribution of different processes to cell fate, prognosis, response to different treatments and inform novel personalized treatment strategies to improve cancer patient outcome. Mutant p53 is one of the first to describe tumor specific, immune stimulatory neoantigens. Excitingly, there is increasing evidence to support important roles for p53 in tumor immunology and immune homeostasis, which once better understood will hopefully inform novel treatment strategies in combination with burgeoning number of immune-modulatory therapies.

See also: Li–Fraumeni Syndrome.

Further Reading

Bieging, K.T., Mello, S.S., Attardi, L.D., 2014. Unraveling mechanisms of p53-mediated tumor suppression. Nature Reviews. Cancer 14, 359–370.
Fischer, M., 2017. Census and evaluation of p53 target genes. Oncogene 36, 3943–3956.
Fischer, M., Grossmann, P., Padi, M., DeCaprio, J.A., 2016. Integration of TP53, DREAM, MMB-FOXM1 and RB-E2F target gene analyses identifies cell cycle gene regulatory networks. Nucleic Acids Research 44, 6070–6086.
Garcia-Alonso, L.M., Iorio, F., Matchan, A., Fonseca, N.A., Jaaks, P., Peat, G., Pignatelli, M., Falcone, F., Benes, C.H., Dunham, I., et al., 2017. Transcription factor activities enhance markers of drug sensitivity in cancer. Cancer Research 1679, 2017.
Green, D.R., Llambi, F., 2015. Cell death signaling. Cold Spring Harbor Perspectives in Biology 7, a006080.
Hoe, K.K., Verma, C.S., Lane, D.P., 2014. Drugging the p53 pathway: Understanding the route to clinical efficacy. Nature Reviews. Drug Discovery 13, 217–236.
Joerger, A.C., Fersht, A.R., 2016. The p53 pathway: Origins, inactivation in cancer, and emerging therapeutic approaches. Annual Review of Biochemistry 85, 375–404.
Kastan, M.B., Bartek, J., 2004. Cell-cycle checkpoints and cancer. Nature 432, 316–323.
Kastenhuber, E.R., Lowe, S.W., 2017. Putting p53 in context. Cell 170, 1062–1078.
Kim, M.P., Lozano, G., 2018. Mutant p53 partners in crime. Cell Death and Differentiation 25, 161–168.
Kruiswijk, F., Labuschagne, C.F., Vousden, K.H., 2015. P53 in survival, death and metabolic health: A lifeguard with a license to kill. Nature Reviews. Molecular Cell Biology 16, 393–405.
Leroy, B., Anderson, M., Soussi, T., 2014. TP53 mutations in human cancer: Database reassessment and prospects for the next decade. Human Mutation 35, 672–688.
Levine, A.J., Oren, M., 2009. The first 30 years of p53: Growing ever more complex. Nature Reviews. Cancer 9, 749–758.
McDade, S.S., Patel, D., Moran, M., Campbell, J., Fenwick, K., Kozarewa, I., Orr, N.J., Lord, C.J., Ashworth, A.A., McCance, D.J., 2014. Genome-wide characterization reveals complex interplay between TP53 and TP63 in response to genotoxic stress. Nucleic Acids Research 42, 6270–6285.
Sullivan, K.D., Galbraith, M.D., Andrysik, Z., Espinosa, J.M., 2018. Mechanisms of transcriptional regulation by p53. Cell Death and Differentiation 25, 133–143.
Zaika, A.I., Wei, J., Noto, J.M., Peek, R.M., 2015. Microbial regulation of p53 tumor suppressor. PLoS Pathogens 11, e1005099.

Relevant Websites

http://p53.iarc.fr/.
https://p53.fr/.
http://www.targetgenereg.org/.

Tumor-Associated Macrophages

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Glossary

Branching morphogenesis Growth and branching of epithelial tubules during embryogenesis.
Chemokine A type of cytokine that stimulates chemotaxis of cells.
Cytokine An immunoregulatory protein often secreted by immune cells to regulate other immune cells.
Extravasate Invasion of a cancer cell through a blood or lymphatic vessel wall and into the surrounding tissue.
Intravasate Invasion of a cancer cell through the basement membrane and into a blood or lymphatic vessel.
Plasticity The adaptability of a cell to its environment.
Ramified A highly branched morphology.

Abbreviations

CCL2 Chemokine (C–C motif) ligand 2
CSF-1 Colony-stimulating factor-1
CSF-1R Colony-stimulating factor-1 receptor
CTLA-4 Cytotoxic T-lymphocyte-associated antigen 4
ECM Extracellular matrix
EGF Epidermal growth factor
GM-CSF Granulocyte-macrophage colony-stimulating factor
HSC Hematopoietic stem cell
IFNγ Interferon γ
IGF-1 Insulin-like growth factor-1
IL-4 Interleukin-4
LPS Lipopolysaccharide
MDSC Myeloid-derived suppressor cells
PD-1 Programmed death-1
TAM Tumor-associated macrophage
TMEM Tumor microenvironment of metastasis
TNF-α Tumor necrosis factor-α
Treg Regulatory T cell
VEGF Vascular endothelial growth factor

Introduction

For well over a century, macrophages, as their name implies, have been recognized for their ability to phagocytose foreign particles and dying cells. Early observations of macrophages ingesting microbes suggested a role in host defense in infection and injury but Élie Metchnikoff was the first to recognize the physiological importance of macrophage phagocytosis in organismal development and homeostasis. Despite Metchnikoff's prescience, throughout most of the 20th century the primary function of macrophages was considered to be immune surveillance. However, the recent development of powerful molecular biological and imaging techniques combined with macrophage-specific stains has allowed us to study the origins, distribution and functions of tissue resident macrophages in much greater detail. It is now evident that macrophages have a much broader role than just immune protection. They form an integral part of every organ system from the earliest stages of embryonic development and contribute to normal development and maintenance of their host tissues. Indeed, macrophages have been aptly described as housekeepers or accessory cells to the highly specialized parenchymal cells in each tissue as they support optimal functioning of the primary cells. For nascent macrophages to successfully integrate into different tissues throughout the body, they adapt their form and function in response to specific

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physiological demands of each tissue or organ. In addition, as recognized by the earliest macrophage researchers, macrophages respond to tissue damage and contribute to tissue repair mechanisms through a process of inflammation to eventually restore homeostasis. This intimate connection with host tissues in health and disease can also have deleterious outcomes as macrophage functions can be co-opted by the disease process, leading to progression of the pathological condition. This maladaptive pathophysiological response is particularly true of tumor-associated macrophages (TAMs).

Origins, Distribution and Diversity of Macrophages

Just as our understanding of the many immunophysiological roles of macrophages has developed rapidly in recent years, our knowledge of the origins of different macrophage populations has undergone a paradigm shift. Previously, all tissue macrophages were thought to originate from circulating monocytes derived from hematopoietic stem cells (HSC) in the adult bone marrow. A corollary of this notion was that tissue macrophages were considered to be terminally differentiated and unable to divide. Macrophages are now known to arise from at least three different sources; yolk sac-derived erythro-myeloid progenitor cells, fetal liver HSCs and post-natal bone marrow HSCs (Fig. 1). Depending on its particular demands and functions, each tissue or organ has its own distinct mix of embryonic or adult derived macrophage populations. Furthermore, most tissue macrophage populations are embryonically derived and maintained by self-renewal without replenishment by circulating monocytes.

Whether embryonic or adult in origin, macrophages are highly plastic and rapidly change their expression profile to adapt to the diverse environments and demands of tissues throughout the body. Parenchymal cells instruct newly arrived macrophages to alter their morphology and behaviour, including secretion of specific trophic factors required for development and homeostasis of the host tissue. For example, yolk sac derived microglia develop a ramified shape in the brain where they secrete brain derived neurotrophic factor to promote synapse formation. Tissue macrophages are also required for remodeling of the extracellular matrix (ECM) and removal of apoptotic cells as exemplified in the breast, which undergoes striking changes not only at puberty but also during each menstrual cycle and pregnancy with phagocytosis of large numbers of dying epithelial cells required during involution. Considering the variety and functions of different tissues, the heterogeneity of tissue macrophages is not surprising.

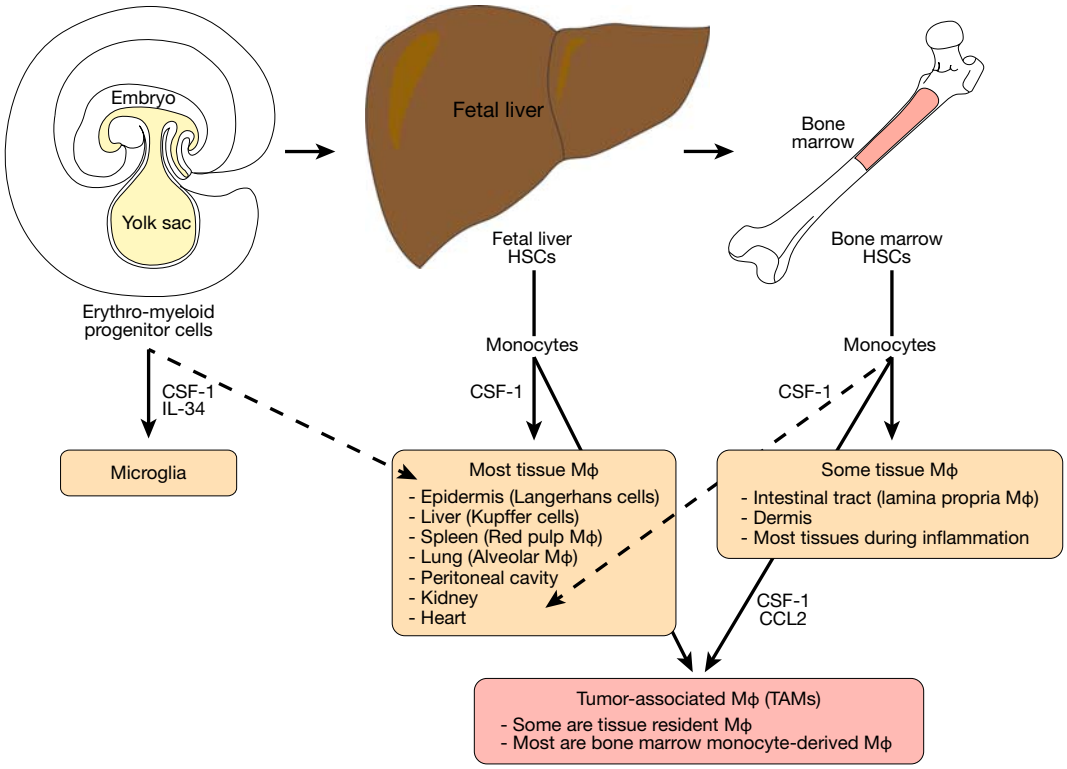

Fig. 1 Origins of macrophages in tissues and tumors. Macrophages arise from three different sources. Yolk sac-derived erythro-myeloid progenitor cells give rise to 100% of microglia in the brain and some Langerhans cells in the epidermis. Most tissues are seeded during embryogenesis with macrophages that are self-renewing and develop from fetal liver hematopoietic stem cells (HSC) and differentiate into monocytes. Macrophages in the intestines and dermis are continually replaced by differentiating monocytes derived from adult bone marrow HSCs. There is some evidence that bone marrow-derived macrophages may also contribute to kidney and heart macrophage populations. Inflammatory conditions lead to an influx of bone marrow-derived macrophages. Irrespective of their origins, all tissue macrophages differentiate under the influence of CSF-1 although IL-34, another ligand for the CSF-1R, drives development of microglia and Langerhans cells. While some TAMs are derived from tissue resident macrophages, the majority are recruited from bone marrow HSCs under the influence of CSF-1 and CCL2.

Nevertheless, macrophages share the common capacities of phagocytosis and migration as they also patrol their host tissues to detect and respond to infection or injury.

Macrophages undergo additional transcriptional reprogramming or "activation" in tissues as a result of exposure to cytokines, growth factors and other local influences. The best known of these activating factors are interferon γ (IFNγ) which, along with the microbial stimulus lipopolysaccharide (LPS), induces a pro-inflammatory, classically activated state in macrophages while the cytokines interleukin (IL)-4 and IL-13 induce an anti-inflammatory, alternatively activated state. Earlier studies of macrophage activation gave rise to the simplistic binary concept of M1 (classically) versus M2 (alternatively) activated macrophages. However, more recent transcriptomic analyses of macrophages derived from different tissues or exposed to various cytokine combinations demonstrate remarkable plasticity and diversity in their activated phenotypes. Thus, a more appropriate framework in which to consider macrophage activation is that of a spectrum, which allows for the presence of different macrophage populations within the same tissue depending on the precise mix of cytokines and conditions such as oxygenation in the immediate vicinity (Fig. 2). In this context, the primary macrophage lineage regulator, colony-stimulating factor-1 (CSF-1, also known as macrophage-CSF, M-CSF), is considered a baseline cytokine that is essential for the production of most tissue macrophages. Consistent with this, macrophages derived from the yolk sac, fetal liver and adult bone marrow progenitors all express the receptor for CSF-1 (CSF-1R). Macrophages that have differentiated under the influence of CSF-1 but have not yet been further influenced by other cytokines are sometimes described as M0 macrophages (Fig. 2). CSF-1 not only induces macrophage proliferation and differentiation but also stimulates macrophage migration, both acutely as a chemokine and through increased expression of motility-regulating proteins. CSF-1-induced macrophage migration is not only important for normal development and homeostasis but also the promotion of tumor invasion by TAMs.

Tumor-Associated Macrophages

Background

Cancers do not consist solely of tumor cells but contain an abundance of nonmalignant host cells, including large numbers of immune cells along with other cell types such as endothelial cells, fibroblasts and other mesenchymal cells. Immune infiltrates are found to a greater or lesser extent in all tumors with innate and adaptive immune cells and their cytokine products flooding the tumor microenvironment. Indeed, chronic, non-resolving inflammation has long been recognized as a premalignant condition and is now recognized as an enabling characteristic of the malignant process itself. Untreated, long-term infections caused by microbes ranging from viruses to parasites produce chronic inflammation in the infected tissues, which leads to cancer in those tissues, for example Hepatitis B and C viruses in the liver, *Helicobacter pylori* in the stomach and *Schistosoma haematobium* in the urogenital tract. It is thought that at least 15% of cancers world-wide are caused by chronic infections. Non-infectious causes of chronic inflammation also lead to cancer, including inflammatory bowel disease, which predisposes to colorectal cancer, and tissue irritants such as inhaled asbestos fibers and now perhaps carbon nanotubes, which can work their way through to the pleural cavity where they drive chronic inflammation to induce malignant mesothelioma.

As macrophages normally constitute up to 20% of tissue mass in organs such as the liver, it is not altogether surprising that TAMs are usually the most abundant immune cell in cancers where they can make up more than 50% of the tumor mass. Growing tumors have extensive metabolic and housekeeping demands, causing macrophage numbers to swell further. However, the considerable

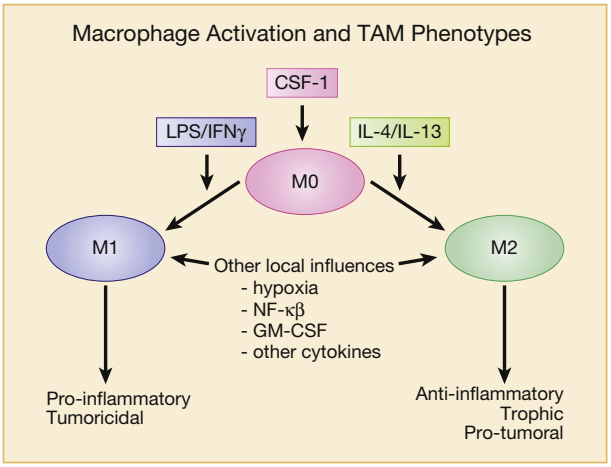

Fig. 2 Macrophage precursor cells, including erythro-myeloid progenitors and mononuclear phagocytic lineage cells are induced to mature into adherent, migratory baseline macrophages (M0) under the influence of CSF-1. M0 macrophages then differentiate under instruction by the specific mix of cytokines and other conditions in the local environment of their tissue of residence. While the binary notion of M1/M2 activation in macrophages is highly simplistic, conceptually it demonstrates the primary influences driving phenotypic changes towards tumoricidal or tumor supporting behavior in macrophages.

accumulation of TAMs was only routinely detected in tumors with the advent of antibodies specific for macrophage markers. Subsequently, TAMs were found to exist in large numbers in solid tumors such as breast, ovarian, bladder, stomach, head and neck and non-small cell lung cancer. Moreover, their density was shown to correlate with poor outcomes. TAMs are now known to accumulate in hematological tumors such as lymphoma, both Hodgkin's and non-Hodgkin's, and chronic lymphocytic leukemia also, with deleterious outcomes in each. Thus, increased TAM density correlates with poor outcomes in tumors of widely different origins, although a striking exception exists in the case of colorectal cancer in which increased numbers of TAMs are associated with positive outcomes.

Origin, Recruitment and Activation of TAMs

In steady state conditions, most tissue macrophage populations are maintained by self-renewal of the largely yolk sac or fetal liver-derived macrophages. However, in inflammatory conditions where demands for macrophages are dramatically increased, the self-renewal capacity of resident macrophages is overwhelmed and their numbers are augmented by circulating monocytes. Tumors themselves are chronic, sterile inflammatory environments where there is evidence that both infiltration of monocytes and proliferation of local tissue macrophages play a role in the accumulation of TAMs during tumor growth. Nevertheless, the balance of evidence in several mouse models of cancer, including breast cancer, lung cancer and glioblastoma multiforme, indicates an influx of monocyte-derived TAMs predominates over proliferation of locally-derived macrophages in the accumulation of TAMs (Fig. 1).

Circulating monocytes are recruited to tumors by a range of chemokines, the most important of which are CSF-1, vascular endothelial growth factor (VEGF) and chemokine (C−C motif) ligand 2 (CCL2) and CCL5. Interestingly, monocytes entering tumors may not always come directly from the bone marrow as some appear to be deployed from a splenic reservoir. In addition, not all incoming monocytes differentiate fully into macrophages although they do respond to influences in the tumor microenvironment to become tumor-induced effector monocytes, which are also known as myeloid-derived suppressor cells. It is important to note that the studies contributing to these observations used mouse models of cancer and much less is understood with respect to the origins and recruitment of macrophages and other myeloid cells in human tumors. Nevertheless, elevated circulating levels of CSF-1 are associated with poor outcomes in several human cancers, including breast, ovarian and pancreatic cancer as well as Hodgkin's lymphoma.

Consistent with the plasticity of macrophages, TAMs evolve with tumors as they progress to invasion and metastasis. There is strong evidence that, early in the evolution of a tumor, TAMs are pro-inflammatory and anti-tumoral in their activities as they try to eliminate tumor cells. However, as the tumor progresses and produces signals to subvert the behavior of its resident macrophages, TAMs transition to a pro-tumoral, immunosuppressive phenotype. Anti-tumoral TAMs are commonly described as M1 macrophages while pro-tumoral TAMs express many markers of an M2 phenotype. However, TAMs often show a mixed phenotype with both M1 and M2 markers, supporting the notion of a spectrum of activation depending on particular signals produced in the immediate vicinity of each TAM. Consequently, TAMs exhibit significant heterogeneity in function and gene expression within a single tumor according to local influences such as the level of hypoxia or whether the TAM resides within tumor nests or are found at the invasive front. In addition to this intra-tumoral plasticity, populations of TAMs exist that express specific, fixed phenotypic markers such as perivascular TAMs, which express Tie2. These macrophages have a particular patho-physiological function to stimulate angiogenesis in ischemic conditions to thereby restore homeostasis. Tumors exploit their angiogenic capacity to produce a neovascular network for the tumor. Despite the heterogeneity of TAM phenotypes, secretion of IL-4, IL-10, IL-13 and other cytokines by tumor immune cells, especially T cells, and tumor cells themselves as the tumor progresses, leads to a strong pro-tumoral TAM phenotype. Interestingly, the phenotype of TAMs in human colorectal cancer is anti-tumoral, supporting the correlation of high TAM numbers with positive outcomes in this cancer. Pro-tumoral TAMs contribute to the promotion of malignant progression in a number of ways, culminating in tumor dissemination.

Pro-Tumoral Mechanisms of TAMS

Almost 20 years ago, Hanahan and Weinberg described a set of six capabilities that tumors must acquire to become fully invasive and metastatic. More recently their conceptual framework was extended to include additional hallmarks and enabling characteristics. TAMs have been shown to contribute to several of these hallmarks of cancer via a range of mechanisms, the most important of which are described in more detail below (Fig. 3). In many ways, TAMs recapitulate in tumors the behavior of embryonic macrophages in development. As well as phagocytosis of cells that are no longer required during organogenesis, the trophic, interstitial migratory and matrix remodeling capacities of macrophages are needed for processes as diverse as blood vessel anastomosis during vascular development and branching morphogenesis in the developing lung, pancreas, kidney, and mammary gland. In the developing brain, microglia are essential for normal neuronal patterning. Central to these trophic functions, embryonic macrophages secrete a number of growth factors and, similar to their embryonic counterparts, TAMs are capable of producing a range of growth factors such as epidermal growth factor (EGF), VEGF and other angiogenic factors, and insulin-like growth factor (IGF)-1 and IGF-2, to support tumor cells. Both EGF and IGF-1/2 can stimulate tumor cell survival and proliferation, which comprise two hallmarks of cancer; sustained proliferative signaling and resistance to cell death. Furthermore, exposure of TAMs to either CSF-1 or IL-4 leads to increased secretion of EGF.

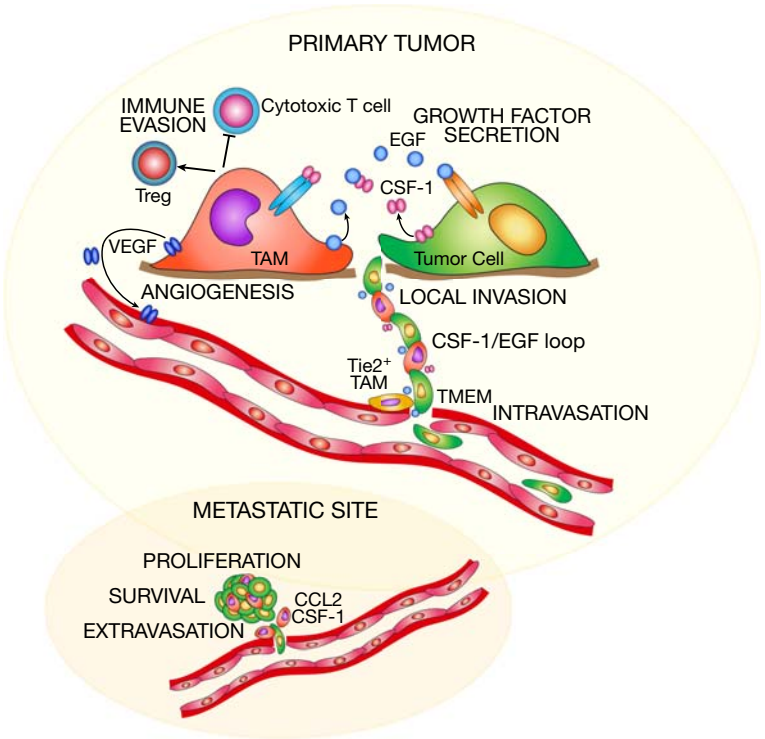

Fig. 3 Contribution by tumor-associated macrophages (TAM) to tumor progression. In the primary tumor, TAMs secrete growth factors, including epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) to support tumor cell survival and proliferation, and angiogenesis respectively. TAMs also inhibit cytotoxic T cells and recruit regulatory T cells (Treg) to suppress the anti-tumor immune response. Tumor cells secrete colony-stimulating factor-1 (CSF-1) to set up a CSF-1/EGF paracrine loop between tumor cells and TAMs that drives tumor invasion. Co-migrating TAMs and tumor cells migrate towards a blood vessel where $Tie2^{+}$ perivascular TAMs interact with endothelial cells and tumor cells in the tumor microenvironment of metastasis (TMEM) to facilitate tumor cell intravasation. At the metastatic site, TAMs help prepare the pre-metastatic niche then, through secretion of CCL2 and CSF-1, encourage tumor cell extravasation, survival and proliferation.

Angiogenesis

TAMs have long been known to regulate tumor angiogenesis through secretion of VEGF and other pro-angiogenic factors such as placental growth factor. VEGFA in particular drives the angiogenic switch that is necessary for continued tumor growth. The strategically placed perivascular $Tie2^{+}$ macrophages are particularly important for this hallmark capability in tumor progression. Unlike normal vascular development, tumor vessel patterning is aberrant with highly variable vascular branching and caliber. This results in leakiness and dead-end vessels, which impacts interstitial pressure, oxygenation and drug delivery in tumors. Consistent with this, therapies that normalize tumor vasculature rather than ablate it have been shown to enhance cytotoxic drug delivery and response to treatment. Since at least two populations of TAMs, $Tie2^{-}$, and $Tie2^{+}$ macrophages, contribute to tumor angiogenesis and therapies differentially affect them, targeting TAMs in general to normalize tumor vasculature does not appear to be a useful therapeutic approach.

Invasion

Interstitial migration, which requires both motility and matrix degradation, is another capacity of tissue macrophages that is integral to the deleterious behavior of TAMs. Indeed, the ability of TAMs to move through tissues and degrade basement membranes and other barriers is arguably their most important contribution to tumor progression. It was shown some time ago in a mouse model of breast cancer that a lack of TAMs, due to CSF-1 deficiency, significantly slowed malignant progression from early adenomas to late stage carcinomas in the primary mammary tumors and almost completely abrogated further progression to invasion and subsequent metastasis to the lung. These findings highlighted the central importance of TAMs in tumor invasion and metastasis, at least in this model. Subsequent studies revealed the presence of a paracrine interaction between CSF-1-secreting tumor cells and EGF-secreting TAMs that stimulated directed motility and invasion of both cell types along collagen fibers towards blood vessels (Fig. 3). Consistent with this, high TAM densities correlate strongly with metastatic breast cancer, and high CSF-1 levels are measured alongside dense collections of TAMs at the invasive fronts of human breast cancers. Once invasive tumor cells have reached the blood vessel, they intravasate into the circulation at specific sites called "tumor microenvironment of metastasis" (TMEM). TMEMs are a three-cell structure comprising a tumor cell, a $Tie2^{+}$ macrophage and an endothelial cell that functions as a trapdoor to release the tumor cell into the circulation. Importantly, human correlates for this metastasis facilitating structure have been demonstrated.

Metastasis

Once in the circulation, tumor cells acquire a sheath of platelets that facilitates attachment to the endothelium at the secondary site. Just as in the primary tumor, macrophages play several important roles at the metastatic site. Firstly, they prepare the pre-metastatic niche, perhaps under instruction by tumor-derived exosomes. Subsequently they are essential for tumor extravasation and seeding. In the lung for example, once the tumor cell has lodged in the pulmonary vasculature, they secrete CCL2 to attract monocytes to the lung. The recruited monocytes differentiate into metastasis associated TAMs, which facilitate extravasation of the tumor cells from the blood vessel into the lung parenchyma and then support their survival and proliferation. Importantly, inhibition of the macrophage CCL2 receptor or ablation of macrophages at the metastatic site blocks tumor cell extravasation and metastatic growth in the lung. Similar processes occur in other tissues that are common sites for metastatic seeding. In these tissues, macrophages or other mononuclear phagocytic lineage-derived cells, such as osteoclasts in the bone, facilitate several of the steps required for successful colonization by circulating tumor cells.

Immune suppression

Another powerful mechanism by which TAMs (and MDSCs) contribute to tumor progression is through suppression of the immune response to tumors. Consistent with this, tumors containing high numbers of TAMs and $CD4^+$ regulatory T cells (Treg) and fewer cytotoxic $CD8^+$ T cells are associated with poor outcomes. This is true of a range of different human cancers, including melanoma, breast, lung, pancreatic and gastric cancers among others although again colorectal cancer is a standout with increased infiltrating Tregs associated with a better prognosis. As part of their immunosuppressive action in tumors, TAMs secrete cytokines such as CCL17 and CCL22 to recruit Tregs, which are highly immunosuppressive. In addition, TAMs express ligands for inhibitory regulators of T cell function. By expressing ligands for both programmed death 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), TAMs activate these negative regulators of T cell function to prevent T cells from attacking tumor cells, despite a frequently high burden of foreign antigens expressed by tumor cells. An increased immunosuppressive tumor environment through a combination of these mechanisms may also contribute to the higher incidence of cancer in the elderly.

Treatment resistance

As well as contributing to multiple processes driving tumor progression, TAMs contribute to the development of resistance to cytotoxic therapies. Considering a central purpose of tissue macrophages is to service their host tissues, it follows that TAMs will work to help tumor cells to survive treatments such as chemotherapy and radiotherapy. They do so by providing survival factors and blocking apoptosis in the case of chemotherapy. This notion was confirmed when neoadjuvant chemotherapy in women with breast cancer was shown to increase TAM numbers. Examination of the phenomenon in a mouse model of breast cancer revealed that paclitaxel treatment induced tumor cell secretion of CSF-1 and IL-34 and TAM recruitment. Importantly, combining CSF-1R inhibition with paclitaxel slowed tumor progression. There is also evidence for another type of paracrine interaction between tumor cells and TAMs that stimulates IL-6 production by TAMs and leads to malignant cell survival and proliferation. In addition, TAMs may secrete tumor necrosis factor (TNF)-α to mediate chemoresistance through NF-$\kappa\beta$ signaling.

Similar to chemotherapy, radiotherapy induces recruitment of large numbers of TAMs to the irradiated region in response to tissue damage inflicted by irradiation. Once again CSF-1 appears to be important for TAM recruitment as radiotherapy increases tumor and circulating CSF-1 levels. The recruited TAMs adopt a pro-tumoral phenotype, which limits treatment efficacy, and prevention of their recruitment enhances the therapeutic response to radiotherapy. Both radiotherapy and anti-angiogenic therapies disrupt the tumor vasculature and thereby cause hypoxia, which stimulates TAMs, especially $Tie2^+$ TAMs, to secrete VEGF. As a consequence, tumor vasculature and growth are restored. Consistent with this, ablation of $Tie2^+$ TAMs improves the therapeutic response to anti-angiogenic therapies.

TAMs as Therapeutic Targets

Considering the involvement of TAMs in several critical areas of cancer progression, as well as therapy resistance, it is not surprising that they have been the focus of a number of strategies aimed at either reducing their numbers or re-educating their pro-tumoral phenotype. Targeting stromal cells in tumors has the additional benefit that they are not malignant and thus are unlikely to acquire mutations leading to treatment resistance. It is clear that specific cytokines and chemokines are key drivers of TAM recruitment and activation towards a pro-tumoral phenotype. Hence, current therapeutic strategies have targeted those key molecules.

CSF-1/CSF-1R inhibition

An obvious pathway to target in macrophage recruitment to and subsequent function in tumors is the CSF-1/CSF-1R axis. The pleiotropic signaling of the CSF-1R supports diverse macrophage functions, including survival, proliferation, differentiation, and migration. Furthermore, CSF-1 was unequivocally shown to promote tumor progression to metastasis in a mouse model of breast cancer where CSF-1 secretion by tumor cells leads to macrophage recruitment and activation into a pro-tumoral TAM phenotype. TAMs recruited by tumor-secreted CSF-1 trigger angiogenesis, block anti-tumoral immune responses and secrete EGF to activate the CSF-1/EGF motility loop. This paracrine chemokine interaction underpins co-invasion of tumor cells and TAMs away from the tumor towards blood vessels, where tumor cells intravasate and begin their march towards metastasis. At the metastatic site,

CSF-1 is again important, this time in combination with CCL2, where they enable tumor cells to extravasate and colonize the secondary site. Hence, a number of clinical trials are currently underway to investigate the effects of CSF-1R inhibitors or anti-CSF-1/CSF-1R antibodies on a variety of advanced solid tumors, either as monotherapy or in combination with chemotherapy or radiotherapy. Despite encouraging therapeutic responses to targeted CSF-1R therapies in several experimental models of cancer, CSF-1R inhibitor-induced depletion of TAMs in preclinical models of breast cancer did not reduce primary tumor growth unless additional chemotherapeutic agents were administered concomitantly. Nevertheless, notable clinical success has been achieved with emactuzumab, an anti-CSF-1R antibody, in the treatment of a rare synovial cell-derived tumor where uncontrolled CSF-1 production by synovial cells leads to a massive influx of macrophages. However, there is concern that indiscriminate ablation of TAMs by CSF-1R blockade may have deleterious effects through depletion of tumoricidal pro-inflammatory TAMs or due to subsequent accumulation of other tumor promoting immune cells such as tumor-associated neutrophils. Thus, new drug development is aimed at either reprogramming pro-tumoral TAMs or targeting specific CSF-1R signals that support pro-tumoral TAM behavior. Unexpectedly, a blood-brain barrier penetrant small molecule CSF-1R inhibitor, BLZ945, did not deplete TAM numbers in a mouse model of glioblastoma multiforme. Instead, the phenotype of the tumor promoting TAMs was switched to a tumor inhibitory one through secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) by glioma cells, leading to tumor shrinkage. This finding suggests that CSF-1R inhibition may re-educate TAMs in some tumors and ablate TAMs in others. The outcomes of clinical trials examining the effects of CSF-1R inhibition in solid tumors, particularly in combination with immune checkpoint inhibitor therapies, are awaited with interest.

CCL2/CCR2 inhibition

CCL2 has also been shown to be a key chemokine for recruitment of TAMs, which express its cognate receptor CCR2, particularly to metastatic tumor sites. Consistent with this, inhibition of CCL2 or its receptor has been shown to reduce tumor growth in several experimental models. However, concerns were raised when withdrawal of CCL2 inhibition in several breast cancer models led to rebound tumor growth and accelerated rates of pulmonary metastasis. Furthermore, clinical trials of an antibody targeting CCL2, carlumab, showed that it failed to produce a sustained reduction in CCL2 levels and may even have provoked higher rebound levels. Consistent with these observations, carlumab failed to produce any evidence of tumor response. Alternatively, the CCR2 could be targeted but outcomes from a completed clinical trial that tested an anti-CCR2 antibody in patients with bone metastases have not been disclosed. Thus, results of CCL2/CCR2 targeting are not encouraging.

Other therapeutic strategies

Other strategies to selectively target or reprogram TAMs have been tested. Although the transcription factor NF-κβ is well known to be a driver of cancer-associated inflammation, it also causes TAMs to maintain a pro-tumoral, immune suppressive phenotype. Consequently, blocking NF-κβ activation in TAMs may increase their tumoricidal activity and experimental models support this notion. Other mechanisms to re-educate TAMs towards a cytotoxic rather than a trophic phenotype include use of inflammatory agents such as CpG-DNA or agonists of CD40, which is a member of the TNF receptor superfamily expressed on macrophages and is a key driver of tumor-specific T cell priming.

An important consideration in all TAM-specific therapies is that their successful use in the treatment of cancer is likely to rely on co-administration of other therapies such as chemotherapy or radiotherapy.

Conclusion and Future Directions

Macrophages integrate with parenchymal cells in every tissue to facilitate development, function and repair. It is not surprising, therefore, that macrophages in cancers are subverted by tumor cells to turn their trophic functions towards supporting the enemy from within, making TAMs compelling targets in the treatment of cancer. Consequently, the list of clinical trials investigating the effects of anti-TAM therapies is long but the results are thus far underwhelming, indicating a need for more selective therapies targeting TAMs.

To support their remarkable plasticity, macrophages have a complex transcriptome and selectively express a number of proteins or isoforms not expressed in other immune cells. Selectively blocking these unique proteins and the pathways they regulate may prove to be Achilles heels for macrophages. Thus, while reprograming TAMs to enhance their tumoricidal activity may prove successful, drug development aimed at blocking particular macrophage functions that are more important for pro-tumoral than anti-tumoral functions, such as TAM motility, may prove to be more successful.

On a final note, the disappointing clinical results of therapies aimed at reducing TAMs illustrate a core problem of research directed at identifying molecular targets for cancer treatment. Robust responses to newly developed cancer therapies in preclinical models frequently do not translate to clinical cases, particularly where immune cells are involved. Perhaps this is not surprising considering the highly variable expression profiles of key proteins and their homologues in human immune cells compared to those in mice and other animals. Much work remains to be done to tease apart the complexities of the immune environment in tumors and to identify core differences between species.

See also: Cancer Vaccines: Dendritic Cell-Based Vaccines and Related Approaches.

Further Reading

Biswas, S.K., Allavena, P., Mantovani, A., 2013. Tumor-associated macrophages: Functional diversity, clinical significance, and open questions. Seminars in Immunopathology 35, 585–600.
Condeelis, J., Pollard, J.W., 2006. Macrophages: Obligate partners for tumor cell migration, invasion and metastasis. Cell 124, 263–266.
Epelman, S., Lavine, K.J., Randolph, G.J., 2014. Origin and functions of tissue macrophages. Immunity 41, 21–35.
Franklin, R.A., Li, M.O., 2016. Ontogeny of tumor-associated macrophages and its implication in cancer regulation. Trends in Cancer 2, 21–24.
Gomez Periguero, E., Geissmann, F., 2016. Development and maintenance of resident macrophages. Nature Immunology 17, 2–8.
Gordon, S., Plüddemann, A., 2017. Tissue macrophages: Heterogeneity and functions. BMC Biology 15, 53.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: The next generation. Cell 144, 646–674.
Mantovani, A., Marchesi, F., Malesci, A., Laghi, L., Allavena, P., 2017. Tumor-associated macrophages as treatment targets in oncology. Nature Reviews. Clinical Oncology 14, 399–416.
Murray, P.J., Allen, J.E., Biswas, S.K., et al., 2014. Macrophage activation and polarization: Nomenclature and experimental guidelines. Immunity 41, 14–20.
Noy, R., Pollard, J.W., 2014. Tumor-associated macrophages: From mechanisms to therapy. Immunity 41, 49–61.
Okabe, Y., Medzhitov, R., 2016. Tissue biology perspective on macrophages. Nature Immunology 17, 9–17.
Pollard, J.W., 2009. Trophic macrophages in development and disease. Nature Reviews. Immunology 9, 259–270.
Ruffell, B., Coussens, L.M., 2015. Macrophages and therapeutic resistance in cancer. Cancer Cell 27, 462–472.

Relevant Websites

Clinicaltrials, n.d., https://clinicaltrials.gov/—current clinical trials targeting TAMs.

Tumors and Blood Vessel Interactions: A Changing Hallmark of Cancer

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Glossary

Angiogenesis The formation of new vessels from the existing one. There are three types of angiogenesis: sprouting, intussusceptive, and glomerular.

Nonangiogenic tumors Tumors growing in the absence of *angiogenesis* or *postnatal vasculogenesis*. The commonest mechanism is exploitation of preexisting vessels by *cooption*, the alternative is *vasculogenic mimicry*.

Perivascular niche Perivascular microenvironment(s) including distinct nonmalignant cell types and stroma, where cancer cells find specific cues for survival, invasion, proliferation, stemness, and therapy resistance.

Postnatal vasculogenesis The formation of tumor-associated new vessels from normal stem cells.

Vascular cooption Is defined as the mechanism(s) by which nonangiogenic cancer cells relate to preexisting normal vessels.

Vascular mimicry The ability of tumor cells to form vessel-like channel networks inside tumors. According to the degree of "stemness" of these cancer cells, the spectrum goes from rough channels lined by neoplastic-looking cells up to normal-looking vessels which are however derived from the cancer stem cells rather than normal.

Introduction

Awareness that cancers contain, and have a close relationship with, blood vessels goes back to the dawn of medicine. According to legend, Paul of Aegina reported in the 7th century AD that some diseases had been called "cancer" (karkinos, i.e., crab) because these lesions "has the veins stretched on all sides as the animal the crab has its feet, whence it derives its name." However, the first historical description of blood vessels related to cancer is recognized to Galen of Pergamon (AD 129—c. 200/c. 216) in his treaty "On abnormal swellings": "Whenever black bile attacks the flesh, because it is corrosive, it eats into the surrounding skin producing an ulcer. If it arises with less intensity, it causes a cancerous swelling without ulceration. As I have said before, the veins are distended by black bile to a greater extent than in inflammatory swelling, regardless of the colors that they appear to be."

In 1787 the concept of new vessel growth in tumors was formally introduced by Horton, who illustrated the process by which new blood vessels originated from the existing ones. In 1903 the word "angiogenesis" was introduced by JM Flint to describe this phenomenon and has been used since. In the first decade of the 20th century the first papers were published describing that blood vessels multiplies inside tumors. In 1971 Judah Folkman formally introduced the hypothesis that tumor growth is always and strictly dependent on angiogenesis, at least when the tumor wants to outgrowth a microscopic state where the limits of diffusion of oxygens and nutrients are reached. Folkman's hypothesis was based not only on his work but was following also a series of earlier studies. In 1939 Ide described that tumor implants in the ears of rabbits were accompanied by the formation of new capillaries and later on, between 1943 and 1945, a series of investigations using wound chambers in mice demonstrated that new vessels sprout and migrate toward tumors. In 2000, the concept of "Sustained angiogenesis" was included as one of the six hallmarks of cancer by Hanahan and Weinberg and 11 years later was modified to "Inducing angiogenesis."

The Case for Angiogenesis Dependence and the First Two Angiogenesis Hallmarks

In 1971 Folkman published a seminal paper in which the idea that "the growth of solid neoplasm is always accompanied by neovascularization" was put forward. This hypothesis was generated mostly on the ground of in vitro work and animal models. Animal experiments had been conducted usually in a-vascular sites, such as the work on the cornea of a rabbits, regarded as classic proof of concept. Following undertakings on mice had not only confirmed the need for angiogenesis but also indicated that its induction is an early occurrence. Immunohistochemical studies of "in situ" breast carcinomas and cervical carcinomas on human biopsies showed an increased number of microvessels in the basal membrane leading to suppose that angiogenesis may be an intermediate event between the appearance of an "in situ" lesion and the progression as infiltrating carcinoma. A large series of studies also started to appear indicating that the higher microvessel density in a tumor, the worse the outcome, further strengthening the idea of a link between angiogenesis and tumor growth. However, as more studies appeared, such a strict association has been put in question.

The Different Types of Angiogenesis

Angiogenesis is defined as formation of new vessels originating from the preexisting ones. It follows the hypoxia suffered by cancer cells as the neoplastic lesion becomes larger outgrowing the blood supply. Three different types have been described which can coexist in the same neoplastic lesion: sprouting (classic) angiogenesis, intussusceptive microvascular growth (IMG), and glomeruloid microvascular proliferation (Fig. 1).

Classic Angiogenesis: Vascular Sprouting

This appears the most common type of angiogenesis occurring in neoplasm. Mature vessels in the body are quiescent and their homeostasis is mainly controlled by the Angiopoietin family (ANG1, ANG2—which however can also destabilize vessels—and ANG4) and their receptors (TIE-1 and TIE-2).

Following hypoxia, transcription and secretion of angiogenic factors is induced. The most important among these factors are proteins belonging to the VEGF family: four ligands, VEGF A, B, C, and D and three receptors, VEGF1R/flt-1, VEGFR2/kdr, and VEGFR3/flt4. The VGFA isoforms 121 and 165 plus the VGFR-2, a type III receptor tyrosine kinase, are the most relevant. Another membrane protein, Neuropilin, links to VEGFA165 to produce an angiogenic effect similar to that observed following activation of VEGFR-2.

At the same time, endothelial cells loosen their junctions and detach by the basal membrane through the action of matrix metalloproteases (MMPs) making the vessels leaky. Endothelial cells can break through the basal membrane and migrate toward the areas of higher concentration of angiogenic proteins. As the endothelial cells migrates, they start to secrete ANG2, which competes, with ANG1 for the TIE2 receptor, further inducing endothelial cell detachment, vascular permeability and proliferation among the nearby endothelium.

As the VEGFR-2 is activated it triggers the PLC gamma/PKC/RAF MEK/MAPK pathway inside the endothelial cell, inducing cell proliferation, and the PI3K/AKT pathways, maintaining cell survival. As the endothelial cells proliferate and accumulate they form a cord of cells in which two types can be seen: the Stalk cells, forming most of the growing new vessel, and the most advanced cell, the Tip cell, leading the migration as they follow the gradient of angiogenic molecules. The differentiation into either Stalk or Tip cells is regulated by the NOTCH/WNT signaling pathway. All the endothelial cells express NOTCHs (1, 2, 3, and 4) and NOTCH ligands (Jag 1, 2 Dll1, 3, and 4). Activated endothelial cells transcribe higher levels of DLL4: the Tip cells, those microenvironment is richer in VEGFA, will accumulate more DLL4 resulting into a higher DLL4: NOTCH ratio compared to the endothelial cells further back along the vascular sprout.

In these tip cells, the intracellular domain of NOTCH, freed from the extracellular portion, acquires transcription factor activity and moves from membrane to cytoplasm where induces transcription of factors belonging to pathways that reduce the proliferative effect of VEGF. As VEGF is inactivated, the endothelial cells move to a mature phenotype. In summary, the Tips cells have a phenotype characterized by low proliferation and lack of vascular lumen formation, but VEGFA-dependent production of filopodia rich in actins, resulting in increased motility. The Stalk cells will have instead a phenotype characterized by proliferation which is progressively switched off as NOTCH activation increases leading to vascular lumen formation, establishment of inter endothelial junctions, and deposition of basal membrane. Therefore the number of stalk cells increases, enlonging the vessels, but the number of tips remains small. As a lumen is formed, oxygenated blood starts flow and eliminates the hypoxia and its proliferative effect,

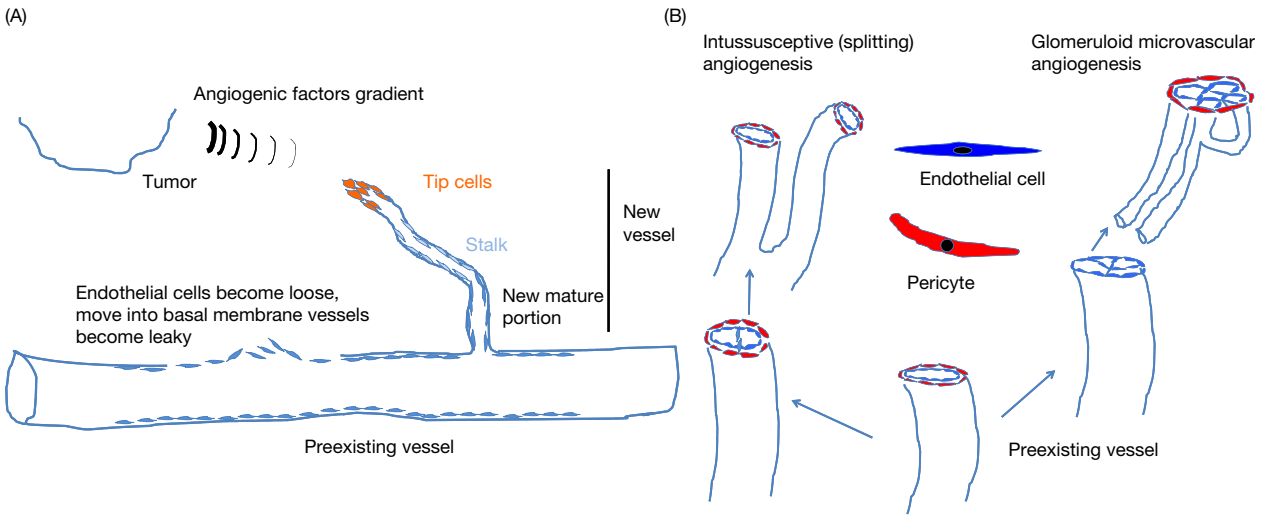

Fig. 1 The different types of angiogenesis (A) classic sprouting. (B) Intussusceptive and glomeruloid (original picture).

pericyte recruitment follows, and vessels maturation is completed. Eventually the newly formed branches will fuse with other vessels and a new vascular network is ready.

Intussusceptive Microvascular Growth (Splitting Angiogenesis)

In this type of angiogenesis, the longitudinal splitting of a preexisting vessel in two, forms the new vessels. This appears to be the fastest way of increasing the number of blood vessel, more rapid than the induction of sprouting angiogenesis. The first step is the growth of endothelial cells inside the vascular lumen, forming a transluminal septum. Endothelial cells than reorganization in order to cover the two newly formed channels. Then connective tissue moves into the septum, completing the division into two vessels. A variation can occur: rather than starting with the creation of the endothelial bridge, the process can also begin with the two sides of the vessel getting into contact. Fenestration follows in the opposite endothelial cells getting into touch, and through these holes the stroma infiltrates, creating a connective septa ultimately completely covered by new endothelial cells. During the whole process blood is circulated and actually the way it flows can affect how the process occurs. The biology underlying IMG is still not very well understood, but some data are available. In the chicken embryo chorioallantoic membranes model, VEGF121 isoform and recombinant human erythropoietin preferentially induce IMG rather than sprouting angiogenesis. TIE1 and TIE2 are also involved, as TIE2 induces endothelial cell stretching, motility, and adhesion to the extracellular matrix while TIE1 inhibits motility.

Glomeruloid Microvascular Proliferation

This type of angiogenesis leads to the formation of the so-called glomeruloid bodies: small vascular structures which morphologically resemble glomeruli in the kidney, hence their name. This type of angiogenesis occurs frequently, but not only, in glioblastomas (GBM). They are formed by small vessels lined by high endothelial cells surrounded by a discontinuous layer of pericytes and by a basal membrane. Few capillaries with flat endothelium are also seen, possibly the vessels from which the glomeruli are generated. The VEGF pathway also mediates their formation.

Postnatal Vasculogenesis

There is a fourth way in which new vessels can appear in tumors. It differs form angiogenesis as the vessels do not originate from the preexisting ones, but from circulating endothelial progenitor cells (EPCs). It is a process that recapitulates the formation of vessels from stem cells during embryogenesis (vasculogenesis). EPCs are not only mainly found in the bone marrow but also found in the peripheral blood, fetal liver, and umbilical cord blood. They have a variable phenotype, according to which tissue they are isolated from but expression of CD34 and VEGFR2 is always present. EPCs are attracted to the growing tumors by the secretion of hypoxia-related molecules and cytokines. Once recruited inside the growing neoplasm the EPCs differentiate into mature endothelial cells and ultimately, into vessels. This model however is rather an exemplification as the biology of the intratumor vasculogenesis is a rather complex event. There are also other bone marrow-derived cells, other than EPCs, which are possibly able to generate endothelial cells like Tie-2 positive monocytes, CD11b positive myeloid cells, VEGFR1 positive hemangiocytes, VE cadherine positive leukocytes, lymphocytes stimulated by pleiotrophin, and intratumor macrophages, but their role is still speculative. It is not yet clear whether these cells can truly form endothelium or just stay closely adjacent to the vessels influencing their formation by a paracrine effect. It also not established how much intratumor vasculogenesis relays on the marrow-derived stem cells rather than those from other tissues.

Can Tumor Cells Become Parts of Blood Vessels?

Finally, two groups have suggested that tumor stem-like cells in GBM can transdifferentiate into bonafide endothelial cells, contributing to tumor vascularization. Using different animal models, other groups have found it difficult to recapitulate many of these findings, questioning the relevance of these observations for the formation of new blood vessels in human tumors.

Tumor Growth Without Angiogenesis

Nonangiogenic tumors are defined as neoplasm growing in the absence of new vessel formation. Two main mechanisms exist: exploitation of preexisting vessels and vascular mimicry.

An increasing amount of data, initially from histopathology studies and subsequently from preclinical and clinical studies has revealed that the formation of new vessels is not essential for a tumor to grow. There are malignant neoplasms able to develop and progress in the absence of neoangiogenesis by exploiting the preexisting vasculature. Such “nonangiogenic” cancers, primaries and secondaries, have been first identified and described in the lung and, subsequently, in several other organs.

Evidence That Tumors Can Be Nonangiogenic

"Nonangiogenic" cancers were first described, as such, in the lung where they grow by filling the alveolar space (Fig. 2). The only vessels present in such tumors are those trapped ones from the alveolar septa and highlight the alveolar structure of the lung tissue by neoplastic cells. The fact that the vessels are the preexisting alveolar vessels is suggested not only by their pattern but also by the conservation of anthracitic pigment alongside the vascular septae, which is similar to the surrounding uninvolved lung parenchyma. Neither endothelial cells nor tumor-associated stroma is present among the neoplastic cell masses within the alveolar sacs. Besides being purely nonangiogenic, tumors having both angiogenic and nonangiogenic areas are a common features.

These initial findings were further supported by a three-dimensional reconstruction, and comparison of normal lung, nonangiogenic and angiogenic lung cancers.

Serial sections were cut and immunostained both for cytokeratin, present in the malignant epithelial cells and normal pneumocytes, and CD34, expressed by the endothelial cells. When looking at the blood vessels in the 3D reconstruction, vascular architecture from normal lungs was indistinguishable from that of the vessels in the nonangiogenic tumor. In contrast, a disorganized network of randomly disposed vessels was seen in the angiogenic tumors.

More details have been subsequently unveiled of how cancer cells dispose themselves in these tumors: animal models have shown that the metastatic cells, after having extravasated, form small aggregates within the alveolar wall in the space between the epithelial cells and the basal membrane of the vessels. The cancer cells then move inside the alveolar space and fill it, moving then to nearby spaces throughout the alveolar pores. Once the alveolar space is filled, some cells transit again between the alveolar cells and infiltrate again the basement membrane dissecting the normal alveolar epithelium from the endothelial cell/alveolar wall. Eventually the alveolar epithelial cells detach and die, and the neoplastic cells lie directly in continuity with the alveolar capillaries.

The absence of newly formed vessels and the persistence of preexisting lung vasculature have been confirmed by immunohistochemical studies fort the expression of LH39 and aVb3 which demonstrated that the vessels present in the nonangiogenic tumors had the phenotype of those in the normal lung with only occasional proliferating cells.

Nonangiogenic tumors have been since also formally described in several other organs in particular in the liver, brain, and lymph nodes. Of note, these organs (including, and particular, the lung) share a very characteristic blood vessel tissue architecture, which

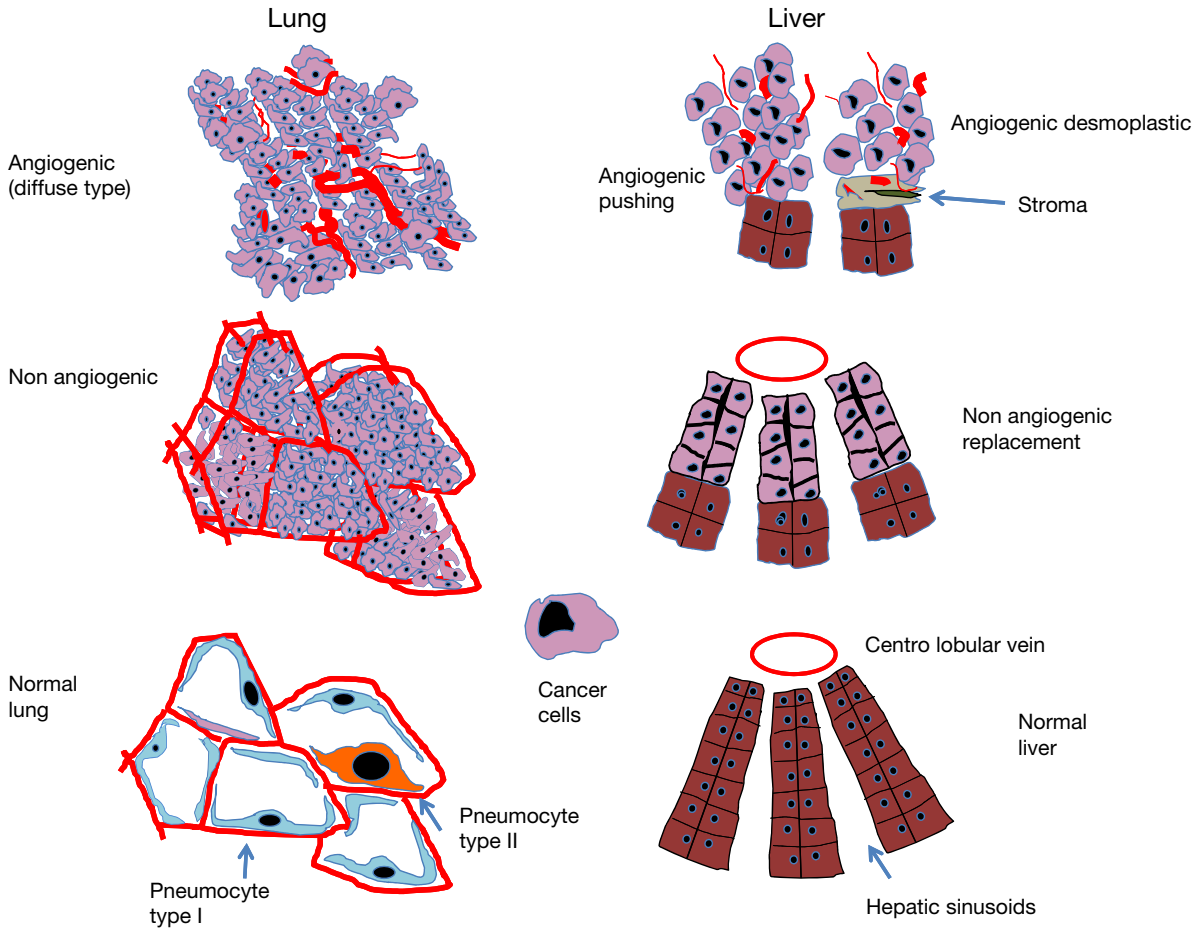

Fig. 2 Architecture of the nonangiogenic tumors compared to angiogenic in the lung and in the liver (original picture).

has probably made it much easier to detect preservation of the normal vasculature in growing tumors. It is likely that vascular cooption can also be found in other organs; this will require careful histological studies, but—if possible—intravital imaging studies that have already greatly contributed to the study of vascular cooption. For melanoma, for example, the dorsal skinfold chamber has contributed to the identification of vascular cooption, which has later been confirmed by careful histology. Likewise, long-time imaging through the brain cranial window had an important role to detect vascular cooption, including the mandatory role it plays during the early stages of brain metastasis formation.

In the liver, three different patterns of hepatic metastatic growth of colorectal and breast adenocarcinomas have been identified. In the desmoplastic and the pushing types, the architecture of the liver parenchyma was not preserved and an angiogenic tumor is present. However, in the replacement growth pattern, the metastatic cells infiltrate the liver parenchyma without any disturbance of the preexisting liver structure. There is no fibrosis, minimal inflammation, and tumor cells, and hepatocytes have intimate cell–cell contact. The tumor cells are growing by coopting the stroma with the sinusoidal blood vessels. As in the lung, liver metastases can have a pure or mixed pattern of growth with some patients who have multiple metastases displaying both "pure" angiogenic and nonangiogenic tumors. The patients with nonangiogenic metastases appeared to have a slower but still aggressive disease as they had a better prognosis at 24 but not at 60 months. The incidence of these liver patterns of metastases depends on the tissue of origin: breast, pancreatic, and urothelial secondaries have a prevalent nonangiogenic growth pattern while only one-third of colorectal metastases grow in this fashion.

A second nonangiogenic growth pattern, in which the neoplastic cells colonized the sinusoid, rather than replace the hepatocytes, has also been identified.

It is a long-standing neuropathological wisdom that diffuse and malignant gliomas, including GBM multiforme, consistently show a highly diffuse infiltrative growth pattern, even of otherwise normal appearing brain, next to a core region which is typically highly angiogenic in GBM, mainly depending on VEGF and angiopoietin signaling. From early on, this makes high-grade gliomas to a whole-brain diseases with both angiogenic and nonangiogenic tumor parts. Glioma cells coopt preexisting brain blood vessels to slowly infiltrate the brain, even single cells far away from the main tumor mass, which is contributing to the very diffuse growth pattern, next to alternative infiltration routes along axonal tracks. The perivascular niche is a preexisting anatomical space that allows easy mechanical passage of cells, next to providing crucial cues for survival, proliferation, cancer cell stemness, and therapy resistance. However, in contrast to the extensive study of molecular factors contributing to brain tumor angiogenesis, it is much less clear which pathways are involved in vascular cooption in the brain; VEGF-A is apparently not involved, while serpins and c-MET have been suggested as candidates that can drive cooptive growth in brain tumors.

In the direct peritumoral region, in vivo imaging discovered IMG, and subsequent cooption of these newly formed blood vessels in the brain, which provides an interesting mechanism how tumors can combine angiogenesis and subsequent vascular cooption in the very same microenvironment.

Furthermore, the early growth of many brain metastatic tumor cells is also purely cooptive, particularly in melanoma and breast cancer animal models, but also some lung cancer cell lines. Of note, strict perpetuation of a perivascular niche position is mandatory to survive in the brain metastatic process, particularly directly after extravasation when the alien tumor cells have to adapt to the new "soil" of the brain.

Finally nonangiogenic metastases from solid tumors can also grow in lymph nodes as demonstrated using a mouse with a lymph node window installed. TRITC-Dextran angiography was performed to visualize the lymph node vessels, whereas the metastatic carcinoma cells were detectable since they were transfected with a gene-encoding green fluorescent protein. The first neoplastic cells are detected in the subcapsular sinuses, initially as single cells than as small aggregates after which they invade deeper in the lymph node cortex where they start to dispose themselves along the vessels when they reach a depth of 509–100 μm. Monitoring by angiography of the vessels did not reveal any change or evidence of angiogenic blood vessel sprouting while the metastatic cells colonized lymph nodes over a period of 40 days. The vascular counts showed consistently that the reactive nodes have the same or even a higher vascular density than the nodal metastases and detected using immunohistochemistry by the expression of Ki67 in the endothelial cells.

Nonangiogenic Tumor Growth: Why and How Does It Happen?

As this is a newly discovered field only some first data are available and we are still far away from fully understanding how tumor cells take the nonangiogenic path (and vice versa!). Different nonangiogenic patterns, intraalveolar in the lung, hepatocyte replacement in the liver, and parenchyma infiltration in the brain, suggest that different mechanisms are at the basis of these patterns of growth.

In the lung, an histological comparison of lung nonsmall cell carcinomas, only minor differences were found as far as necrosis is concerned whereas chronic inflammation and fibrosis characterized the angiogenic tumors, but no differences with respect to microvessel density or cells with morphological features suggesting apoptosis. Immunohistochemical studies failed to demonstrate any major difference in the expression of markers of angiogenesis and hypoxia, the only exception being differences in stromal thrombospondin, almost absent in nonangiogenic tumors but widely present in the angiogenic ones. A high level of thrombospondin (an antiangiogenic protein) in stroma where vascular remodeling occurs is easily explained by its well-known involvement in the remodeling activity of newly generated vessels.

mRNA expression profiling by microarray studies on tumor samples confirmed that no differences in classic hypoxia/angiogenesis pathways could be found with the exception, again, of thrombospondin-1 higher in angiogenic cancers. An unexpected finding

was the increased expression in nonangiogenic tumors of a set of genes linked to oxidative phosphorylation, suggesting the possibility of metabolic reprogramming in the nonangiogenic tumors. The second major finding was the decreased level of a set of adhesion molecule genes, raising the hypothesis that diminished cell-to-cell contact could again be associated with failure to develop a vascular.

A third finding was the association between expression of cytoplasm p53 and nonangiogenic tumors. A pilot study on a small number of these cases demonstrated a higher incidence of P53 mutations in these cases. If confirmed, these data would be consistent with the report that, in animal models, inactivation of P53 leads to resistance to antiangiogenic drugs by increasing the ability of the cells to survive in hypoxia.

Less data are available as far as the biological characterization of the nonangiogenic liver metastases is concerned. However the different type of spreading compared to the lung (replacement of normal cells versus filling empty spaces) and the finding so far that very scanty CA9 expression at the edge of the replacement pattern in the liver indicates that these metastases are not very hypoxic which suggests that liver and lung nonangiogenic tumors could have different underlying mechanisms of development and biology.

The key question however is why and how a cell becomes nonangiogenic rather than angiogenic. We still do not know about "why" and very little on "how."

The gene that has so far better been described as able to regulate such a choice is IRE1. In a murine orthotopic and in a chorioallantoic membrane model of GBM using the cell line U87, the IRE1 gene, which encodes a stress-sensing protein that triggers the unfolded protein response, is a key regulator for angiogenesis. Wild type of U87 glioma cells expressing this gene grows as a discrete, highly angiogenic mass, while the U87 cells, in which the expression of IRE1 has been blocked, grow as nonangiogenic and more infiltrative tumors coopting preexisting vessels. These nonangiogenic cells have a lower expression of proangiogenic genes encoding proteins such as IL1beta, IL6, IL8, BEFGA, MMP1, MMP9, and PLAU while there is upregulation of SPARC. Whether this gene has a role in actual human tumors is not known. It has been reported to carry missense mutation in several types of tumors but whether it causes an effect on angiogenesis is not known.

In addition, there are several studies consistently showing that inhibition or downregulation of the VEGF-A pathway leads to a more invasive growth pattern, partly via vascular cooption. This mechanism of resistance against anti-VEGF therapies, called evasive resistance, has been found for not only primary brain tumors but also other tumor entities. From analyzing the published data carefully, it is impossible to say whether this invasive growth pattern is merely a default, fall-back option for tumors that are deprived of rapid angiogenic growth, or whether interference with VEGF-A signaling by itself is inducing this growth pattern.

Vascular Cooption

Vascular cooption is defined as the mechanism by which nonangiogenic cancer cells exploit preexisting normal vessels, and this is the commonest way nonangiogenic tumors grow. Cells come into contact with the abluminal side of the blood vessels in order to thrive. This is a physiological phenomenon, for example, plasma cells can be seen to coopt vessels in bone marrow. In tumors, so far, cooption has been identified as a mechanism by which nonangiogenic neoplastic cells "parasitize" normal vessels to obtain oxygen and nutrients. However, as cooption is an active mechanism that allows the cancer cell to exploit the presence of vessels, it is likely angiogenic cancer cells to interact and exploit with the newly formed vessels also use that cooption. This is also a newly opened field and only a few studies are so far available addressing the mechanism of vascular cooption. These studies are predominantly focused on brain growth models.

In primary brain tumors the first step is the attraction of neoplastic cells toward the vessel. An important player is a bradykinin-signaling pathway: the glioma cells express bradykinin 2 receptor (B2R), which is activated by the endothelial cells through secretion of bradykinin. Once activated by its ligand, B2R induces intracellular Ca^{2+} oscillations, which mobilize the neoplastic cells toward the vessel along the gradient of secreted bradykinin.

The second step is the interaction with the vessels, once it has been reached. GBM cells produce cytoplasm extensions, denominated flectopodia, which rely on CDC42, a GTPAse that regulates the actins-dependent cytoplasm extension. Once the cell has reached the abluminal side of the vessel, flectopodia adhere to the pericytes through the adhesion molecule CD44. Fusion between the cytoplasm of the GBM cells and the pericytes occurs, with some hybrid cells formation demonstrated "in vitro." If CDC42 activity is blocked, vascular cooption does not occur in this model with some pericytes acquiring a macrophage-like phenotype and showing antitumor activity. The possibility that this occurs in actual human primary brain malignancies in humans is supported by immunohistochemical stains demonstrating increased levels of CDC42 and CD44 expression in perivascular GBM cells as assessed by GBM histological sections.

With respect to brain metastases by other types of tumor, the question is why the cells, after extravasation, stick to the vessel and coopt it. From animal models, serpin seems to play a key role. Early metastatic cells resist apoptosis and coopt brain vessels by expressing the protein neuroserpin, which blocks the generation of plasmin. Plasmin is a protein able to protect the brain from metastatic cells by inducing their apoptosis and inhibiting their ability to move along the vasculature. Astrocytes link these two events, the entering of metastatic tumor cells and the production of plasmin. Following metastatic cancer cells appearance, inflammation, and parenchyma damage astrocytes express high levels of Fas ligand (FasL) and plasminogen activator (PA). PA cleaves the plasminogen, secreted by neurons, into the active form, plasmin, which subsequently acts on several other proteins, including FasL and the adhesion molecule L1CAM. FasL is bound to the membrane of astrocytes, but plasmin is able to cleave and release it as soluble form, sFasL, which in turn can link to its receptor, Fas, on the tumor cell and trigger apoptosis. However, in the presence

of cancer cells expressing high levels of neuroserpin, the astrocyte's production of PA is inhibited, thereby blocking the release of plasmin from plasminogen, and suppressing the secretion of sFasL and thus apoptosis of tumor cells.

Once the increased survival of the cancer cells is achieved, there is the problem of coopt the vessels: a few different mechanisms have started to be unveiled. One mechanism follows again neuroserpin expression: by inhibiting generation of plasmin, the L1CAM molecule expressed by the tumor cell is not cleaved and remains intact, thus allowing the metastatic cells to adhere to the outbound surface of the vessels. In metastatic breast cancer and melanoma, vascular cooption in vivo is reported to be dependent upon the induction of connexin 26 and connexin 43 expression by the "twist" transcription factor. These two connexins induce the formation of functional gap junctions between cancer cells and endothelium. Silencing of these connexins in both breast adenocarcinoma and melanoma cells results in loss of the ability to coopt vessels in zebra fish, chicken embryos, and mouse models, while the presence of the beta1 integrin mediates the adhesion between neoplastic cells and the vascular basal membrane.

Vasculogenic Mimicry

Alongside exploitation of normal vessels, vasculogenic mimicry (VM), that is, the ability of tumor cells to form vessel-like networks, is the second, less common, way for tumors to grow in the absence of angiogenesis. It was discovered and described for the first time in uveal melanoma but now is known to occur in several different tumors. Tumors growing by VM are not angiogenic as formation of new vessels originating by preexisting vessels does not occur, nor are vasculogenic has there is no vascular formation from the normal stem cells of the body.

In these malignancies there are channels in which blood can flow; however these channels originate from the cancer cells themselves. VM appears to recapitulate vasculogenesis network as the neoplastic cells possibly reverse to an embryonic-like phenotype therefore becoming competent to mimic endothelial cells. According to how much of cancer stem cell properties these cancer cells maintain, the intratumor channels can go from resembling morphologically a normal vessels, although carrying the same genetic abnormalities of the tumor, up to rogue channels lined up by clearly malignant cells.

Little is known about the underlying biology. The main signaling pathway regulating VM is ignited by upregulation of VE-cadherin through HIF2. VE-cadherin colocalizes with and phosphorylates EphA2 which in turn activates PI3K both directly and throughout the FAK/ERK1/2 pathway. PIK3 induces the cleavage of pro-MT1-MMP and pro-MMP2 into their corresponding active forms which promote the cleavage of laminin 5γ2 to the promigratory fragments 5γ2′ and 5γ2x. These two latter protein fragments induce and guide the arrangement of the neoplastic cells into channel structures. This pathway can be on one side inhibited by cAMP, inhibiting ERK1/2 function. However cAMP can also sustain VM by positively regulating VE-cadherin expression throughout the Nodal/Notch 1/4 pathway with Gal-3 having a similar positive effect on VM.

The Perivascular Niche: Where It All Happens

When tumor cells coopt preexisting blood vessels of the host in order to grow, and/or when single tumor cells invade the host tissue, they regularly colonize a highly specific microenvironment: the perivascular niche (Fig. 3).

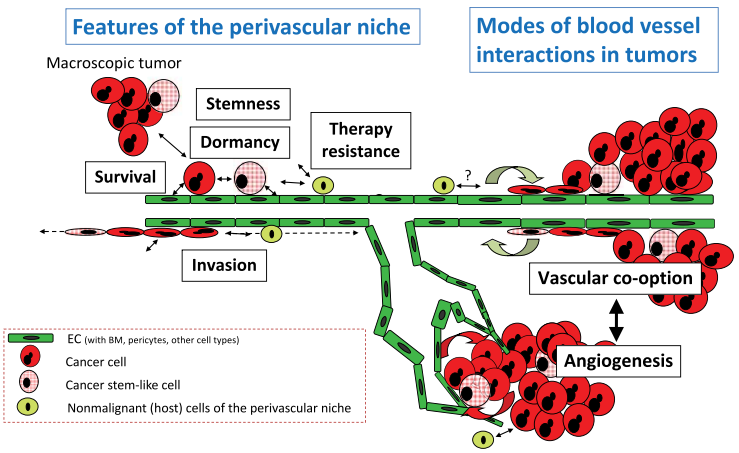

Fig. 3 The perivascular niche and blood vessel exploitation, either by vascular cooption or by angiogenesis, is one hallmark of cancer. (Right) Two principle ways of how tumors get access to blood vessels exist: first, the formation of new blood vessels by sprouting and other types of angiogenesis. Second, cooption of preexisting blood vessels of the host. Incorporation of existing blood vessels and the formation of new blood vessels can occur simultaneously in tumors, but can also be regarded as alternative mechanisms of tumor progression when one of these basic mechanisms is blocked. (Left) The perivascular niche can provide central cues for tumor initiation, progression, and therapy resistance. *EC*, endothelial cell; *BM*, vascular basement membrane (original picture).

In this particular anatomical, physiological, and molecular space that also fosters colonization by new cells due to preformed loose tissue structure in many organs, tumor cells certainly find optimal access to oxygen and nutrients from the blood stream. However, both in the brain and in the lung, it has been demonstrated that neoplastic metastatic cells need to reside in this perivascular niche in order to survive and thrive despite the fact that oxygen and nutrient would be available also away from those blood vessels. Not only the presence of angiocrine factors seems to be one of the reasons but also the presence of special cell types like pericytes, endothelial cells, immune cell subtypes, and others (e.g., astrocyte in the brain). Furthermore, the presence of a specific extracellular matrix including the vascular basement membrane (e.g., collagens, laminin) appears to be another crucial factor. As perivascular niches are important for normal stem cells, it is therefore been raised the hypothesis that these microenvironments are also essential for survival of cancer stem cells. A particular stemness of tumor cells and resistance of tumor cells against established therapies have been best demonstrated for the perivascular niche in the brain yet. The perivascular niche as a place that allows efficient invasion of the host tissue at the tumor borders has been demonstrated for many organs (see earlier). It is therefore very likely that conditions in this perivascular space could decide tumor cell destiny.

Prospective Vision

The discovery of nonangiogenic tumors has opened a new field in cancer biology, in which several important new research areas are present. Several new questions are now facing cancer biologists and clinicians:

- The first question is why and subsequently how a neoplastic cell decides in favor of the angiogenic, or nonangiogenic status.
- Is this process different in different organs?
- As the angiogenic status can change from angiogenic to nonangiogenic and vice versa, is this due to selections of clones, or some neoplastic cells can switch their behavior?
- Which are the mechanism of vascular cooption of preexisting vessels and, again, are they different throughout the body?
- Are there also specific biological mechanisms regulating the interaction between cancer cells and newly formed vessels?
- What exact advantage has cancer cells from perivascular niche colonization?
- And finally, how can we best identify new targets for effective treatment?

A Therapeutical Outlook

It is clear that therapeutic targeting of vessel exploitation has the potential to add to the armamentarium of cancer therapy. This has been extensively shown for antiangiogenic drugs, like antibodies targeting VEGF-A, and tyrosine kinase inhibitors that often target multiple angiogenic pathways. Primary and adaptive resistance to those antiangiogenic drugs, however, has compromised the efficacy of these therapies in patients. From what is known today, a rational attack on the cooptive growth that appears to be a frequent mechanism of primary and adaptive resistance against antiangiogenic therapies might provide even more benefits: it holds the promise of interfering with multiple central traits of tumor biology, since the tumor-promoting factors of the perivascular niche are targeted. The molecular pathways and cell types that have already been suggested as being relevant for vascular cooption and perivascular niche habitation can provide a cue for future directions of research and drug development.

Cancer and Blood Vessels: The New Hallmark

Robust evidences have now confirmed the finding that tumors can grow in the absence of angiogenesis. Contrary to the original theory of Folkman, it is now well established that the requirement of induction or sustainability of angiogenesis for tumors to growth is not essential. The blood supply of a tumor can also be provided by the ability of tumors to exploit the preexisting vasculature by coopting it, or by VM. We have now a more complete view of the role of blood perfusion in cancer, and therefore the current hallmark of cancer, "Inducing angiogenesis," needs to be modified. As the role of blood perfusion remains central to cancer biology we proposed a new hallmark:

"Exploitation of blood vessels, preexisting and/or newly formed."

Further Reading

Döme, B., Hendrix, M.J., Paku, S., Tóvári, J., Tímár, J., 2007. Alternative vascularization mechanisms in cancer: pathology and therapeutic implications. American Journal of Pathology 170, 1–15.

Donnem, T., Hu, J., Ferguson, M., Adighibe, O., Snell, C., Harris, A.L., Gatter, K.C., Pezzella, F., 2013. Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment? Cancer Medicine 2, 427–436.

Folkman, J., 1971. Tumor angiogenesis: therapeutic implications. New England Journal of Medicine 285, 1182–1186.

Folkman, J., 1971. What is the evidence that tumors are angiogenesis dependent? Journal of the National Cancer Institute 82, 4–6.

Frentzas, S., Simoneau, E., Bridgeman, V.L., Vermeulen, P.B., Foo, S., Kostaras, E., Nathan, M.R., Wotherspoon, A., Gao, Z.H., Shi, Y., Van den Eynden, G., Daley, F., Peckitt, C., Tan, X., Salman, A., Lazaris, A., Gazinska, P., Berg, T.J., Eltahir, Z., Ritsma, L., van Rheenen, J., Khashper, A., Brown, G., Nyström, H., Sund, M., Van Laere, S., Loyer, E., Dirix, L., Cunningham, D., Metrakos, P., Reynolds, A.R., 2016. Vessel co-option mediates resistance to anti-angiogenic therapy in liver metastases. Nature Medicine 22, 1294–1302.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.

Harris, A.L., 2002. Hypoxia—a key regulatory factor in tumour growth. Nature Reviews Cancer 2, 38–47.

Kerbel, R.S., 2008. Tumour angiogenesis. New England Journal of Medicine 358, 2039–2049.

Maniotis, A.J., Folberg, R., Hess, A., Seftor, E.A., Gardner, L.M., Pe'er, J., Trent, J.M., Meltzer, P.S., Hendrix, M.J., 1999. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. The American Journal of Pathology 155, 739–752.

Pezzella, F., Pastorino, U., Tagliabue, E., Andreola, S., Sozzi, G., Gasparini, G., Menard, S., Gatter, K.C., Harris, A.L., Fox, S., Buyse, M., Pilotti, S., Pierotti, M., Rilke, F., 1997. Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. The American Journal of Pathology 151, 1417–1423.

Seftor, R.E., Hess, A.R., Seftor, E.A., Kirschmann, D.A., Hardy, K.M., Margaryan, N.V., Hendrix, M.J., 2012. Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. American Journal of Pathology 181, 1115–1125.

Weinberg, R., 2013. The biology of cancer, 2nd edn. Garland Science Taylor and Francis Group, New York.

Winkler, F., 2015. The brain metastatic niche. Journal of Molecular Medicine 93, 1213–1220.

Winkler, F., 2017. Hostile takeover: how tumors hijack pre-existing vascular environments to thrive. Journal of Pathology 242, 267–272.

Tunneling Nanotubes (TNTs): Intratumoral Cell-to-Cell Communication

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Intercellular Connections in Tumors

Solid tumors and hematological malignancies are considerably heterogeneous tissues. This heterogeneity comes from the tumor cells themselves that include cancer stem cells (CSCs), believed to be responsible for tumor progression and recurrence following therapy, and cancer cells stratified at different stages of differentiation. Neoplastic tissues also include non-cancer cells. These comprise residing mesenchymal, epithelial and endothelial cells, as well as cells recruited by the tumor such as immune cells and mesenchymal stem cells (MSCs). Importantly, although non-cancerous, these cells nonetheless often present a modified and abnormal phenotype due to their location in the tumor microenvironment and consequently favor tumor progression, metastasis and resistance of the cancer cells to therapy.

Cell communication within the tumor, amongst cancer cells themselves and between cancer and non-cancer cells is now fully acknowledged as widely used by the tumor to grow and circumvent therapeutic treatments. In the last decades, this intercellular communication was believed to heavily rely on secreted cytokines/chemokines, metabolites and extracellular vesicles. In the past few years, a new means of cell-to-cell communication that uses tunneling nanotubes (TNTs) was shown to enable cells to connect to far-off cells and to transfer them biological cargos, ranging from ions to whole organelles, as it will be detailed in this chapter. This donation is qualified horizontal, to distinguish it from the vertical donation from a parental cell to its offspring during mitosis. The number of scientific publications describing this TNT-mediated new mode of communication between cells, including cancer cells, steadily increased since 2004, when they were initially described (Fig. 1). Importantly, TNTs involving cancer cells were also observed in situ, in patient resected solid tumors from both malignant pleural mesothelioma and lung adenocarcinoma, demonstrating their relevance in the cancer pathology.

The occurrence of TNTs in tumors and the ensuing intercellular trafficking are now bringing about a radical turmoil in the current paradigm of the intercellular communications that take place in tumors as TNTs guide and allow the dynamic fluxes of biological cargos, notably mitochondria, that are literally passed from the cytoplasm of the donor cell to that of the recipient cell. This TNT-mediated trafficking occurs from cells of the microenvironment to the cancer cells, modifying the functional properties and response to therapy of the tumor cells. It also occurs in the reverse direction, from the cancer cells to non-cancer cells of the tumor microenvironment, likely contributing to the observed changes in phenotype of these normal cells that ultimately further contribute to tumor progression and resistance to therapy.

General Features of TNT-Dependent Cell-to-Cell Exchanges

TNTs are long tubular structures, with diameters ranging from 50 and 1500 nm and lengths that can span several tens to hundreds of microns. The most important feature of TNTs is definitely the fact that they allow cytoplasm continuity between the connected cells

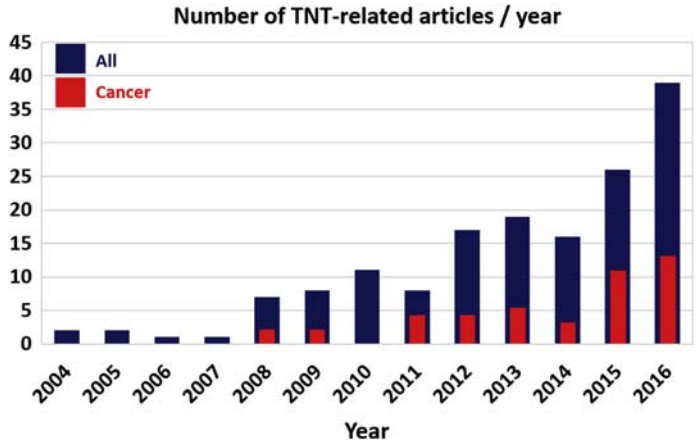

Fig. 1 Number of TNT-related scientific publications. The scheme shows the number of original research articles related to tunneling nanotubes, including studies involving cancer cells, published between 2004 and 2016.

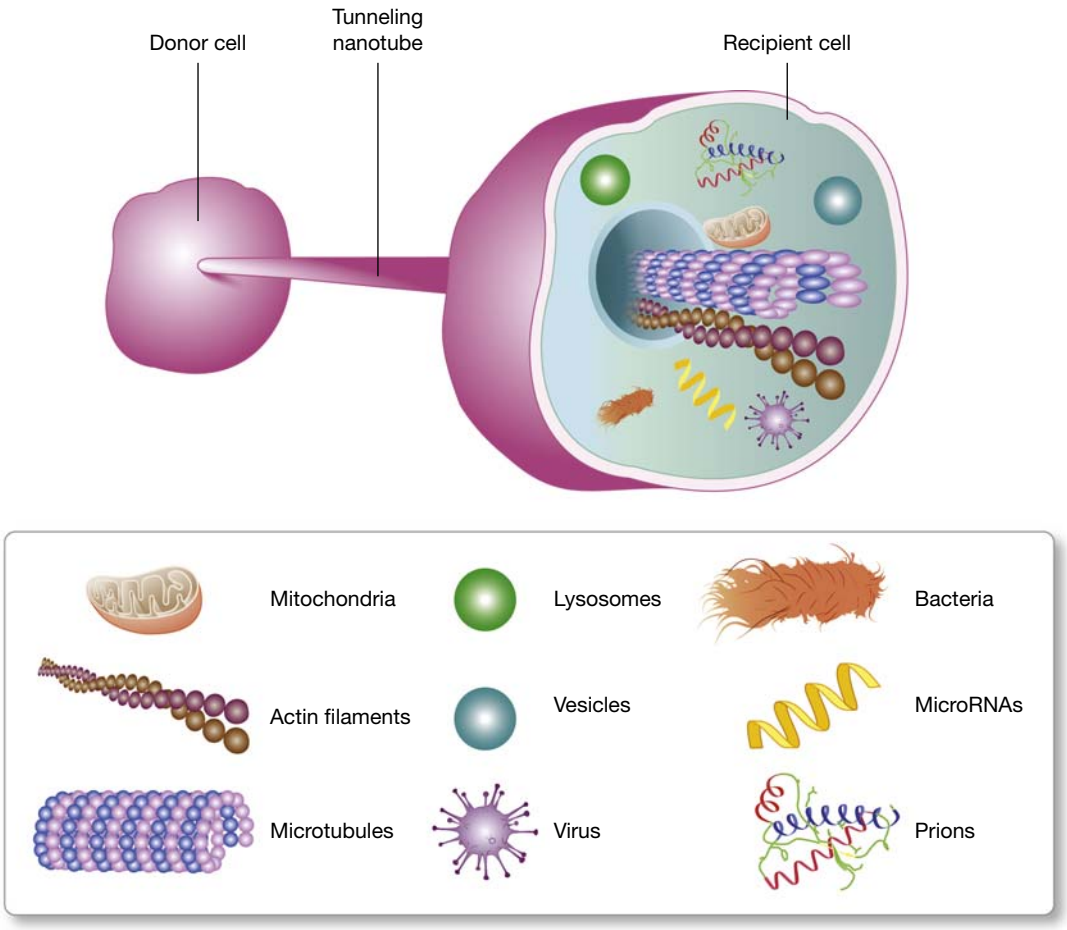

Fig. 2 Tunneling nanotube (TNT). Tunneling nanotubes mediate physical connections between different cells. TNTs allow the horizontal transfer between these cells of diverse cargos, such as mitochondria organelles, viruses, bacteria, proteins, miRNAs and ions. The intercellular trafficking occurs along cytoskeleton fibers of actin microfilaments, microtubules, or both.

and consequently enable the transport of cellular components between these cells. The transported cargos include a whole panel of cellular components, from ions, miRNAs, proteins and virus to whole organelles like lysosomes and mitochondria, as schematized in Fig. 2.

In this review, we provide a general overview of what is currently known about tunneling nanotubes: the cells involved, the cargos transported and the mechanisms that allow this intercellular trafficking. We further focus on the specific signals that induce the formation of nanotubes in tumors and on the biological outcome of these TNT-dependent communications, that ranges from metabolic reprogramming to induced survival in response to therapy. A special attention will be given to the TNT-mediated intercellular transfer of mitochondria as it has been the focus of intense scrutiny over the past few years and gives rise to remarkable biological effects in the recipient cells.

Cell Types Involved in TNT Connections

Tunneling nanotubes were first described, in 2004, in pheochromocytoma-derived PC12 cancer cells and in normal immune cells. Ever since, the number of cell types described to undergo such connecting processes has grown to more than 50, comprising both normal and cancerous cells, as shown in Table 1. These include bladder, breast, colon, and ovarian cancer cells as well as laryngeal squamous cell carcinoma, malignant mesothelial, pheochromocytoma, and osteosarcoma cells. TNTs can provide connections between cells of the same type as for astrocytomas. TNT formation was also observed between normal and cancer cells, including human ovarian epithelial benign and cancer cells, murine stromal osteoblast and osteosarcoma cells, fibroblasts, and HeLa breast cancer cells. A number of the TNTs described between normal and cancer cells involve mesenchymal stem cells (MSCs), known to be recruited to tumor sites. MSCs are stem cells characterized by their capacity to differentiate along several lineages including

Table 1 TNT-connected cells and cargos

Authors	*Cells involved in the tunneling nanotube connection*	*Transported cargoes*
Onfelt et al. (2004)	Human NK cells/EBV transformed human B-cells Human macrophages Human EBV transformed human B-cells Murine J774 macrophages	GFP-tagged cell surface class I MHC
Rustom et al. (2004)	Rat pheochromocytoma PC12 Human embryonic kidney (HEK) Normal rat kidney (NRK)	Microvesicles Organelles
Castro et al. (2005)	Colon carcinoma cell line SW620	ND
Koyanagi et al. (2005)	Human endothelial progenitor/rat cardiomyocytes	Mitochondria
Watkins and Salter (2005)	Human dendritic cells/THP-1 cells Human THP-1 monocytes	Calcium flux Major-histocompatibility proteins (MHC class I)
Onfelt et al. (2006)	Human macrophages	Bacteria Mitochondria Vesicles (endosomes, lysosomes)
Chinnery et al. (2008)	Murine MHC class II dendritic cells	ND
Gurke et al. (2008)	Normal rat kidney cells (NRK)	Endocytic organelles
Sowinski et al. (2008)	Jurkat and primary T cells Primary T cells	HIV viral particles
Bukoreshtliev et al. (2009)	Pheochromocytoma PC12 cells	Intracellular organelle
Eugenin et al. (2009)	Human macrophages	HIV viral particles
Plotnikov et al. (2010)	Human mesenchymal stromal cells/rat renal tubular cells	Mitochondria
Acquistapace et al. (2011)	Human mesenchymal stem cells/cardiomyocytes	Mitochondria and intracellular material
Domhan et al. (2011)	Human proximal tubular epithelial cells (RPTEC)	Microvesicles
Wang et al. (2011)	Rat hippocampal astrocytes and neurons	Endoplasmic reticulum Mitochondria Golgi fragments Endosomes Amyloid β
Yasuda et al. (2011)	Human umbilical vein endothelial cells (HUVEC)	Lysosomes Mitochondria
Islam et al. (2012)	Murine MSCs and alveolar epithelial cells	Mitochondria
Lou et al. (2012)	Human primary cancer cells Human mesothelial lines (MSTO-211H, VAMT, H-Meso)	Mitochondria
Vallabhaneni et al. (2012)	Human MSCs and vascular smooth muscle cells	Mitochondria
Wittig et al. (2012)	Human retinal pigment epithelial (ARP-19) cells	
Costanzo et al. (2013)	CAD cells Primary cerebellar granule neurons (CGNs)	Htt aggregates
Pasquier et al. (2013)	Human mesenchymal stem cells (MSCs) Human endothelial cells (HECs) Human ovarian cancer cells (SKOV3, OVCAR3, HTB-161) human breast cancer cells (MDA-MB231 and MCF7)	Mitochondria
Rainy et al. (2013)	Human B cells and T cells	Plasma membrane associated proteins (H-Ras)
Schiller et al. (2013)	HeLa	Transmembrane HLA-A2-EGFP protein
Ady et al. (2014)	VAMT (sarcomatoid mesothelioma cell line) H2052 (mesothelioma cell line) MSTO-211H (derived from mesothelioma patient) Met5A (immortalized mesothelioma cell line)	ND
Ahmad et al. (2014)	Murine MSCs and lung epithelial cells	Mitochondria
Antanaviciūtė et al. (2014)	Laryngeal squamous cell carcinoma	Mitochondria DAPI-positive vesicles
Figeac et al. (2014)	Murine cardiomyocytes and human MSCs	
Liu et al. (2014)	Human MSCs and umbilical vein endothelial cells (HUVEC)	Mitochondria
Thayanithy et al. (2014a,b)	Murine K7 M2 osteosarcoma cells and MC3T3 osteoblasts Ovarian epithelial cells and SKOV3 ovarian cancer cells	microRNAs (miR-199a)
Thayanithy et al. (2014a,b)	Human biphasic mesothelioma MSTO-211H cells	Exosomes from other cells
Astanina et al. (2015)	Epithelial cells	Lipid droplets
Biran et al. (2015)	NK cells	Proteins
Burtey et al. (2015)	HeLa and NRK fibroblasts	Tf-R (transferrin receptor), endosomes
Caicedo et al. (2015)	Human MSCs and breast cancer cell line MDA-MB-231	Mitochondria
Osswald et al. (2015)	Astrocytoma	Intercellular calcium waves (ICWs)

(Continued)

Table 1 TNT-connected cells and cargos—cont'd

Authors	*Cells involved in the tunneling nanotube connection*	*Transported cargoes*
Polak et al. (2015)	Human MSCs and acute lymphoblastic leukemia cells Human MSCs and B-cell precursors	Vital dyes
Wang and Gerdes (2015)	PC12 cells (−/+ ultraviolet light treatment)	Mitochondria
Zhu et al. (2015)	CAD neuronal cells	Prions Lysosomes Early endosomes
Ady et al. (2016)	Herpes simplex virus (NV1066) infected and non-infected cells	Activated ganciclovir (Bystander effect)
Desir et al. (2016)	Chemoresistant ovarian cancer cells (SKOV3, C200) Resistant (SKOV3) and sensitive (A2780) ovarian cancer cells Resistant ovarian cancer cells (SKOV3) and benign epithelial ovarian cells (IOSE)	Mitochondria
Hashimoto et al. (2016)	Monocyte-derived macrophage	HIV-1
Hayakawa et al. (2016)	Astrocytes and neurons	Mitochondria
Jackson et al. (2016)	Human MSCs and macrophages	Mitochondria
Jiang et al. (2016)	Mesenchymal stem cells and corneal epithelial cells	Mitochondria
Moschoi et al. (2016)	BM-MSCs and acute myeloid leukemic cells	Mitochondria
Reichert et al. (2016)	Hematopoietic progenitors	CD133
Tardivel et al. (2016)	Neurons	Tau protein
Victoria et al. (2016)	Astrocytes and neurons	Prions
Zhang et al. (2016)	iPSC-MSCs, BM-MSCs and cardiomyocytes	Mitochondria
Bittins and Wang (2017)	Apoptotic and non-apoptotic rat pheochromocytoma cells (PC12)	Phosphatidylserine (PS) Oxidized phospholipids Calreticulin
Claus et al. (2017)	Murine macrophage-like cells (J774A.1)	SAA1 protein
Dieriks et al. (2017)	Neuroblastoma cells (SH-SY5Y) Parkinson's disease pericytes	α-Synuclein
de Rooij et al. (2017)	ALL cells and MSCs	Autophagosomes mitochondria ICAM1
Kumar et al. (2017)	Lung epithelial cells (infected and uninfected)	Mitochondria Ribosomes Influenza virus proteins and genome
Lu et al. (2017)	Bladder cancer cells	Mitochondria
Mahrouf-Yorgov et al. (2017)	Cardiomyocytes and MSCs	Mitochondria
Marlein et al. (2017)	Bone marrow stromal cells and leukemic blasts	Mitochondria
Nzigou Mombo et al. (2017)	Human MSCs and glioblastoma stem cells	Mitochondria
Patheja and Sahu (2017)	Breast adenocarcinoma cells (MCF-7)	Mitochondria Cytoplasmic fragments
Sáenz-de-Santa-María et al. (2017)	Squamous cell carcinoma	Endosomal/lysosomal vesicles Autophagosomes Mitochondria
Sanchez et al. (2017)	Wharton's jelly mesenchymal stem cells	Mitochondria

osteocytes, adipocytes and chondrocytes. They are found in many tissues, notably the bone marrow and the adipose tissue. They are recruited to the inflammatory tumor microenvironment where they have been shown to make TNT connections in vivo.

It is worth noting that the occurrence of TNTs is not limited to pathological and cancerous tissues. TNTs also constitute a means of intercellular connection in physiological conditions. This was shown for renal tubular, kidney and retinal pigment epithelial cells. TNT formation was also reported for connecting endothelial progenitor cells with endothelial cells and cardiac myocytes. Likewise, TNTs were described between hippocampal neurons and astrocytes. The immune system, notably macrophages, monocytes, dendritic cells, natural killer and B cells, appear particularly prone to use TNT-mediated communication, with measurable effects on the immune response. MSCs are also recruited to damaged or inflammatory tissues where they contribute to tissue repair. TNT-dependent connections have been observed between MSCs and diverse cell types such as renal tubular cells, cardiomyocytes, bronchial epithelial cells, macrophages, endothelial cells, and vascular smooth muscle cells. Even though TNT-mediated connections appear as a widespread process, not all cell types are endowed with this connecting capacity. For instance, astrocytic brain tumors can develop TNT connections that allow intercellular calcium waves and resistance to radiotherapy, but oligodendroglial tumors do not.

Beyond Humans and the rat and mouse mammalian models, it is worthwhile noting that TNTs were also described in *B. subtilis* and *E. coli* bacteria, in Drosophila and in the zebrafish, thus underlining the evolutionary conservation of this cell-to-cell connecting mechanism.

Cargos Transported Between TNT-Connected Cells

Mitochondria

Mitochondria have been the TNT cargos most extensively studied so far. This stems from the extent of the biological effects of the transported mitochondria, both on the metabolism and functional capacities of the target cells but also, and more practically, from the available tools to observe this mitochondria trafficking, both in vivo and in vitro. Detection of the mitochondria transfer was performed using fluorescent mitochondrial vital dyes and viral expression of GFP-tagged proteins targeting the mitochondria (Fig. 3). In heterologous systems, human mitochondria could be detected with antibodies specific for human mitochondrial organelles. At the genetic level, taking advantage of the SNPs present in the mitochondrial DNA (mtDNA), mitochondria originating from different donors could be detected and their concentration evaluated on the basis the mtDNA SNP quantification. In addition, and independently of TNTs, mitochondria (isolated beforehand) display the remarkable capacity to be directly internalized by cells in a macropinocytosis-like process. This provided a useful tool to analyze the biological effects of the transferred mitochondria in the target cells.

TNT and gap junction-mediated mitochondria trafficking involves both normal and cancer cells, and cells as diverse as renal tubular epithelial cells, endothelial cells, macrophages, neuronal cells, astrocytes, astrocytomas, laryngeal squamous cell carcinoma, and leukemia cells. A vast majority of studies so far focused on mitochondria transport originating from MSCs and targeting a whole series of different cells, notably cardiomyocytes, endothelial cells, pulmonary alveolar epithelial cells, renal tubular cells, macrophages as well as cancer cells such as acute myeloid leukemia cells, breast cancer cells and glioblastoma stem cells (Fig. 4).

Other Cargos

Lots of cargos, outside from mitochondria, can be transported between TNT-connected cells. This is the case for the lysosomal organelles. Transported cargos also include the prion and the Tau infectious proteins, possibly contributing to the related pathologies. The HIV virus was described to traffic through TNTs from infected to non-infected cells, thus precluding the need for a fully mature HIV virus to infect neighboring cells. The variety of TNT-transported cargos is amazingly large as, in addition to the above, it also includes microRNAs, lipid droplets and Ca^{2+} calcium ions.

Mechanisms of TNT and Gap Junction-Dependent Cell-to-Cell Exchanges

Gap junctions (GJCs) are intercellular channels that connect the cytoplasms of different cells and allow the exchange of molecules and ions between these cells. First observed in 1958, their role in cardiac tissue has been largely studied. GJCs are composed

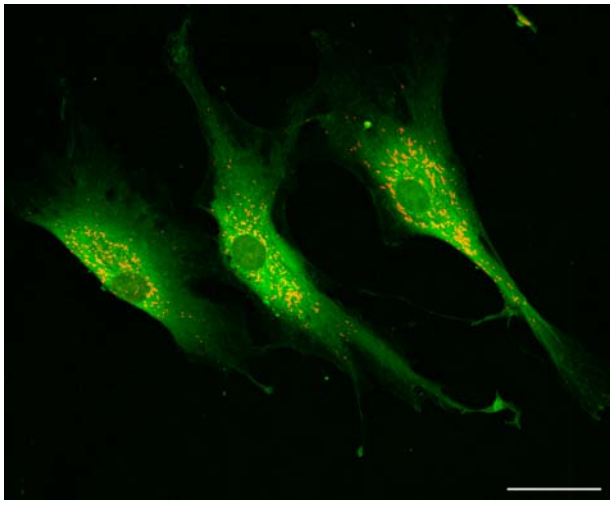

Fig. 3 Mesenchymal stem cell mitochondria network. MSCs were labeled with the green CellTracker CMFDA while their mitochondria were labeled with the MitoTracker Red CMXRos. The MSC mitochondria networks as well as thin intercellular connections can be observed using these vital dyes. Scale bar, 50 μm.

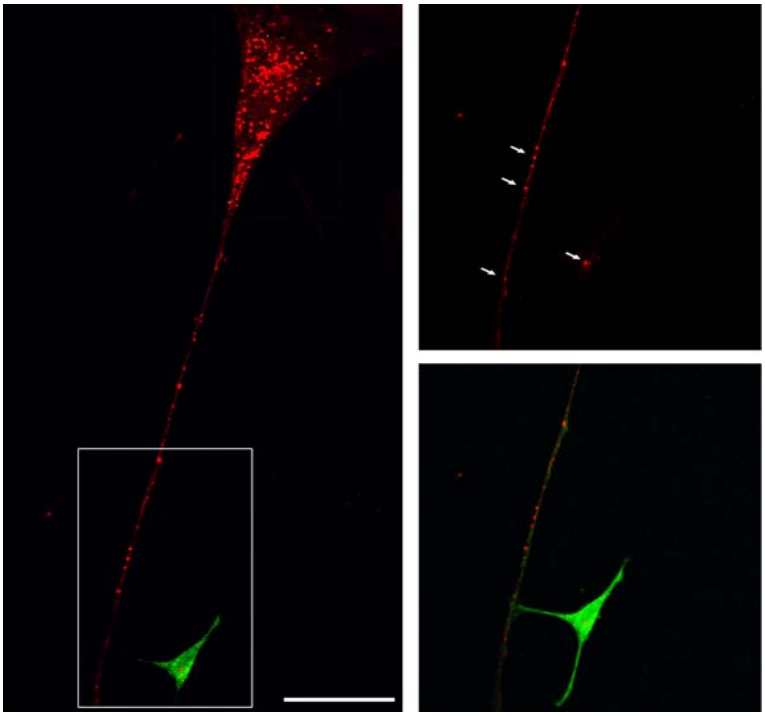

Fig. 4 Mitochondria transfer between mesenchymal stem cells (MSCs) and glioblastoma stem cells (GSCs). Tunneling nanotube formation and mitochondria trafficking between MSCs (labeled with the MitoTracker Red CMXRos) and GSCs (labeled with the green CellTracker CMFDA) after 72 h of coculture. In the enlarged images: MSC mitochondria in the MSC-GSC TNT and inside the GSC (*arrows*). Scale bar, 50 μm.

of two hemi-channels (one for each cell), each of them composed of a hexamer of transmembrane connexin proteins. The connexin 43 (Cx43) was shown, both in vitro and vivo, to play an essential role in the formation of the TNT and gap junction-dependent cell-to-cell connections and subsequent cargo trafficking. Since GJCs only allow passage of molecules smaller than ATP, their precise implication in the TNT-mediated intercellular transfer of organelles like mitochondria obviously deserves further investigation. Additional mechanisms for TNT-mediated intercellular cargo trafficking have also been described to involve M-Sec/TNFaip2 and the exocyst complex as well as various GTPases. However, the precise role of the different GTPases, notably Cdc42, RalA, Rab8, that have been analyzed so far for their involvement in TNT elongation and cargo trafficking, still needs to be fully clarified. Importantly though, the Rho GTPase Miro1 (also called RhoT1/2), well known for taking part with the Milton adapter protein and the kinesin molecular motor in mitochondria transport in axons, is now established to play a key role in the TNT and gap junction-dependent mitochondria transport. Cargo transport within the TNTs occurs along cytoskeleton fibers. The nature of this cytoskeleton depends on both the types of cells connected and the cargos transported. Both microtubules and actin microfilaments, either alone or together inside the same tunneling nanotubes, mediate the active cargo trafficking inside the TNTs.

Signals That Regulate TNT Formation and Cargo Trafficking

Identifying the factors that promote, or inhibit, the formation of TNTs and the subsequent cargo trafficking is of great importance to fully understand the role of this process in tissue homeostasis and, most of all, in pathologies. Different genetic and chemical stresses initiate or stimulate TNT formation and intercellular organelle exchange. These stimuli can be classified in two categories of stress: those affecting the cellular energy metabolism and those related to cell defense in response to inflammation or DNA damage.

Mitochondrial deficiency is a cellular stimulus sufficient to trigger exchange of mitochondria between mitochondria-competent and deficient cells. For instance, lung carcinoma cells treated over long periods with the mitochondrial DNA damaging agent ethidium bromide have non-functional mitochondria but are still viable. These carcinoma cells trigger TNT formation from bone marrow MSCs toward the carcinoma cells leading to the exchange of functional mitochondria and to the recovery of mitochondrial function in the cancer cells. Mitochondrial chemical poisoning with agents such as rotenone or antimycin, that block the electron transport chain, also increases the mitochondria donation from untreated cells to the chemically treated cells. In a consistent manner, glucose starvation and oxygen deprivation also constitute inducer stimuli. Reactive oxygen species (ROS) that are produced during metabolic or physical stress conditions are also involved in the cross talk signaling between the requesting

and donor cells. Actually, the experimental increase in cellular ROS, upon cell exposure to hydrogen peroxide for instance, is a strong TNT inducer.

Alternatively, inflammatory signaling induced by bacterial lipopolysaccharides or TNFα treatment of the recipient cells are also strong stimuli identified as promoters of mitochondria exchange. In addition, a number of DNA damaging agents, mostly employed for cancer treatment, are also potent inducers of TNT-mediated mitochondria exchange. Zeocin, a DNA intercalating agent related to bleomycin, was found to increase by a factor of 10 the number of TNTs formed between renal proximal tubular epithelial cells. Cytarabine (ARA), a nucleoside analog used for the treatment of acute myeloid leukemia (AML), as well as the topoisomerase II inhibitor etoposide and the anthracycline doxorubicin were found to promote the acquisition of MSC mitochondria by AML cells.

Inhibition of the horizontal mitochondrial organelle exchange can be achieved by targeting different steps of the TNT exchange process including: (1) the initial stress signal produced by the cargo-requesting cells, (2) the plasma membrane protrusion formation, (3) the contact/docking between the TNT-involved cells, and (4) the "railway motorization" allowing the cargo trafficking along the cytoskeleton fibers contained within these TNTs. Stress signals leading to local ROS increase can be lessened with antioxidizing agents such as *N*-acetylcysteine. Cell directional polarization and TNT formation can be opposed by virtually all cytoskeleton inhibitors, including agents that either stabilize or depolymerize microtubules and actin microfilaments. Inhibiting the connexin mediated cell-cell docking or shielding the cargo-recipient cells with annexin-V, a protein that recognizes phosphatidylserines abnormally exposed extracellularly by the stressed cargo-requesting cells, can target the cell-cell docking. Finally, even when bridging TNTs have already been established, inhibiting the actin or microtubule-based molecular motors can block cargo trafficking between the donor and acceptor cells.

TNT/Gap Junction-Dependent Connections in Cancer

The occurrence of intercellular organelle exchange is established for solid tumors as well as for hematological malignancies, using immortalized cell lines or fresh primary cancer cells isolated from patients resected tumors. With regard to the cancer cells, organelle exchange can be either inbound (organelle intake by the cancer cell) or outbound (organelle unloading toward the cellular microenvironment). The tumor microenvironment, although not transformed and mutated, plays a direct role in cancer initiation, progression and response to therapeutic treatments. In particular, cells of the microenvironment interact with the cancer stem cells (CSC) present in the tumor and display instructive and protective functions to maintain CSC quiescent and immature properties. Cancer cells educate their microenvironment for the benefit of the CSCs and at the expense of the normal tissue adult stem cells present within the tissue. In this particular context of microenvironment remodeling, cancer cells can use the outbound transfer of mitochondria or lysosomes, to the non-cancer cells of the microenvironment and consequently modify their cytokine secretion pattern. Acute lymphoblastic leukemic cells use TNTs to signal to MSCs and drive the stroma secretion of the pro-inflammatory cytokines CXCL10, CCL2, and interleukin 8. Beyond the outbound transfer to modify their microenvironment, cancer cells can use this unloading system to detoxify during chemotherapy exposure. The outbound transfer of lysosomal vesicles containing the chemotherapeutic agents was shown to reduce the concentration of these toxic agents in leukemic cells.

Regarding the intake of mitochondria, its main reported outcome is a survival benefit for the recipient cells, for both normal or cancer cells. For instance, leukemic cancer stem cells that capture mitochondria during cytarabine exposure become more resistant to cell death and boosted with a long-term regrowth potential. In line with these observations, mitochondria horizontal transfer directly modifies the energetic metabolism of the recipient cells. Many cancer cells predominantly produce energy through a high rate of glycolysis, which converts glucose to pyruvate, followed by the production of lactic acid from this pyruvate. On the contrary, most normal cells use an alternative pyruvate metabolic pathway that takes place in the mitochondria where the oxidation of pyruvate ultimately leads to the phosphorylation of ADP in ATP, in a pathway called oxidative phosphorylation (OXPHOS). The complete eradication of OXPHOS for a cancer cell is a disadvantage. This can be achieved experimentally by a long-term exposure to mitochondrial DNA damaging agents, leading to cells named ρ0 cells. These OXPHOS-incompetent ρ0 cancer cells have a lower tumorigenic and metastatic potential compared to the mitochondria-competent parental cancer cells. Over time ρ0 cancer cells, placed in vivo within a mitochondria-competent surrounding environment, recover their OXPHOS activity through mitochondria horizontal transfer and retrieve their tumorigenic potential. Independently of the organelle exchange, different anticancer radiation or chemical therapies can switch cancer cell glycolytic metabolism toward an OXPHOS metabolism. In different cancer models, an OXPHOS metabolism was shown to confer cancer cells an increased drug resistance compared to a glycolytic metabolism. Antineoplastic agents stimulate intercellular mitochondria exchange, which further favors the OXPHOS metabolism in the recipient cancer cells. This survival benefit associated with the intake of exogenous organelles is observed not only for differentiated cancer cells but also for cancer stem cells. These observations, while questioning our long-standing view of cancer metabolism, offer new opportunities for developing innovative and more efficient therapeutic treatments. For instance, blocking the microenvironment-mediated drug resistance acquisition by interfering with the TNT-based intercellular organelle exchange combined with the inhibition of the OXPHOS metabolism is expected to improve the current treatment standards and tackle cancers at their roots.

Noteworthily, outside from cancer, TNT and gap junction-dependent cargo exchange also has important biological effects, resulting notably in tissue repair. As demonstrated in murine models of lipopolysaccharide and rotenone-induced damage of pulmonary alveoli, lung instillation of MSCs leads to in situ connections between the MSCs and the damaged pulmonary alveolar epithelial cells and to the subsequent transfer of MSC mitochondria, resulting in the regeneration of the affected alveoli and mice

survival. In another context, that of *E. coli*-induced pneumonia (murine model), acquisition of MSC mitochondria by lung alveolar macrophages results in increased phagocytic activity and antimicrobial response. Consequently, in non-tumor injured tissues, therapeutic strategies would aim at increasing the mitochondria transfer occurrence and efficacy, both by increasing the local number of mitochondria donor cells in the wounded environment and by pharmacologically promoting cell–cell interactions and organelle trafficking from these cells.

Prospective View

The discovery of TNT-mediated organelle exchange provides new perspectives in the field of cancer cell biology. The discovery of this new mode of intercellular communication stems from the efforts of the scientific community, in the past two decades, to improve the physiological relevance of the studied cancer models. Although immortalized cancer cell lines were helpful to study the intrinsic cellular and molecular deregulations responsible for cancer cell transformation, these models were less suited for studying the biology of the primary tumors and their interactions with their microenvironment. For example, standard cancer cell lines are generally cultured in vitro with high glucose and oxygen concentrations, at least double their actual physiological concentrations. The recent advances in engineering organotypic co-culture systems, in 2- or 3-dimensions, as well as the development of humanized xenograft systems using immunodeficient mice, are prompting the discovery of new features in cancer biology.

It is worth mentioning, at this point, that most of our current knowledge of the TNT-mediated intercellular exchanges originates from in vitro observations. Even though TNT connections and mitochondria intercellular trafficking were also demonstrated to occur in vivo, it will be worthwhile extending these in vivo studies. For instance, it is presently unknown whether current anti-cancer therapies do increase TNT formation in vivo. A number of likewise unanswered questions remain to be explored, such as the actual amplitude of the TNT phenomenon in the different types of cancer, its frequency as well at its prognostic value. The time scale during which cells remain biologically modified, following TNT-mediated mitochondria organelle acquisition, needs as well to be better assessed. It also remains to be determined whether there are "quality control" checkpoints for mitochondria donated by the donor cells as well as for those accepted by the recipient cells. It will most likely depend on both the donor and recipient cells biological properties. Furthermore, technical issues will need to be settled, notably the monitoring of chemical probes and the development of novel markers for selectively tracking the exchanged mitochondria organelles over longer periods of time.

As a conclusion, it now clearly appears that the discovery and characterization of TNT intercellular connections, and of cargos transported from one cell to the other through these connections, bring a novel understanding of the cancer cell biology that takes place within tumors. As this cargo trafficking has consequences on tumor progression and resistance to therapy, the challenge will now be to exploit this new knowledge to conceive and develop novel anticancer therapeutic strategies.

References

Acquistapace, A., Bru, T., Lesault, P.-F., Figeac, F., Coudert, A.E., le Coz, O., Christov, C., Baudin, X., Auber, F., Yiou, R., et al., 2011. Human mesenchymal stem cells reprogram adult cardiomyocytes toward a progenitor-like state through partial cell fusion and mitochondria transfer. Stem Cells 29, 812–824.

Ady, J.W., Desir, S., Thayanithy, V., Vogel, R.I., Moreira, A.L., Downey, R.J., Fong, Y., Manova-Todorova, K., Moore, M.A.S., Lou, E., 2014. Intercellular communication in malignant pleural mesothelioma: Properties of tunneling nanotubes. Frontiers in Physiology 5, 400.

Ady, J., Thayanithy, V., Mojica, K., Wong, P., Carson, J., Rao, P., Fong, Y., Lou, E., 2016. Tunneling nanotubes: An alternate route for propagation of the bystander effect following oncolytic viral infection. Molecular Therapy - Oncolytics 3.

Ahmad, T., Mukherjee, S., Pattnaik, B., Kumar, M., Singh, S., Kumar, M., Rehman, R., Tiwari, B.K., Jha, K.A., Barhanpurkar, A.P., et al., 2014. Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. The EMBO Journal 33, 994–1010.

Antanavičiūtė, I., Rysevaitė, K., Liutkevičius, V., Marandykina, A., Rimkutė, L., Sveikatienė, R., Uloza, V., Skeberdis, V.A., 2014. Long-distance communication between laryngeal carcinoma cells. PLoS One 9, e99196.

Astanina, K., Koch, M., Jüngst, C., Zumbusch, A., Kiemer, A.K., 2015. Lipid droplets as a novel cargo of tunnelling nanotubes in endothelial cells. Scientific Reports 5, 11453.

Biran, A., Perelmutter, M., Gal, H., Burton, D.G.A., Ovadya, Y., Vadai, E., Geiger, T., Krizhanovsky, V., 2015. Senescent cells communicate via intercellular protein transfer. Genes & Development 29, 791–802.

Bittins, M., Wang, X., 2017. TNT-induced phagocytosis: Tunneling nanotubes mediate the transfer of pro-phagocytic signals from apoptotic to viable cells. Journal of Cellular Physiology 232, 2271–2279.

Bukoreshtliev, N.V., Wang, X., Hodneland, E., Gurke, S., Barroso, J.F.V., Gerdes, H.-H., 2009. Selective block of tunneling nanotube (TNT) formation inhibits intercellular organelle transfer between PC12 cells. FEBS Letters 583, 1481–1488.

Burtey, A., Wagner, M., Hodneland, E., Skaftnesmo, K.O., Schoelermann, J., Mondragon, I.R., Espedal, H., Golebiewska, A., Niclou, S.P., Bjerkvig, R., et al., 2015. Intercellular transfer of transferrin receptor by a contact-, Rab8-dependent mechanism involving tunneling nanotubes. The FASEB Journal 29, 4695–4712.

Caicedo, A., Fritz, V., Brondello, J.-M., Ayala, M., Dennemont, I., Abdellaoui, N., de Fraipont, F., Moisan, A., Prouteau, C.A., Boukhaddaoui, H., et al., 2015. MitoCeption as a new tool to assess the effects of mesenchymal stem/stromal cell mitochondria on cancer cell metabolism and function. Scientific Reports 5, 9073.

Castro, M.A.A., Grieneisen, V.A., de Almeida, R.M.C., 2005. Disruption and de novo formation of nanotubular membrane extensions in SW620 colon carcinoma cell line during cell division. Cell Biology International 29, 929–931.

Chinnery, H.R., Pearlman, E., McMenamin, P.G., 2008. Cutting edge: Membrane nanotubes in vivo: A feature of mhc class II+ cells in the mouse cornea. Journal of Immunology 180, 5779 (Baltimore, Md.: 1950).

Claus, S., Puscalau-Girtu, I., Walther, P., Syrovets, T., Simmet, T., Haupt, C., Fändrich, M., 2017. Cell-to-cell transfer of SAA1 protein in a cell culture model of systemic AA amyloidosis. Scientific Reports 7, 45683.

Costanzo, M., Abounit, S., Marzo, L., Danckaert, A., Chamoun, Z., Roux, P., Zurzolo, C., 2013. Transfer of polyglutamine aggregates in neuronal cells occurs in tunneling nanotubes. Journal of Cell Science 126, 3678–3685.

Desir, S., Dickson, E.L., Vogel, R.I., Thayanithy, V., Wong, P., Teoh, D., Geller, M.A., Steer, C.J., Subramanian, S., Lou, E., 2016. Tunneling nanotube formation is stimulated by hypoxia in ovarian cancer cells. Oncotarget 7, 43150–43161.

Dieriks, B.V., Park, T.I.-H., Fourie, C., Faull, R.L.M., Dragunow, M., Curtis, M.A., 2017. α-synuclein transfer through tunneling nanotubes occurs in SH-SY5Y cells and primary brain pericytes from Parkinson's disease patients. Scientific Reports 7, 42984.

Domhan, S., Ma, L., Tai, A., Anaya, Z., Beheshti, A., Zeier, M., Hlatky, L., Abdollahi, A., 2011. Intercellular communication by exchange of cytoplasmic material via tunneling nanotube like structures in primary human renal epithelial cells. PLoS One 6, e21283.

Eugenin, E.A., Gaskill, P.J., Berman, J.W., 2009. Tunneling nanotubes (TNT) A potential mechanism for intercellular HIV trafficking. Communicative and Integrative Biology 2, 243–244.

Figeac, F., Lesault, P.-F., Le Coz, O., Damy, T., Souktani, R., Trébeau, C., Schmitt, A., Ribot, J., Mounier, R., Guguin, A., et al., 2014. Nanotubular crosstalk with distressed cardiomyocytes stimulates the paracrine repair function of mesenchymal stem cells: Cardiomyocyte crosstalk improves stem cell healing. Stem Cells 32, 216–230.

Gurke, S., Barroso, J.F.V., Hodneland, E., Bukoreshtliev, N.V., Schlicker, O., Gerdes, H.-H., 2008. Tunneling nanotube (TNT)-like structures facilitate a constitutive, actomyosin-dependent exchange of endocytic organelles between normal rat kidney cells☆. Experimental Cell Research 314, 3669–3683.

Hashimoto, M., Bhuyan, F., Hiyoshi, M., Noyori, O., Nasser, H., Miyazaki, M., Saito, T., Kondoh, Y., Osada, H., Kimura, S., et al., 2016. Potential role of the formation of tunneling nanotubes in HIV-1 spread in macrophages. Journal of Immunology 196, 1832–1841.

Hayakawa, K., Esposito, E., Wang, X., Terasaki, Y., Liu, Y., Xing, C., Ji, X., Lo, E.H., 2016. Transfer of mitochondria from astrocytes to neurons after stroke. Nature 535, 551–555.

Islam, M.N., Das, S.R., Emin, M.T., Wei, M., Sun, L., Westphalen, K., Rowlands, D.J., Quadri, S.K., Bhattacharya, S., Bhattacharya, J., 2012. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. Nature Medicine 18, 759–765.

Jackson, M.V., Morrison, T.J., Doherty, D.F., McAuley, D.F., Matthay, M.A., Kissenpfennig, A., O'Kane, C.M., Krasnodembskaya, A.D., 2016. Mitochondrial transfer via tunneling nanotubes is an important mechanism by which mesenchymal stem cells enhance macrophage phagocytosis in the in vitro and in vivo models of ARDS. Stem Cells 34, 2210–2223.

Jiang, D., Gao, F., Zhang, Y., Wong, D.S.H., Li, Q., Tse, H., Xu, G., Yu, Z., Lian, Q., 2016. Mitochondrial transfer of mesenchymal stem cells effectively protects corneal epithelial cells from mitochondrial damage. Cell Death & Disease 7, e2467.

Koyanagi, M., Brandes, R.P., Haendeler, J., Zeiher, A.M., Dimmeler, S., 2005. Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: A novel mechanism for cell fate changes? Circulation Research 96, 1039–1041.

Kumar, A., Kim, J.H., Ranjan, P., Metcalfe, M.G., Cao, W., Mishina, M., Gangappa, S., Guo, Z., Boyden, E.S., Zaki, S., et al., 2017. Influenza virus exploits tunneling nanotubes for cell-to-cell spread. Scientific Reports 7, 40360.

Liu, K., Ji, K., Guo, L., Wu, W., Lu, H., Shan, P., Yan, C., 2014. Mesenchymal stem cells rescue injured endothelial cells in an in vitro ischemia-reperfusion model via tunneling nanotube like structure-mediated mitochondrial transfer. Microvascular Research 92, 10–18.

Lou, E., Fujisawa, S., Morozov, A., Barlas, A., Romin, Y., Dogan, Y., Gholami, S., Moreira, A.L., Manova-Todorova, K., Moore, M.A.S., 2012. Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma. PLoS One 7, e33093.

Lu, J., Zheng, X., Li, F., Yu, Y., Chen, Z., Liu, Z., Wang, Z., Xu, H., Yang, W., 2017. Tunneling nanotubes promote intercellular mitochondria transfer followed by increased invasiveness in bladder cancer cells. Oncotarget 8, 15539–15552.

Mahrouf-Yorgov, M., Augeul, L., Da Silva, C.C., Jourdan, M., Rigolet, M., Manin, S., Ferrera, R., Ovize, M., Henry, A., Guguin, A., et al., 2017. Mesenchymal stem cells sense mitochondria released from damaged cells as danger signals to activate their rescue properties. Cell Death and Differentiation 24, 1224–1238.

Marlein, C.R., Zaitseva, L., Piddock, R.E., Robinson, S., Edwards, D., Shafat, M.S., Zhou, Z., Lawes, M., Bowles, K.M., Rushworth, S.A., 2017. NADPH oxidase-2 derived superoxide drives mitochondrial transfer from bone marrow stromal cells to leukemic blasts. Blood 130, 1649–1660.

Moschoi, R., Imbert, V., Nebout, M., Chiche, J., Mary, D., Prebet, T., Saland, E., Castellano, R., Pouyet, L., Collette, Y., et al., 2016. Protective mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemic cells during chemotherapy. Blood 128, 253–264.

Nzigou Mombo, B., Gerbal-Chaloin, S., Bokus, A., Daujat-Chavanieu, M., Jorgensen, C., Hugnot, J.-P., Vignais, M.-L., 2017. MitoCeption: Transferring isolated human MSC mitochondria to glioblastoma stem cells. Journal of Visualized Experiments.

Önfelt, B., Nedvetzki, S., Yanagi, K., Davis, D.M., 2004. Cutting edge: Membrane nanotubes connect immune cells. The Journal of Immunology 173, 1511–1513.

Onfelt, B., Nedvetzki, S., Benninger, R.K.P., Purbhoo, M.A., Sowinski, S., Hume, A.N., Seabra, M.C., Neil, M.A.A., French, P.M.W., Davis, D.M., 2006. Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. Journal of Immunology 177, 8476–8483.

Osswald, M., Jung, E., Sahm, F., Solecki, G., Venkataramani, V., Blaes, J., Weil, S., Horstmann, H., Wiestler, B., Syed, M., et al., 2015. Brain tumour cells interconnect to a functional and resistant network. Nature 528, 93–98.

Pasquier, J., Guerrouahen, B.S., Al Thawadi, H., Ghiabi, P., Maleki, M., Abu-Kaoud, N., Jacob, A., Mirshahi, M., Galas, L., Rafii, S., et al., 2013. Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. Journal of Translational Medicine 11, 94.

Patheja, P., Sahu, K., 2017. Macrophage conditioned medium induced cellular network formation in MCF-7 cells through enhanced tunneling nanotube formation and tunneling nanotube mediated release of viable cytoplasmic fragments. Experimental Cell Research 355, 182–193.

Plotnikov, E.Y., Khryapenkova, T.G., Galkina, S.I., Sukhikh, G.T., Zorov, D.B., 2010. Cytoplasm and organelle transfer between mesenchymal multipotent stromal cells and renal tubular cells in co-culture. Experimental Cell Research 316, 2447–2455.

Polak, R., de Rooij, B., Pieters, R., den Boer, M.L., 2015. B-cell precursor acute lymphoblastic leukemia cells use tunneling nanotubes to orchestrate their microenvironment. Blood 126, 2404–2414.

Rainy, N., Chetrit, D., Rouger, V., Vernitsky, H., Rechavi, O., Marguet, D., Goldstein, I., Ehrlich, M., Kloog, Y., 2013. H-Ras transfers from B to T cells via tunneling nanotubes. Cell Death & Disease 4, e726.

Reichert, D., Scheinpflug, J., Karbanová, J., Freund, D., Bornhäuser, M., Corbeil, D., 2016. Tunneling nanotubes mediate the transfer of stem cell marker CD133 between hematopoietic progenitor cells. Experimental Hematology 44, 1092–1112.e2.

de Rooij, B., Polak, R., Stalpers, F., Pieters, R., den Boer, M.L., 2017. Tunneling nanotubes facilitate autophagosome transfer in the leukemic niche. Leukemia 31, 1651–1654.

Rustom, A., Saffrich, R., Markovic, I., Walther, P., Gerdes, H.-H., 2004. Nanotubular highways for intercellular organelle transport. Science 303, 1007–1010.

Sáenz-de-Santa-María, I., Bernardo-Castiñeira, C., Enciso, E., García-Moreno, I., Chiara, J.L., Suarez, C., Chiara, M.-D., 2017. Control of long-distance cell-to-cell communication and autophagosome transfer in squamous cell carcinoma via tunneling nanotubes. Oncotarget 8, 20939–20960.

Sanchez, V., Villalba, N., Fiore, L., Luzzani, C., Miriuka, S., Boveris, A., Gelpi, R.J., Brusco, A., Poderoso, J.J., 2017. Characterization of tunneling nanotubes in Wharton's jelly mesenchymal stem cells. An intercellular exchange of components between neighboring cells. Stem Cell Reviews and Reports 13, 491–498.

Schiller, C., Huber, J.E., Diakopoulos, K.N., Weiss, E.H., 2013. Tunneling nanotubes enable intercellular transfer of MHC class I molecules. Human Immunology 74, 412–416.

Sowinski, S., Jolly, C., Berninghausen, O., Purbhoo, M.A., Chauveau, A., Köhler, K., Oddos, S., Eissmann, P., Brodsky, F.M., Hopkins, C., et al., 2008. Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. Nature Cell Biology 10, 211–219.

Tardivel, M., Bégard, S., Bousset, L., Dujardin, S., Coens, A., Melki, R., Buée, L., Colin, M., 2016. Tunneling nanotube (TNT)-mediated neuron-to neuron transfer of pathological tau protein assemblies. Acta Neuropathologica Communications 4, 117.

Thayanithy, V., Dickson, E.L., Steer, C., Subramanian, S., Lou, E., 2014a. Tumor-stromal cross talk: Direct cell-to-cell transfer of oncogenic microRNAs via tunneling nanotubes. Translational Research 164, 359–365.

Thayanithy, V., Babatunde, V., Dickson, E.L., Wong, P., Oh, S., Ke, X., Barlas, A., Fujisawa, S., Romin, Y., Moreira, A.L., et al., 2014b. Tumor exosomes induce tunneling nanotubes in lipid raft-enriched regions of human mesothelioma cells. Experimental Cell Research 323, 178–188.

Vallabhaneni, K.C., Haller, H., Dumler, I., 2012. Vascular smooth muscle cells initiate proliferation of mesenchymal stem cells by mitochondrial transfer via tunneling nanotubes. Stem Cells and Development 21, 3104–3113.
Victoria, G.S., Arkhipenko, A., Zhu, S., Syan, S., Zurzolo, C., 2016. Astrocyte-to-neuron intercellular prion transfer is mediated by cell-cell contact. Scientific Reports 6, 20762.
Wang, X., Gerdes, H.-H., 2015. Transfer of mitochondria via tunneling nanotubes rescues apoptotic PC12 cells. Cell Death and Differentiation 22, 1181–1191.
Wang, Y., Cui, J., Sun, X., Zhang, Y., 2011. Tunneling-nanotube development in astrocytes depends on p53 activation. Cell Death and Differentiation 18, 732–742.
Watkins, S.C., Salter, R.D., 2005. Functional connectivity between immune cells mediated by tunneling nanotubules. Immunity 23, 309–318.
Wittig, D., Wang, X., Walter, C., Gerdes, H.-H., Funk, R.H.W., Roehlecke, C., 2012. Multi-level communication of human retinal pigment epithelial cells via tunneling nanotubes. PLoS One 7, e33195.
Yasuda, K., Khandare, A., Burianovskyy, L., Maruyama, S., Zhang, F., Nasjletti, A., Goligorsky, M.S., 2011. Tunneling nanotubes mediate rescue of prematurely senescent endothelial cells by endothelial progenitors: Exchange of lysosomal pool. Aging (Albany NY) 3, 597–608.
Zhang, Y., Yu, Z., Jiang, D., Liang, X., Liao, S., Zhang, Z., Yue, W., Li, X., Chiu, S.-M., Chai, Y.-H., et al., 2016. iPSC-MSCs with high intrinsic MIRO1 and sensitivity to TNF-α yield efficacious mitochondrial transfer to rescue anthracycline-induced cardiomyopathy. Stem Cell Reports 7, 749–763.
Zhu, S., Victoria, G.S., Marzo, L., Ghosh, R., Zurzolo, C., 2015. Prion aggregates transfer through tunneling nanotubes in endocytic vesicles. Prion 9, 125–135.

Further Reading

Berridge, M.V., McConnell, M.J., Grasso, C., Bajzikova, M., Kovarova, J., Neuzil, J., 2016. Horizontal transfer of mitochondria between mammalian cells: Beyond co-culture approaches. Current Opinion in Genetics & Development 38, 75–82.
Torralba, D., Baixauli, F., Sánchez-Madrid, F., 2016. Mitochondria know no boundaries: Mechanisms and functions of intercellular mitochondrial transfer. Frontiers in Cell and Developmental Biology 4.
Vignais, M.-L., Caicedo, A., Brondello, J.-M., Jorgensen, C., 2017. Cell connections by tunneling nanotubes: Effects of mitochondrial trafficking on target cell metabolism, homeostasis, and response to therapy. Stem Cells International 2017, 1–14.

Unprogrammed Gene Activation: A Critical Evaluation of Cancer Testis Genes

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Glossary

BRDT Bromodomain testis-specific is a gene encoding a double bromodomain protein of the bromodomain and extra terminal (BET) protein family.
Cancer testis (C/T) genes Testis-specific genes that are aberrantly active in tumor cells.
Cancer testis antigens (CTA) The products of cancer testis genes that are unknown to the immune system and hence are antigenic.
Cell identity Molecular systems that ensure a specific pattern of gene expression conferring functional specificities to a given cell type.
CT-X genes C/T genes that are localized on the X chromosome.
Quiescent stem cells Non-proliferative resting stem cells.
Supervised transcriptomic analysis Comparison of transcriptomes from sample groups defined a priori.
Tissue-specific genes Genes within the genome that are exclusively expressed in a given tissue.
Tissue-restricted genes A term that has the same meaning as tissue-specific genes.
Tissue-privileged genes Genes within the genome that are predominantly expressed in a given tissue but are also expressed in other tissues at a lower or background level.
Transcriptome The repertoire of cellular RNAs in a given cell.

Abbreviations

ALL Acute lymphoblastic leukemia
AML Acute myeloblastic leukemia
CAR-T Chimeric-antigen receptor T cell therapy
CLL Chronic lymphocytic leukemia
CML Chronic myeloid leukemia
DLBCL Diffuse large B-cell lymphoma
MDS Myelodysplastic syndrome
MHC Major histocompatibility complex
MLL Mixed lineage leukemia

Introduction

Cell differentiation corresponds to a physiological cell programming process that instructs the genome for a stepwise control of specific gene expression. A tight interconnection between the genome, metabolism and the epigenome is of primordial importance in correctly driving cell differentiation. The stability of gene expression that characterizes adult cells and their different functional states or, in other words, their identity, largely relies on the stable relationship between the metabolism and the epigenome. Specific epigenetic barriers prevent unprogrammed changes in gene expression and ensure the stability of the differentiated cell states. Pre-cancerous situations and malignant cell transformation involving changes in cell metabolism and hence in the epigenome, destabilize gene expression states and favor events that may also affect genome integrity. A direct consequence of these alterations is the aberrant desilencing of hundreds of tissue-specific genes in various considered tumors. Cancer cells aberrantly express a large number of tissue-restricted or tissue-privileged genes. The expression of some of these normally silent genes, such as testis-specific genes, has already attracted attention. Indeed, one of the most visible awakening of normally dormant genes in cancer concerns the so-called testis-specific genes, also known as C/T genes. Sporadic identification of these genes in various cancers has led the investigators to see them as a particular category of genes identified and studied over time and listed as such. However, other investigations indicate that the C/T genes are not the only tissue-specific genes activated in cancer and that desilencing could actually affect a variety of dormant genes. In fact, a systematic identification of genes with tissue-restricted patterns of expression by

Rousseaux and colleagues revealed that testes express the highest number of restricted genes, which suggested that this could also be a reason why testis-specific genes are the ones most frequently identified as aberrantly expressed in cancer. Whether cancer cells express testis-specific genes in a biased or unbiased manner remains one of the important open questions that will be specifically discussed here.

Another important point is that both the establishment of the list of testis-specific genes and the systematic consideration of their ectopic expression in various cancers are now demonstrating that the list of genes already classified as C/T genes actually only corresponds to a subset of the genes that are potentially activated in cancer. It is therefore important that the notion of C/T genes is revised not only to highlight the fact that C/T genes could potentially correspond to any testis-specific genes but also to consider that ectopic gene activation includes other tissue-restricted genes. In fact, cancer cells express genes that are normally active in placenta, in embryos and during development, in ovary and trophoblasts, in fetal ovaries or in various differentiated cell types, including the nervous system and megakaryocytes/platelets, as well as in many other tissues that will be discussed in more details below.

A global vision of the phenomenon of cancer cell identity loss and of large-scale aberrant activations of tissue-specific or tissue-privileged genes in all tumors now appears as an essential issue. It is important to place C/T gene activation in the true context of general cell identity loss in cancer cells, and specifically apprehend the biology which is behind the ectopic activation of genes and more specifically behind the aberrant male germ cell genes activation during malignant transformation. Here, answers to some of these crucial questions regarding the nature of the ectopic activation of genes in cancer and the specific place of testis-specific genes are provided. Indeed, it is essential to establish once and for all, whether, among all the tissue-restricted genes, cancers preferentially express testis-specific genes and whether or not the extent of ectopic gene activation in cancer depends on the type of cancers. It is also of importance to establish whether some genomic regions/chromosomes are more prone than others to desilencing and ectopic gene activations in cancers. These are all unanswered questions and their consideration could open a new avenue to the understanding of the biology of cancer and to envision new therapeutic approaches based on the general phenomenon of ectopic gene activations.

Revisiting the Concept of Cancer Testis Genes

Cancer-associated expressed genes encoding antigenic proteins were identified and named “cancer testis antigens” (CTA) genes, and have attracted attention for several years. The interest for this category of genes is now becoming more acute with the possibility to use them as antigens for anti-cancer immunotherapy in improved therapeutic proocols in combination with inactivation of the immune checkpoints. The original discovery of C/T genes was based on the use of T-cell or antigen recognition by antibodies from melanoma patients. The analysis of the normal pattern of expression of the corresponding genes revealed that they are mostly testis-specific gene, hence the name cancer testis (C/T) genes. Since that time, sporadic reports on the identification of testis-specific genes expressed in tumors has fed the growing list of C/T genes which can be consulted on dedicated web sites.

However, a careful consideration of the tissue-of-origin of the expression of the genes listed in the database shows that actually they are not all testis-restricted, and that many of them could also be testis/brain restricted or be preferentially expressed in testis but also expressed at lower levels in other tissues, as noted by Hofmann and colleagues. The accumulation of transcriptomic data from various normal tissues and cancers prompted several investigators first to establish a list of testis-specific genes and second to systematically consider their expression in cancers. The resulting data indicate that the previously established list needs to be reconsidered to include new members as noted by Rousseaux and colleagues and different groups including McFarlane’s.

Additionally, other investigations that aimed at the unbiased identification of genes or proteins aberrantly expressed in tumors compared to their normal counterparts using transcriptomic and proteomic data, revealed that the identified mRNAs and proteins could correspond to genes normally expressed in testis as expected, but also to genes expressed in other tissues types. The question therefore arises whether C/T activation is a specific property of cancer cells or whether C/T gene activation is merely one aspect of the general desilencing of tissue-restricted genes in cancer.

Testis-Specific Genes Are Preferentially Activated in Cancer

Taking into account the aberrant activation of a variety of tissue-restricted genes in cancer, it becomes important to establish whether tumors specifically activate C/T genes rather than other normally silenced genes, and if the apparent enrichment for testis-specific gene activation in cancers is a biologically meaningful phenomenon.

An element that needs to be considered to answer these questions is that, compared to other human cell and tissue types, male germ cells express the highest number of genes with a restricted pattern of expression at both mRNA and protein levels, as established by Rousseaux and colleagues.

In order to have a more precise vision of the genes with tissue-restricted expression, taking into account recent data that are now publically available, including RNA-seq of various normal tissue and cancers, we re-established a list of tissue-restricted genes. Fig. 1A confirms our previous observation showing that indeed testes express the highest number of tissue-restricted genes and that the number of tissue-specific genes drops when somatic tissues are considered. In addition, the number of testis-restricted genes found with this new approach ($n = 1597$) largely exceeds that of the genes recorded in the C/Tdatabase ($n = 243$).

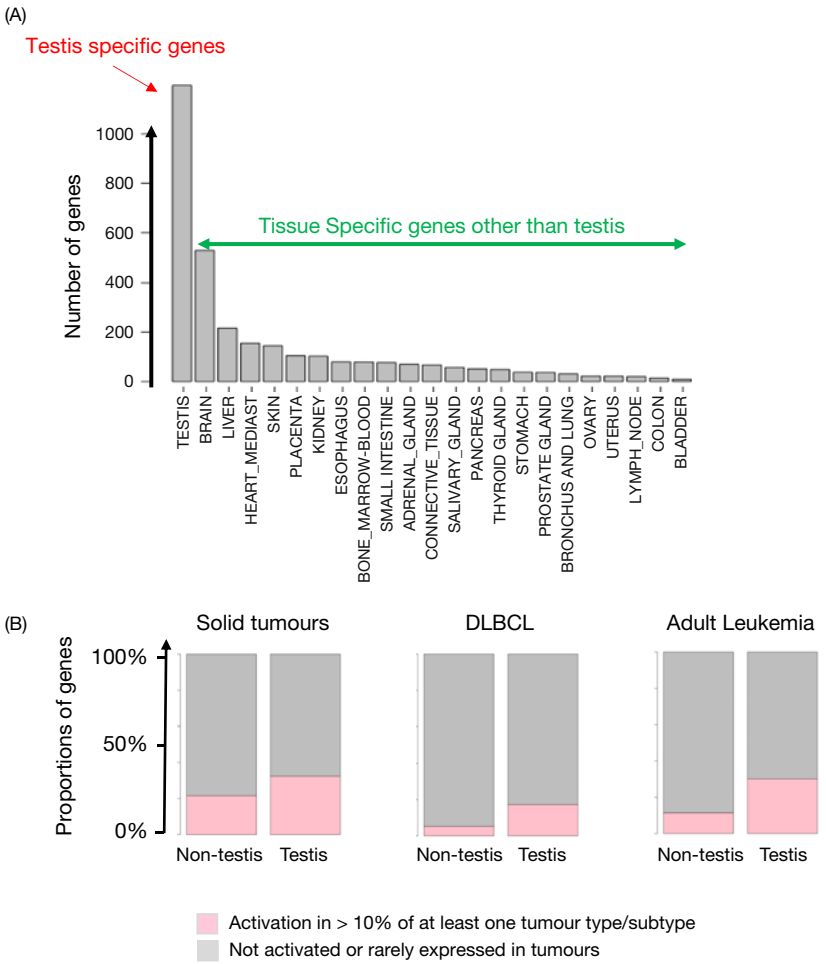

Fig. 1 (A) Numbers of genes with a tissue restricted expression as a function of the tissue of expression (based on RNAseq data, E-MTAB-1733). A gene was considered tissue specific if the corresponding normalized value of read counts (FPKM: Fragments Per Kilobase Million) was above a threshold defined by the mean value of read counts in all tissues +10 standard deviations (std) in this particular tissue. Of the 3823 identified tissue restricted genes, 1597 were testis-specific. (B) Respective proportions of tissue restricted genes, of testis or non-testis origin, which are activated in at least 10% of the cases of at least one of the cancer types/subtypes according to the transcriptomic data (obtained with the Affymetrix array Human Genome U133 Plus 2.0) from three series of malignancies, including a large series of solid tumors (multicancer study, GSE2109), Diffuse Large B-cell Lymphomas (DLBCL, GSE10846) and adult Leukemia (GSE13159). After matching with updated gene identifiers, 3270 genes out of the 3823 tissue-restricted genes identified in A were found represented on the array. For each gene the threshold of activation corresponds to the mean value +3 std. of the signal obtained on the same array in a series of normal tissues (GSE3526), following a protocol published by Rousseaux and collaborators.

Therefore the question arises whether the observed predominancy of C/T genes among genes ectopically activated in cancers merely reflects the larger number of testis-restricted genes as compared to the numbers of silenced genes of other tissues of origin. In other words, a higher probability of silent gene reactivation due to a larger number rather than to a specific biology could explain the high frequency of C/T gene activation.

To answer this important question the respective numbers of testis-specific genes or of tissue restricted genes of other origin activated in cancers were calculated and compared. The observation of a higher proportion of testis-specific genes in cancer would argue for a biased activation of male germ cell genes in cancer. Taking into account the rather low number of tissue-specific genes compared to testis-specific genes, we pooled all non-germline tissue-specific genes and compared the proportion of these that are frequently activated in various cancer types with the proportion of testis-specific genes expressed with similar frequencies in the same tumor types.

Interestingly, Fig. 1B shows that cancer cells clearly preferentially activate testis-specific genes compared to all other tissue-restricted genes, irrespectively of the studied cancer sample series of solid tumors or hematological malignancies.

This observation has important biological significance. It may indicate that the mechanisms that are used to repress testis-specific genes are more sensitive to deregulation in cancer compared to the other tissue-restricted silenced genes. It may also indicate that the activation of male germ cell-specific genes could selectively contribute or stimulate malignant transformation and cancer

progression. Although there is not much evidence for a particular sensitivity of testis-specific genes to desilencing in cancer, an important body of data is now accumulating that clearly demonstrates the oncogenic activities of testis-specific genes (see below).

Revisiting the Notion of CT-X

Considering the established list of C/T genes, several authors noticed an important enrichment of these genes on the X chromosome. These genes named CT-X genes attracted attention and their preferential localization on the X chromosome fired many discussions and speculations as presented in a timely review by Simpson and colleagues (Fig. 2, left panel).

The new data showing that C/T genes are actually only part of a larger number of genes with a testis-specific pattern of expression also invites us to reconsider the notion of CT-X genes. Indeed the list of CT-X genes was based on the list of established C/T genes, which, as discussed above, is incomplete.

In order to evaluate the possibility of a chromosome-biased localization of the identified C/T genes, we used the list of C/T genes that we found active in tumors (identified in Fig. 1A and B) and considered their distribution on chromosomes.

Fig. 2 (right panel) shows that the updated list of deregulated C/T genes actually does not show any significant enrichment on the X chromosome. This figure also indicates that, at the chromosome scale, there are no specific genomic regions which seem more prone to ectopic gene activation in cancer, taking into account differences between chromosomes in gene densities.

These data demonstrate that a localisation on a specific chromosome does not account for ectopic gene activation and suggest that the mechanisms involved could hit various regions of the genome. It is therefore important to revise the discussions concerning the preferential activation of C/T-X genes in cancer.

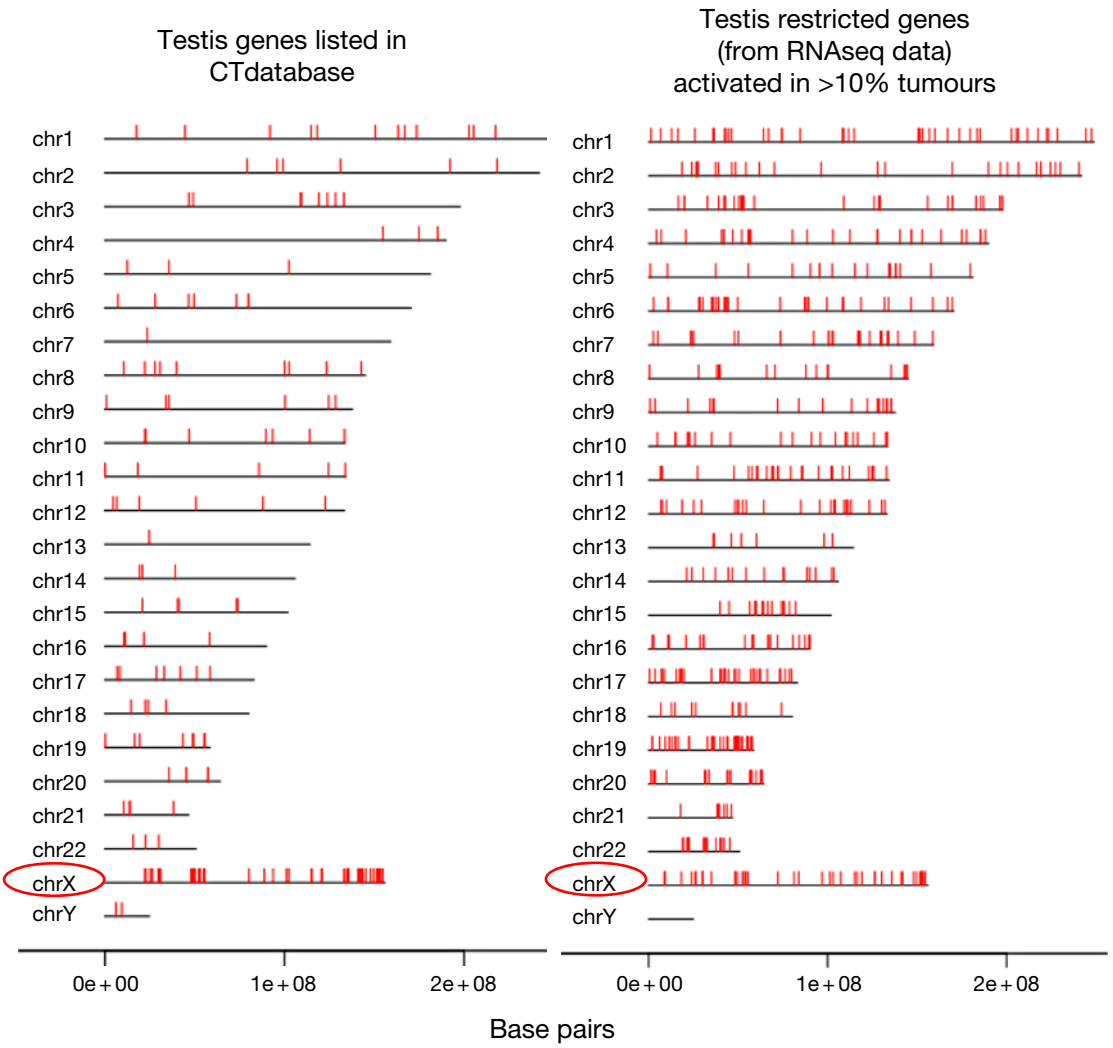

Fig. 2 Distribution of testis-restricted genes on chromosomes (using Stoda R Package, Ycart et al. personal communication) comparing the positions of C/T genes listed in the CT database (*left panel*) and of the testis-restricted genes selected using RNAseq data as explained in Fig. 1A and activated in at least 10% of tumor types/subtypes as explained in Fig. 1B (*right panel*).

Cancer Type-Biased Activation of C/T and Tissue-Restricted Genes

Another question is the relationship between cancer types and the ectopic gene activation. Do all cancer types activate tissue- or testis-specific genes to the same extent? To this end we calculated the respective proportions of all genes with a restricted expression in testis or other tissue (identified in Fig. 1A), that are frequently activated in various solid tumors types and hematological malignancies subtypes. A gene was considered "frequently" activated when it was aberrantly expressed in 10% or more of the samples of each tumor type/subtype.

Fig. 3 confirms that all solid tumors types and hematological malignancies activate tissue- and testis- specific genes. In addition it shows that various types of solid tumors and hematological malignancies of different origins have similar proportions of aberrantly reactivated genes.

However, it is important to note that an individual C/T gene may become active in a tumor-dependent manner, as recently shown by Bourova-Flin and colleagues. In fact, *BRDT*, a particular testis-specific gene, was recently shown to be more frequently activated in lung cancers compared to other solid tumors. Additionally, within lung cancers, this gene is preferentially activated in adenocarcinomas whereas its aberrant activation is not observed in carcinoid tumors or in small cell carcinomas. These observations suggest that the biology of the tissue-of-origin of cancers that leads to the activation of tissue-specific genes could somehow condition the ability of cells to particularly activate a given set of C/T or tissue-specific genes. An interesting issue to address and explore in future research remains the impact of the tissue-of-origin in gene desilencing.

C/T Gene Expression and Immunotherapy

The discovery of C/T genes relied on melanoma patients immune response to the expression of a series of testis-specific genes. Indeed, C/Ts are thought to be normally expressed in the immune isolated environment of the seminiferous epithelium, which is protected against immune cells by the so-called blood-testis barrier. Additionally, spermatogenic cells are unable to present their proteins to the immune cells because they lack the MHC class I molecules. Finally, the interstitial epithelium is unfavorable to the survival and growth of immune cells. All these characteristics make testis an immune-privileged site and consequently proteins specifically expressed in spermatogenic cells should remain unknown to the immune system. However, the situation is more

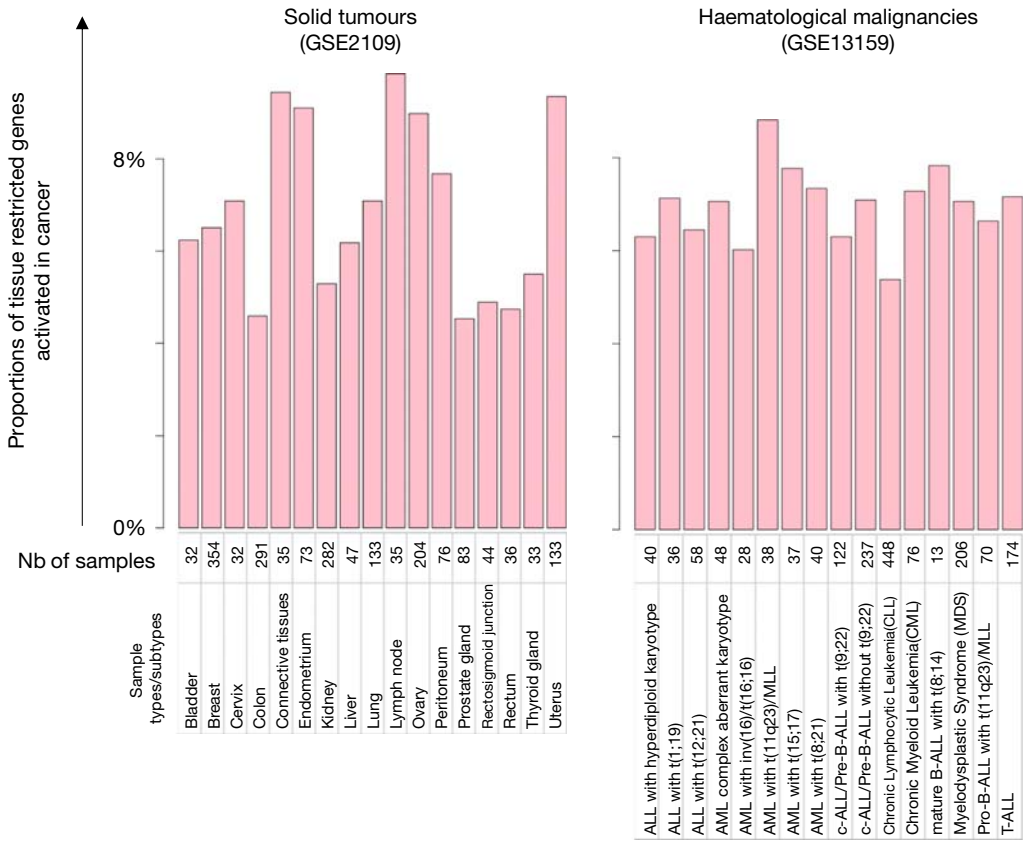

Fig. 3 Proportions of tissue-restricted genes expressed in at least 10% of tumor samples considering each of the tumor types or subtypes in solid tumors (*left panel*) or in hematological malignancies (*right panel*).

complex, since the thymus appears to express a large number of tissue-specific antigens due to the expression of a particular transcription factor, named autoimmune regulator (AIRE), which turns on a variety of normally silent genes in the thymic epithelial cells, exposing the immune cells to the corresponding antigens. The question arises whether C/T antigens are really unknown to the immune system and whether they can be considered as privileged targets for immunotherapy. However, the recent advent of Chimeric-Antigen Receptor T (CAR-T) cell therapy brings the interest in the products of C/T genes at the forefront of the collective efforts to define new antigens as targets of cancer immunotherapy.

Unbiased Discovery of Ectopic Gene Activation in Cancer

The early identification of tumor antigens as testis-specific genes aberrantly activated in cancers, prompted investigators to develop various approaches to identify ectopically activated genes in cancer, which were all biased towards the identification of testis-specific genes. It is interesting to note that unbiased approaches were also considered to identify ectopically active genes in cancer and to define their normal tissue-of-origin.

An example is the use of proteomic data from a well-characterized diffuse large B-cell lymphoma (DLBCL) cell line, B593, by Emadali and colleagues to pinpoint ectopically expressed proteins and evaluate their values as a biomarker and as an oncogenic driver.

In this study, to gain sensitivity and deepness in sequencing for a better protein identification, the nuclear acid soluble (NAS) protein fraction was considered. NAS proteins are basic proteins and hence have the potential to interact with the negatively charged nucleic acids, DNA and RNA. Mass spectrometry sequencing of these proteins and their identification proved that the majority of these proteins, 66%, are annotated as DNA-binding, i. e., histones or RNA binding proteins, including a number of ribosomal proteins. An analysis pipeline was established to identify, among these proteins, those that are in fact the result of ectopic gene activation. As part of this pipeline, the data from a published lymphoma transcriptomic study with survival data was recovered and analyzed to identify, within the out-of-context expressed proteins, the encoding genes whose expression is associated with poor prognosis (shorter survival probability). This approach identified a protein that is highly expressed in Diffuse Large B-cells Lymphomas (DLBCL), known as CYCLON, whose encoding gene expression is associated with poor prognosis in lymphoma patients. The consideration of the tissue-of-origin of CYCLON gene expression showed that the encoding gene is, in fact, a testis-specific gene that is aberrantly active in lymphoma.

The identification of a testis-specific gene following an unbiased approach as an oncogenic ectopically activated gene in lymphoma brings an additional argument supporting a preferential activation of C/T genes in cancer and their contribution to oncogenesis.

Ectopically Activated Testis-Restricted Genes Are Robust Drivers of Cancer

The analysis shown in Fig. 1B, demonstrates that tumor cells preferentially activate testis-specific genes. A reason for this observation could be that, compared to other tissue-restricted genes, male germ cell-specific genes more efficiently contribute to oncogenesis. In fact this hypothesis is now strongly supported by many experimental data showing a direct oncogenic role for C/T gene products.

An elegant study using the induced tumorogenesis in Drosophila reported by Janic and colleagues clearly demonstrated the determinant contribution of ectopically activated germline genes in cancer development. Indeed, the inactivation of a Drosophila tumor suppressor gene leading to brain tumor development was accompanied by the activation of germ cell-specific genes that corresponded to almost 25% of the significantly activated genes. This study also showed that these activations did not occur only as by-products of malignant transformation and that the products of these genes were directly involved in tumorogenesis.

In another global approach that aimed at testing the contribution of CT genes in oncogenesis, Whitehurst and colleagues first identified cancer cell lines corresponding to six different cancer types that significantly expressed a chosen list of 135 C/T genes. Most of the considered C/T genes were expressed in at least two of the eleven selected cell lines and 20% of the C/T genes were expressed in all cell lines.

Systematic knock-down of these CT genes identified 26 genes that were essential for cell viability or were involved in various oncogenic mechanisms.

All published data on the cell transforming role of C/Ts, in addition to the two comprehensive studies described above, definitively support the wide-spread involvement of the ectopic activation of male germ cell-specific genes in oncogenesis. It is therefore possible now to confirm that C/T gene activation is part of the malignant cell genome reprogramming process and this conclusion should prompt investigators to set up research programs in order to decipher the underlying oncogenic mechanisms.

C/T Gene Activation: A Homogenous Ground over Cancer Heterogeneity

A systematic evaluation of the association of ectopic tissue-restricted gene activation with prognosis in lung cancer and in acute lymphoblastic leukemia (ALL) conducted by Wang and Rousseaux and colleagues led to the discovery of specific gene expression signatures that biologically identify lung cancer and leukemic cells respectively which, despite their apparent heterogeneous nature,

share some fundamental and homogeneous properties closely associated with their aggressiveness. This observation suggests that the aberrant activation of some of the tissue-restricted genes, although apparently heterogenous, is associated with the profound nature of cancer cells and is not merely a manifestation of malignant transformation.

The approach carried out to investigate the biological/molecular profile of tumor aggressiveness is based on a supervised comparison of the whole transcriptomes between tumors classified as aggressive according to their ectopic gene expression signature and other tumors. Remarkably, this strategy identifies thousands of genes whose expression differs between the two compared tumor populations, highlighting a specific biology associated with the aggressive tumors ectopically expressing a specific set of C/T genes. For instance, aggressive lung tumors identified according to the ectopic expression of genes of a subset of 26 classifying testis- or placenta- restricted genes, also upregulate genes whose products are nuclear and associated with cell proliferation while down-regulating other genes that are responsible for the immune response.

In ALL cells, the identity of some of the genes up- and down- regulated in aggressive forms of leukemia associated with the ectopic expression of genes of a specific tissue-restricted genes signature supports the hypothesis that the nature of the corresponding malignant cells is close to that of quiescent stem cells.

A major issue in cancer biology is the notoriously heterogenous nature of tumors. The investigations discussed above could represent a way to apprehend common shared properties between tumors beyond their obvious heterogenous nature. In fact one could propose that tumor heterogeneity is fueled by a common and relatively homogenous nature of malignant cells and that the expression of some of the C/T genes could be associated with or indicative of this ground nature of tumor cells.

Prospective Vision

The function of most the C/T genes, including those within the prognostic classifying gene signatures, remains unknown and functional investigations of these factors should open new doors to a better understanding of yet unexplored oncogenic mechanisms. Additionally, a knowledge of the activity of the encoded factors at the molecular level would set a basis for new therapeutic approaches that would directly target the nature of malignant cells rather than the consequences of the oncogenic transformation such as cell proliferation.

In addition to their use for prognosis purposes, cancer-specific ectopic gene activations constitute a repertoire of excellent biomarkers which could be primarily used for diagnosis and follow-up of cancer through non-invasive approaches. Indeed, since under normal conditions the products of these genes should be mostly unknown to the immune system, serological approaches could be used to detect their activation and hence to develop simple and efficient diagnostic tests. Finally, the immunogenic nature of these gene products also makes them excellent candidate targets for new immunotherapy approaches, which hence could constitute the basis for future developments of anticancer therapy or anti-cancer vaccine approaches.

See also: DNA Methylation Changes in Cancer: Mechanisms.

Further Reading

Bourova-Flin, E., Chuffart, F., Rousseaux, S., Khochbin, S., 2017. The role of Bromodomain testis-specific factor, BRDT, in cancer: A biomarker and a possible therapeutic target. Cell Journal 19, 1–8.

Emadali, A., Rousseaux, S., Bruder-Costa, J., Rome, C., Duley, S., Hamaidia, S., Betton, P., Debernardi, A., Leroux, D., Bernay, B., Kieffer-Jaquinod, S., Combes, F., Ferri, E., McKenna, C.E., Petosa, C., Bruley, C., Garin, J., Ferro, M., Gressin, R., Callanan, M.B., Khochbin, S., 2013. Identification of a novel BET bromodomain inhibitor-sensitive, gene regulatory circuit that controls rituximab response and tumor growth in aggressive lymphoid cancers. EMBO Molecular Medicine 5, 1180–1195.

Feichtinger, J., Aldeailej, I., Anderson, R., Almutairi, M., Almatrafi, A., Alsiwiehri, N., Griffiths, K., Stuart, N., Wakeman, J.A., Larcombe, L., McFarlane, R.J., 2012. Meta-analysis of clinical data using human meiotic genes identifies a novel cohort of highly restricted cancer-specific marker genes. Oncotarget 3, 843–853.

Hofmann, O., Caballero, O.L., Stevenson, B.J., Chen, Y.-T., Cohen, T., Chua, R., Maher, C.A., Panji, S., Schaefer, U., Kruger, A., Lehvaslaiho, M., Carninci, P., Hayashizaki, Y., Jongeneel, C.V., Simpson, A.J.G., Old, L.J., Hide, W., 2008. Genome-wide analysis of cancer/testis gene expression. Proceedings of the National Academy of Sciences of the United States of America 105, 20422–20427.

Janic, A., Mendizabal, L., Llamazares, S., Rossell, D., Gonzalez, C., 2010. Ectopic expression of germline genes drives malignant brain tumor growth in drosophila. Science (New York, N.Y.) 330, 1824–1827.

Mathieu, J., Ruohola-Baker, H., 2017. Metabolic remodeling during the loss and acquisition of pluripotency. Development (Cambridge, England) 144, 541–551.

Maxfield, K.E., Taus, P.J., Corcoran, K., Wooten, J., Macion, J., Zhou, Y., Borromeo, M., Kollipara, R.K., Yan, J., Xie, Y., Xie, X.-J., Whitehurst, A.W., 2015. Comprehensive functional characterization of cancer-testis antigens defines obligate participation in multiple hallmarks of cancer. Nature Communications 6, 8840.

Rousseaux, S., Debernardi, A., Jacquiau, B., Vitte, A.-L., Vesin, A., Nagy-Mignotte, H., Moro-Sibilot, D., Brichon, P.-Y., Lantuejoul, S., Hainaut, P., Laffaire, J., de Reyniès, A., Beer, D.G., Timsit, J.-F., Brambilla, C., Brambilla, E., Khochbin, S., 2013. Ectopic activation of germline and placental genes identifies aggressive metastasis-prone lung cancers. Science Translational Medicine 5, 186ra66.

Rousseaux, S., Wang, J., Khochbin, S., 2013. Cancer hallmarks sustained by ectopic activations of placenta/male germline genes. Cell Cycle (Georgetown, TX) 12, 2331–2332.

Salmaninejad, A., Zamani, M.R., Pourvahedi, M., Golchehre, Z., Hosseini Bereshneh, A., Rezaei, N., 2016. Cancer/testis antigens: Expression, regulation, tumor invasion, and use in immunotherapy of cancers. Immunological Investigations 45, 619–640.

Sammut, S.J., Feichtinger, J., Stuart, N., Wakeman, J.A., Larcombe, L., McFarlane, R.J., 2014. A novel cohort of cancer-testis biomarker genes revealed through meta-analysis of clinical data sets. Oncoscience 1, 349–359.

Simpson, A.J.G., Caballero, O.L., Jungbluth, A., Chen, Y.-T., Old, L.J., 2005. Cancer/testis antigens, gametogenesis and cancer. Nature Reviews. Cancer 5, 615–625.

Wang, J., Mi, J.-Q., Debernardi, A., Vitte, A.-L., Emadali, A., Meyer, J.A., Charmpi, K., Ycart, B., Callanan, M.B., Carroll, W.L., Khochbin, S., Rousseaux, S., 2015. A six gene expression signature defines aggressive subtypes and predicts outcome in childhood and adult acute lymphoblastic leukemia. Oncotarget 6, 16527–16542.
Wang, J., Rousseaux, S., Khochbin, S., 2014. Sustaining cancer through addictive ectopic gene activation. Current Opinion in Oncology 26, 73–77.
Whitehurst, A.W., 2014. Cause and consequence of cancer/testis antigen activation in cancer. Annual Review of Pharmacology and Toxicology 54, 251–272.

Relevant Websites

http://www.cta.lncc.br—C/T gene database.

Uterine Cervix Cancer: Diagnosis and Treatment

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Abbreviations

5-FU 5-Fluorouracil
CIN Cervical intraepithelial neoplasia
EBRT External beam radiation therapy
ECC Endocervical curettage
FDA US Food and Drug Administration
FIGO International Federation of Gynecology and Obstetrics
HPV Human papilloma virus
LEEP Loop electrosurgical excision procedure
LVSI Lymphovascular space invasion
MRI Magnetic resonance imaging
NCCN US National Comprehensive Cancer Network
PET/CT Positron emission tomography with computer tomography
RT Radiotherapy
SCC Squamous cell carcinoma
SNL Sentinel lymph node
SNLD Sentinel lymph node dissection

Definition

The most common type of uterine cervix cancer is squamous cell carcinoma (SCC; about 80% of cases), followed by adenocarcinoma (20%). This entry does not cover other, rare, cervical cancer types.

Presentation and Diagnosis

Precancerous lesions of the uterine cervix are asymptomatic and are usually detected by routine cytological screening and colposcopy. The earliest stages of cervical carcinoma are frequently asymptomatic as well. They may also be associated with a watery vaginal discharge and postcoital bleeding or intermittent spotting. These symptoms are frequently not recognized by the patient. In more advanced stages, vaginal discomfort, pelvic pain, and/or dyspareunia may also appear. The lateral growth of the tumor into the parametrium may cause ureteral obstruction leading to anuria and uremia. Pelvic side wall involvement may manifest as sciatic pain and, less commonly, lymphedema of the lower extremities.

Palpation can detect induration or nodularity of the cervix or of the parametria in more advanced lesions. Tumor gross appearance is variable. Some early cancers are not easily detected and even deeply invasive tumors may be somewhat deceptive on gross examination. Visual inspection of the cervix can reveal gray, discolored areas as well as visible bleeding and/or evidence of cervicitis. The tumor may appear as a red, friable, exophytic or ulcerated lesion.

As the uterine cervix is easily accessible, accurate diagnosis can usually be made based on a cytological examination (Papanicolaou (Pap) smear) and a cervical biopsy. However, cytological screening methods are less useful for detecting adenocarcinoma as adenocarcinoma in situ develops in areas that are less accessible (like upper parts of the endocervical canal) and so more difficult to sample. Moreover, the accuracy of the Pap smear test may be questionable as it depends on a subjective morphological evaluation of the sample and a high proportion of inadequate specimens has been reported. Since cervical cancer frequently develops in association with a human papilloma virus (HPV) infection and HPV DNA can be found in nearly all cases of cervical cancer, testing for high-risk HPV DNA is now widely advocated as a mean to improve screening sensitivity.

Abnormal cervical cytology and/or a positive high-risk HPV test should lead to colposcopy and biopsy or excisional procedures, such as loop electrosurgical excision or conization. Biopsy specimens of all visibly abnormal areas should be taken, regardless of the findings on the Pap smear.

Endocervical curettage (ECC) is required when the Pap smear shows a high-grade lesion but colposcopy does not reveal a lesion, when the entire squamocolumnar junction cannot be visualized, when atypical endocervical cells are present on the Pap smear, or when women previously treated for cervical intraepithelial neoplasia (CIN) show new high-grade findings on cytology. If the ECC reveals a high grade squamous intraepithelial lesion, patients should undergo cervical conization or

a loop electrosurgical excision procedure (LEEP). Diagnostic conization (cone biopsy) may also be indicated if adenocarcinoma in situ is suspected from cytology, if standard cervical biopsy is not sufficient to define invasiveness, or if accurate assessment of microinvasive disease is required.

Management and Therapy

Risk assessment is based on tumor size, stage, depth of tumor invasion, lymph node status, and lymphovascular space invasion (LVSI). Lymph node status and number of lymph nodes involved are the most important prognostic factors. There are conflicting reports on whether the tumor histological type has some prognostic value. However, a majority of studies suggest that adenocarcinoma is associated with a worse prognosis than SCC.

High-grade precancerous lesions (CIN) can often be treated with cryotherapy, laser ablation, or other ablative procedures. Conization with a scalpel is preferred for lesions which are not entirely visible on colposcopy or in case adenocarcinoma in situ is suspected.

The management of cervical cancer usually involves some kind of surgery, although definitive radiotherapy (RT) may be an option in early-stage disease. The kind of surgical procedure applied is strictly correlated with disease stage as defined by the TNM and the International Federation of Gynecology and Obstetrics (FIGO) classification. Conservative fertility-sparing approaches may be considered in highly selected patients who have been thoroughly counseled on the disease risks and also on related prenatal and perinatal issues.

Adenocarcinoma in situ can be successfully treated with loop excision and a close cytological follow-up in most cases. Microinvasive cancer without LVSI can be managed with conization or simple trachelectomy to preserve fertility. A conservative approach may also be an option for stage IA patients with evidence of LVSI, in which case conization with sentinel lymph node (SLN) mapping algorithm or pelvic lymphadenectomy is performed. The aim of conization is the *en bloc* removal of the ectocervix and the endocervical canal. The expert panel of the US National Comprehensive Cancer Network (NCCN) recommends cold knife (scalpel) conization as the preferred approach. If properly performed, loop electrosurgical excision procedure (LEEP) is also acceptable. LEEP is often recommended for excising high-grade precancerous lesions. Vaginal or abdominal radical trachelectomy may be a fertility-sparring option for selected patients with stage IA-II disease, with the exception of the minimal deviation adenocarcinoma whose aggressive nature makes it inappropriate for conservative treatment. Radical trachelectomy consists in removing the cervix, vaginal margins, and supporting ligaments, while preserving the main body and fundus of the uterus, with simultaneous laparoscopic pelvic lymphadenectomy.

If the patient does not wish to preserve fertility or is not eligible for fertility-sparring treatment, hysterectomy is usually performed, either simple (extrafascial), modified radical, or radical. Radical hysterectomy with extensive parametrial resection and bilateral lymph node dissection is standard treatment, with minimally invasive approaches (laparotomy or laparoscopy) becoming increasingly common. As some studies suggest the para-aortic lymph node involvement is closely related to the presence of metastases to the pelvic and iliac lymph nodes in patients with stage I or II disease, para-aortic lymph node sampling during pelvic lymph node dissection may also be performed. Radical hysterectomy is preferred to simple hysterectomy due to its wide paracervix resection margin. However, some studies suggest that radical hysterectomy may be an overtreatment in patients with small localized tumors and that radiotherapy (RT) may be equally effective. In the US, chemoradiation is typically preferred over radical surgery for selected patients with bulky FIGO stage IB2-IIA2 tumors. In European countries, radical hysterectomy with or without prior neoadjuvant chemotherapy is a frequent choice.

The clinical benefit of sentinel lymph node dissection (SNLD) in cervical cancer is not perfectly established and no international consensus exists. However, recent data suggest that SNL biopsy may be useful in decreasing the need for pelvic lymphadenectomy in early-stage cervical cancer patients and most guidelines suggest at least considering SNLD in patients with lower stage (FIGO I, tumors no bigger than 4 cm) disease. The NCCN panel recommends considering a SNL mapping algorithm for patients with tumors smaller than 2 cm.

Patients who are not eligible for hysterectomy or who refuse surgery, may be treated with chemoradiation as a definitive therapy. In this case, external beam RT (EBRT) with brachytherapy is usually performed concurrent with the administration of a cisplatin-containing chemotherapeutic regimen, most commonly cisplatin alone or cisplatin with 5-fluorouracil (5-FU). Cisplatin may be replaced by carboplatin or gemcitabine in patients with renal dysfunction. The concept of delivering chemotherapy before RT (neoadjuvant or induction chemotherapy) has also been explored, however with conflicting results. Pelvic RT and chemoradiation invariably lead to ovarian failure in premenopausal women. In order to preserve intrinsic hormonal function in younger women, ovarian transposition prior to RT may be considered.

Performing completion surgery (adjuvant hysterectomy) following primary chemoradiation in patients with lower-stage disease (I–II) is controversial. Adjuvant hysterectomy after RT has been shown to improve pelvic control but not overall survival. It is also associated with increased morbidity. However, this approach may be considered in case the disease extent or uterine anatomy precludes adequate coverage by brachytherapy.

Surgery with concurrent chemoradiation is usually the primary treatment of choice for patients with advanced disease. If surgery is not possible, chemoradiation remains the primary treatment. Of note, the meaning of "advanced disease" is not clearly

defined. In particular stage IB2 and IIA2 disease may be classified as early-stage or advanced-stage disease, depending on the oncologist and the center. Radiological imaging, including positron emission tomography with computer tomography (PET/CT) and often pelvic magnetic resonance imaging (MRI) are essential for defining disease local extent and for adequate RT planning. The treatment consists of EBRT with concurrent cisplatin-based chemotherapy (usually cisplatin alone or cisplatin with 5-FU) and brachytherapy.

Women with a high risk of recurrence should receive adjuvant therapy following hysterectomy. The exact criteria for selecting high-risk patients slightly differ between different guidelines. Overall, women with early-stage disease, small primary tumors, negative margins, negative parametria and no lymph node involvement are usually considered to be at low risk of recurrence, whereas higher-stage disease, large primary tumor, parametrial involvement, positive lymph nodes, deep stromal invasion and/or LVSI qualify for post-surgical adjuvant treatment. The usual choice is chemoradiation consisting of EBRT with concurrent cisplatin-based chemotherapeutic regimen. Additional vaginal brachytherapy may be useful in patients with positive vaginal resection margins. Adjuvant therapy is also indicated for patients with cervical cancer that was found incidentally at hysterectomy performed for other reasons. However, no consensus guidelines so as to optimal regimens for these patients exist.

Recurrent cancer is usually manifested by pelvic pain, particularly in the sciatic nerve distribution, vaginal bleeding, malodorous discharge, or leg edema. Recurrence must be confirmed by a pathological evaluation of a biopsy specimen because these symptoms and even physical findings can be similar to those associated with radiation changes. Patients with a localized recurrence after initial treatment may be candidates for radical re-treatment, that is RT and/or chemotherapy, or surgery, depending on the recurrence localization, extent, and previous treatments. Lower vaginal recurrence can occasionally be cured by RT or exenteration. Pelvic exenteration may also be considered for central pelvic recurrent disease after primary RT when the spread is confined to the vagina, bladder, or rectum. RT is usually not offered if the patient develops recurrence following previous RT, in particular if it appears within a previously irradiated field. Typical chemoradiation regimen for the treatment of recurrence includes cisplatin or cisplatin/5-FU and EBRT, with or without brachytherapy. In patients who have relapsed soon after completing initial chemoradiation with this regimen, alternative chemotherapeutics, such as carboplatin, paclitaxel, or gemcitabine, may be considered.

Distant metastatic disease, whether at initial presentation or following treatment of the primary tumor, is rarely curable. However, occasional log-term survival has been reported for patients with isolated distant metastases amendable to local treatment with surgical resection or local ablative therapies with or without EBRT, and EBRT alone or with concurrent chemotherapy. Still, for most metastatic patients the treatment is palliative. It involves either chemoradiation, or chemotherapy alone.

Cisplatin is considered to be most effective in treatment of metastatic cervical cancer. However, most patients with metastases had been treated with concurrent cisplatin RT and may have become insensitive to single-agent platinum therapy. Therefore, a number of cisplatin combinations have been tested. Cisplatin-based doublets with paclitaxel and topotecan have been shown to give better outcomes than cisplatin monotherapy. The cisplatin/topotecan regimen has been approved by the US Food and Drug Administration (FDA) for treatment of advanced cervical cancer. However, this regimen may be more toxic than the two other cisplatin-based doublets. The addition of bevacizumab, an angiogenesis inhibitor, to the cisplatin-based doublets may improve overall survival. However, bevacizumab is associated with higher toxicities and may lead to, among others, hypertension and an increased risk of venous thromboembolic events, which is why the overall quality of life of patients is not necessarily improved with this treatment regimen. For patients who are not candidates for cisplatin-based therapy, carboplatin with paclitaxel may be a reasonable option. For those who may not be treated with a taxane, cisplatin with topotecan or with gemcitabine may be considered. A number of single-agent chemotherapeutic regimens is used for palliative treatment of recurrent and/or metastatic cervical cancer. The NCCN expert panel recommends cisplatin, carboplatin, and paclitaxel as the first-line treatment to this effect, whereas bevacizumab, docetaxel, 5-FU, gemcitabine ifosfamide, irinotecan, mitomycin, albumin-bound paclitaxel (nab-paclitaxel), topotecan, permetrexed, and vinorelbine may be considered as second-line choices.

Cervical cancer survivors are at risk of second cancers. Moreover, patients who had undergone RT for pelvic cancers are at an increased risk of cancers at irradiated sites (e.g., colon or urinary bladder). Therefore, appropriate surveillance not only for cervical cancer recurrence/metastasis but also for primary cancers at other sites is of utmost importance. Of note, abnormal Pap smears on follow-up may represent post-irradiation dysplastic changes and not a new primary cancer. Therefore, suspected areas should be diagnosed by biopsy.

Compared to other cancers, personalized medicine for cervical cancer patients seems a rather far perspective, with a limited number of targeted molecular therapeutics that are currently in clinical development. A combination of a MEK inhibitor (trametinib) and an Akt inhibitor (GSK2141795) for treatment of recurrent and/or persistent cervical cancer is being investigated in a phase II trial. Another phase II trial tests apatinib, a tyrosine kinase inhibitor, for treatment of recurrent and metastatic cervical cancer in a sequential regimen with standard chemotherapy (phase II).

See also: Uterine Cervix Cancer: Pathology and Genetics.

Further Reading

Chmielowski, B., Territo, M., 2017. Manual of clinical oncology, 8th edn. Wolters Kluwer, Philadelphia.
Kurman, R.J., Carcangiu, M.L., Herrington, C.S., Young, R.J. (Eds.), 2014. WHO Classification of tumors of female reproductive organs, 4th edn. International Agency for Research on Cancer, Lyon, France.
Marth, C., et al., 2017. Cervical cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Annals of Oncology 28 (Suppl 4), iv72–iv83.
National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Cervical Cancer. Version 1.2018 – October 25, 2017: https://www.nccn.org/professionals/physician_gls/default.aspx#site

Relevant Website

https://www.figo.org/—International Federation of Gynecology and Obstetrics (FIGO).

Uterine Cervix Cancer: Pathology and Genetics

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Glossary

Carcinoma A malignant tumor of epithelium.
Genome The complete set of genetic material present in a cell or organism.
Grade The degree to which a tumor resembles its putative tissue of origin.
Integration The insertion of DNA (usually viral) into the chromosomes of a human cell.
Stage The extent to which a tumor has spread.

Carcinoma of the Cervix

Burden

Cervical cancer is relatively uncommon in developed nations. For example, cervical cancer was the 13th most common cancer in women in the United Kingdom in 2014, with 3224 new cases; and the 17th commonest cause of cancer-related death, with 890 deaths. Cervical cancer mortality has fallen by 72% since the early 1970s. However, worldwide, cervical cancer was the 6th commonest cancer and the 8th commonest cause of cancer death in women in 2012, with approximately 520,000 new cases and 265,000 deaths. A number of factors underly these differences, including the presence of a quality-controlled cervical screening program in many developed nations. HPV vaccination is also likely to impact the incidence and mortality statistics as implementation varies across the world, being much less in resource-poor settings.

Risk Factors and Population Health

The major risk factor for cervical cancer is high-risk HPV infection, particularly infection with either HPV type 16 or HPV type 18. The strength of association between HPV infection and cervical carcinoma is extremely high (relative risk/odds ratio >100), surpassing that for smoking and lung cancer; and hepatitis B infection and liver cancer. A significant secondary risk factor is smoking. This has prompted consideration of the use of HPV testing in cervical screening, both for follow-up of patients after treatment, and as the primary screening modality, in place of cytology. Thus, rather than using HPV testing to triage women with cytologically abnormal cervical smears, HPV testing will soon be implemented in some countries as the primary screening test, with cytology performed only on those samples that are positive for high-risk HPV. HPV vaccination has been offered in the United Kingdom since 2008 and has been associated with a reduction in the incidence of preinvasive disease. It is likely that this will translate over time into a reduction the incidence of invasive disease.

Table 1 Classification of common cervical carcinomas and their precursors[a]

Carcinoma	*Precursor lesion*
Associated with HPV infection	
Squamous cell carcinoma	CIN/SIL
Adenocarcinoma, usual type	CGIN/AIS
High grade neuroendocrine carcinoma (small and large cell types)	Often CGIN/AIS
Not associated with HPV infection	
Gastric-type adenocarcinoma	Lobular endocervical glandular hyperplasia
Clear cell carcinoma	Unknown
Mesonephric carcinoma	Mesonephric duct remnants

[a]Classification is according to association with HPV infection. Only the common tumor types are shown. See text for explanation of abbreviations.

Classification and Pathology (See Table 1)

Precursor lesions

There are well-defined precursors to invasive cervical carcinoma and cervical screening programs are designed to detect and treat these precursor lesions, particularly squamous precursors, in order to prevent mortality from invasive disease.

Squamous cell carcinoma is preceded by non-invasive abnormality of the squamous epithelium, the terminology of which has been debated over many years. In the 1960s, a three-tier system using the term cervical intraepithelial neoplasia (CIN) was introduced to reflect the then preferred concept of a continuum of abnormality, which could be subdivided into grades that correlated with progression risk. Improved understanding of the biology of HPV infection, however, has led more recently to the adoption by many of a two-tier system based on the concept that low-grade abnormalities (termed low-grade squamous intraepithelial lesions (SIL)) represent productive HPV infections, whereas high-grade abnormalities (high-grade SIL) represent transforming HPV infections with a much greater potential to progress to invasive disease. In North America this two-tier system is used both for cytology (smears) and for histopathology (biopsies), whereas in the United Kingdom it is currently used only for cytology, with the three-tier CIN system still used for histopathology.

There is also an HPV-associated precursor lesion for most cervical adenocarcinomas, termed adenocarcinoma in situ (AIS) in North America and cervical glandular intraepithelial neoplasia (CGIN) in the United Kingdom. AIS is not graded. CGIN is subdivided into low and high-grade categories although there is debate about the existence of a reproducible and clinically meaningful category of low-grade CGIN. The current WHO recommendation is that only p16 positive CGIN lesions are considered significant (see below and the WHO classification given in section "Further Reading").

Rarer precursor lesions are associated with the less common types of cervical carcinoma. Gastric type adenocarcinomas are associated with lobular endocervical gland hyperplasia (thought to represent gastric foveolar metaplasia) and its atypical form; and mesonephric carcinomas are often associated with mesonephric duct remnants, with or without mesonephric duct hyperplasia. These precursors are not associated with HPV infection.

Invasive carcinoma

Squamous cell carcinoma

Squamous cell carcinoma (Fig. 1) differs from CIN/SIL by the presence of invasion into the cervical stroma and/or metastasis to distant sites, particularly lymph nodes. Invasive squamous cell carcinomas retain squamous morphological features, for example the formation of intercellular bridges between tumor cells and keratinization; and express markers of squamous differentiation such as cytokeratin 5, cytokeratin 14 and p63 (Fig. 1). A number of different histological types are recognized, although these do not influence clinical management. Thus, squamous cell carcinomas can be classified as keratinizing, non-keratinizing, papillary, basaloid, warty and squamotransitional types. Rarely, the tumor may be associated with a marked inflammatory cell infiltrate,

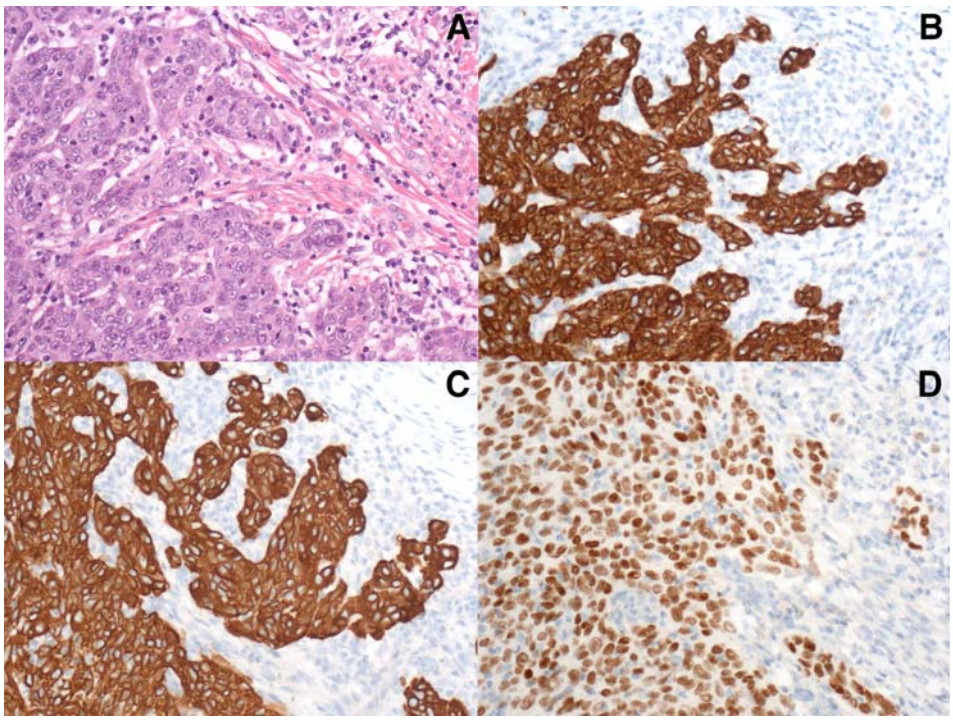

Fig. 1 Squamous cell carcinoma showing the invasion of tongues of malignant squamous epithelium into an inflamed stroma (A). The malignant cells express cytokeratin 5 (B), cytokeratin 14 (C) and p63 (D), which are markers of squamous differentiation.

which obscures the infiltrating epithelial cells—this variant is termed lymphoepithelioma-like carcinoma. These types are all associated with high-risk HPV infection and behave similarly, their clinical progression being largely related to invasion of local structures rather than distant metastasis.

Verrucous carcinoma is also described in the cervix, but is much less frequent here than in the vulva. Most lesions traditionally referred to as verrucous carcinomas are either usual type squamous cell carcinomas with a pushing infiltration pattern; or giant condylomas (warts), the latter being benign and associated with low-risk HPV infection.

Adenocarcinoma

Adenocarcinoma of usual type

Most adenocarcinomas of the cervix are of "usual type" (Fig. 2). These tumors show evidence of glandular differentiation with, typically, formation of glandular structures lined by endocervical type epithelium with varying degrees of mucin depletion. This mucin depletion can impart an "endometrioid" appearance, which does not however reflect true endometrioid differentiation. Well differentiated tumors may form papillary structures and, when prominent, this has been termed villoglandular type of adenocarcinoma which, in the past, was thought to be associated with well differentiated indolent tumors. However, it is now recognized that these tumors often show a more typical infiltration pattern elsewhere, particularly at the invasive front, and are not necessarily associated with a good clinical outcome, so this feature is best regarded as a pattern rather than a type. Endocervical adenocarcinoma of usual type is associated with HPV infection and AIS/CGIN is its precursor. Adenocarcinomas are less common than squamous cell carcinomas, as might be predicted from the relationship between HPV infection and squamous differentiation (see below). However, cervical screening is less effective for adenocarcinoma as the tumors are more often located further up the cervical canal, in a less accessible position for cervical smear sampling, and typically grow into the cervix, sometimes producing a "barrel cervix" (Fig. 2) as a result of this endophytic growth pattern. Adenocarcinomas appear to be more common now than in the past: whether this represents a true rise in incidence rather than an increase in proportion due to the prevention of cervical squamous cell carcinoma by cervical screening is not clear.

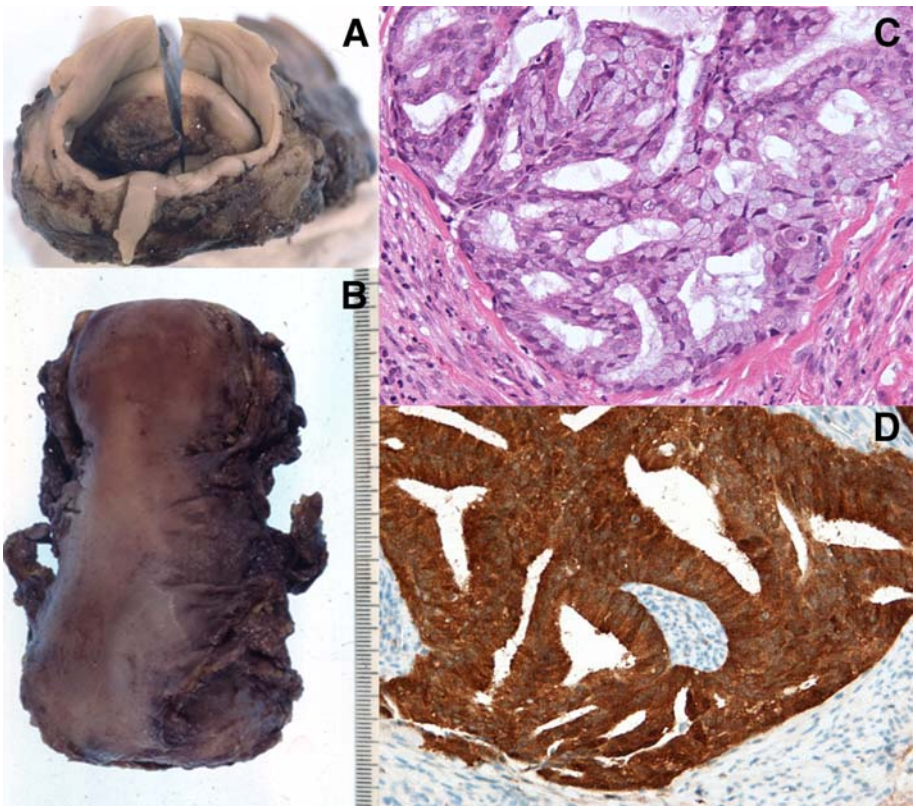

Fig. 2 Adenocarcinoma of the cervix in which there is a visible tumor at the ectocervical surface (A) but the bulk of the tumor extends into the cervical wall, producing the appearance of a "barrel cervix," seen here from the posterior aspect (B). Histologically, the tumor forms glandular structures lined by mucinous epithelial cells (C) that strongly express p16 (D), consistent with an HPV-driven lesion. Immunostaining for estrogen receptor (ER) and vimentin was negative in this case, providing further support for a primary endocervical adenocarcinoma of usual type. The smallest divisions in the ruler in B are millimeters.

Gastric type adenocarcinoma

Some cervical adenocarcinomas show evidence of gastric differentiation. These tumors are not associated with HPV infection and are typically negative for markers of high-risk HPV infection such as p16. It has been recognized in recent years that these tumors form a spectrum, with the very well differentiated adenocarcinoma previously termed "adenoma malignum" representing the best differentiated end of this spectrum. The importance of this phenomenon lies in the difficulty of diagnosis of this tumor type in superficial cervical biopsies: their well differentiated morphology can be extremely difficult to distinguish from normal endocervical glands. Increasing awareness of this tumor type, and the use of immunohistochemical markers such as HIK1083 and MUC6, is leading to its more frequent identification. This is important as it has been shown that this tumor type behaves more aggressively than usual-type adenocarcinoma, presenting at higher stage and with lower survival, even when very well differentiated. It is also of note that these tumors will not be identified by HPV testing, as they are not associated with HPV infection.

Other adenocarcinomas

Rare adenocarcinomas include intestinal type and signet-ring cell carcinomas, which show intestinal differentiation. Clear cell carcinoma can also occur as a primary tumor in the cervix, although it is rare. Most serous carcinomas presenting in the cervix represent extension from a uterine serous carcinoma. Mesonephric carcinomas are rare. They are typically found in the lateral wall of the cervix, where mesonephric duct remnants are most commonly found, but can present as tumors involving the endocervical canal, where they can mimic other tumor types.

Neuroendocrine tumors

Neuroendocrine tumors of the cervix are classified into low-grade tumors and high-grade carcinomas. Low-grade neuroendocrine tumors (carcinoid and atypical carcinoid tumors) are extremely rare as primary neoplasms of the cervix. High-grade neuroendocrine carcinomas are also uncommon but are encountered reasonably regularly in clinical practice. They are most frequently of small cell type, analogous to the tumors found more commonly in the lung, but may be of large cell type. These carcinomas are associated particularly with AIS and adenocarcinoma of usual type, and are almost always HPV positive. Their importance lies in their aggressive behavior and they are typically treated initially with chemotherapy rather than surgery.

Other epithelial tumors

Some tumors show both squamous and glandular differentiation and are termed adenosquamous carcinomas. These are usually associated with HPV infection. When poorly differentiated they can have a typical morphological appearance, termed glassy cell carcinoma.

Adenoid basal carcinoma is considered by some not to be a carcinoma, but rather a basaloid proliferation. This lesion typically occurs in post-menopausal women and is associated with the presence of CIN 3/HSIL.

Adenoid cystic carcinoma has a classical appearance, similar to that found in the salivary glands. Some basaloid carcinomas can have a rather adenoid cystic appearance but others appear to be true adenoid cystic carcinomas and are associated with the *myb-NFIB* translocation found in the equivalent salivary gland tumors.

Molecular Pathology and Genetics

The dominant molecular events in squamous cell carcinoma of the cervix relate to HPV infection. Persistent infection with high-risk HPV types (particularly HPV16 and HPV18) is associated with lesion progression, often accompanied by integration of the viral genome into host chromosomes. Integration leads to disruption of the long control region of the viral genome, with loss of coordinated expression of viral genes, loss of viral capsid production, and failure of productive viral infection. Continued expression of viral oncogenes, particularly E6 and E7, in cycling basal cells induces genetic instability and accumulation of secondary genetic events. The E6 and E7 proteins have multiple cellular effects, which represent the equivalent of multiple genetic events. For example, the E6 protein binds to and inactivates p53; and the E7 protein binds to and inactivates pRB1. In addition, E6 activates telomerase. These molecular drivers underpin the strong association between high-risk HPV infection and the development of carcinoma. The predominance of squamous cell carcinoma follows from the link between productive HPV infection and squamous differentiation, which is required for HPV replication and virus production. However, most cervical adenocarcinomas are also associated with HPV infection, as are neuroendocrine carcinomas. It is likely that these types derive from infection of reserve/stem cell populations that have the ability also to differentiate along non-squamous pathways.

Staging and Grading

The grading of cervical carcinomas is type-specific but the staging of all types of cervical carcinoma follows the FIGO and TNM systems. Grading of squamous cell carcinoma of the cervix usually follows the poorly reproducible Broder system, whereby nuclear grade is assessed in order to assign the tumor to well (grade 1), moderately (grade 2) and poorly differentiated (grade 3) categories. Keratinization does not influence grade. Similarly, grading of adenocarcinomas is determined by the degree of glandular differentiation (formation of glandular structures) and nuclear atypia but, again, is subjective. Neuroendocrine carcinomas are by definition high grade. Less attention has been paid to grading than to staging, as the latter is strongly associated with clinical outcome and is

the primary parameter used to determine patient management. Staging follows both the FIGO and the TNM systems, TNM being used in addition to FIGO as the latter does not assess lymph node status. Table 2 details and compares the FIGO and TNM systems.

Microenvironment Including Immune Response

Little is know about the influence of the microenvironment and immune response on cervical carcinoma. Recently, there has been renewed interest in this area in view of the availability of checkpoint inhibitors for therapy but, to date, our knowledge is poorly developed. This area is likely to develop more rapidly in the near future.

Diagnostic, Prognostic and Predictive Biomarkers

The most important and commonly used biomarker in this context is p16 protein expression. Diffuse strong expression of this protein, detected by immunohistochemistry, is a marker of retinoblastoma pathway disruption and, as high-risk HPV infection leads to abrogation of pRB1 function, p16 overexpression can be used to identify lesions associated with these viruses. The specificity of this overexpression is, however, dependent on context. For intraepithelial lesions, either CIN/SIL or CGIN/AIS, p16 overexpression in a "block-type" pattern for squamous lesions or diffuse consistent expression for glandular lesions is an excellent marker of lesions driven by high-risk HPV infection. However, interpretation of this biomarker requires care as focal expression is common in the absence of high-risk HPV infection. "Block-type" positivity refers to strong nuclear (with or without cytoplasmic) positivity consistently involving basal keratinocytes and extending at least one third up the involved epithelium: this pattern correlates with expression of the high-risk HPV E7 protein. When accurately defined, the correlation with high-risk HPV infection is high. However, "block-type" staining does not aid in the grading of CIN/SIL, which should be performed using standard histopathological criteria. For CGIN/AIS, true positivity requires diffuse and consistent overexpression throughout the lesion; more focal, "patchy" expression can be seen in a number of contexts, for example tubo-endometrioid metaplasia, which is common in cervices that have been previously treated, particularly by surgical excision therapies. p16 immunohistochemistry is often combined with staining for Ki67 as coordinate overexpression of p16 and Ki67 provides useful confirmation that the biomarkers are identifying a significant HPV-associated lesion.

For invasive tumors, the situation is more complex as p16 can be overexpressed by a wider range of tumors. It should therefore be used as part of a panel rather than being relied upon as a single marker. Primary cervical adenocarcinoma of usual type (see Fig. 2), for example, is typically negative for estrogen receptor (ER) and vimentin, whereas low-grade endometrioid carcinoma of the endometrium, which is often the differential diagnosis, would normally express both of these proteins.

No prognostic or predictive biomarkers are currently routinely used for cervical carcinomas.

Malignant Non-epithelial and Mixed Epithelial/Mesenchymal Tumors

Almost any sarcoma can occur in the cervix. None is common. Leiomyosarcoma may arise in the cervix or the cervix may be involved by a leiomyosarcoma arising elsewhere in the uterus. Embryonal rhabdomyosarcoma can present as a cervical mass (sarcoma botryoides). Other sarcomas include alveolar soft-part sarcoma, angiosarcoma, malignant peripheral nerve sheath tumor, liposarcoma, and Ewing sarcoma. Mixed tumors include adenosarcoma and carcinosarcoma, both of which are rare.

Malignant melanoma can occur in the cervix, as can other rare tumors such as germ cell tumors, lymphomas and myeloid neoplasms. Finally, tumors may metastasise to the cervix, for example lobular carcinoma of the breast.

Prospective Vision

The classification of tumors of the cervix is currently based on traditional morphological features. As data accumulate, and the non-HPV-associated tumors are better defined, it is likely that the classification will be based on their association with HPV, as given in simplified form in Table 1. This is not just an academic exercise as HPV testing will only detect, and HPV vaccination will only prevent, HPV-associated tumors. It therefore seems logical to segregate the tumors accordingly.

HPV testing is already used in some screening programs for the triage of women with low-grade cervical cytological abnormalities, and for follow-up after treatment. Extension of HPV testing to become the primary screening modality is likely to be adopted in the very near future in some countries. This will change the spectrum of lesions identified by screening as cervical cytology analysis will only be performed in samples that are HPV positive. Non-HPV-associated lesions will therefore not be detected directly. HPV vaccination is already reducing the incidence of HPV-associated precursor lesions but what effect that will have on HPV-driven lesions that are not associated with the HPV types present in the vaccine is not yet clear. Expansion of the HPV types included in the vaccine through use of the more recently developed multivalent vaccines will also alter the demographics of HPV-associated lesions in the countries in which they are used.

There is increasing interest in the use of immune checkpoint inhibitors, which target for example cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), programmed cell-death 1 (PD-1) and programmed cell-death ligand 1 (PD-L1), for the treatment of

Table 2 TNM and FIGO pathological staging of cervical carcinoma

TNM category	*FIGO stage*	*Definition*
TX	–	Primary tumor cannot be assessed
T0	–	No evidence of primary tumor
Tis	–	CIN (carcinoma in situ)
T1	I	Cervical carcinoma confined to the uterus (extension to the corpus should be disregarded)
T1a	IA	Invasive carcinoma, diagnosed by microscopy only (all macroscopically visible lesions even those with superficial invasion are pT1b/Stage IB)
T1a1	IA1	Stromal invasion 3.0 mm or less in depth[a] and 7.0 mm or less in horizontal spread
T1a2	IA2	Stromal invasion more than 3.0 mm in depth and not more than 5.0 mm with a horizontal spread 7.0 mm or less
T1b	IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than T1a2/IA2[b]
T1b1	IB1	Clinically visible lesion 4.0 cm or less in greatest dimension
T1b2	IB2	Clinically visible lesion more than 4.0 cm in greatest dimension
T2	II	Tumor invades beyond the uterus but not to pelvic wall or to lower third of vagina
T2a	IIA	Tumor without parametrial invasion
	IIA1	Clinically visible lesion 4.0 cm or less in greatest dimension
	IIA2	Clinically visible lesion more than 4.0 cm in greatest dimension
T2b	IIB	Tumor with parametrial invasion
T3	III	Tumor extends to the pelvic wall and/or involves the lower third of the vagina, and/or causes hydronephrosis or non-functioning kidney[c]
T3a	IIIA	Tumor involves lower third of vagina, no extension to pelvic wall
T3b	IIIB	Tumor extends to pelvic wall and/or causes hydronephrosis or non-functioning kidney
T4	IVA	Tumor invades the mucosa[d] of bladder or rectum and/or extends beyond true pelvis
M1	IVB	Distant metastasis

Regional lymph nodes (N) (TNM staging system): NX, regional lymph nodes cannot be assessed; *N0*, no regional lymph node metastasis; *N1*, regional lymph node metastasis. Regional lymph nodes include paracervical, parametrial, hypogastric (obturator); common, internal and external iliac; presacral and lateral sacral nodes. Metastasis to lymph nodes outside the regional nodal group is classified as distant metastasis.

Distant metastasis (M) (TNM staging system): MX, distant metastasis cannot be assessed; *M0*, no distant metastasis; *M1*, distant metastasis (excludes peritoneal metastasis).

Additional/optional descriptors in TNM classification

Sentinel lymph node: The following designations are applicable for sentinel lymph node assessment: *pNX(sn)*, sentinel lymph node could not be assessed; *pN0(sn)*, no sentinel lymph node metastasis; pN0(sn), sentinel lymph node metastasis.

Isolated tumor cells: Isolated tumor cells (ITCs) are single tumor cells or small clusters of cells no more than 0.2 mm in greatest dimension detected by routine H&E stains or immunohistochemistry. Cases with ITC in lymph nodes or distant metastatic sites should be classified as N0 or M0 respectively. This also applies to findings suggestive of tumor cells or tumor cell components detected by non-morphological techniques such as flow cytometry or DNA analysis.

The following designations are applicable to ITC in regional lymph nodes: *pN0*, no regional lymph node metastasis histologically; no examination for ITCs. *pN0(i−)*, no regional lymph node metastasis histologically; negative morphological findings for ITCs. *pN0(i+)*, no regional lymph node metastasis histologically; positive morphological findings for ITCs. *pN0(mol−)*, no regional lymph node metastasis histologically; negative non-morphological findings for ITCs. *pN0(mol+)*, no regional lymph node metastasis histologically; positive non-morphological findings for ITCs.

The following designations are applicable to ITCs in sentinel lymph nodes: *pN0(i−)(sn)*, no sentinel lymph node metastasis histologically; negative morphological findings for ITCs. *pN0(i+)(sn)*, no sentinel lymph node metastasis histologically; positive morphological findings for ITCs. *pN0(mol−)(sn)*, no sentinel lymph node metastasis histologically; negative non-morphological findings for ITCs. *pN0(mol+)(sn)*, no sentinel lymph node metastasis histologically; positive non-morphological findings for ITCs.

Multiple primary tumors: The suffix "m," in parentheses, is used to indicate the presence of multiple primary tumors at a single site.

Classification following multimodality therapy: The prefix "y" is used to categorize tumors examined following multimodality therapy. This indicates the extent of tumor present at the time of that examination and is not an estimate of the extent of tumor prior to multimodality therapy.

Recurrent tumors: Recurrent tumors classified after a disease-free interval, are identified by the "r" prefix.

Classification at autopsy: The prefix "a" indicates that classification is first determined at autopsy.

Lymphatic invasion—L: LX, lymphatic invasion cannot be assessed; *L0*, no lymphatic invasion; *L1*, lymphatic invasion.

Venous invasion—V: VX, venous invasion cannot be assessed; *V0*, no venous invasion; *V1*, microscopic venous invasion; *V2*, macroscopic venous invasion. Note: Macroscopic involvement of the wall of veins (with no tumor within the lumen of the veins) is classified as V2.

Perineural invasion—Pn: PnX, perineural invasion cannot be assessed; *Pn0*, no perineural invasion; *Pn1*, perineural invasion.

[a]The depth of invasion is measured from the base of the epithelium, either surface or glandular, from which it originates and should always be reported in mm, even in those cases with "early (minimal) stromal invasion" ($\sim$ or <1 mm). The depth of invasion is defined as the measurement of the tumor from the epithelial-stromal junction of the adjacent most superficial epithelial papilla to the deepest point of invasion. Vascular space involvement, either venous or lymphatic, does not alter the staging. Further guidelines on lesion measurement, including multifocal disease, are given in the Royal College of Pathologists dataset document, referenced below.

[b]All macroscopically visible lesions—even with superficial invasion—are allotted to stage IB carcinomas.

[c]On rectal examination, there is no tumor-free space between the tumor and the pelvic wall. All cases with hydronephrosis or non-functioning kidney are included, unless they are known to be due to another cause.

[d]Presence of bullous edema is not sufficient evidence to classify a tumor as T4 or IVA. The lesion should be confirmed by biopsy.

cervical carcinoma and a number of clinical trials that incorporate these therapies are underway. It is likely that these agents will become incorporated into therapeutic strategies in the future.

See also: Uterine Cervix Cancer: Diagnosis and Treatment.

Further Reading

Datasets for the histopathological reporting of cervical neoplasia, 3d edn, 2011. Royal College of Pathologists, London. http://www.rcpath.org/publications).
Heong, V., Ngoi, N., Tan, D.S.P., 2017. Update on immune checkpoint inhibitors in gynaecological cancers. Journal of Gynecologic Oncology 28, e20.
Herrington, C.S. (Ed.), 2017. Pathology of the Cervix. Essentials of Diagnostic Gynecological Pathology, vol. 3. Springer.
Kurman, R.J., Carcangiu, M.L., Herrington, C.S., Young, R.H. (Eds.), 2014. World Health Organisation classification of tumours of the female reproductive organs. IARC, Lyon.
Pecorelli, S., Zigliani, L., Odicino, F., 2009. Revised FIGO staging for carcinoma of the cervix. International Journal of Gynaecology and Obstetrics 105, 107–108.

Relevant Websites

http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/cervical-cancer—Cancer Research UK statistics.
http://www.figo.org/—International Federation of Gynecology and Obstetrics (FIGO).
http://www.iccr-cancer.org/datasets/published-datasets/female-reproductive-organs/cervical-carcinoma—International Collaboration on Cancer Reporting dataset.
https://pave.niaid.nih.gov/—Papillomavirus episteme (PAVE).
http://publications.iarc.fr/Book-And-Report-Series/Who-Iarc-Classification-Of-Tumours/Who-Classification-Of-Tumours-Of-Female-Reproductive-Organs-2014—WHO classification of tumours of the female reproductive organs.
http://www.uicc.org/resources/tnm—TNM classification of malignant tumours.

Wilms Tumor: Pathology and Genetics

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Glossary

Genetic predisposition A genetic makeup which leads to a person being at increased risk of (Wilms) tumor.
Histologic types Different histologic appearances which depend on the presence of different tumor's components.
Molecular markers Chromosomal or genetic abnormalities that can be used in stratification of treatment.
Wilms tumor—Nephroblastoma A malignant embryonal tumor of the kidney histologically resembling fetal phase of renal development.

Wilms tumor or nephroblastoma, an embryonal renal tumor, is one of the most common solid malignant tumors in children. It comprises 85%–90% of all renal tumors under 15 years of age, with more than 80% of children diagnosed under the age of 5 years (median age 3.5 years). They very rarely also occur in adults. Other pediatric renal tumors include mesoblastic nephroma (2%–3%), clear cell sarcoma (2%–3%), renal cell carcinoma (4%–5%), rhabdoid tumor (2%), and others (2%–4%). Wilms tumor is one of the most curable human tumors, with an overall survival of over 90% for unilateral non-metastatic cases.

Clinical Features

Wilms tumor is one of the rare tumors in children with a female preponderance among Caucasian patients. It usually presents as an asymptomatic abdominal mass discovered incidentally, but in 20%–30% of cases it presents with abdominal pain, malaise, hematuria, and hypertension. The most commonly used investigation to detect a renal mass is ultrasound examination but cross-sectional imaging, such as computed tomography and magnetic resonance imaging, is also considered standard practice.

The incidence of Wilms tumor varies significantly between ethnic groups. In the United States, the incidence is slightly higher among Black compared to Caucasian children. In the United Kingdom Wilms tumor is considerably more frequent among children of West Indian ethnic origin compared with Caucasians. The lowest rates are in South and East Asia, but also among children of East and South East Asian ethnicity in the United States and among South Asian children in the United Kingdom. Data from the National Wilms Tumor Study (NWTS) in North America indicates that among Asian children with renal tumors, 17.5% are non-Wilms tumors (clear cell sarcoma and rhabdoid tumor), compared with 4.6%–6.4% of non-Hispanic white, Black, and Hispanic children.

The presence of ethnic rather than geographical variation in incidence suggests a role of genetic predisposition in its etiology. A significant number of patients have a recognizable underlying predisposing genetic syndrome. Over 50 syndromes have been described in association with Wilms tumor, including various congenital anomalies and constitutional chromosomal abnormalities.

Genetics

Most Wilms tumors are sporadic, but very rarely they occur with autosomal dominant inheritance. The first gene discovered to be mutated in Wilms tumor was the *WT1* gene, located on chromosome 11p13, and somatic and germ-cell line mutations of *WT1* have been found in Wilms tumor. Other genes implicated in Wilms tumor genetics include β-Catenin (*CTNNB1*) and *WTX* gene. *WT1* mutations are often associated with monoallelic *CTNNB1* mutations and Wilms tumors which are early onset, often bilateral, stromal type, and associated with prominent rhabdomyogenesis, intralobar nephrogenic rests and genitourinary anomalies in males. Another type of Wilms tumor is associated with epigenetic alterations in the 11p15 region, and is characterized by later onset, blastemal or epithelial type histology, and perilobar nephrogenic rests.

Although only 1%–2% of Wilms tumor patients have a relative with Wilms tumor, the underlying constitutional predisposition accounts for a substantial proportion of cases. Many syndromes, congenital anomalies and constitutional chromosomal abnormalities have been reported as being associated with WT in 9%–17% of cases, which is higher than in any other malignancy.

The conditions with increased risk of Wilms tumor may be of high risk (>20%), moderate risk (5%–20%), and low risk (<5%). The high risk group includes familial WT, *WT1* deletions, *WT1* mutations, Perlman syndrome, Fanconi anemia, and mosaic variegated aneuploidy. The moderate risk group includes 11p15 overgrowth syndromes, Beckwith–Wiedemann syndrome (BWS),

Fraiser syndrome, and Simpson–Golabi–Behmel syndrome. The low risk group includes isolated hemihypertrophy, Bloom syndrome, Li–Fraumini syndrome, Mulibrey nanism, trisomy 18, trisomy 13, and 2q37 deletion.

There are at least 50 known familial Wilms tumor pedigrees, and they usually present with nonsyndromic Wilms tumor, with no recognized underlying cause. In a minority of cases they are associated with syndromes such as *WT1* mutations/deletions, 11p15 defects, mosaic variegated aneuploidy, and biallelic *BRCA2* mutations. At present, two familial Wilms tumor genes have been recognized. *FWT1* is an autosomal dominant nonsyndromic familial WT gene, mapped to 17q21 but not yet characterized. The *FWT2* gene, associated with an autosomal dominant nonsyndromic condition, has been proposed to be at 19q13 but there is no firm evidence favoring this locus. However, the finding of families not linked to *FWT1*, *FWT2*, *WT1*, or 11p15 indicates further genetic heterogeneity in familial Wilms tumor.

The group of *WT1*-associated syndromes includes those which are caused by mutations or deletions in the *WT1* gene predisposing to Wilms tumor. Individuals are diagnosed at younger age, with bilateral tumors (38% vs. 5% in unselected Wilms tumor cases), which are often stromal predominant/stromal type, and with intralobar nephrogenic rests. Constitutional *WT1* defects are associated with a variety of overlapping phenotypes, showing various combinations of three features: Wilms tumor predisposition, genitourinary abnormalities, and renal dysfunction.

WAGR syndrome (Wilms tumor–aniridia–genitourinary abnormalities–mental retardation) is found in about 7–8 per 1000 individuals with Wilms tumor. It is caused by monoallelic deletions at 11p13 including the *WT1* and *PAX6* genes—deletion of *WT1* results in Wilms tumor predisposition and deletion of *PAX6* results in aniridia. Some 30% of individuals with aniridia harbor *WT1–PAX6* deletions, and many of the remainder harbor point mutations or intragenic deletions of *PAX6*. Individuals with isolated *PAX6* defects are not at increased risk of Wilms tumor.

Denys–Drash syndrome is the combination of Wilms tumor, nephropathy (mesangial sclerosis), and genitourinary abnormalities which in males vary in severity from mild hypospadias to pseudohermaphroditism. Some females have gonadal dysgenesis, but the majority has normal genitourinary development.

Frasier syndrome consists of nephropathy, gonadal dysgenesis, and gonadoblastoma. It is caused by mutations in intron 9 of *WT1* that alter splicing and prevent formation of *WT1* isoforms. The nephropathy (focal segmental glomerulosclerosis) progresses to renal failure by the second or third decade of life. In males, genitourinary abnormalities are severe and sex reversal is common.

In some patients only one or two characteristic features of *WT1*-associated syndromes occur (such as Wilms tumor with cryptorchidism, or Wilms tumor with isolated nephropathy). They may also have *WT1* mutations but are more likely to have intragenic truncating mutations than missense mutations in the zinc finger domains. Most germline *WT1* mutations are de novo, but rare families have been reported with *WT1* defects that have presented with familial Wilms tumor.

The risk of Wilms tumor in individuals with *WT1* deletions, truncating mutations, or pathogenic missense mutations targeting the zinc finger domains is probably at least 50%. The risk of Wilms tumor in individuals with *WT1* intron 9 splice mutations (Frasier syndrome) is considerably lower than for other mutations—only 6% of patients with Frasier syndrome develop Wilms tumor, but the risk of gonadoblastomas is high.

Perlman syndrome is a rare, high mortality, autosomal recessive overgrowth disorder caused by biallelic mutations in *DIS3L2* gene. It is characterized by prenatal overgrowth with polyhydramnios, visceromegaly, cryptorchidism, facial dysmorphism, developmental delay, renal dysplasia, and Wilms tumor (reported in ~30% of cases).

Fanconi anemias are recessive chromosome breakage disorders with overlapping clinical and cellular phenotypes. Characteristic clinical features include short statute, microcephaly, radial ray defects, hyper- and hypopigmentated skin lesions, and bone marrow failure. There are 13 types and only Fanconi anemia types D1 and N are associated with an increased risk of Wilms tumor (~30%). Fanconi anemia type D1 is caused by biallelic mutation in *BRCA2*, whereas biallelic mutations in *PALB2* cause type N.

Mosaic variegated aneuploidy is a rare autosomal disorder characterized by constitutional mosaicism for gains and losses of whole chromosomes resulting in variable clinical features such as microcephaly, developmental delay, congenital heart defects, cataracts, and others.

In the moderate risk group the most common disorders are overgrowth syndromes. They represent a heterogeneous group of disorders characterized by pre and/or postnatal overgrowth often in association with other abnormal phenotypic features. Several of them are associated with Wilms tumor. Beckwith–Wiedemann syndrome is caused by dysregulation of various imprinted growth-regulating genes at 11p15 and carries an elevated (4%–21%) risk for several tumor types, Wilms tumor being the most frequently reported (in 1%–8% of affected children). Conversely, about 1% of children with Wilms tumor have Beckwith–Wiedemann syndrome. Beckwith–Wiedemann syndrome is characterized by pre- and postnatal overgrowth, macroglossia, anterior abdominal wall defects, ear lobe creases and posterior helical pits, neonatal hypoglycemia, and hemihypertrophy. The individuals may also have other abnormalities, including nephromegaly, renal cysts, medullary sponge kidney, medullary dysplasia, and hydronephrosis.

Simpson–Golabi–Behmel syndrome is an X-linked overgrowth syndrome characterized by coarse facial features, cardiac abnormalities, urinary tract abnormalities (renal dysplasia, nephromegaly) polydactyly, accessory nipples and, in some individuals, learning difficulties. Wilms tumor has been reported in 9% of patients with *GPC3* mutations. The condition is caused by loss of function mutations or deletions of *glypican-3* (*GPC3*) located at Xq26, which are identifiable in about 70% of affected individuals. The cause of the remainder is unknown.

The low risk group includes some syndromes and constitutional chromosomal abnormalities. In Bloom syndrome, hereditary hyperparathyroidism-jaw tumor syndrome and mulibrey nanism, the risk is probably below 5%. There also appears to be an increased risk of Wilms tumor associated with three constitutional chromosomal abnormalities including trisomy 18, trisomy

13, and 2q37 deletion. Although their prevalence at birth is relatively high, the first two of these account for a very small proportion of all cases of Wilms tumor as infant mortality is very high.

Isolated hemihypertrophy carries a low risk of Wilms tumor (~3%). Approximately 20% of individuals with hemihypertrophy have an identifiable 11p15 defect. Interestingly, tumors occur at similar frequency in the larger and smaller kidney in asymmetric individuals.

Somatic Mutations in Wilms Tumor

Wilms tumors usually show few genetic changes, even by deep sequencing. Karyotypes may show trisomies (chromosomes 18 and 12), but translocations are uncommon in spite of several reports of t(1;16). Tumors with the most genetic changes are aneuploid in association with anaplasia. Some chromosomal losses (1p and 16q) and 1q gain are associated with a poorer prognosis (without features of anaplasia), but the genetic basis of this are not yet known. Loss of heterozygosity is seen at various sites, especially at 11p15 (the Beckwith–Wiedemann syndrome locus which is also found in sporadic hepatoblastoma and rhabdomyosarcoma) and 11p13. Changes at these two loci can be considered as somatic mutation in sporadic tumors, as well as in cases with syndromic features a second (somatic) hit.

In Wilms tumor a variety of genetic pathways disturb the normal development of committed nephrogenic mesenchyme, and these can be grouped into about five different types, arising at different stages of development and with involvement of different genes. Some, such as epigenetic loss of imprinting at 11p15 (as in children with BWS), serve as a background for subsequent changes, as they can be found in histologically normal kidney around the tumor. The first identified pathway involves *WT1* but *WT1* mutations are only seen in around 15% of Wilms tumors. More recently mutations of *WTX* identified a second pathway and also the WNT pathway is involved with mutations of the *CTNNB1* gene. Another group involves pathways involving noncoding RNA, as in the Perlman syndrome and *DICER1* mutations. Furthermore, mutations of the nuclear homologue to *DICER1*, *DROSHA* are also found in a significant number of tumors. Dominant mutations of *SIX1* and *SIX2*, which are highly expressed in fetal nephrogenic blastema and in blastema of WTs also, appear to be early drivers of Wilms tumor development. Rather than considering Wilms tumor pathogenesis as a result of mutation of individual genes, it is becoming more likely that Wilms tumors arise through genetic and epigenetic changes in common gene pathways such as the Wnt pathway, with a crucial role of noncoding RNA in gene regulation in these pathways. *TP53* mutations are found usually in association with anaplasia, and should be considered a late event in Wilms tumor oncogenesis.

Next generation sequencing is starting to identify and confirm other changes, including low level N-myc amplification and new potential oncogenes, as causal factors of Wilms tumor. Wilms tumors are genetically heterogeneous, containing various clones with different genome changes, some of which are likely to be passenger mutations, others representing drivers of clonal evolution of the tumor. This implies that a tumor tissue sample may not be representative for all important genome abnormalities, which may impact on treatment planning. Furthermore, Wilms tumors conceivable contain a small population of cancer stem cells that might have to responsible for secondary chemoresistance.

Nongenetic Causes of Wilms Tumor

In adult tumors, environmental factors usually play a dominant role in oncogenesis but in childhood tumors the genetic background is usually more important. There have been some reports of environmental exposure inducing Wilms tumors but the evidence is not strong. A rat model of triphasic Wilms tumor has been developed following nitrosamine exposure in utero, affecting *CTNNB1* in the Wnt pathway. In humans Wilms tumor incidence is higher with increased birthweight, suggesting increased growth factors in utero as factor, which is supported by few reports of increased risk for Wilms tumor in infants of diabetic mothers, as insulin is a potent growth factor for the fetus.

Future Developments

Wilms tumor pathogenesis clearly recapitulates normal nephrogenesis. As nephrogenesis becomes better understood, it will help us understand Wilms tumor development. The final pathogenetic pathway for Wilms tumor will involve in the cells of origin not only driver mutations in genes but also epigenetic changes, such as chromatin structure, histone architecture, DNA methylation, and noncoding RNA, as well as external cellular factors including cell adhesion molecules and external growth factors. Tumors rarely arise in older children and adults, and understanding normal differentiation pathways may provide novel insight guiding new treatment modalities.

Pathologic Features

Macroscopically, Wilms tumors are usually large and distort the renal contours. They are rarely multicentric (5%–10% of cases) or bilateral (5%–8% of cases)—unique features which may be helpful in differentiating them from other childhood renal tumors. Cut surface appearance varies, depending on the histological composition of the tumor—blastemal tumors are soft and fragile, whereas stromal tumors are firm. If treated with preoperative chemotherapy, large areas of necrosis and cystic changes are often seen.

Histologically, Wilms tumors show a wide range of patterns with three histological components—blastemal, epithelial, and stromal—represented in variable proportions. Classical, triphasic tumors contain all three components (Fig. 1), but cases with only two (Fig. 2) or even just one component are not rare.

Blastema represents an undifferentiated component and is regarded as the major malignant element. It consists of small, round, blue cells with overlapping nuclei and usually high mitotic activity. The blastemal cells may be arranged in several patterns (diffuse, serpentine, nodular, or basaloid) but these have no prognostic significance. Unlike other histological Wilms tumor types, blastemal tumors frequently have no capsule separating them from the renal parenchyma. Sometimes blastema may show signs of early epithelial differentiation, with cells arranged in very primitive epithelial structures such as rosettes, and this may cause diagnostic difficulties when assessing the percentage of blastemal relative to epithelial components, which is of significance in the SIOP histological classification (see below). Similarly, blastema may contain areas composed of spindle cells, which may be difficult to distinguish from the stromal component. As no exact histological or immunohistochemical criteria exist to discriminate between the components, Wilms tumor subtyping is subjective and dependent on the experience of the pathologist. This justifies central pathology review, which has been part of all multicenter studies around the world in the last 40–50 years.

The epithelial component may display both nephrogenic and heterologous epithelial elements. The former includes the whole spectrum of differentiation from primitive epithelial rosette-like structures to well differentiating tubules or glomeruli-like structures resembling different stages of nephrogenesis. The heterologous epithelial elements include squamous and mucinous epithelium.

The stromal component may also show a variable architecture between loose, hypocellular, myxoid areas and densely packed undifferentiated mesenchymal cells. Heterologous differentiation in the stroma includes well-differentiated skeletal or smooth muscle cells, fat tissue, cartilage, bone, and even glial tissue (in rare cases), especially in tumors which have undergone preoperative chemotherapy.

Preoperative chemotherapy influences the histological presentation of Wilms tumors due to chemotherapy-induced changes, including induction of maturation of the pretreatment components. Chemotherapy induced changes include necrosis, fibrosis, and areas with foamy macrophages and/or hemosiderin-laden macrophages. Usually it is the blastema, representing the least differentiated component, that responds to chemotherapy, whereas mature epithelial and stromal components are often less

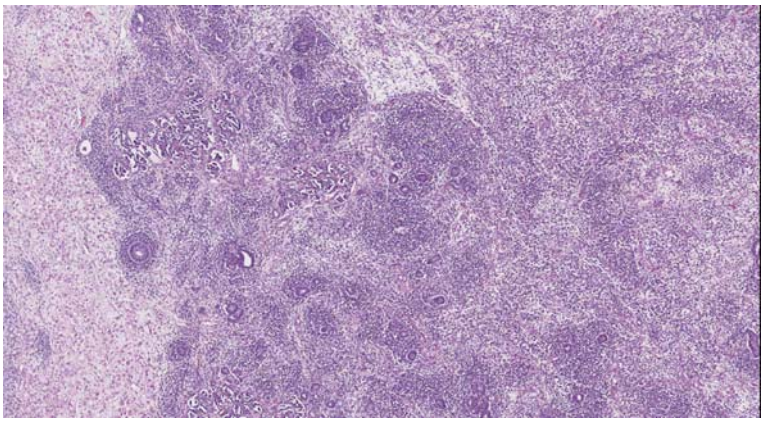

Fig. 1 Mixed (triphasic) Wilms tumor showing blastemal, epithelial, and stromal components.

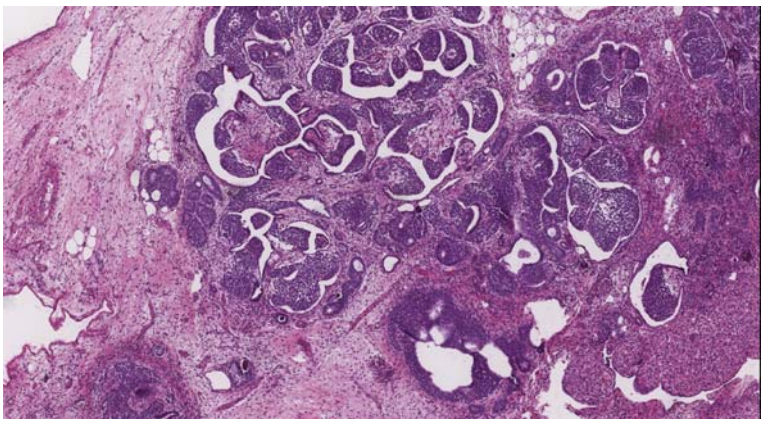

Fig. 2 Mixed (biphasic) Wilms tumor consisting of blastemal and stromal components.

chemotherapy sensitive. Tumors predominantly composed of mature epithelial or stromal components often show no preoperative therapy modifications.

Cystic partially differentiated nephroblastoma is a cystic variant of Wilms tumor which occurs as a solitary, unilateral, multilocular cystic lesion that usually measures 5–10 cm in diameter, with cysts ranging in size from a few mm to 4 cm. It is sharply demarcated from surrounding renal parenchyma and secondary changes such as necrosis and hemorrhage are rare. Diagnostic criteria for cystic partially differentiated nephroblastoma are clear demarcation from the non-cystic renal parenchyma, uniquely composed of cysts and their septa, septa representing the only solid portion of the tumor as composed of fibrous tissue with blastemal cells in any amount, and lining of the cysts by attenuated cuboidal or hobnail epithelium. Neoplastic tubules, variably differentiated glomeruli, striated muscle, cartilage and fat may also be found along with blastemal cells in the septa (**Fig. 3**). A macroscopically and histologically similar lesion—cystic nephroma—used to be regarded as a lesion related to cystic partially differentiated nephroblastoma, but recent molecular studies showed that they are likely separate entities since cystic partially differentiated nephroblastoma shows no association with *DICER1* mutations, which are reported in 90% of cystic nephroma. From a management perspective, however, both lesions are treated with surgery only, and both have excellent prognosis (100% survival), but it is important to distinguish them from Wilms tumor with prominent cystic component.

An important histological feature of Wilms tumor, with major therapeutic and prognostic implications, is anaplasia which is defined as the presence of large, multipolar, atypical mitotic figures, marked nuclear enlargement and hyperchromasia (**Fig. 4**). Anaplastic tumors are generally aneuploid. Anaplasia is found in 5%–8% of Wilms tumors, and the patients are usually older than those with non-anaplastic tumors. Anaplasia never occurs in the first 6 months of life, it is exceptionally rare in the first year of life, and only 5% of patients with anaplastic tumors are under 2 years of age. Anaplasia may occur in any of the histological components of the tumor, but it is critical for its diagnosis that all three criteria are fulfilled. A rhabdomyoblastic stromal component in a tumor without anaplasia may show considerable cellular pleomorphism, but if atypical mitoses are absent, this is not regarded as genuine anaplasia. Anaplasia is further subclassified as focal and diffuse. Focal anaplasia is localized, usually as a sharply demarcated focus (or two to three small foci) showing the features mentioned above. All other cases with anaplasia should be considered as diffuse anaplasia, including when multiple foci of anaplasia are found or focal anaplasia with marked nuclear

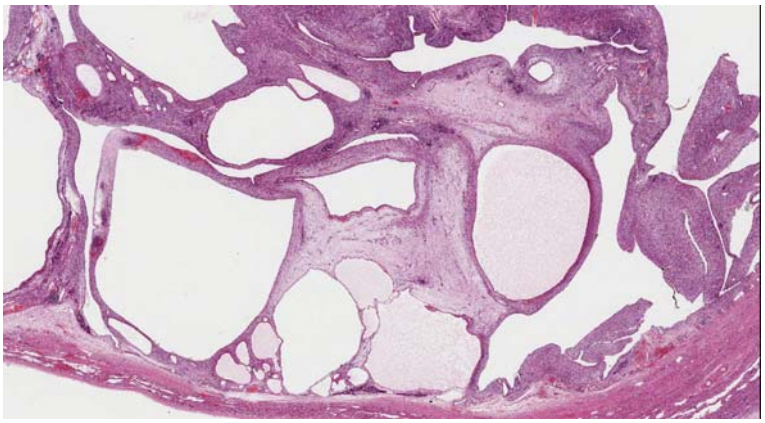

Fig. 3 Cystic partially differentiated nephroblastoma—composed entirely of cysts and septa which contain some immature tubules and foci of blastema.

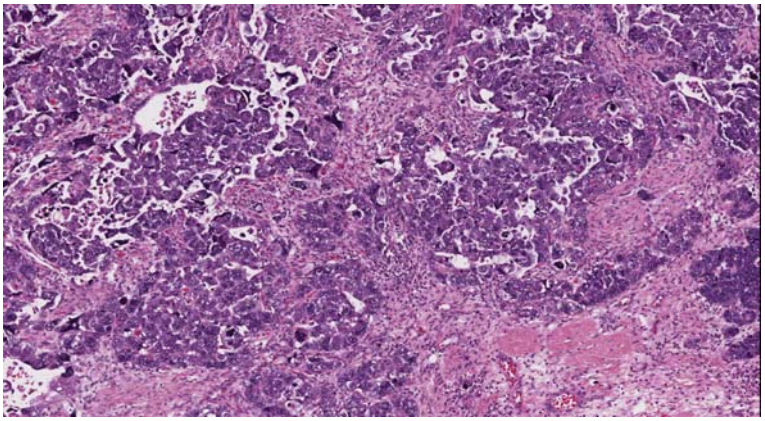

Fig. 4 Wilms tumor with diffuse anaplasia showing atypical mitoses, enlarged, pleomorphic, and hyperchromatic nuclei.

enlargement and hyperchromasia elsewhere in the tumor, when the anaplasia is seen in distant metastases diagnosed in a biopsy. Anaplastic clones are more chemotherapy-resistant than aggressive, and high stage tumors with anaplasia have a poor prognosis with only ~35% overall survival in stage 4. Anaplastic tumors often immunohistochemically express p53 (in ~80% of cases) and are *TP53* mutated, but this is also found in non-anaplastic Wilms tumors and does not distinguish between anaplastic and non-anaplastic Wilms tumors. Anaplastic Wilms tumors also show dysregulation of the *MYCN* gene, which is involved in the development of tumors with adverse outcome.

Further subclassification of Wilms tumors is based on semiquantitative assessment of different tumor components, but the criteria for subtyping differ between the two major groups which have been running renal tumor trials in the last 50 years—in the Children's Oncology Group (COG, former National Wilms Tumor Study Group), where surgery is used as the first step in treatment, followed by chemotherapy and, in higher stages, radiotherapy, Wilms tumors are classified as non-anaplastic and anaplastic types. Non-anaplastic Wilms tumors are further assigned on the basis of a predominant component (which makes more than 2/3 of the tumor) as epithelial predominant, stromal predominant or mixed (if no component makes more than 2/3 of tumor). In the International Society of Paediatric Oncology (SIOP) approach, preoperative chemotherapy is given as the first line of treatment, followed by surgery and further chemotherapy and, in higher stages, radiotherapy. Since preoperative chemotherapy often results in chemotherapy-induced changes, they are also taken into account when subclassifying tumors: if there is no viable tumor left, it is completely necrotic type. If more than 66% (2/3) of tumor is nonviable (i.e. shows chemotherapy-induced changes), it is regarded as regressive type irrespectively of the presence of remaining viable tumor components (except anaplasia). If viable tumor comprises more than 1/3 of the tumor mass, subtyping depends on the percentage of the viable components: in mixed type no component comprises more than 66% of tumor while in epithelial (or stromal) type more than 66% of the tumor is composed of epithelial (or stromal) elements, and in addition only up to 10% of blastema is allowed. When this exceeds 10% the tumor is regarded as mixed type. Blastemal type is regarded as a high risk tumor in the SIOP classification (**Table 1**).

For these reasons, it is important to bear in mind that although the terms used in COG and SIOP subtyping of Wilms tumor are similar, they do not necessarily describe the same histology and complicate a direct comparison of different studies.

Similarly, the staging criteria between the SIOP and COG differ since in the SIOP criteria chemotherapy-induced changes are taken into account for some stages (**Tables 2** and **3**).

The diagnosis is usually relatively easy in triphasic or even biphasic Wilms tumors, but their subclassification may be challenging. However, monophasic Wilms tumors may be very difficult to separate from other renal tumors with similar histological features. Pure blastemal type Wilms tumors have to be distinguished from other undifferentiated tumors such as neuroblastoma, primitive neuroectodermal tumor/Ewing sarcoma of the kidney, and desmoplastic small round cell tumor. It is particularly important to consider non-Wilms tumors in older patients and adults—Wilms tumor does occur in adults, but many of the renal tumors which in the past were labeled as adult Wilms tumors proved to be other entities. In order to reach the correct diagnosis in such cases, it is critical to perform immunohistochemical and molecular investigations looking for characteristic feature of Wilms tumors. Although blastemal components may show focal CD99 positivity, it is usually not diffuse and membranous as in Ewing sarcoma of the kidney, where genetic studies also show characteristic translocations, with t(11;22)(q24;q12) being the most common. Desmoplastic small round cell tumor shares many immunohistochemical features with blastemal type Wilms tumor, but is extremely rare and the diagnosis should only be made if genetic analysis identifies the *EWS-WT1* t(11;22)(q13;q12) translocation. Neuroblastoma usually shows elevated levels of catecholamines, and on histological examination its cells reveal nonoverlapping nuclei and coarse "salt and pepper" chromatin. Neuron-specific enolase and CD56 are positive in both tumors, but WT1 marker is negative in neuroblastoma, and chromogranin, synaptophysin and NB84a markers are negative in Wilms tumor. Rhabdoid tumor can be

Table 1 The SIOP working classification of renal tumors of childhood

	Pretreated cases[a]	*Primary nephrectomy cases*[b]
Low risk	Mesoblastic nephroma	Mesoblastic nephroma
	Cystic partially differentiated nephroblastoma	Cystic partially differentiated nephroblastoma
	Completely necrotic nephroblastoma	
Intermediate risk	Nephroblastoma—epithelial type	Non-anaplastic nephroblastoma
	Nephroblastoma—stromal type	Nephroblastoma—focal anaplasia
	Nephroblastoma—mixed type	
	Nephroblastoma—regressive type	
	Nephroblastoma—focal anaplasia	
High risk	Nephroblastoma—blastemal type	
	Nephroblastoma—diffuse anaplasia	Nephroblastoma—diffuse anaplasia
	Clear cell sarcoma of the kidney	Clear cell sarcoma of the kidney
	Rhabdoid tumor of the kidney	Rhabdoid tumor of the kidney

[a]Diagnostic criteria for cases treated with preoperative chemotherapy differ from those used for cases that are treated with primary nephrectomy.
[b]For cases treated with primary nephrectomy, the only subclassification needed is to distinguish between non-anaplastic and anaplastic types.

Table 2 SIOP staging criteria (2016) (for tumors treated with preoperatively chemotherapy)

Stage I
(a) The tumor is limited to kidney or surrounded with a fibrous (pseudo)capsule if viable tumor is outside of the normal contours of the kidney. The renal capsule or pseudocapsule may be infiltrated by the tumor but it does not reach the outer surface of the capsule.
(b) The tumor may be protruding (bulging) into the pelvic system and "dipping" into the ureter but it is not infiltrating their walls.
(c) The vessels or the soft tissues of the renal sinus are not involved.
(d) Intrarenal vessel involvement may be present.
Stage II
(a) Viable tumor is present in the perirenal fat and it is not covered by a (pseudo)capsule, but it is completely resected (resection margins "clear").
(b) Viable tumor infiltrates the soft tissues of the renal sinus.
(c) Viable tumor infiltrates blood and lymphatic vessels of the renal sinus or renal veins or is present in the perirenal tissue but it is completely resected.
(d) Viable tumor infiltrates the ureter's wall.
(e) Viable tumor infiltrates adjacent organs or vena cava but is completely resected.
Stage III
(a) Viable tumor extends to the resection margins. If there is only nonviable tumor at inked resection line, it is regarded as stage III only if viable tumor is <5 mm to the inked margin. If the viable tumor is >5 mm from the resection line and only regressive changes are found at inked margin it does not upstage the tumor (the minimal distance of 5 mm tissue without viable has to be documented with several blocks of this area).
(b) Any abdominal lymph nodes are involved with either viable or nonviable tumor.
(c) Pre- or intraoperative tumor rupture, if visible at pathological examination (irrespective of other criteria for staging).
(d) Tumor thrombus is present at resection margins of ureter, renal vein or vena cava inferior (always discuss resection margins with a surgeon).
(e) Tumor thrombus which is attached to the IVC wall is removed piecemeal by surgeon.
(f) Tumor has been biopsied (wedge/open biopsy) prior to preoperative chemotherapy or surgery.
(g) Tumor implants (viable and/or nonviable) are found anywhere in the abdomen.
(h) Tumor (viable and/or nonviable) has penetrated through the peritoneal surface.
Stage IV
Hematogenous metastases (lung, liver, bone, brain, etc.) or lymph node metastases outside the abdomino-pelvic region.
Stage V
Bilateral renal tumors at diagnosis. Each side should be substaged according to the above criteria.

Table 3 COG staging system (for tumors treated with primary surgery)

Stage I
(a) Tumor limited to the kidney and completely resected.
(b) Renal capsule intact.
(c) The tumor was not ruptured or biopsied prior to removal.
(d) Renal sinus vessels not involved.
(e) No evidence of tumor at or beyond the margins of resection.
Stage II
(a) Tumor completely resected.
(b) No evidence of tumor at or beyond the margins of resection.
(c) Tumor extends beyond the kidney, as evidenced by one of the following:
- Penetration through the renal capsule.
- Extensive invasion of the soft tissue of renal sinus.
- Blood vessels within the nephrectomy specimen outside the renal parenchyma, including those of the renal sinus, contain tumor.

Stage III
(a) Residual non-hematogenous tumor confined to the abdomen is present after surgery as evidenced by any of the following:
- Involvement of lymph nodes within the abdomen or pelvis
- Penetration through the peritoneal surface
- Tumor implants on the peritoneal surface
- Tumor present at the margins of surgical resection
- Tumor not resectable because of local infiltration into vital structures
- Biopsy of tumor prior to removal of kidney
- Tumor spillage of any degree or localization occurring before or during surgery
- Tumor removed in greater than one piece

Stage IV
(a) Hematogenous metastases (lung, liver, bone, brain, etc.)
(b) Lymph node metastases outside of the abdomino-pelvic region
Stage V
(a) Bilateral renal involvement at the time of initial diagnosis
(b) Each side should be separately staged according to the above criteria

distinguished from Wilms tumor by marker immunohistochemistry, as nuclear INI1 expression is not seen in rhabdoid tumor. Pure epithelial type Wilms tumor may be difficult to distinguish from metanephric adenoma, renal cell carcinoma, and hyperplastic perilobar nephrogenic rest. Highly differentiated epithelial type Wilms tumor may be composed of small, well differentiated, and closely packed tubules similar to metanephric adenoma, but the latter can be diagnosed by the lack of a capsule between the tumor and renal parenchyma and absent mitotic activity. The combination of CK7–, AMACR–, WT1+, and CD57+ is the typical immunohistochemical marker profile of metanephric adenoma. Histological features of renal cell carcinomas in children associated with translocations are usually distinctive and immunohistochemistry or fluorescence in situ hybridization to detect *TFE3* gene rearrangement is of huge help, but papillary renal cell carcinoma (as seen in adults) may be more difficult to diagnose. Immunohistochemistry using the epithelial markers CK7 and CD10 and cytogenetic analysis may be very helpful. In the differential diagnosis of pure stromal type Wilms tumor, clear cell sarcoma of the kidney and mesoblastic nephroma and in older children monophasic synovial sarcoma should be considered. In Wilms tumors treated with preoperative chemotherapy, the stroma may show a striking clear cell sarcoma-like appearance, and extensive sampling may be required in order to find foci with other Wilms tumor components.

The diagnosis and staging of renal tumors of childhood is challenging for a number of reasons, including their rarity, extreme morphological heterogeneity, which varies from case to case, histological patterns of certain Wilms tumor subtypes which may appear similar to other rare pediatric renal tumors, lack of objective criteria distinguishing Wilms tumor from nephrogenic rests, and the fact that assessment of the tumor and determination of the pathology stage is a multistep and time-consuming process. For all these reasons, a significant number of misdiagnosed and mis-staged cases have been identified in the previous SIOP and COG studies, and in order to ensure that the patients who are entered in the studies are treated according to their precise diagnosis and stage, rapid central pathology review has been introduced within a time-frame that assures that the treating physician will dispose of the review results.

Nephrogenic Rests

Nephrogenic rests are defined as abnormal foci of embryonic tissue persisting beyond 36 weeks of intrauterine life. They are found in 40% of kidneys with Wilms tumor and very rarely in routinely examined kidneys at autopsy. Nephrogenic rests are regarded as precursor lesions of Wilms tumor. There are two main types of nephrogenic rests—perilobar and intralobar nephrogenic rests, and they have different histological features and clinical importance. In about 5% of cases, both perilobar and intralobar nephrogenic rests are present. They are further subclassified as obsolescent, dormant, sclerosis, and hyperplastic types. Perilobar nephrogenic rests are found at the periphery of the renal lobe, they are typically multifocal and demarcated from the renal parenchyma. Histologically, they usually contain blastema and tubules (Fig. 5) and are associated with overgrowth Wilms tumor syndromes (hemihypertrophy and Beckwith–Wiedemann syndrome) and with blastemal and epithelial Wilms tumors. Intralobar nephrogenic rests are found within the renal lobe, they are poorly defined and merge with the surrounding renal parenchyma, as they are composed of stroma, blastema, and tubules (Fig. 6). They are associated with WAGR and Denis–Drash syndromes and stromal Wilms tumors. If perilobar or intralobar nephrogenic rests are hyperplastic, they may be impossible to distinguish from a Wilms tumor, since there are no histological or immunohistochemical features which provide definitive diagnosis. The vast majority of the rests do not develop into a Wilms tumor. Nephroblastomatosis is term used to describe multiple nephrogenic rests. A special type of nephroblastomatosis is diffuse hyperplastic perilobar nephroblastomatosis, in which the renal cortex is completely replaced by proliferating perilobar rests. It requires treatment as it virtually always progresses into a Wilms tumor. Similar to Wilms tumor, there are also racial differences in the prevalence of nephrogenic rests, with fewer perilobar rests seen in the Japanese and Indian population.

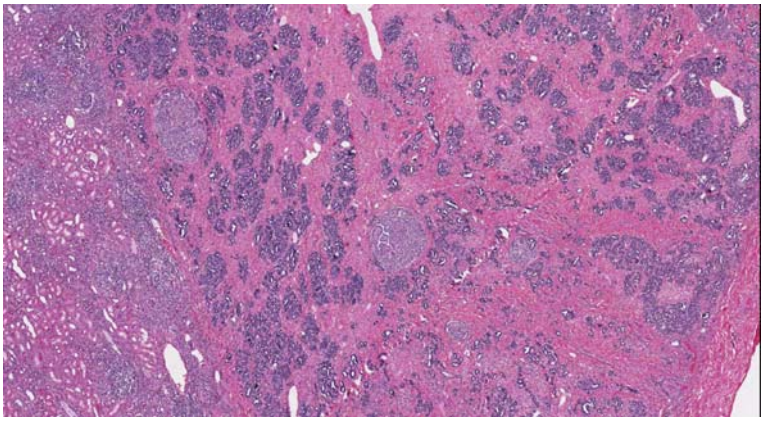

Fig. 5 Perilobar nephrogenic rests showing a mixture of sclerosing and hyperplastic (adenomatous) rests without sharp demarcation from the renal parenchyma.

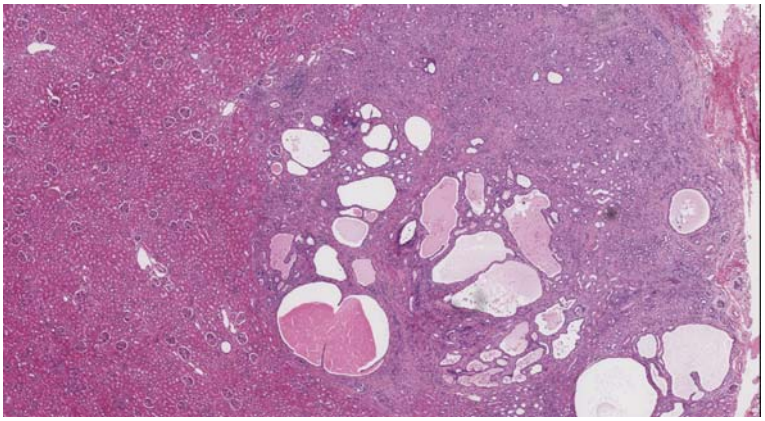

Fig. 6 Intralobar nephrogenic rest with cystically dilated tubules blending with the surrounding renal parenchyma.

Prognostic Features

The prognosis of Wilms tumors depends on a number of factors which, again, are not identical between the two big groups. In SIOP, prognostic factors include stage, histology, tumor volume, response to treatment, and (potentially) volume of blastema in postchemotherapy specimen.

An initial stage is assigned at the time of diagnosis in terms of localized, metastatic or bilateral, and this provides the basis for preoperative chemotherapy. A final stage is determined after preoperative chemotherapy, following nephrectomy, applying the staging criteria as outlined above and taking into account both chemotherapy-induced changes and viable tumor components.

Histological subtyping and risk group assignment are done following nephrectomy as in **Table 1**. Completely necrotic tumors are regarded as low risk, and no further treatment is given for stage 1, whereas other stages are treated with milder therapy. The high risk group contains, in addition to diffuse anaplasia, blastemal type Wilms tumor. Although it represents only 5%–8% of Wilms tumors, it is responsible for about 30% of events, and therefore it is treated with more aggressive postoperative therapy which has resulted in improved 5-year event-free survival from 67% to 80% (for all stages of localized disease), and for stage 1 from 71% to 96%. The last SIOP 2001 Trial showed that treatment of stage 2 and stage 3 tumors from the intermediate risk group without doxorubicin (a known cardiotoxic drug) is just as effective, which is important for avoiding late sequelae of treatment. Focal anaplasia is regarded as conferring intermediate risk and treated accordingly.

Preoperative chemotherapy usually results in decreased tumor volume, but when tumors remain large (with a volume of 500 mL or more after preoperative chemotherapy) and are not of stromal or epithelial subtype (which are known to be chemotherapy-resistant), this is regarded as a bad prognostic sign, and such tumors receive more intense postoperative treatment. Response to preoperative treatment is assessed on the basis of reduction in tumor volume, different distribution of tumor types, and clearance of metastases.

Age has no significance in treatment stratification in SIOP, although the results of previous studies showed that patients under 2 years of age with localized disease had 92% 5-year OS, compared to patients between 2 and 4 years (88% 5-year OS) and patients older than 4 years (80% 5-year OS).

The results of the SIOP 2001 Wilms tumor Trial indicated that patients could be better stratified on the basis of blastemal volume rather than the percentage of the blastemal component, and that patients with localized disease and a blastemal volume of more than 20 mL did significantly worse than those with a smaller blastemal volume. This criterion is now being prospectively assessed in the new SIOP Study (UMBRELLA 2016).

In COG, the prognostic factors are stage, histology, patient age and tumor weight, response of lung metastases, and molecular markers (loss of heterozygosity at 1p and 16q, and 1q gain).

Tumor stage has been an important risk factor since the first NWTSG Study, and over the years the criteria for staging have been modified, taking into account the results and findings from the previous studies. It is worth emphasizing that the staging criteria in COG and SIOP are not exactly the same, since in SIOP there are chemotherapy-induced changes which are taken into account for certain stages, whereas in COG tumors are staged on the basis of viable components only. The staging criteria are presented in **Table 3**.

The only histological feature impacting on treatment and with prognostic significance is anaplasia (see above), and there is a difference in treatment of focal and diffuse anaplasia. Treatment and prognosis of a tumor with focal anaplasia is intermediate between that of tumors with favorable histology and those with diffuse anaplasia.

Previous studies showed that increased age is associated with increased risk of relapse and less favorable outcome, but this is only taken into account for risk stratification of small (<550 g), stage 1, favorable histology Wilms tumors in patients younger than 2 years of age, who are treated with surgery only.

If there is no complete response of lung metastases on initial chemotherapy, this is regarded as a bad prognostic sign and treatment is intensified by adding radiotherapy of the lung while this is not given to patients with complete response.

Previous NWTSG studies showed that in a small number of patients (~5%) concurrent loss of heterozygosity for chromosomes 1p and 16q is associated with increased relative risk of relapse and death.

Recent COG and SIOP studies showed that gain of chromosome 1q is present in about 30% of Wilms tumors, and as it is associated with poor outcome it will be used as a risk stratification criterion in coming COG studies.

Management

As already mentioned, management of Wilms tumors is according to two different approaches: COG and SIOP. The COG approach is followed mainly in the North America and consists of primary surgery, followed by further treatment if necessary, including chemotherapy and advanced stages radiotherapy. The last COG trial included the following four categories: (a) very low and standard risk favorable histology; (b) higher risk favorable histology; (c) high risk (anaplastic); and (d) bilateral, multicentric, or bilaterally predisposed unilateral Wilms tumor. The assignment to treatment groups was based on the above mentioned prognostic factors. For surgical and pathological staging this is more straightforward since tumor histology is not modified by chemotherapy. Furthermore, non-Wilms tumors are not treated with potentially inappropriate preoperative chemotherapy, and patients from a very low risk group will not receive chemotherapy.

In the SIOP approach, tumors are treated with preoperative chemotherapy, followed by postoperative chemotherapy and radiotherapy, if necessary. The advantages of this approach are that tumors respond to treatment and are reduced in size, are better encapsulated, and easier to remove, which reduces the risk of pre- or intraoperative rupture and spillage. This reduces overall treatment in all stages. Preoperative chemotherapy also results in down-staging of tumors. Studies showed that of Wilms tumors primarily treated by surgery, only 62% could be completely resected (stage I–II), whereas this was 84% in patients who underwent preoperative chemotherapy. Finally, preoperative therapy provides in vivo information on treatment response, and allows identification of tumors which are chemotherapy resistant.

In the United Kingdom, the SIOP approach has been followed since 2001, but including pre-chemotherapy biopsy for the sole purpose of pre-therapy confirmation of the tumor as Wilms tumor. The percutaneous cutting needle biopsy is not associated with an increased risk of local relapse and therefore does not imply a higher stage.

These two different approaches have resulted in remarkably similar outcome, with overall survival over 90% making Wilms tumor one of the childhood cancers with excellent prognosis. In Britain, 5-year survival of children with renal cancer was 59% for those diagnosed in 1971–75 and 75% for those diagnosed in 1976–80. In population-based data from Europe and North America, 5-year survival has generally been over 85% for Wilms tumor and over 80% for all renal tumors combined. In Cuba, the 5-year survival for renal tumors diagnosed in 1988–89 was 62%. In Bangalore, India, 5-year survival for Wilms tumor in 1982–89 was only 27%. Treatment has a high success rate in specialist centers in developing countries, but overall survival is adversely affected because patients discontinue treatment.

As a result of increasing survival rates there are now substantial numbers of long-term survivors of Wilms tumor in the general population. As with other childhood cancers, one of the most serious sequelae of Wilms tumor is development of a subsequent primary neoplasm. Large studies of subsequent malignancies after Wilms tumor showed that leukemia, skin carcinoma, and solid tumors of the digestive organs, breast, thyroid, bone, and CNS are the most common second tumors. Occurrence of a subsequent malignancy has a severe effect on survival. Two-thirds of patients who developed leukemia have died, with median time from diagnosis of leukemia to death of 10 months. Age-specific mortality was increased 15-fold for subjects who developed a solid second malignancy. Risk of leukemia was highest in the most recently diagnosed patients, whereas the risk of solid tumor decreased for more recent decades of Wilms tumor diagnosis. Late causes of death among 5-year survivors are subsequent malignancies, cardiomyopathy and congestive heart disease, and end-stage renal disease.

Conclusion

The last decades have shown a remarkable progress in classification, treatment, and understanding of the pathology and molecular biology of Wilms tumor. As a rare tumor, it continues to represent a diagnostic problem and awareness of the potentially complex pathological features of this malignancy is required for accurate diagnosis, subtyping, and staging to allow appropriate treatment. Preoperative chemotherapy affects histological and staging features, and diagnostic pathologists should be familiar with these when assessing such tumors. Molecular biology markers are likely to play an even more important role for diagnosis and prognosis in the future but pathological examination continues to represents the gold standard for diagnosis, subtyping, treatment, and prognosis.

Further Reading

Beckwith, J.B., 1993. Precursor lesions of Wilms tumor: Clinical and biological implications. Medical and Pediatric Oncology 21, 158–168.

Breslow, N.E., Beckwith, J.B., Perlman, E.J., Reeve, A.E., 2006. Age distributions, birth weights, nephrogenic rests, and heterogeneity in the pathogenesis of Wilms tumor. Pediatric Blood and Cancer 47, 260–267.

Dome, J.S., Perlman, E.J., Graf, N., 2014. Risk stratification for Wilms tumor: Current approach and future directions. American Society of Clinical Oncology Educational Book 215–223.
Dome, J.S., Graft, N., Geller, J.I., et al., 2015. Advances in Wilms tumor treatment and biology: Progress through international collaboration. Journal of Clinical Oncology 33, 1–9.
Graf, N., Tournade, M.F., de Kraker, J., 2000. The role of preoperative chemotherapy in the management of Wilms tumor. The SIOP studies. International Society of Pediatric Oncology. Urological Clinics of North America 27, 443–454.
Hohenstein, P., Pritchard-Jones, K., Charlton, J., 2015. The yin and yang of kidney development and Wilms tumors. Genes & Development 29, 467–482.
Israels, T., Riberio, R.C., Molyneux, E.M., 2010. Strategies to improve care for children with cancer in sub-Saharan Africa. European Journal of Cancer 46, 1960–1966.
Perlman, E.J., 2005. Pediatric renal tumors: Practical updates for the pathologist. Pediatric and Developmental Pathology 8, 320–338.
Pritchard-Jones, K., Dome, J.S. (Eds.), 2016. Renal tumors of childhood. Biology and therapy. Springer-Verlag, Berlin-Heidelberg.
Royer-Pokora, B., 2013. Genetics of pediatric renal tumors. Pediatric Nephrology 28, 13–23.
Scott, R.H., Stiller, C.A., Walker, L., et al., 2006a. Syndromes and constitutional chromosomal abnormalities associated with Wilms tumour. Journal of Medical Genetics 43, 705–715.
Scott, R.H., Walker, L., Olsen, Ø.E., et al., 2006b. Surveillance for Wilms tumour in at-risk children: Pragmatic recommendations for best practice. Archive of Disease of Childhood 91, 995–999.
Vujanić, G.M., Sandstedt, B., 2010. The pathology of Wilms tumour (nephroblastoma): the International Society of Paediatric Oncology approach. Journal of Clinical Pathology 63, 102–109.
Vujanić, G.M., Sandstedt, B., Harms, D., et al., 2002. Revised International Society of Paediatric Oncology (SIOP) working classification of renal tumors of childhood. Medical and Pediatric Oncology. 38, 79–82.
Weirich, A., Leuschner, I., Harms, D., et al., 2001. Clinical impact of histologic subtypes in localized nonanaplastic nephroblastoma treated according to the trial and study SIOP-9/GPOH. Annals of Oncology 12, 311–319.

Relevant Websites

http://www.cancer.net/cancer-types/wilms-tumor-childhood/introduction - Cancer.Net. Wilms Tumor - Childhood: Introduction.
https://www.childrensoncologygroup.org/index.php/wilmstumorandotherkidneycancers - Children's Oncology Group. Wilms Tumor and Other Kidney Cancers.
http://www.healthline.com/health/wilms-tumor - Healthline. Wilms' Tumor.
https://medlineplus.gov/wilmstumor.html - MedlinePlus. Wilms Tumor.
https://www.cancer.gov/types/kidney/patient/wilms-treatment-pdq - National Cancer Institute. Wilms Tumor and Other Childhood Kidney Tumors Treatment (PDQ®)–Patient Version.
http://www.siop-rtsg.eu/ - SIOP-Renal Tumour Study Group (SIOP-RTSG).
https://www.stjude.org/disease/wilms-tumor.html - St. Jude Children's Research Hospital. Wilms Tumor.
https://patient.info/doctor/wilms-tumour-pro - Patient.info. Wilms' Tumour.
https://www.cancer.org/cancer/wilms-tumor/about/what-is-wilms-tumor.html - American Cancer Society. Wilms Tumor.

Wnt Signaling in Intestinal Stem Cells and Cancer

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Glossary

Adenoma A benign tumor formed from glandular structures in epithelial tissue.

Carcinoma A cancer arising from epithelial tissues, such as the intestinal epithelium.

Epithelium The layer of cells that lines the surface of an organ. Epithelial cells attach to a basement membrane that separates them from connective tissue and are held together by specialized cell adhesions. Intestinal epithelial cells are a single layer of columnar cells that face the lumen of the intestinal tract, are involved in absorption of nutrients and water, and form a protective barrier.

Germline mutation A mutation that is passed from parent to offspring.

Homeostasis Maintenance of a stable equilibrium; in the case of an epithelium, generation of new cells to replace dying cells without a change in the total number of cells. If too many new cells form, this leads to neoplasia. If insufficient cells form to replace dying cells, epithelial functions fail.

Ligand A molecule that binds to a receptor to activate or inhibit signaling.

Malignant A cancer that can invade outside of its tissue of origin, leading to metastasis.

Neoplasia New cell growth out of proportion to normal tissues. This can be benign, as in adenomas, or malignant, as in cancer.

Oncogene A gene that causes cancer when activated, for example, by mutation.

Palmitoylation The covalent attachment of palmitic acid, a fatty acid, to proteins, causing the protein to localize to hydrophobic surfaces such as the plasma membrane. More generally, acylation is the attachment of fatty acids to proteins through an acyl linkage.

Somatic mutation A mutation that occurs after fertilization, that is, not a germline mutation.

Tumor suppressor gene A gene that normally prevents cancer progression and when mutated allows for cancer progression.

Wnt The name of the signaling pathway described here *and* the family of secreted proteins that serve as ligands for the Wnt pathway. "Wnt" is a portmanteau of *Wingless* (the first *Wnt* gene identified in *Drosophila*) and *Int-1*, the first *Wnt* gene discovered in mammalian cells.

Introduction

Wnt signaling regulates cell fate specification and embryonic pattern formation throughout development and maintains self-renewal of somatic stem cells in multiple tissues, including intestinal, neural, and epidermal stem cells. Unrestrained activation of canonical Wnt signaling due to loss of function mutations in pathway inhibitors or gain of function mutations in pathway activators promotes carcinogenesis. For example, loss of the tumor suppressor *Adenomatous Polyposis Coli* (*APC*) causes the inherited cancer predisposition syndrome familial adenomatous polyposis (FAP) and somatic mutations in *APC* underlie the pathogenesis of most colorectal carcinomas. This article will describe the canonical Wnt signaling pathway and then summarize the role of APC and other Wnt pathway components in the regulation of intestinal stem cells and colorectal cancer.

Canonical Wnt/β-Catenin Signaling

The canonical Wnt signaling pathway is activated by the binding of Wnts to receptors on the cell surface, leading to stabilization of the transcription activator β-catenin and culminating in the transcription of Wnt target genes (Fig. 1). This "canonical" pathway, as delineated by genetic and biochemical studies in model organisms including *Drosophila*, *Xenopus*, mice, as well as human colorectal cancer cells, is characterized by a conserved complement of intracellular signaling components that includes the Wnt coreceptors Frizzled and LRP5/6, the β-catenin destruction complex, β-catenin, and a conserved DNA binding complex that is activated by β-catenin. In the absence of Wnts, the destruction complex promotes rapid, proteasomal degradation of β-catenin. When the pathway is activated, β-catenin is stabilized, translocates to the nucleus, and activates target gene transcription. The core components of the Wnt signal transduction machinery will be introduced in more detail in the following section.

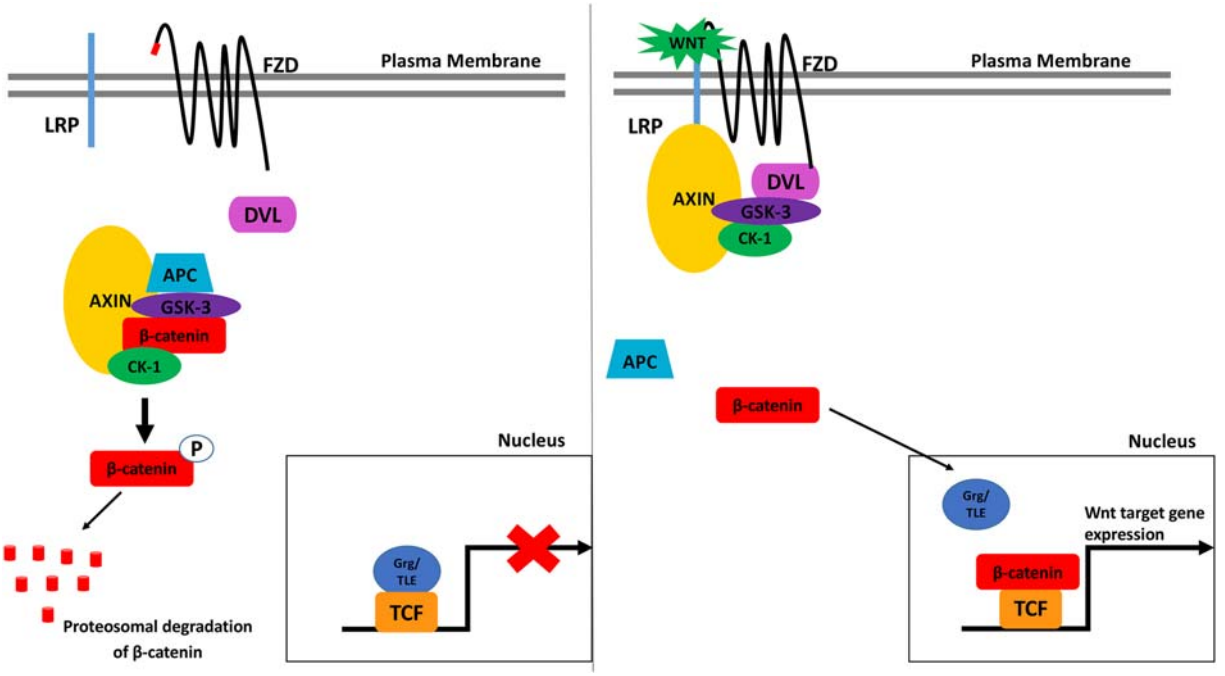

Fig. 1 The canonical Wnt signaling pathway. Panel A: In the absence of Wnts, β-catenin is targeted for destruction by a complex comprising APC, Axin, CK1α, and GSK-3. Sequential phosphorylation by CK-1α and GSK-3 marks β-catenin for ubiquitination by the βTrCP ubiquitin ligase complex and subsequent proteasomal degradation. Panel B: The pathway is activated when Wnts bind to the Fzd CRD and the extracellular domain of LRP, which results in the recruitment of the Axin complex, phosphorylation of the LRP5/6 C-terminus, and inhibition of GSK-3 mediated phosphorylation of β-catenin. Unphosphorylated β-catenin translocates into the nucleus, binds to TCF (or the related factor LEF), displaces the repressor Groucho/TLE, recruits coactivator proteins, and thereby activates transcription of downstream target genes.

Wnt Signaling Components

Wnts

The Wnt family in mammals consists of 19 secreted glycoproteins with conserved spacing of multiple cysteine residues. Wnts are posttranslationally modified, including N-linked glycosylation and palmitoylation at a unique serine residue within a highly conserved sequence motif. Palmitoylation has been proposed to play a role in secretion, receptor binding, and tethering of the ligand to extracellular surfaces. Wnt palmitoylation occurs in the endoplasmic reticulum and requires Porcupine (Porc); Wnts are then escorted to the membrane for secretion by Wntless (Wls). The significance of multiple Wnt genes is still not well understood, although they have been broadly subcategorized into "canonical" Wnts (e.g., Wnt1, Wnt3a), which activate β-catenin-dependent transcriptional programs, transform mammary cells, and induce dorsal development in *Xenopus*, and "noncanonical" Wnts (e.g., Wnt5a/b), which have diverse functions, including regulation of polarized cell movements, and appear to activate distinct intracellular signaling pathways. However, this distinction may depend more on the complement of Wnt receptors present in the receiving cell as well as potential synergistic interactions between different Wnt proteins.

The palmitoylation of Wnts markedly reduces their solubility and has limited the biochemical analysis of Wnt proteins, but a crystal structure was recently solved for *Xenopus* Wnt8 in complex with the extracellular domain of the Wnt receptor Frizzled8 (Fzd8). This structure revealed that Wnt8 binds to the Fzd extracellular domain at two sites with the palmitoyl group (site 1) partially bound to a hydrophobic groove in Fzd. This groove only buries 60% of the palmitoyl group, however, with the remainder of this hydrophobic surface apparently exposed to solvent. Unexpectedly, mutation of the acylation site reduces but does not eliminate the binding or activity of Wnt8, Wnt3a, and the *Drosophila* Wnt1 homolog wingless (wg). Furthermore, non-Wnt ligands such as Norrin, which is not acylated, and "surrogate" Wnt ligands that lack the acylation site also bind the Fzd CRD and activate signaling. The activity of Wnt proteins lacking the conserved palmitoylation site raises the possibility that acylation serves an alternative function, such as restriction of diffusion by tethering the ligand to the plasma membrane.

Frizzleds and LRP5/6

In the canonical pathway, Wnts bind to a surface receptor complex that includes Frizzled (Fzd) and the coreceptor LDL receptor-related proteins 5 or 6 (LRP5/6). There are 10 mammalian Fzds, each containing an extracellular cysteine-rich domain (CRD) that binds Wnts, 7 transmembrane domains, and an intracellular carboxy-terminal domain that is not well conserved among

the different family members. Although Fzds are topologically similar to G-protein coupled receptors, the sequence similarity is limited.

LRP5/6 are type 1 membrane receptors that are 70% identical to each other and are functionally redundant in most instances. These single transmembrane receptors contain an extracellular domain made up of four tandem β-propeller/epidermal growth factor repeats and three LDLR type A repeats. Wnts bind initially to Fzd with high affinity and then bind to the extracellular domain of LRP to form a bridge between LRP and Fzd. This interaction leads to recruitment of Disheveled, phosphorylation of the LRP5/6 by glycogen synthase kinase-3 (GSK-3) and casein kinase-1, and recruitment of the destruction complex to the intracellular plasma membrane.

Disheveled

Disheveled (Dvl, Dsh) functions downstream of Frizzled and is required for the transmission of Wnt signals. It contains a Disheveled–Axin interaction (DIX) domain that can bind to Axin or to itself and a central PDZ domain that interacts with Frizzled. When Wnts bind to Frizzled and LRP, Disheveled is recruited to Frizzled. Disheveled can then oligomerize via the DIX domain, and this has been proposed to form a docking site for the Axin complex, described below.

Axin

Axin is a cytosolic scaffold for core Wnt signaling components, including Dvl, APC, casein kinase 1α (CK1α), GSK-3, and β-catenin. The Axin complex serves two, seemingly opposing functions in the Wnt pathway. Recruitment of the Axin complex to LRP5/6 is essential for pathway activation, yet, in the absence of Wnts, the complex inhibits the pathway by facilitating the degradation of β-catenin. Thus, loss of function mutations in *Axin* activate Wnt signaling, induce dorsal axis duplication in mouse and *Xenopus* embryos (hence the name "Axin" for axis inhibitor), and promote carcinogenesis. Axin binds to APC through the N-terminal RGS domain, which bears sequence similarity to regulators of G protein signaling but lacks a key residue conserved in RGS proteins. The GSK-3 interaction domain (GID) and the CK1α binding sites are adjacent to the β-catenin interaction domain, facilitating sequential phosphorylation of β-catenin. In addition to β-catenin, GSK-3 phosphorylates Axin to regulate its stability and increase binding to APC and β-catenin. Axin also interacts with itself through a dimerization domain in the C-terminus and has been reported to interact with multiple other signaling molecules, including protein phosphatases-1 and 2A and the tuberous sclerosis complex.

Mammals have two *Axin* genes; *Axin1* and *Axin2*. *Axin1* is expressed ubiquitously and is likely the predominant form involved in basal Wnt suppression and LRP phosphorylation. *Axin2* expression (also known as *Axil* or *conductin*) is generally induced by Wnt pathway activation and is considered a feedback inhibitor of the pathway. *Axin2* also serves as a useful expression marker for pathway activation.

APC

APC is a classical tumor suppressor gene that encodes an approximately 310 kd protein. In addition to antagonizing Wnt signaling by promoting β-catenin degradation, APC regulates cell adhesion, migration, chromosome segregation, spindle assembly, apoptosis, and neuronal differentiation. APC contains multiple conserved functional domains (Fig. 2). The domains involved in Wnt signaling are located in the central part of the protein. In vertebrate APC, these include three 15-amino acid repeats and seven

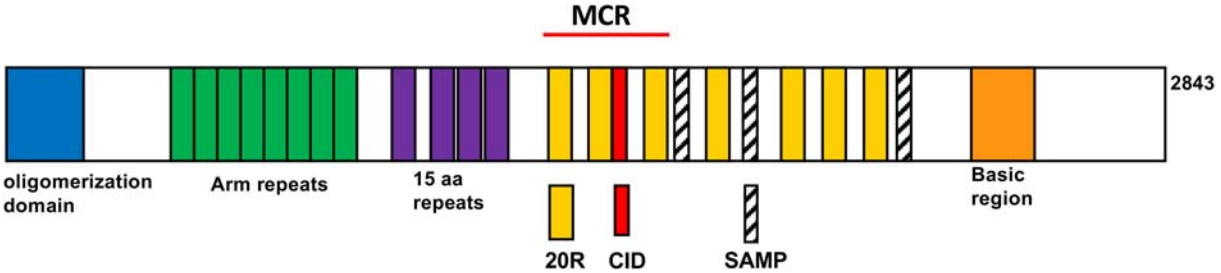

Fig. 2 APC conserved functional domains. APC contains multiple conserved functional domains involved in the regulation of pathways such as cell adhesion, migration, chromosome segregation, spindle assembly, apoptosis, and neuronal differentiation. The domains involved in Wnt signaling include (in vertebrate APC) four 15-amino acid repeats (*purple*) and seven 20-amino acid repeats (*yellow*), all of which bind to β-catenin except the second 20 amino acid repeat; three Ser-Ala-Met-Pro (SAMP) repeats (*hatched*), which bind to the RGS domain of Axin; and a β-catenin inhibitory domain (CID; *red*). Most truncating mutations in *APC* occur in the mutation cluster region (MCR), indicated here by a *solid line*. APC also contains an N-terminal oligomerization domain and a C-terminal basic region that interacts with microtubules.

20-amino acid repeats, all of which bind to β-catenin except the second 20-amino acid repeat; three Ser-Ala-Met-Pro (SAMP) repeats, which bind to the RGS domain of Axin; and a β-catenin inhibitory domain (CID). The CID and second 20-amino acid repeat are required for suppression of Wnt signaling, although neither motif binds to β-catenin. Truncating mutations in APC associated with Wnt pathway activation and carcinogenesis frequently occur in the mutation cluster region (MCR), preventing expression of these motifs. Furthermore, a fragment of APC encoding these motifs can restore normal levels of Wnt signaling and reverse APC mutant phenotypes.

However, the mechanism by which APC suppresses Wnt signaling is not well understood. Several hypotheses to explain APC's role in the destruction complex have been put forward. APC may recruit β-catenin to the destruction complex, protect β-catenin from dephosphorylation to ensure subsequent ubiquitination and degradation, act as an additional scaffold for the complex, or facilitate the transfer of phosphorylated β-catenin from the Axin scaffold to the proteasome, as APC competes with Axin for the same binding sites on β-catenin. Alternatively, APC may enhance the enzymatic activity of GSK-3 to increase phosphorylation of β-catenin; as Wnt pathway activation causes dissociation of APC from the Axin complex, this latter hypothesis can also explain how Wnt signaling reduces GSK-3 enzymatic activity and thereby reduces β-catenin phosphorylation.

Protein Kinases GSK-3α/β and CK1α

The Axin complex drives β-catenin turnover by supporting sequential phosphorylation by CK1α and GSK-3. CK1α provides a priming phosphorylation at serine-45 of β-catenin that is essential for subsequent phosphorylation by GSK-3 at threonine-41, serine-37, and serine-33. GSK-3 also phosphorylates APC and Axin; phosphorylation of Axin increases binding with β-catenin and increases the stability of Axin. Mammals have two GSK3 genes, *GSK3A* and *GSK3B* which encode the highly homologous and mostly redundant isoforms GSK-3α and GSK-β (here referred to collectively as GSK-3). Both isoforms of GSK-3 are constitutively active serine/threonine kinases that suppress Wnt signaling, glycogen synthesis, and signaling through Akt, hedgehog, Hippo, and mechanistic target of rapamycin complex-1 (mTORC1) dependent pathways. Thus, inhibition of GSK-3 activates downstream signaling in each case. For example, insulin signaling inhibits GSK-3 by a phosphatidylinositol 3-kinase (PI3K)/Akt-dependent mechanism; Akt phosphorylates GSK-3 on an N-terminal serine (serine-21 in GSK-3α and serine-9 in GSK-3β), which inhibits GSK-3 activity by creating a pseudo-substrate. Wnt signaling inhibits GSK-3 activity through a completely separate mechanism unrelated to N-terminal phosphorylation. GSK-3 involved in Wnt signaling is sequestered in the Axin complex and inaccessible to Akt. Several distinct but nonexclusive mechanisms for Wnt-mediated GSK-3 inhibition have been proposed. As above, Wnt-induced dissociation of APC from the Axin complex may reduce GSK-3 activity within the complex; alternatively, the phosphorylated C-terminus of LRP5/6 can inhibit GSK-3 directly; additionally, sequestration of Axin and GSK-3 into multivesicular bodies has been proposed to suppress GSK-3 phosphorylation of β-catenin although this is likely too slow to account for the rapid inhibition of GSK-3 by Wnts.

β-Catenin

β-Catenin, encoded by the *CTNNB1* gene, was first recognized for its role in cadherin-based adherens junctions that stabilize cell–cell contacts where the vast majority of β-catenin is sequestered (in epithelial cells). β-Catenin was subsequently shown to have an independent role in activation of transcription of Wnt target genes. β-Catenin contains 12 armadillo repeats that bind to both APC and Axin. As described above, phosphorylation of Axin-bound β-catenin by CK-1 and GSK-3 marks β-catenin for ubiquitination and proteasomal degradation. This pool of rapidly turned over β–catenin is distinct from cadherin-associated β-catenin, which has a substantially longer half-life. Wnt activation stabilizes cytosolic β-catenin, which then enters the nucleus where it binds TCF/LEF family proteins and activates transcription. β-Catenin contains both N- and C-terminal activation domains. The N-terminal activation domain binds the coactivator Pygopus through the adaptor protein Bcl9. The C-terminal activation domain, distal to the armadillo repeats, binds the histone acetyltransferase CBP or p300. β-Catenin also recruits the protein arginine methyltransferase PRMT2, which is required for Wnt signaling in *Xenopus* and colorectal cancer cell lines.

TCF/LEF

T-cell factor (TCF)/lymphoid enhancer factor transcription factors are the major nuclear mediators of the Wnt signaling pathway. They contain a conserved high mobility group (HMG) box and a basic tail which together comprise the DNA-binding domain. In the absence of β-catenin (e.g., Wnt signaling off), TCF/LEF repress transcription of Wnt target genes due to recruitment of a repressor complex that includes Groucho/transducin-like enhancer of split (TLE) and histone deacetylases. When the pathway is activated, β-catenin binds to a conserved motif near the amino terminus, displaces Groucho/TLE, and activates transcription of target genes. TCF/LEF proteins display considerable heterogeneity as a result of alternative promoters and mRNA splicing, and this allows for Wnt signals to be interpreted differently in terms of gene targeting, as well as interacting with specific repressors or activating cofactors.

Pathway Inhibitors

This section describes core components of the canonical Wnt signaling machinery, but it should be emphasized that many more factors have been implicated at each step of this signaling cascade. Although a full description is beyond the scope of this article, it should be noted that multiple Wnt pathway inhibitors and coactivators have been described. For example, the secreted factors dickkopf (dkk), frizzled related proteins (sFRPs), Wnt inhibitory factor (WIF), Notum, and sclerostin interfere with Wnt binding to the Fzd CRD or to LRP5/6, whereas R-spondin is a secreted factor that potentiates canonical Wnt signaling in intestinal stem cells through its interaction with the Lgr receptor family. In addition, several Wnt induced intracellular antagonists have been described, including Axin2 (see above), Naked cuticle (Nkd), and, in intestinal cells, the transmembrane E3 ubiquitin ligases Rnf43 and Znrf3.

Putting It Together: Activation of Wnt Signaling

In the absence of Wnts, β-catenin is targeted for destruction by a complex comprising APC, Axin, CK1, and GSK-3. Sequential phosphorylation by CK-1 and GSK-3 marks β-catenin for ubiquitination by the β-transducin repeat–containing protein (βTrCP) ubiquitin ligase complex and subsequent proteasomal degradation. The pathway is activated when Wnts bind to coreceptors Fzd and LRP which results in the recruitment of the Axin complex to LRP5/6, phosphorylation of the LRP5/6 C-terminus, and inhibition of GSK-3 mediated phosphorylation of β-catenin. Unphosphorylated β-catenin translocates into the nucleus, binds to TCF/LEF, displaces the repressor Groucho/TLE, recruits coactivator proteins, and thereby activates transcription of downstream target genes, including Axin2, cyclinD1, and many others.

Wnt Signaling and Intestinal Stem Cells

Intestinal Stem Cells

Intestinal epithelial cells are repeatedly exposed to pathogens, xenobiotics, and other toxic agents and must be able to regenerate rapidly after damage. This ability to regenerate depends on a robust intestinal stem cell (ISC) compartment that includes actively proliferating crypt base columnar (CBCs) stem cells, in which canonical Wnt signaling induces expression of *Lgr5* and mitotic self-renewal, as well as a distinct, rare population of slowly cycling ISCs lacking canonical Wnt pathway activity referred to as reserve stem cells.

$Lgr5^+$ CBCs give rise to cycling transit amplifying progenitors and all differentiated cell types in the intestinal epithelium, as shown by lineage tracing. Although genetic ablation of $Lgr5^+$ cells indicated that they are dispensable for intestinal homeostasis under basal conditions, they are essential for a robust regenerative response to genotoxic treatments such as irradiation. The expendable nature of $Lgr5^+$ CBCs during intestinal homeostasis as well as the sensitivity of CBCs to DNA damage suggested that a distinct population of intestinal epithelial cells compensate for CBC loss.

Reserve ISCs are distinct from CBCs and are indispensable for epithelial homeostasis under basal conditions. These cells were first identified by insertion of a Cre recombinase dependent reporter into the *Bmi1* gene, but cells that share characteristics of $Bmi1^+$ cells have since been identified, including those marked by insertion of CreER into *Hopx*, *mTert*, and subpopulations of much larger groups of cells marked by broadly expressed reporters inserted into *Krt19*, *Lrig1*, *Axin2*, and *Sox9*. Reserve ISCs are characterized by limited response to Wnt signals, slow cycling relative to CBCs under basal conditions, resistance to DNA damage, and a strong proliferative response to such injury. They are also referred to as "quiescent" stem cells and as '+4' cells based on their position above the crypt base, although several different cell types have been observed at this position.

Although reserve stem cells are refractory to Wnt signaling, they give rise to Wnt-responsive CBCs during intestinal homeostasis and after injury consistent with a model in which reserve stem cells reside upstream of more active and shorter-lived, Wnt-driven CBC stem cells. Interconversion between these active and dormant stem cell states, however, remains a possibility.

Wnt Signaling in ISC Homeostasis

Wnt signaling is required for crypt base proliferation, differentiation into secretory cells, and migration along the crypt-villus axis. The role of Wnt signaling in intestinal crypt biology was demonstrated in one of the earliest functional studies through disruption of the *Tcf7l2 (Tcf4)* gene, which caused loss of proliferative compartments in the intestinal epithelium of neonatal mice. Similarly, inhibition of Wnt signaling in adult intestinal epithelial cells, either by deletion of *Tcf7l2* or overexpression of the Wnt inhibitor *Dickkopf 1* (*Dkk1*), caused loss of CBCs and decreased epithelial proliferation. Conversely, CBC-specific activation of the Wnt pathway, for example, by inactivating *APC*, enhances proliferation of ISCs and leads to adenoma formation, as described in greater detail below.

The importance of Wnt signaling for maintenance of the intestinal epithelium raises questions about the source of the Wnt ligand in the ISC niche and its range of action. Paneth cells secrete Wnt3 and were hypothesized to form the CBC niche. However, genetic deletion of Paneth cells had no effect on CBCs or intestinal homeostasis under basal conditions and did not affect regeneration following radiation injury. Furthermore, selective deletion of *Wnt3* or inhibition of Wnt secretion in epithelial cells had no effect on ISC activity, suggesting a nonepithelial source of Wnt. In contrast to Paneth cell ablation, ablation of a rare population of $Foxl1^+$ subepithelial cells (termed telocytes), which express *Wnts* and other niche factors, impairs Wnt signaling and proliferation in

the ISC compartment. Furthermore, knockout of *porcupine*, which is required for Wnt processing and secretion, in *Foxl1*$^+$ telocytes blocks Wnt signaling responses in intestinal crypt cells and impairs ISC maintenance and renewal, demonstrating that *Foxl1*$^+$ telocytes are a critical source of Wnts for ISCs.

Wnt target gene expression is graded in the intestinal epithelium, declining as cells progress away from the crypt base. Although this might suggest a gradient of extracellular Wnt protein, the palmitoylation of Wnts markedly limits their solubility and likely their diffusion in the intestinal epithelium. Consistent with this, newly secreted Wnt3 in intestinal organoid cultures appears to bind only to adjacent cells, and distribution of Wnt3 away from its source was proposed to occur through cell division rather than extracellular diffusion. In *Drosophila*, a membrane tethered form of Wingless (Wg) can replace the endogenous protein in the imaginal disk; apparent action at a distance from the source of ligand was shown to be due to exposure from adjacent cells at an earlier stage of development, followed by separation of secreting and responding cells due to cell division, and, at a later stage of development, delayed activation of Wg target genes through "memory" of the prior exposure to Wg. We hypothesize that this memory could be explained by Wnt-dependent chromatin modifications conferring epigenetic memory for prior Wnt exposure, as demonstrated for Wnt responses in *Xenopus* embryos. A similar "memory" of Wnt exposure could also account for Wnt responses in cells that were once adjacent to, but are now distant from the source of Wnt in the intestinal crypt.

These studies have established the hierarchical nature of the ISC within the intestinal crypt, the complexity of the ISC niche, the importance of epithelial and mesenchymal cell interactions, and the central role of Wnt signaling (as well as other secreted factors) in intestinal epithelial homeostasis. Disruption of this complex regulatory system interferes with intestinal homeostasis. For example, the *Clostridium difficile* toxin TcdB, binds to the extracellular domain of Fzds in intestinal cells and inhibits Wnt signaling, and this has been proposed as a pathogenic mechanism for *C. difficile* colitis. In contrast, mutations that inappropriately activate Wnt signaling lead to adenoma formation and cancer, as described in the next section.

Wnt/β-Catenin Signaling in Cancer

Genetic analysis of inherited cancer predisposition syndromes together with molecular, biochemical, and developmental biology studies have established the central importance of genetic lesions that activate key signaling pathways in multiple cancers including colorectal cancers. A key to this line of investigation was the study of Familial Adenomatous Polyposis (FAP), an autosomal dominant disorder characterized by formation of numerous colonic polyps at a young age, transformation to adenocarcinoma, and multiple extracolonic neoplasms. Genetic analyses of families with FAP identified the *APC* gene on the long arm of chromosome 5 and showed that the disorder is caused by truncating mutations in *APC* followed by loss of the wild-type allele (loss of heterozygosity or LOH). Similar somatic mutations in *APC* were found in approximately 80%–90% of sporadic colon cancers.

The Wnt pathway was linked to colon cancer when it was found that APC interacts with both β-catenin and GSK-3, and additionally that truncating mutations in *APC* stabilize β-catenin protein and constitutively activate downstream Wnt signaling. *APC* is the most commonly mutated gene in sporadic colorectal cancer, and *APC* mutations are among the earliest driving mutations in the transformation to cancer. The majority of these mutations occur in the "Mutation Cluster Region" (MCR), a region spanning the central region of the protein and containing the 20 amino acid repeats, the SAMP repeats, and the CID (**Fig. 2**). These mutations are often frameshift or point mutations leading to premature termination and expression of a truncated protein lacking the SAMP repeats and other Wnt signaling motifs. Subsequent loss of the wild-type copy of APC then activates Wnt signaling. Importantly, expression of full-length *APC* can rescue colorectal cancer cells back to functioning "normal" cells in vivo even in the presence of activated *KRAS* and *p53* loss of function mutations. Furthermore, expression of an *APC* gene fragment containing the Wnt signaling motifs restores turnover of β-catenin, suppresses Wnt signaling, and rescues *Apc* loss of function phenotypes in animal models. There may be selective pressure for mutations in the MCR in colorectal cancer, as larger deletions in *APC* are uncommon, possibly because the N-terminal region of APC serves a vital function and/or that the truncated *APC* alleles achieve an intermediate level of Wnt/β-catenin signaling that is critical for neoplastic transformation.

Although *APC* mutations are found in ~80% of colon cancers, a substantial fraction of colon cancers with wild-type *APC* have gain of function mutations in *β-catenin/CTNNB1* that prevent phosphorylation and thereby stabilize the protein, providing compelling genetic support for the core roles of APC and β-catenin in Wnt signaling and colon cancer. *CTNNB1* mutations are also found in cancers that occur outside the gut, such as endometrioid ovarian cancer.

Somatic mutations in *Axin (Axin1)* and *Axin2* have also been found in sporadic cancers, including hepatocellular, colorectal, and other cancers. As reviewed elsewhere, the role of *AXIN1* loss of function mutations in HCCs is well supported, but the functional significance of *AXIN1* and *AXIN2* sequence variants in colorectal cancer remains to be established.

Constitutive activation of Wnt signaling is an essential early step in the development of colorectal cancer, but additional steps are required for malignancy. This was evident from the observations that FAP patients develop hundreds to thousands of adenomas due to LOH for *APC*, but only a few of these transform to cancer. Similarly, up to 50% of adults will develop colonic adenomas associated with LOH for *APC*, but only a fraction of these will develop cancer. Furthermore, *APC* mutations initially cause microadenomas and dysplastic aberrant crypt foci without morphological evidence of malignancy, indicating that additional steps are required for progression to cancer.

These and other observations led to the model that *APC* serves a gatekeeper function in the pathogenesis of colon cancer and that additional mutations are required in the progression to malignancy. The most common mutations include activating mutations in *KRAS* (found in 40% of sporadic CRCs), loss of the tumor suppressor *p53*, as well as mutations that either inactivate transforming

growth factor-β signaling or activate phosphatidylinositol-3 kinase (PI3K) signaling pathways. These mutations by themselves do not transform colon epithelia; for example, *p53* mutations are found in 80% of CRCs but germline mutations in *p53* do not predispose to CRC in the absence of *APC* mutations. Similarly, activating mutations in *KRAS* by themselves do not lead to CRC. A whole exome sequencing study of breast and colon tumors confirmed the high frequency of these mutations in CRC and revealed additional recurrent and potential driver mutations that occur at low frequency in CRC.

Divergence in Canonical Wnt Signaling

Studies of the "canonical" Wnt pathway have generally focused on the regulation of β-catenin stability and activation of Wnt target genes (the "Wnt/β-catenin pathway"). However, accumulating evidence suggests that the Axin/APC/GSK-3 complex is a signaling hub with β-catenin as just one of its multiple effectors (Fig. 3). Wnt signaling through Axin, APC, and GSK-3 stabilizes other proteins, determines mitotic spindle polarity in *C. elegans*, modulates transcription factors in the Hippo pathway, and activates mammalian target of rapamycin complex-1 (mTORC1) signaling. A truncating mutation in zebrafish *apc* also causes multiple β-catenin independent phenotypes.

These findings raise the possibility that β-catenin independent pathways contribute to the effects of *APC* loss in early adenoma formation. The central role of β-catenin is firmly established and further supported by the observations that overexpression of *β-catenin/CTNNB1* generates adenomas in mice and knockout prevents adenoma formation in *APC* mutant mice. However, nuclear β-catenin is observed infrequently in early FAP adenomas, sporadic human colonic polyps, or microadenomas from a rat FAP model, despite confirmed loss of heterozygosity of *APC* and increased levels of cytosolic β-catenin. Although this may indicate that β-catenin is active in the nucleus below the limit of detection by immunofluorescence, these results also raise the possibility that an increase in β-catenin following *APC* loss is not sufficient to initiate hyperproliferation and microadenoma formation.

An additional signal downstream of *APC* loss may be provided by activation of mTORC1, a nutrient sensor that regulates translation, metabolism, proliferation, and survival and is aberrantly activated in many cancers. mTORC1 is inhibited by the tuberous sclerosis complex (TSC1 and TSC2), and phosphorylation of TSC2 by GSK-3 enhances this inhibitory function. Therefore, inhibition of GSK-3 by loss of APC or upstream activation of Wnt signaling activates mTORC1. Wnt/β-catenin signaling can also increase transcription of TOR, a core component of the mTORC1 complex, and this may also contribute to increased mTORC1 activity in *APC* mutant neoplasms. Furthermore, mTORC1 activity is required for adenoma formation downstream of *APC* loss, as

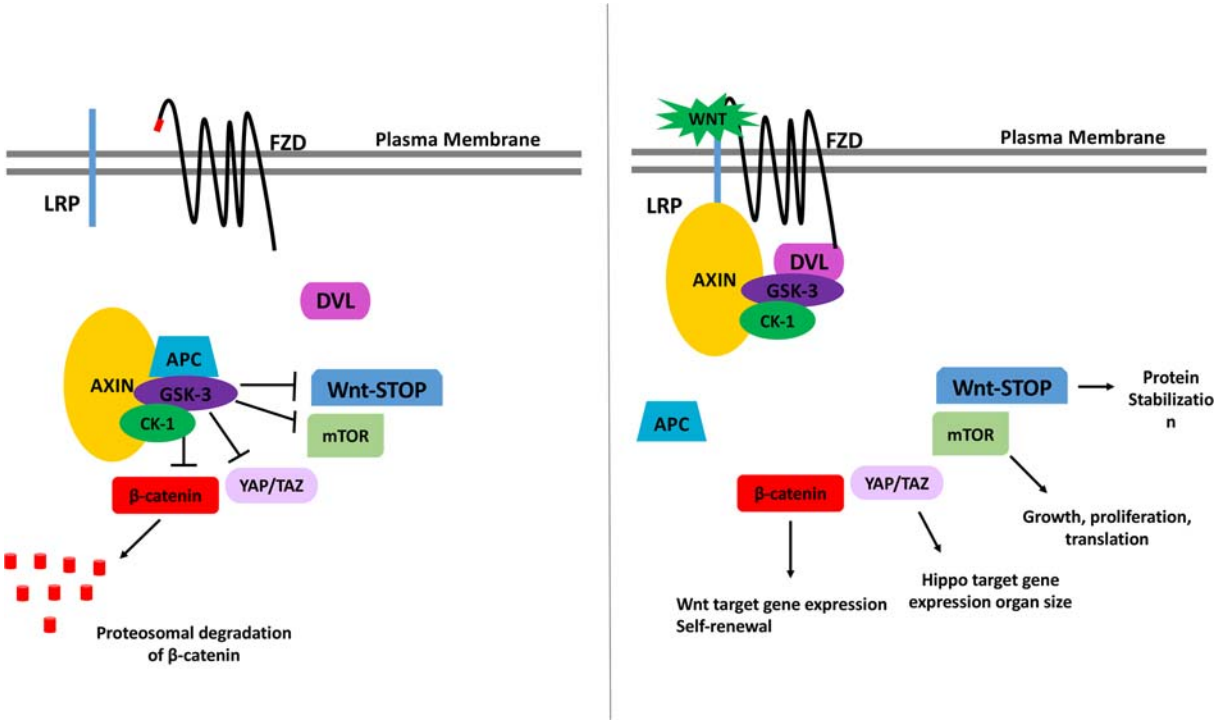

Fig. 3 The canonical Wnt signaling pathway diverges at the destruction complex to regulate β-catenin-independent pathways. In addition to stabilization of β-catenin, Wnt signaling through Axin, APC, and GSK-3 stabilizes other proteins through the Wnt-STOP pathway, modulates transcription factors in the Hippo pathway (e.g., YAP), and activates mammalian target of rapamycin complex-1 (mTORC1) signaling to regulate growth and proliferation. The "T" symbols in the left panel indicate inhibition and the *arrows* in the right panel indicate activation.

pharmacologic inhibition of mTORC1 blocks adenoma formation in *Apc* mutant mice. Thus, loss of *APC* may activate mTORC1 through β-catenin independent as well as β-catenin-dependent signaling.

The activation of mTORC1 by *APC* truncation mutations suggests an intriguing parallel between FAP and other hereditary disorders that are associated with mTORC1 activation and which cause neoplasias in multiple organs, including tuberous sclerosis, Peutz–Jeghers syndrome, and Cowden's syndrome. Tuberous sclerosis complex (TSC) is an autosomal dominant disorder associated with hamartomatous tumors of multiple organs caused by germline loss of either *TSC1* or *TSC2* which in turn activates mTORC1. Peutz–Jeghers and Cowden's syndromes similarly cause hamartomatous polyposis in multiple organ systems. Peutz–Jeghers syndrome is caused by mutations in LKB, an upstream positive regulator of TSC2, so that loss of LKB indirectly activates mTORC1. Similarly, Cowden's syndrome is caused by loss of function mutations in *PTEN*, which activate AKT and thereby also activate mTORC1. Although these syndromes are clinically and pathologically distinct, they share mTORC1 activation. This also raises the possibility that mTORC1 inhibitors currently in use clinically could be repurposed to control the extracolonic manifestations of FAP.

Hippo signaling regulates organ size through inhibition of protein kinases resulting in stabilization of the transcription factors YAP and TAZ. YAP is necessary for adenoma formation resulting from the loss of *APC* in mice and is activated in tubular adenomas of FAP patients. APC regulates other β-catenin-independent processes that may contribute to *APC* loss of function pathology, including retinoic acid synthesis, protein stability (Wnt/STOP), microtubule stability and polarity, as well as the BMP and ERK signaling pathways.

Therapeutic Targeting of the Wnt Pathway in Cancer

The Wnt pathway has been considered difficult to target with small molecule inhibitors, as the most "druggable" targets, enzymes such as GSK-3, CK1α, and ubiquitin ligase, inhibit the pathway, Indeed, GSK-3 inhibitors are used extensively to activate Wnt signaling, for example, in the culture of pluripotent stem cells, maintenance of somatic stem cells, and organoid culture. In contrast, positive regulators function mostly through protein–protein interactions (Wnt, Fzd, LRP, β-catenin), which are inherently more difficult to target with small molecules. However, a number of small molecules as well as protein-based inhibitors have been developed that target multiple steps in the pathway, from processing and secretion of Wnts to transcriptional activation by the β-catenin/TCF complex. For example, small molecules that inhibit the acyltransferase activity of Porc and thereby reduce secretion of Wnts have been tested in animal models and are in phase I clinical trials. Small molecule inhibitors of tankyrase, a poly ADP-ribose polymerase that targets Axin for degradation, inhibit Wnt signaling by increasing Axin levels to promote β-catenin turnover. Several tankyrase inhibitors have been tested in preclinical models and while significant gastrointestinal toxicity has limited their clinical use, inhibitors such as XAV939 have been useful in the laboratory for in vitro pathway analysis. PRI-724 is a small molecule inhibitor of β-catenin/CBP interaction that has been tested in preclinical models and a phase I clinical trial for pancreatic cancer, and other small molecules that interfere with β-catenin/TCF and β-catenin/Bcl9 interactions have been tested in preclinical models. Neutralizing antibodies to the Fzd CRD as well as Fzd-mimetic polypeptides have also advanced to phase I trials. An in-depth description of Wnt pathway inhibitors can be found in Krishnamurthy and Kurzrock (see Further Reading).

Conclusions

Years of research on a variety of model organisms as well as genetic and molecular investigation of human cancers have delineated the canonical Wnt signaling pathway, which is activated by secreted ligands termed Wnts resulting in the transcription of Wnt target genes. This highly conserved pathway is critical in normal development as well as stem cell homeostasis, and yet overactivation is an early step in cancer pathogenesis, as first established through characterization of mutations in *APC* and *β-catenin/CTNNB* in both hereditary and sporadic colon cancer in humans. The field is now at a point where our understanding of the pathway can be exploited for therapeutic interventions. Currently pharmacological activation of the pathway is a key tool in regenerative medicine and in the maintenance of embryonic and somatic stem cells. Conversely, inhibiting the pathway is a major goal in the treatment of colon cancer and other Wnt-driven malignancies. The Wnt pathway had traditionally been thought of as difficult to inhibit pharmacologically because most of the activating steps involve protein–protein interactions rather than more easily targeted enzymatic steps. However, numerous drugs targeting various points along the pathway are now at advanced stages of development including drugs that inhibit processing of Wnts, binding to the receptors, stability of β-catenin, or function of the Wnt-dependent transcription complex. Cancers associated with Wnt activation invariably require multiple mutations in other genes, including activating mutations in proto-oncogenes and inhibitory mutations in tumor suppressor genes, raising concern that targeting the Wnt pathway alone may not be sufficient to control an advanced malignancy. However, reexpression of *APC* in mouse intestinal tumor models ameliorates the cancer phenotype, providing hope that Wnt pathway inhibitors will have a beneficial clinical impact on the course of human malignancies.

See also: Colorectal Cancer: Pathology and Genetics. Gastric Cancer: Pathology and Genetics.

Further Reading

Aoki, K., Taketo, M.M., 2007. Adenomatous polyposis coli (APC): A multi-functional tumor suppressor gene. Journal of Cell Science 120 (Pt 19), 3327–3335.
Barker, N., van Es, J.H., Kuipers, J., et al., 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449 (7165), 1003–1007.
Barker, N., Ridgway, R.A., van Es, J.H., et al., 2009. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 457 (7229), 608–611.
Cadigan, K.M., Waterman, M.L., 2012. TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harbor Perspectives in Biology 4 (11).
Doble, B.W., Patel, S., Wood, G.A., Kockeritz, L.K., Woodgett, J.R., 2007. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. Developmental Cell 12 (6), 957–971.
Fearon, E.R., 2011. Molecular genetics of colorectal cancer. Annual Review of Pathology: Mechanisms of Disease 6, 479–507.
Fearon, E.R., Vogelstein, B., 1990. A genetic model for colorectal tumorigenesis. Cell 61 (5), 759–767.
Huang, H.C., Klein, P.S., 2004. The Frizzled family: Receptors for multiple signal transduction pathways. Genome Biology **5** (7), 234.
Janda, C.Y., Waghray, D., Levin, A.M., Thomas, C., Garcia, K.C., 2012. Structural basis of Wnt recognition by Frizzled. Science 337 (6090), 59–64.
Kinzler, K.W., Vogelstein, B., 1996. Lessons from hereditary colorectal cancer. Cell 87 (2), 159–170.
Korinek, V., Barker, N., Morin, P.J., et al., 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. Science 275 (5307), 1784–1787.
Krishnamurthy, N., Kurzrock, R., 2018. Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. Cancer Treatment Reviews 62, 50–60.
Liu, C., Li, Y., Semenov, M., et al., 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108 (6), 837–847.
Morin, P.J., Sparks, A.B., Korinek, V., et al., 1997. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275 (5307), 1787–1790.
Nusse, R., Clevers, H., 2017. Wnt/beta-catenin signaling, disease, and emerging therapeutic modalities. Cell 169 (6), 985–999.
Rustgi, A.K., 2007. The genetics of hereditary colon cancer. Genes & Development 21 (20), 2525–2538.
Shoshkes-Carmel, M., Wang, M.J., Wangensteen, K.J., Tóth, B., Kondo, A., Massassa, E.E., Itzkovitz, S., Kaestner, K.H., 2018. Subepithelial telocytes are an important source of Wnts that supports intestinal crypts. Nature 557 (7704), 242–246.
Su, L.K., Vogelstein, B., Kinzler, K.W., 1993. Association of the APC tumor suppressor protein with catenins. Science 262 (5140), 1734–1737.
Wehrli, M., Dougan, S.T., Caldwell, K., et al., 2000. Arrow encodes an LDL-receptor-related protein essential for wingless signaling. Nature 407 (6803), 527–530.
Zeng, L., Fagotto, F., Zhang, T., et al., 1997. The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell 90 (1), 181–192.

Relevant Websites

http://web.stanford.edu/group/nusselab/cgi-bin/wnt/—The WNT Homepage.

Xeroderma Pigmentosum: When the Sun Is the Enemy

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Glossary

Lentigines Dark spots that appear in the skin as a result of sunlight exposure.
Nucleotide excision repair (NER) Molecular pathway that acts on the removal of bulky DNA damage. Defective in most XP patients.
Translesion DNA synthesis Molecular pathway that allows the replication of a damaged DNA template. Defective in XP variant patients.
Unscheduled DNA synthesis DNA synthesis induced by NER, and not due to S-phase DNA replication.
Transcription coupled repair NER subpathway, where DNA damage is removed in actively transcribed genes.
Global Genome repair NER subpathway, where DNA damage is removed in the genome in general, independent of transcription.
Chromophores Molecules inside the cells that may absorb light with various wavelengths and generate reactive molecules, damaging many cell structures.

Introduction

Life is not easy for patients who suffer with Xeroderma Pigmentosum (XP), nor for their relatives, who take care of them. Their main enemy is outside their houses: just opening the door as sunlight hurts them. The same sunlight that provides energy and life for the living beings is what they have to avoid, living as much as possible in the dark, changing day activities to night and day, whenever this is feasible. Their skin is dry (from the Greek words *xero* and *derma*) and highly pigmented (from the Latin word *pigmentosum*), but these manifestations are restricted to the areas of the skin that are commonly exposed to sunlight. This often implies the face, which is highly affected, together with their limbs and hands. The skin of XP patients presents hyperpigmentation with lentigines and freckles, although hypopigmentation is also observed in highly exposed individuals and in black-skinned patients. Precancerous skin lesions, such as actinic keratosis, occur early in the life of unprotected patients, and they develop skin tumors (nonmelanoma and melanoma) before youth. The frequency of skin tumors was estimated 10,000-fold higher for nonmelanoma and 2000-fold for melanoma before the age of 20 years old, in XP patients compared to the general population. As many frequent tumors may appear in their faces, the surgical removal may leave rough scars, with eventual need of grafts and/or the use of prostheses. Certain areas of the face, such as mouth, tip of the tongue and eyes, may also be affected, including with the development of tumors, especially in persons with darker skin. The skin lesions are the most common clinical phenotype for XP patients, although internal tumors may also have increased prevalence (10-fold). About 20%–30% of these XP patients present developmental problems, as well as neurological degeneration and premature aging phenotypes.

The incidence of this autosomal recessive disease is low, and occurs sporadically in families. The first estimated incidence in United States was 1 case per 250,000 and in a more recent detailed study in Western Europe indicated 2.3 per million of autochthonous livebirths. The North of Africa and Japan have reported higher incidence of XP (1 per 20,000–50,000), and this was associated with the occurrence of founder-mutated alleles in high frequency. Interestingly, at least three isolated communities were described in the world with numerous XP patients, due to consanguineous marriages and closed environment.

The first clinical description of XP patients was made in 1874 in Austria, by the dermatologists Ferdinand Ritter von Hebra and Moritz Kaposi, and the clinical symptoms were associated to sunlight exposure, as an inherited disease. Later, a more severe form of XP associated with microcephaly, progressive neurological degeneration and retarded growth was also described, which was called De Sanctis-Cacchione syndrome (DSC). The molecular defect responsible for this disease was discovered in 1968, when James Cleaver detected that cells from these patients were very sensitive to ultraviolet (UV) light, and unable to perform repair synthesis (unscheduled DNA synthesis-UDS), and thus, deficient in removing UV-induced DNA lesions.

This was the first discovery of a human disease to be associated with a molecular defect in the processing of DNA. The cells from the patients were, in general, very sensitive to UV light, as they are unable to remove the UV-induced lesions on DNA. These cells are also very sensitive to and unable to repair other bulky lesions, as those induced by some human chemical carcinogens, such as aflatoxins, or some drugs used for cancer chemotherapy, such as cisplatin. This lack of DNA repair results in increased mutagenesis in cells exposed to UV, in a clear association with the clinical phenotype of increased skin tumor formation in XP patients. Clever experiments, promoting in vitro fusion of cells from two different patients, directed to the identification of genetic complementation groups, when results indicated the recovery of normal repair synthesis (UDS). These experiments led to the discovery of seven

complementation groups, called XP-A to XP-G, which were, later, confirmed by the isolation of the seven genes that, when mutated and in homozygosis in the same individual, result in the disease. The molecular roles of the proteins encoded by these genes were dissected with in vitro experiments, where the whole DNA repair pathway for lesion removal was reconstituted. Basically, these seven proteins are part of a complex repair machinery, known as Nucleotide Excision Repair (NER) that removes lesions, which distort the DNA helix (including those induced by UV).

Interestingly enough, some XP patients were reported to have cells with normal UDS and, thus, no defects in NER. They were called XP variants, or XP-V. These cells were, however, unable to replicate the DNA after UV-irradiation, although they efficiently removed the lesions. Later, the XPV protein was shown to be a DNA polymerase (Pol η) that promotes the translesion synthesis (TLS) in a damaged DNA template. Thus, cells from XP-V patients are unable to bypass correctly the UV-induced lesions, increasing mutagenesis and cancer incidence.

As commented above, 20%–30% of the XP patients have developmental and progressive neurologic degeneration symptoms, suggesting premature aging clinical phenotypes. These patients may be DSC, but also reproduce the symptoms very similar to the Cockayne syndrome (CS), and are normally called XP/CS. A defect of the recovery of RNA synthesis of UV-irradiated cells from CS patients was identified. These problems in the transcription of damaged DNA allowed the identification of two genes (*CSA* and *CSB)* that encode proteins related to the recognition of DNA lesions in actively-transcribed genes, a subpathway of NER. Cells from these XP/CS patients also present defects in the transcription of damaged DNA, but involving mutations in the *XPB*, *XPD* or *XPG* genes. Other syndromes were also related to defects in NER, such as trichothiodystrophy (TTD), cerebro-oculo-facio-skeletal syndrome (COFS) and XPF-ERCC1 progeroid (XFE) syndrome. These very rare syndromes in general involve transcription impairments, and, clinically, present symptoms related to neurological deficiencies and premature aging. Finally, a mild genetic disorder, where the patients present skin photosensitivity without neurological impairment, was described as UV-sensitive syndrome ($UV^{S}S$), which have also a defect in NER caused by mutations in *CSA* or *CSB* genes.

The Sunlight Produces DNA Damage

Although the UV component of sunlight has many health benefits to human kind, as the main source of Vitamin D in our bodies and induction of sensation of happiness, it also induces DNA damage in the skin with deleterious consequences, such as photoaging and cancer. In fact, UV is part of the spectrum of electromagnetic radiation emitted by the sun, with the following wavelength ranges: UVC (100–280 nm), UVB (280–315 nm) and UVA (315–400 nm) (**Fig. 1**A). The high-energy UVC radiation is completely absorbed by the stratospheric ozone layer, which also absorbs much of UVB. Thus, only UVA and some UVB reach the earth surface and are highly relevant with damaging impacts on human health and ecosystems. Actually, UVA corresponds to 95% of the sunlight UV that reaches humans and it penetrates deeper (compared to UVB) in the skin, up to the basal layer and the dermis (**Fig. 1**B). Therefore, although UVB is highly absorbed by DNA, UVA can also induce severe damage to this molecule, and thus both deserve attention in skin protection, especially for individuals with special sensitivity to sunlight, as the case of XP patients.

The direct absorption of UV by DNA causes covalent reactions especially in adjacent pyrimidines inducing two main types of lesion: cyclobutane pyrimidine dimer (CPD) and pyrimidine (6–4) pyrimidone photoproduct (6-4PP) (**Fig. 1**C). These lesions promote strong distortions on the double helix, and are normally repaired by NER in human cells. Thus, these lesions are considered very dangerous and may be responsible for most of the skin lesions in XP patients, which are unable to remove them. UVB (and the sunlight) irradiation of the DNA molecule induces three-fold more CPDs than 6-4PPs. UVA is little absorbed by DNA, but it was shown to also induce CPDs initially in bacteria and phage DNA, later also shown in human cells and in the human skin, demonstrating the biological relevance of these genotoxic effects by these wavelengths. Interestingly, 6-4PPs were also detected after UVA irradiation in the absence of DNA repair (which is the case for NER-deficient XP patients), promoting increased cell death in XP-A cells.

UVB and, mainly, UVA light may also damage DNA indirectly through the absorption by intermediary molecules, called chromophores, that act as photosensitizers. In fact, many chromophores are present in the human skin and UV absorption may generate organic free radicals, reactive nitrogen species and also reactive oxygen species (ROS), which may react with and damage DNA. Among the endogenous chromophores in the human skin, we find melanin and melanin precursors, porphyrins, heme, bilirubin, tryptophan, etc. The basic mechanisms of photosensitization of these molecules involve the formation of a photoexcited triplet state intermediate, which can directly interact with substrates and generate free radicals and ROS, such as the $O_2^{\bullet -}$, H_2O_2 and hydroxyl radical (•OH) via type I reaction, by energy transfer. This triplet state intermediate can also transfer energy directly to molecular oxygen, via type II reaction, producing singlet oxygen ($^1O_2$), which will react to biomolecules, including DNA. These processes generate different types of lesion, such as strand breaks, formation of base adducts and base (mainly purines) oxidation. One of the most frequent and known products of these reactions is the oxidation of guanine, producing primarily 8-oxo-7, 8-dihydroguanine (8-oxoG). Recently, the formation of $^1O_2$, and consequently 8-oxoG, was described after UVA irradiation of DNA molecule, which acts as a direct photosensitizer.

The induction of DNA damage by UVA and UVB are directly linked to the human risk to develop skin cancer, such as malignant melanoma and the nonmelanoma tumors, basal cell and squamous cell carcinomas. Thus, UV radiation was classified as a class I carcinogen by the Word Health Organization, and one of the most dangerous environmental source of cancer in humans. Thus, exposure to UV radiation depends on individuals' behavior and avoiding the sun, especially in children, is strongly recommended, particularly around noontime where the maximum of UVB is reaching the earth. However, in recent decades, the use of artificial UV

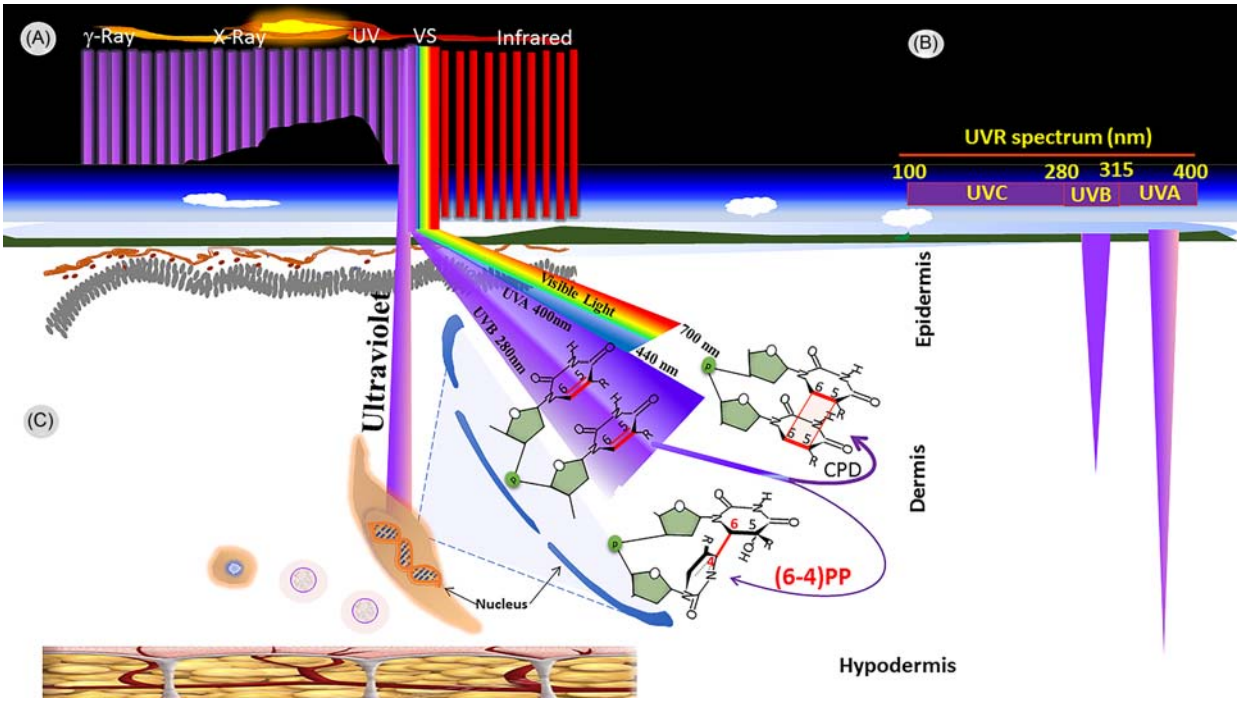

Fig. 1 *Sunlight spectrum and UV-induced DNA damage.* (A) The solar radiation, entering the atmosphere, is composed of mainly infrared light and visible light, with a smaller proportion (10%) of ultraviolet light (UV). Local factors (thickness of the ozone layer, latitude, cloud cover, elevation and season) affect the amount of UV that reaches the ground (roughly 3%). (B) This shows the electromagnetic spectrum of UV radiation at sea level and the relative amount of UVA and UVB that can penetrate differentially into the skin. On average, UVA reaches the earth in the middle of a summer's day 20 times more than UVB. UVA penetrates deeply into the dermis reaching the dermal stratum papillary. Roughly 20% of UVB reaches the epidermal stratum basal, the remainder UVB is almost completely absorbed by the epidermis. UVC are completely filtered within the atmosphere, mainly by absorption in the ozone layer. (C) Direct absorption of UV light by pyrimidine bases next to each other in the same strand can induce damage to the DNA. The most common reaction forms cis-*syn*-cyclobutane pyrimidine dimers (CPD ~ 67%–83%) and pyrimidine (6–4) pyrimidone photoproducts ((6–4) PPs) account for the rest.

radiation for skin tanning is becoming a common behavior, which is normally performed with tanning devices (sunbed). In fact, this indoor tanning is very popular especially in teenagers and women, particularly in Northern Europe and United States. The exposure to high doses of UV through these artificial sources highly increases the risk to develop skin tumors, including melanoma, especially for individuals who received their first tanning session before the age of 30. Thus, there are strong recommendations to avoid the use of sunbed and tan-seeking behavior.

The main symptoms of skin photoaging and tumors of XP patients are most probably related to the main UV photoproducts, CPDs and 6-4PPs, as the mutations observed in XP cells and tumors have signatures typical of those expected from pyrimidine dimers. Moreover, when these lesions are removed by heterologous expression of photolyases, which are specific for these lesions, the XP cells survive better after UV irradiation. These notions fit well with the NER activity of damage that causes distortions in the DNA molecule, such as the pyrimidine dimers. Interestingly, the phenotype of XP cells to UVA irradiation has been little explored, and we still do not know how lesions related to oxidative stress, for example, may affect the cells. This question deserves more investigation and the data may help to understand better how sunlight causes damage in the skin of XP patients, and will also contribute to our knowledge on the mechanisms and deleterious effects of UVA light in the skin of the population in general.

Molecular Mechanisms to Protect From Sunlight-Induced DNA Lesions That Are Deficient in XP Patients

Most of the XP patients are deficient in the ability to remove DNA damage by the NER pathway. This pathway is a very flexible and versatile type of repair that acts in structurally unrelated bulky lesion on DNA, that causes important distortions in the double helix, such as the pyrimidine dimers CPD and 6-4PP, or several chemical carcinogens or antitumoral drugs. Initial experiments detected seven complementation groups for the XP cells, which were confirmed with the cloning of seven genes (named *XPA*, *XPB/ERCC3*, *XPC*, *XPD/ERCC2*, *XPE/DDB2*, *XPF/ERCC4* and *XPG/ERCC5*) that encode the proteins XPA to XPG and participate in NER. In fact, more than 30 proteins are involved directly on this pathway, acting step by step in a concerted mode to remove the lesions from DNA and recover, without mistake, the original structure of the molecule. When the activity of one of the seven XP genes is missing due to mutations, the clinical result leads to a XP phenotype. Moreover, mutations that affect the activity of CSA or CSB proteins

result in CS, while mutations affecting the activity of TTDA/p8 cause TTD. Other more complicated syndromes have also been associated with mutations in genes that participate in the NER pathway: defects in ERCC1 and in XPF may also cause XFE, CS and COFS, and in UVSSA causes UV^{S}S. XP-C is the most common group around the world followed by XP-A and XP-D. The other groups concern a very small number of patients.

Analysis of the recovery of RNA synthesis of UV-irradiated cells from CS patients revealed that two distinct pathways are active for the removal of UV-induced DNA lesions: one, the so called Global Genome Repair (GGR) operates anywhere in the genome through the recognition of single-stranded regions within duplex DNA elicited by UV-induced photodamage, the other one, known as Transcription-Coupled Repair (TCR) is preferentially responsible for repairing damage located on the transcribed strand of actively transcribed genes.

As already pointed out, NER proteins remove DNA lesions that distort the double helix. Nevertheless, although both UV-induced DNA lesions (CPD and 6-4PP) can effectively induce distortions on DNA, CPDs are much less distortive than 6-4PPs and, by implication, much less efficiently recognized by GGR-NER recognition factors. Whereas it is fairly known that the vast majority of 6-4PPs damage are quickly repaired through GGR pathway in human cells, the repair of CPDs occurs in a much slower fashion (up to several days), mainly under the auspices of the TCR, trigged by blocked RNA polymerase II complexes. Actually, in human cells, only approximately 10% of CPDs are removed from the DNA within 4–6 h after UV irradiation, while (6–4)PP lesions are completely repaired during the same period.

The detailed NER pathway of UV-induced lesions is shown in Fig. 2. The early phase of this multiple-step process is intended to ensure the recognition of the lesions, taking into consideration not only the specific degree of distortion imputed on the double helix, relevant to the GGR's recognition factors, but also the positioning of these lesions in the genome, important to the TCR's recognizing proteins. Thus, lesions located anywhere in the genome will be quickly sensed by the XPC-Centrin2 complex, and 6-4PPs are more promptly recognized than CPD lesions. On the other hand, lesions blocking RNA polymerase II, on the transcribed strand of actively-transcribed genes, will be promptly targeted by CSA and CSB proteins. This characterizes the TCR subpathway that operates independently of the GGR subpathway. Subsequent steps of both subpathways (GGR and TCR) converge through the recruitment of the transcription factor (TFIIH). TFIIH is a multifunctional protein complex originally identified by its requirement in transcription initiation by RNA polymerase II. In addition to its role in transcription, TFIIH plays key roles in NER through its

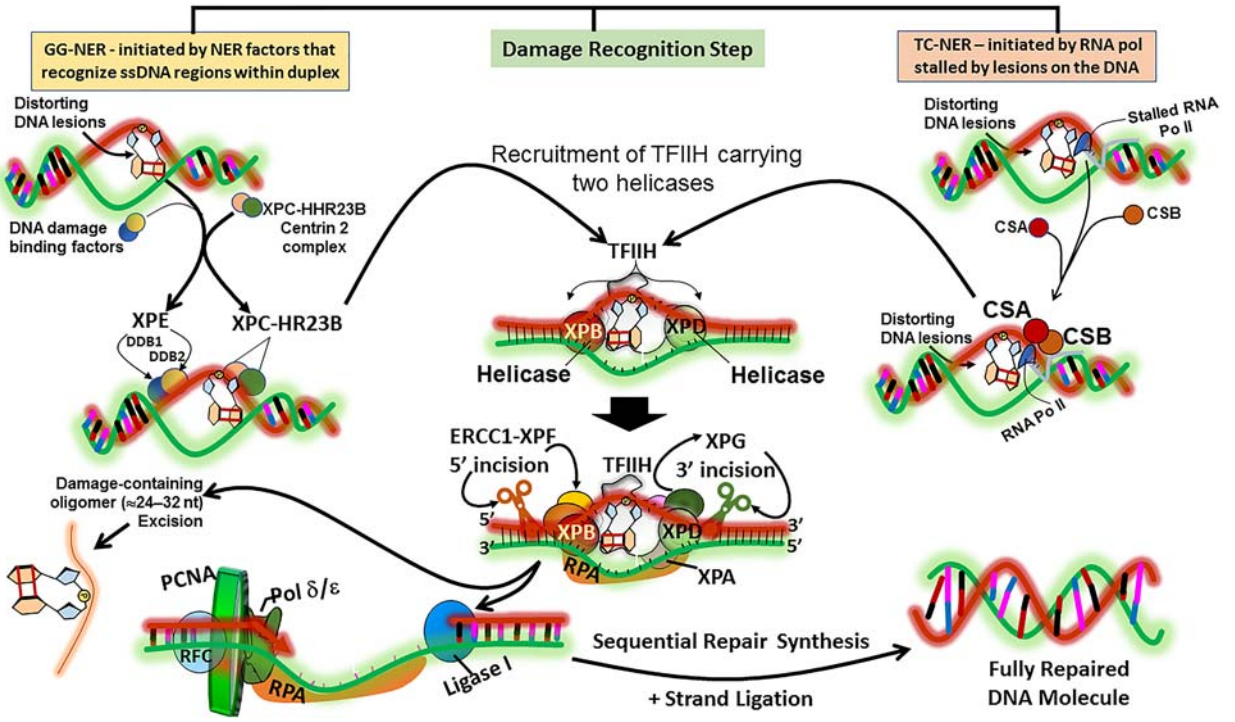

Fig. 2 *Schematic diagram showing the sequential steps of the nucleotide excision repair (NER) process in mammalian cells.* The recognition step proceeds by two independent subpathways: Global genome NER (GG-NER), in the top left-hand corner, can occur anywhere in the genome, except in transcribed strands, is initiated by NER factors (UV-DDB (XPE) and XPC-RAD23B complexes) that recognize ssDNA regions, caused by bulky adducts, within duplex DNA. Transcription-coupled repair (TC-NER), in the top right-hand corner, occurs in actively transcribed genes, due to the blockage of the RNA synthesis machinery, and is initiated by TCR-specific proteins (CSA and CSB). Subsequent steps of both pathways converge through the recruitment of the TFIIH transcription factor, harboring the DNA helicases (XPB/XPD), which induces helix opening stimulated by the XPA protein. The RPA protein stabilizes the single-stranded DNA generated by its local opening and facilitates the localization of XPF-ERCC1 and XPG endonucleases, responsible for 5′ and 3′ incisions, respectively. Finally, a new DNA fragment is synthesized by DNA polymerases epsilon or delta, and DNA ligase I completes the repair process by catalyzing the ligation of the new DNA fragment to the original sequence.

3′-5′ and 5′-3′ ATP-dependent DNA helicases subunits encoded, respectively, by the NER *XPB* and *XPD* genes. Once loaded on the damaged strand, TFIIH unwinds ~30 bp around the damaged DNA site, which become covered by RPA (Replication Protein A), the major eukaryotic single-strand DNA (ssDNA)-binding protein. The local opening of the double helix, afforded by the helicases, is stimulated by the binding of XPA protein, which together with RPA complex stabilizes the single-stranded DNA generated by its local opening and facilitates the localization of the endonucleases (XPF/ERCC1 complex and XPG protein), which cleave the DNA strand at the 5′ and 3′ sides of the lesion, respectively. The 24- to 32-base oligonucleotide containing the DNA damage is removed from DNA leaving a gap. The formed gap is then filled with the synthesis of a new DNA fragment by DNA polymerases epsilon or delta, and the final step is achieved by DNA ligase I, catalyzing the ligation of the newly-synthesized DNA to the original sequence. Thus, at the end of this process, the original DNA sequence is fully recovered in an error-free manner (**Fig. 2**).

XP-E and XP-C patients exhibit a deficient GGR mode but a fully efficient TCR. These patients have a normal development and neurology. XP-A, XP-B, XP-D, XP-F and some XP-G patients exhibit various levels of both TCR and GGR deficiencies associated with various levels of abnormal development and some neurological impairment.

Translesion Synthesis of Damaged DNA: The XP-V Syndrome

Under normal conditions, DNA damage induce a transient arrest of the cell cycle, known as DNA damage checkpoint, which, in turn, activate a plethora of biological processes, including the activation of DNA repair mechanisms to ensure that the lesions are repaired before cell division. However, when bulky DNA injuries are introduced after the cell decision toward DNA replication has already been made, the replication machinery will probably encounter unrepaired DNA lesions, which block DNA synthesis, as replicative polymerases are unlikely to copy damaged templates. By blocking the polymerases, bulky DNA lesions ends up inducing the collapse of the replication fork, then subsequent production of double-strand breaks, and, ultimately, cell death. Therefore, long-term consequences of DNA lesions include different forms of mutations and genomic instability, which, although constituting the driving forces that give rise to evolution, also significantly contribute to carcinogenesis or cell death.

Progress in the past 20 years has unraveled some of the underlying principles of DNA damage tolerance in eukaryotic cells, through which DNA lesions may be overcome during S phase and the repair of these lesions, postponed, allowing the achievement of the DNA replication and cell survival maintenance. In fact, translesion DNA synthesis (TLS), performed by specialized DNA polymerases, constitutes an extremely efficient mechanism of tolerance to ensure replication of damaged DNA template. These TLS polymerases normally have low fidelity of replication into undamaged DNA sequences, but, interestingly act effectively when copying DNA models containing lesions. A summary of the TLS process by the polymerase η is shown in **Fig. 3**.

DNA polymerases, like other enzymes, have their proper structure-function relationship for controlling the substrate access to the active site, which end-up influencing both specificity and catalytic mechanism. Given the importance and complexity of DNA replication, it is not surprising that this process depends on a myriad of factors and accessory molecules. Thus, the human genome, for instance, encodes, nothing less than 15 distinct DNA-dependent DNA polymerases, working in concert to maintain genome stability, including the replicative B-family polymerases, namely, pol alpha (Pol α), pol delta (Pol δ) and pol epsilon (Pol ε) and the specialized Y-family polymerases, pol eta (Pol η), pol iota (Pol ι), pol kappa (Pol κ) and Pol Rev1, characterized by their unique ability to efficiently replicate, not only non-B DNA structures but also particular type of DNA lesions. DNA polymerase η, for example, is proficient in distinguishing and copying past template cyclobutane dimers by incorporating dA residues opposite the lesion. This enzyme is encoded by the *POLH* gene and deficiencies in this protein are unequivocally associated with the variant form of XP: XP-V. The main clinical features of XP-V patients are associated with extreme sensitivity to sunlight and increased risk for developing skin cancer, but normal neurology. Because XP-V patients exhibit a normal NER activity, their UV-induced symptoms usually appeared between the second and the fourth decades of their life, much later than the "classical" XP. XP-V represent about 20% of XP around the world.

The most relevant characteristic for the Y-family DNA polymerases accounts for their catalytic sites, which are much larger and flexible than the replicative polymerase' catalytic sites. Thus, these polymerases contain a more spacious binding pocket at the catalytic site needed to accommodate altered template bases and nucleotide adducts setting up the proper geometry for catalysis. However, despite the high flexibility provided by an enlarged template-binding pocket, these enzymes may present some specificity to different DNA-template adducts. Thus, for instance, pol η is supposed to accurately bypass UV lesions, while pol κ can support nucleotide incorporation opposite and beyond benzopyrene-induced DNA adducts.

Overcoming Obstacles During S Phase: Main Steps

Following UV irradiation, a vastly integrated series of biochemical events takes place through a high degree of specificity of enzymes to ensure that the DNA damage response pathway follows a smooth course. Thus, the formation of bulky adduct DNA lesions, induces a p53-dependent accumulation, chromatin recruitment of DNA pol η and ubiquitination of the DNA polymerase processivity factor, the proliferating cell nuclear antigen (PCNA). Upon encountering a UV-induced DNA lesion, the error-free replicative polymerase is blocked, thereby inducing PCNA (ring) monoubiquitination (Ub). This increases the affinity for Pol η, which inserts correct nucleotides opposite and beyond the DNA damage, thereby bypassing the lesion and allowing the replicative DNA polymerase to resume DNA synthesis (**Fig. 3**).

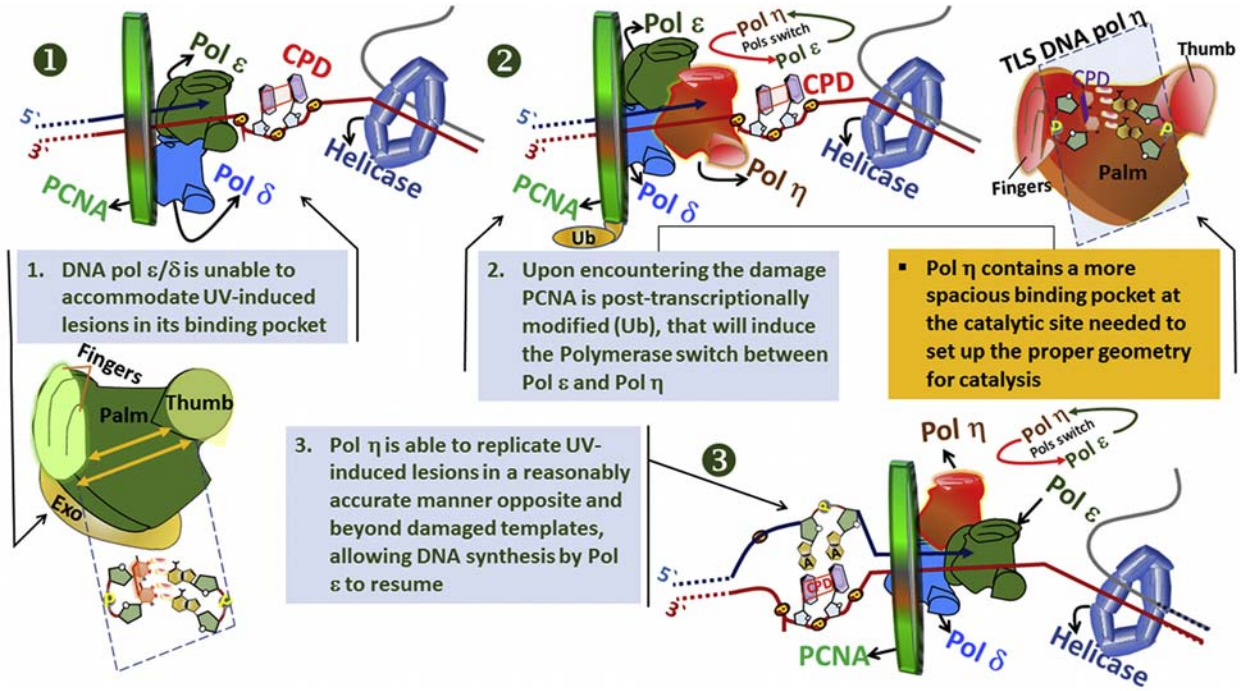

Fig. 3 *Essential steps of UV-induced translesion synthesis* (*TLS*) *process: a hallmark of XPV.* (1) Upon encountering a bulky DNA lesion, the error-free replicative polymerases (Pol epsilon/delta) stall, caused by their inability to insert a nucleotide opposite the lesion, thereby inducing (2) PCNA (ring) monoubiquitination (Ub), which increases the affinity for the specialized DNA polymerase Pol η. (3) Functional Pol η inserts correct nucleotides (A) opposite the thymidine dimers induced by UV light and allows DNA synthesis to resume with replicative polymerases. Pol η is absent in XP-V patients, whereas another error prone TLS DNA polymerases insert incorrect nucleotides opposite the lesion, thereby leading to the clinical pathological changes observed in XP-V patients, mainly sunlight-induced skin cancer.

Functional Pol η is absent in XP-V patients, therefore, mutagenesis and carcinogenesis in these XP patients, probably arise from insertion of incorrect nucleotides opposite UV-induced lesions made by other TLS DNA polymerases incapable of accurately bypass this specific kind of lesions. Indeed, sequencing XP-V skin tumors have shown that the majority of UV-induced mutations on tumor suppressor genes were located opposite Py-Py sequences.

The Psychological Approach of Xeroderma Pigmentosum

The discovery of the disease starts either by a strong sunburn of the newborn after a short exposure to sun or by the appearance of lentigines, freckles or pigmentary changes on the sun-exposed sites of the 1–3 years old child. Then, a long period of wanderings, from general practitioners to dermatologists via allergists or other specialist clinicians, starts can take several years before resumption. The exact diagnosis done by dermatologist and confirmed by molecular biologists occurs usually between the ages of 2–4 in population at risk or up to 6 years for population normally free of the disease. The diagnosis can also be made by neuro-pediatrician for some XP groups associated with neurological abnormalities.

Like most of genetically-transmitted diseases, xeroderma pigmentosum is difficult to explain and to be understood by the parents because at early age of the patient the symptoms are relatively benign. Moreover, some parents feel responsible of their child's disease and try to deny the evolution of it. Parents have to understand and to accept that the only prevention, up to now, is a complete protection of the child from UV exposure as early as possible. These protective measures include covering with UV-resistant films all windows in home, cars and school. Lamps should be everywhere checked for the absence of UV rays. When outside during daylight, XP child should be completely covered with long trousers, long sleeves and gloves as well as UV-resistant and transparent face mask (Fig. 4). For adults a broad-rimmed hat, lip balm and sunglasses are the minimum effort. It is very useful that each family acquires a UV-dosimeter enabling measurement of UVA and UVB radiations in a given place. UV is not detected by human eyes and may be present in places where people don't think about. Patients should use this dosimeter to each new place they are going to.

Parents may pretend that 70%–80% of protection should be good enough but in fact 100% is the only secure number. Even worst, when this is completely understood and that parents have fully protected their child, then a few years later XP children may develop skin cancers on exposed sites. Then the parents don't understand, at all, the arrival of skin tumors because they have fully protected their child in the last couple of years. It is, of course, necessary to explain to them in details that the appearance

Fig. 4 *Evolution of protection of XP patient in 15 years.* Around 2000, the XP patients were completely protected with thick materials and sunglasses used during skiing activities (left picture). Now the full UV-resistant and transparent facial mask with ventilated fresh air allows patients to move and play outside in a sunny day with no problem (right picture). These pictures are a gift from the French Association "Les Enfants de la Lune," with the authorization of the XP families.

of the tumors is due to the very early years of the child's life where he was not protected, independently of the recent full protection. When the family has fully integrated this situation, the future health of the child is insured. For patients without neurological abnormalities (XP-C, XP-E and XP-V), their prognosis is good if stringent protections are followed as suggested.

It is necessary that the members of the family be helped by psychologists to be able to cope with this disease. Beyond the necessary psychosocial follow up of the patient, a particular attention should be given to siblings of the XP child because usually all the family's life is centered on the patient way of living, such as only evening and night outside activities and no outside play during the day. It should be stressed that there is a risk of children with XP that might be borne to brothers and sisters of the XP proband, who might marry a member of the family or someone from the original village. Consanguinity is the highest risk to be borne with a genetic disease.

A usual question asked by XP parents concerns the arrival of a new child in the family. Of course, we explain that the probability for the next baby is 75% to be free of the disease but 25% probability to have another XP child. We and others have developed a prenatal diagnosis based on DNA repair-efficacy following UV irradiation of chorionic villus-derived cells (at 10 weeks pregnancy) or of amniocytes (13 weeks pregnancy) in affected families. When the mutation has been identified in the proband, then it is much easier and faster to sequence the fetal DNA.

Because this is a very rare disease (less than one patient for one million European autochthonous individuals), families think they are the only one in this situation and they feel completely abandoned by medical, administrative and informative authorities. One way to overcome this feeling is to organize meetings between numerous families and XP children. The American, English and French associations of XP patients have organized, for the last 15 years, summer or winter camps where XP families and children meet together and have several days of discussion, comparison and information between them and with medical or paramedical staffs. Plays organized in secure places for sun protection or at night allow the XP children and siblings to spend a very fruitful time. These camps have also been a good way for parents to better understand the disease and to have the opportunity to see some XP patients coming from some countries where children have never been protected from sunlight. The discovery of these very damaged XP patients leads to a painful situation, but a useful one, to completely understand the necessity of full UV-protection for the health of XP children.

Career prospects for XP are thwarted but psychologists should try to explain them it is possible to find a job where patients can be completely protected from sunlight. This limitation may be taking into account during the XP child studies.

Medical Treatment of XP

Photoprotection is a vital preventive measure. Patients must avoid exposure to sun but also to artificial UV irradiation. In the absence of full photoprotection, early skin cancers should be monitored as soon as possible. Physicians should be seen every 2 months and the family should also be taught how to carry out self-examinations. Repeat photographs is a valuable tool to distinguish between a benign pigmented lesion, that does not evolve, and early melanoma or between actinic keratosis (AK) and squamous cell carcinoma. Suspect lesions must systematically be biopsied.

Carcinoma in situ and AK may be treated by the local application of Imiquimod (Aldara) or 5-fluorouracil (Efudix) but complete excision is the treatment of choice for skin cancers when possible. Mohs surgery or surgery associated with histological analysis may

be particularly interesting for XP patients in order to limit the disfigurement associated with numerous cancer removals. If the open wound is too large after surgery, autologous skin grafts using patient skin not previously exposed to the sun should be used.

Systemic chemotherapy and radiation therapy could be prescribed as palliative measures for inoperable tumors. Given the DNA repair deficiency, XP cells are extremely sensitive to antitumoral treatment that damages DNA such as classical chemotherapeutic drugs. It is, therefore, highly recommended to lower the doses as a function of the mechanistic activity of the drug.

Efficient sun protection needs to prescribe regular vitamin D supplementation for XPs but also for their siblings who are usually also prevented from sun exposure. Since vitamin D is rapidly eliminated from the body, it is better to prescribe this supplementation every week or month.

In the past, oral retinoids have been used and shown to be active against skin tumors appearance. The doses used were accompanied of numerous side effects and even worst when the treatment was stopped, the tumors reappeared rapidly. In fact, a sort of rebound effect was observed with a greater number of tumors being obtained after the end of treatment, suggesting that retinoids appear to block only a late step of carcinogenesis. Finally these treatments have been discontinued in routine use.

XP patients should also avoid as much as possible any contact with environmental carcinogens such as cigarette smoke, benzene or polycyclic hydrocarbons. Also, although photodynamic therapy appears as a solution for tumor removal, this type of treatment may be performed carefully and with high surveillance, as the nontumoral tissue may be strongly affected and induce more damage than relief to the patients.

For patients with neurological abnormalities, regular measurement of the head size, assessment of deep tendon reflex, routine audiometry should help to quantify the neurological evolution of the child. A neuro-pediatrician or a neurologist should be implicated in the monitoring and management of these patients. Up to now, there is no prevention and no treatment of neurological deterioration but physical therapy and occupational therapy can help the every-day life. These neurological abnormalities are unfortunately progressive leading to severe disabilities and can result in a shortened lifespan in XP-A and XP-D patients.

Future Treatment of XP

It is necessary to explain to the XP patients and their family that there is no cure available today of their disease. Several laboratories are working on a possible gene therapy of XP. Although all the cells from XP are deficient in NER, in a first step it would be interesting to produce skin cells from an XP patient, which have been complemented or corrected for the XP mutation.

Full complementation of UV-sensitivity and DNA repair in XP-A, XP-C, and XP-D fibroblasts and keratinocytes has been obtained following transduction with recombinant retroviruses expressing the corresponding wild type DNA repair genes. Skins produced in vitro, using XP-C keratinocytes complemented with the *XPC* cDNA transduced with retrovirus, have been grafted on the back of immunodeficient mice and shown to be able to repair UV-induced DNA lesions even after several months of grafting.

As an alternative for genetic therapy of XP patients, DNA virus may carry and transduce the missing gene, with no integration in the cells' genome. This was initially performed with first generation adenovirus, which is a vector that transduces genes in different cell lines and high titers maybe achieved. Several of the NER genes (*XPA*, *XPC*, *XPD*/*ERCC2*, *XPV*/*POLH*) were shown to successfully complement XP cells in vitro: most of the cells in the culture express the transduced gene, become resistant to UV light, as control cells, and recover the ability to remove pyrimidine dimers (NER) or to bypass them (TLS). Strikingly, knockout mice for the *XPA* gene were also treated with this strategy, using AdyXPA recombinant virus. Basically, the mice received subcutaneous injection of a solution of high titer of AdyXPA, in their back, where the hair was shaved before UVB irradiation. The expression of the XPA protein was confirmed in the epidermis, dermis and hair follicle cells, clearly indicating the ability of these viruses to transduce heterologous genes in the skin. Compared to mice that did not receive the adenovirus, those transduced with AdyXPA had the skin strongly protected from the effects of UVB. Moreover, while all untreated 20 XPA mice developed tumors in their skin in the irradiated region, none of those that received the recombinant virus had any sign of tumor. These results demonstrated that recombinant adenovirus prevented the formation of skin cancer, protecting the mice from the harm of UV light, giving hope for future approaches.

However, more experiments indicated that expression of the transduced gene is limited for not more than a week, and a second application did not lead to gene expression. The simplest explanation for these results is that the first application induces an immunological response against the virus, preventing further treatment. Therefore, there is a need to search for other alternatives. The use of recombinant adeno-associated virus (rAAV) appears as a possibility to be tested. AAV are small single-stranded DNA virus, nonpathogenic for humans, inducing low levels of immunological responses, and many data have shown the heterologous expression in animals by these vectors may be maintained for a few years. Recently the European Medicines Agency approved in 2012 the use of Glybera (based on this virus) for commercialization as a pharmaceutical for the gene therapy of the lipoprotein lipase deficiency, a rare human disorder that causes severe pancreatitis. This strategy seems to work well providing good results for the first 3 years after application of the recombinant virus directly in the leg muscles of the patients. Thus the application in the skin still needs to be tested.

More recently, a full correction of the founder mutation of the XPC gene, characteristic of North African patients, has been obtained by using error-free homologous recombination (HR) between the mutated gene and a wild type XPC sequence present in a transduced plasmid (Fig. 5). The HR pathway is initiated following the induction of a double-strand break induced by a specific nuclease (Zn Finger, Meganuclease, TALEN) at a selected given sequence very close to the patient's mutation to be corrected. In this technology, the mutation is directly corrected and replaced by the normal sequence without change of the genomic position of the

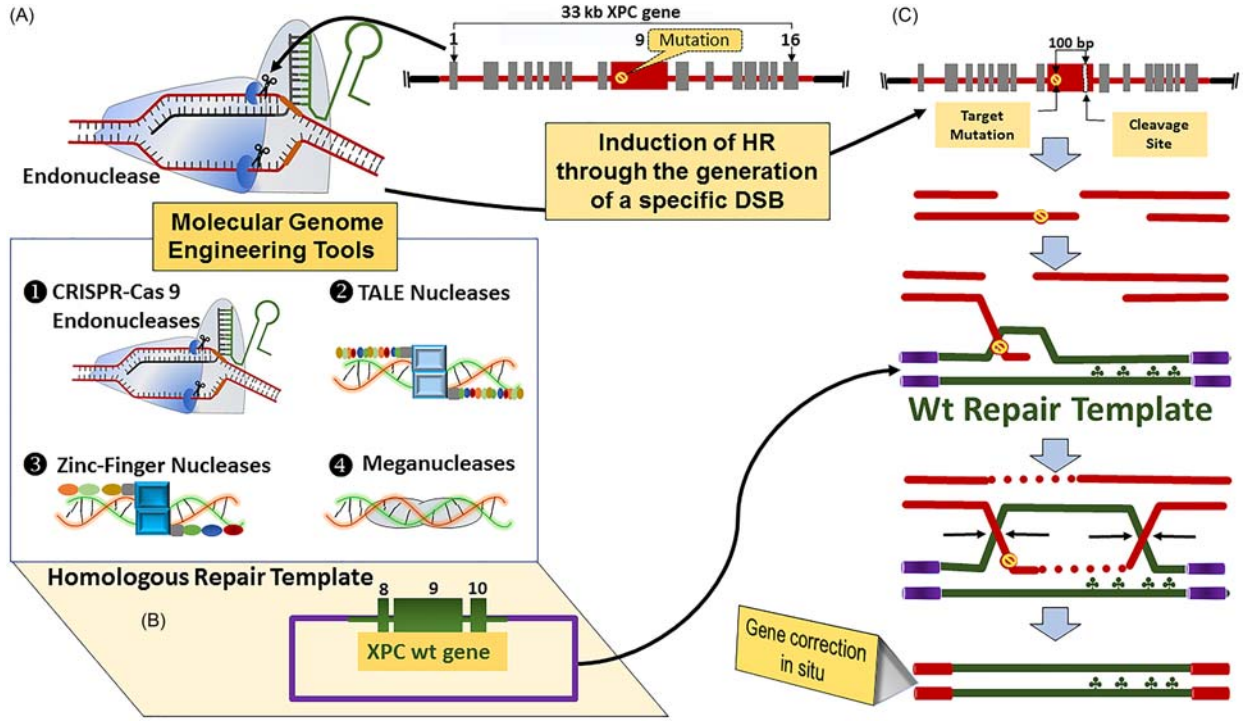

Fig. 5 *Application of high throughput molecular genome engineering tools for a genetic correction of XP cell.* (A) Genome-editing nucleases recognize and cut at specific sequences in the genome, thereby allowing either disruption of an undesirable disease-causing gene or replacement of a mutated allele sequence with a functional new copy. The main endonucleases currently used to introduce double-strand breaks (DSBs) in the genome are: (1) CRISPR-Cas9 nucleases, harboring RNA molecule (guide RNA) to base pair with the target DNA as an RNA-guided nuclease. (2) TALE nucleases, (3) Zinc-Finger nucleases and (4) Meganucleases. The last three mentioned generate sequence recognition specificity via protein-DNA interactions. (B) The presence of suitable homologous repair template (*XPC* wt gene) increases the efficiency of gene editing via homologous recombination. (C) The combination of a specific DSB and the transfection of a homologous donor DNA template corrects the mutation and restores the wt DNA sequence through homologous recombination. In this case, the mutation is fully corrected while the gene is still located at the regular place in the chromosome with its normal transcription regulation. The icons (♣) correspond to silent mutations that we have inserted in the wt *XPC* sequence harbored by the plasmid, in order to disrupt the DNA sequence recognized by the nucleases, to avoid that the plasmid be cut by the nucleases. These silent mutations are found following sequencing in the corrected final *XPC* gene indicating that the correction has been done by homologous recombination.

corrected gene, nor of its normal cellular regulation. The recent use of the CRISPR/Cas9 technology should render much easier this correction.

Adult epidermal stem cells have the capacity to renew epidermis and as already described for severely burned individuals, corrected XP keratinocytes could be used to produce in vitro epidermal sheets that can be grafted on patients such as following a large excision of skin tumors. Although still limited to research laboratories, this technology may be developed and applied to patients in the future.

Moreover from adult somatic XP cells (XP-A, XP-B, XP-C, XP-G and XPV), it has been produced induced Pluripotent Stem Cells (iPSCs) following expression of the four key factors: KLF4, cMyc, Oct3/4 and Sox2 with eventually Lin28 and sh-P53. Interestingly enough, mutations in XP genes did not compromise somatic cellular reprogramming although the XPC protein was reported as an Oct4/Sox2 coactivator. Since these cells have the potential for multilineage differentiation to the three germ layers, one can produce corrected epidermal stem cells that can be used for skin replacement via autologous transplantation. In a research view, these iPSCs can be differentiate in vitro into multiple types of cell lineages that allows researchers to specifically look after the effect of XP mutations in a given organ type. For example, neurological abnormalities in some XP groups that cannot be fully reproduced in mouse models, can be analyzed in iPSCs derived from XP-A patients differentiated into neuronal cells. Such technology should be rapidly used in the future for human therapy.

Conclusion

Xeroderma pigmentosum was first described by the Austrian dermatologist Moritz Kaposi in 1870 as a parchment skin disease. This disease has been "rediscovered" by James Cleaver in 1968 by comparison between the phenotype of XP patients and those of UV-sensitive and DNA repair-deficient bacteria. Since that time, numerous studies have revealed the complicated DNA repair processes

in mammalian cells and the role of at least 30 different proteins to allowing an efficient repair and tolerance of bulky lesions. Interestingly enough, the basic mechanism of DNA repair is similar from bacteria to plants and humans. The cloning between 1985 and 1999 of the 8 XP genes allowed medical doctors and scientists to classify the patients according to the mutated genes and to evaluate better the life expectancy of XP. Genetic counseling started to be easier for XP families.

Around the 2000s, patient associations have been very useful for helping the patients and their families allowing a better evolution for treatment, consideration in the society and interaction with other XP families and foreign patients. It is not clear up to now what will be the next step for treatment. A full protection against sun-exposure, UV-rays and maybe blue light is absolutely necessary and relatively well understood by young patients.

Gene therapy of epithelial and fibroblastic cells in order to produce in vitro XP cells corrected for their mutation is technically available now. Production of reconstituted skin in vitro is also possible and could be used to repair the disfigurement of XP faces following repetitive surgical acts. This process should, however, be carried out by private company authorized for this type of human graft. Unfortunately, the too-few numbers of patients did not guarantee the private company enough money for going into these experiments. Therefore, it is not clear if and when this type of treatment may be available for XP patients.

The use of iPSCs and embryonic stem cells that will be undoubtedly developed in the future is probably the best chance for XP treatment but several years will pass before authorized application in humans. Basic and medical researches are still necessary and the best way for hoping an efficient treatment.

Prospective Vision

As treatment of the Xeroderma Pigmentosum disease still relies mainly on the avoidance of exposure to sunlight and on early skin tumor resection, gene and cell therapy with novel techniques, such as CRISPR-Cas9 to correct the patient's mutation, appear as potential avenues, giving hope for patients and their families. Similarly, embryonic stem cells and iPSCs technology will be used in a near future not only to better understand the disease, but also to propose new therapies. But these opportunities still have long way to go. Meanwhile, the ability to perform fast DNA sequencing (by Next Generation Sequencing) provides in the last few years simple ways for the identification of mutated genes and, thus, molecular diagnosis. This small step may help to detect the disease early in patient's lives, advising the need of sun avoidance, and improving their life quality. The molecular diagnosis, including the prenatal one, may also help the families to take decisions based on genetic counseling.

See also: Melanoma: Pathology and Genetics. Radiation Therapy-Induced Metastasis Promotes Secondary Malignancy in Cancer Patients. Role of DNA Repair in Carcinogenesis and Cancer Therapeutics. Squamous Cell and Basal Cell Carcinoma of the Skin: Diagnosis and Treatment.

Further Reading

Alapetite, C., Benoit, A., Moustacchi, E., Sarasin, A., 1997. The comet assay as a repair test for prenatal diagnosis of xeroderma pigmentosum and trichothiodystrophy. The Journal of Investigative Dermatology 108, 154–159.

Cadet, J., Douki, T., Ravanat, J.L., 2015. Oxidatively generated damage to cellular DNA by UVB and UVA radiation. Photochemistry Photobiology 91, 140–155.

Cleaver, J.E., 1968. Defective repair replication of DNA in xeroderma pigmentosum. Nature 218, 652–656.

Costa, R.M., Chiganças, V., Galhardo, R.S., Carvalho, H., Menck, C.F., 2003. The eukaryotic nucleotide excision repair pathway. Biochimie 85, 1083–1099.

Dupuy, A., Sarasin, A., 2015. DNA damage and gene therapy of xeroderma pigmentosum, a human DNA repair-deficient disease. Mutation Research 776, 2–8.

Greinert, R., de Vries, E., Erdmann, F., Espina, C., Auvinen, A., Kesminiene, A., Schüz, J., 2015. European code against cancer 4th edition: Ultraviolet radiation and cancer. Cancer Epidemiology 39, S75–S83.

Hanawalt, P.C., Spivak, G., 2008. Transcription-coupled repair: Two decades of progress and surprises. Nature Review Molecular Cell Biology 9, 958–970.

Kannouche, P., Stary, A., 2003. Xeroderma pigmentosum variant and error-prone DNA polymerases. Biochimie 85, 1123–1132.

Kraemer, K.H., DiGiovanna, J.J., Moshell, A.N., Tarone, R.E., Peck, G.L., 1988. Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin. New England Journal of Medicine 318, 1633–1637.

Lehmann, A.R., 2003. DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Biochimie 85, 1101–1111.

Magnaldo, T., Sarasin, A., 2004. Xeroderma pigmentosum: From symptoms and genetics to gene-based skin therapy. Cells, Tissues, Organs 177, 189–198.

Marteijn, J.A., Lans, H., Vermeulen, W., Hoeijmakers, J.H., 2014. Understanding nucleotide excision repair and its roles in cancer and aging. Nature Reviews Molecular Cell Biology 15, 465–481.

Menck, C.F., Munford, V., 2014. DNA repair diseases: What do they tell us about cancer and aging? Genetics and Molecular Biology 37, 220–233.

Menck, C.F., Armelini, M.G., Lima-Bessa, K.M., 2007. On the search for skin gene therapy strategies of xeroderma pigmentosum disease. Current Gene Therapy 7, 163–174.

Morice-Picard, F., Cario-André, M., Rezvani, H., Lacombe, D., Sarasin, A., Taïeb, A., 2009. New clinico-genetic classification of trichothiodystrophy. American Journal of Medical Genetics A 149A, 2020–2030.

Moriwaki, S., 2016. Human DNA repair disorders in dermatology: A historical perspective, current concepts and new insight. Journal of Dermatology Sciences 81, 77–84.

Pfeifer, G.P., Besaratinia, A., 2012. UV wavelength-dependent DNA damage and human non-melanoma and melanoma skin cancer. Photochemical and Photobiological Science 11, 90–97.

Saffi, J., Agnoletto, M.H., Guecheva, T.N., Batista, L.F., Carvalho, H., Henriques, J.A., Stary, A., Menck, C.F., Sarasin, A., 2010. Effect of the anti-neoplastic drug doxorubicin on XPD-mutated DNA repair-deficient human cells. DNA Repair 9, 40–47.

Schuch, A.P., Moreno, N.C., Schuch, N.J., Menck, C.F., Garcia, C.C., 2017. Sunlight damage to cellular DNA: Focus on oxidatively generated lesions. Free Radical Biology and Medicine 107, 110–124.

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Notes

Cross-reference terms in *italics* are general cross-references, or refer to subentry terms within the main entry (the main entry is not repeated to save space). Readers are also advised to refer to each article for additional cross-references — not all of these cross-references have been included in the index cross-references.

The index is arranged in set-out style with a maximum of three levels of subheading. Major discussion of a subject is indicated by bold page numbers. Page numbers suffixed by t, f, and b refer to Tables, Figures, and Boxes respectively. *vs.* indicates a comparison.

The index entries are presented in word-by-word alphabetical sequence in which a group of letters followed by a space is filed before the same group of letters followed by a letter. For example, entries beginning 'air density' are alphabetized before 'aircraft.' Prefixes and terms in parentheses are excluded from the initial alphabetization.

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